Reproduction of the root-knot nematode *Meloidogyne arenaria* on flue-cured tobacco possessing resistance genes *Rk1* and/or *Rk2* and the impact of parasitism on the accumulation of nicotine in conventional and low-alkaloid tobacco

Noah R. Adamo

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Plant Pathology, Physiology and Weed Science

Charles S. Johnson

Jonathan D. Eisenback

T. David Reed

Ramsey S. Lewis

December 2020

Blackstone, VA

Keywords: *Meloidogyne, Meloidogyne arenaria, Nicotiana tabacum,* flue-cured tobacco, low-alkaloid tobacco, nicotine, resistance

Reproduction of the root-knot nematode *Meloidogyne arenaria* on flue-cured tobacco possessing resistance genes *Rk1* and/or *Rk2* and the impact of parasitism on the accumulation of nicotine in conventional and low-alkaloid tobacco

Noah R. Adamo

Abstract

Host resistance has become a cornerstone of sustainable production of flue-cured tobacco in regions where root-knot nematodes present a threat to growers. Resistance to races 1 and 3 of *M. incognita*, historically the most significant root-knot nematode threat to tobacco production, is now widely available in commercially available flue-cured tobacco varieties, and is imparted by the gene *Rk1*. The same gene also provides resistance to race 1 of *M. arenaria*. The widespread deployment of this resistance has fostered a shift in root-knot nematode population dynamics, as a result of which *M. arenaria* race 2 has become the predominant root-knot nematode threat in Virginia. A second resistance gene known to impart resistance to *M. javanica*, *Rk2*, has also been incorporated into numerous released cultivars in combination with *Rk1*. This combination has been demonstrated to impart increased resistance to *M. incognita* and *M. javanica* relative to either gene alone. In the present work, eleven greenhouse trials conducted from 2017-2019, as well as two trials conducted in 2018 and 2019 on a cooperating farm, investigated the efficacy of this stacked resistance against *M. arenaria* race 2 and compared the effect of stacking both resistance genes to the effect of either gene alone relative to a susceptible cultivar. We also evaluated how these forms of resistance compare with resistance possessed by a breeding line with resistance reportedly derived from *N. repanda* to determine if additional, novel sources of resistance to root-knot nematodes previously identified from other species in the genus *Nicotiana* could play a role in expanding the genetic diversity of germplasm available for the refinement of

host resistance in flue-cured tobacco. Additionally, in light of potential new rule making from the FDA mandating reduced nicotine content of cured tobacco leaf, we investigated the relationship between alkaloid (nicotine) content of flue-cured tobacco and root-knot nematode parasitism, while also evaluating nematode parasitism effects on carbohydrate content. Despite considerable variability in our results, particularly under field conditions, our results demonstrate that stacking *Rk1* and *Rk2* imparts greater resistance to *M. arenaria* race 2 than either gene alone, but that an entry possessing resistance reportedly derived from *N. repanda* exhibited significantly greater resistance to root-knot nematodes than the combination of *Rk1* and *Rk2* based on root galling, and egg mass and egg production. The alkaloid content of flue-cured tobacco did not appear to have an effect on root-knot nematode parasitism under greenhouse or field conditions, but the presence of the nematode did lead to increased accumulation of nicotine in the roots of plants, while translocation of nicotine to leaves was reduced. Conversely, root-knot nematode parasitism was reduced accumulation of carbohydrates in roots, while having no significant effects on leaf carbohydrate content.

Reproduction of the root-knot nematode *Meloidogyne arenaria* on flue-cured tobacco possessing resistance genes *Rk1* and/or *Rk2* and the impact of parasitism on the accumulation of nicotine in conventional and low-alkaloid tobacco

Noah R. Adamo

General Audience Abstract

Root-knot nematodes (*Meloidogyne* spp.) are microscopic round worms that can cause considerable damage to flue-cured tobacco (*Nicotiana tabacum* L.), and while not typically responsible for killing plants outright, can reduce the quality of cured tobacco leaf and may predispose plants to a host of other issues, resulting in challenges and economic burdens on growers. Chemicals that effectively control nematodes, which are animals, pose inherent threats to human applicators and may harm the environment in a number of ways, so the use of tobacco varieties that are resistant to root-knot nematodes is increasingly common and essential to sustainable tobacco production. One form of root-knot nematode resistance, called *Rk1*, has become common and is found in all commercially grown flue-cured tobacco. This form of resistance is effective against 2 'races' of the root-knot nematode *M. incognita*, which has historically caused tobacco growers the most issues. However, because this resistance is so widely employed, growers have controlled these nematodes, while another species, *M. arenaria*, has become more prevalent, particularly 'race' 2, which is not controlled by *Rk1*. We know from previous research that another gene, *Rk2*, provides resistance to some root-knot nematode that *Rk1* does not effect, and that combining both genes seems to provide even greater root-knot nematode control than either gene alone. We investigated whether *Rk2* is effective at controlling *M. arenaria* race 2 when it is combined with *Rk1* in greenhouse and field experiments. We also investigated how a different, novel type of resistance, which comes from a species of tobacco

related to cultivated tobacco, compares with the *Rk1/Rk2* resistance in greenhouse trials. Additionally, the FDA has recently suggested that nicotine levels in tobacco leaf should be dramatically reduced to help mitigate adverse human health consequences associated with tobacco consumption. Nicotine may play some role in resistance to root-knot nematode in tobacco, and conversely, root-knot nematodes may impact levels of nicotine, as well as other important chemical constituents of tobacco. We also investigated these questions in greenhouse and field experiments. Our results ultimately demonstrate that combining both *Rk1* and *Rk2* gives flue-cured tobacco a higher level of resistance to root-knot nematodes than either gene alone, but also suggests that the form of resistance we evaluated from a related *Nicotiana* species could be even more effective in controlling these nematodes. We observed that the amount of nicotine present in tobacco did not impact nematode parasitism, but that nematode parasitism could lead to lower levels of nicotine in the leaves of plants because the nematodes, which feed on plants roots, cause damage to the plant that interferes in the movement of nicotine from roots to leaves.

Acknowledgements

I have been fortunate to pursue my PhD under the mentorship and guidance of some incredible scientists. I would like to thank Dr. Chuck Johnson for extending the opportunity to work on nematodes in his lab and for helping me mature as a research and extension professional. I thank Dr. Jon Eisenback for recognizing my passion for nematology and joining the project, as well as the staff from his lab, who contributed greatly to the success of this project. Dr. David Reed provided tremendous advice and candor over the past four years. I want to thank all three for teaching me how to be a better nematologist, diagnostician, and plant pathologist. I would also like to acknowledge Dr. Ramsey Lewis from NC State for participating in the project and providing both material support, in the form of tobacco germplasm, and for inspiring a growing interest in plant breeding and crop improvement. Paulo Vieira has been a great source of wisdom over the past four years, and a good friend as well. Elizabeth Bush and Mary Ann Hansen have been huge influences on my continued interest in diagnostic plant pathology and also great friends over the past eight years or so at Virginia Tech. Finally, I would like to acknowledge the support and mentorship of Drs. Anton Baudoin and Rob Wick. Dr. Baudoin was an excellent M. S. advisor and a great mentor. Dr. Wick gave me my first job as a lab technician, showed me my first nematode, and helped give me a direction at a time when I sorely needed one.

I owe Lauren Cogar and Spencer Irby, along with the plant pathology crew as the Southern Piedmont AREC, a tremendous amount of gratitude for their hard work on my behalf both in the lab and under the sun for long days every summer, and my fellow graduate students for their solidarity and friendship. I would like to acknowledge that none of this work would be possible without the rest of the faculty and the incredible staff at SPAREC, particularly Ned

Jones and the farm crew and analytical chemists Brad Ellis and Grace Rogers. I would also like to recognize the financial and material support from Altria Client Services, without which this project would not have come to fruition.

I would finally like to extend my greatest appreciation to my parents, Bethany and Bill Adamo, my brother Tim, and my partner Dayna Guadagno for their steadfast support, love, and humor throughout this process. And I guess that dog is O. K., too.

Table of Contents

Acknowledgements vi

Table of Contents viii

List of Figures x

List of Tables xii

Chapter 1: Literature Review and Research Objectives 1

Research Objectives 14

Literature cited 15

Chapter 2: Reproduction of *Meloidogyne arenaria* race 2 on flue-cured tobacco possessing root-knot nematode resistance genes *Rk1* and/or *Rk2* 26

Abstract 26

Introduction 27

Materials and Methods 30

Results 34

Discussion 45

Literature cited 52

Chapter 3: Reproduction of *Meloidogyne arenaria* race 2 on flue-cured tobacco incorporating a resistance trait introgressed from *Nicotiana repanda* 58

Abstract 58

Introduction 59

Materials and Methods 62

Results 64

Discussion 68

Literature cited 74

Chapter 4: Influence of *Meloidogyne arenaria* race 2 on nicotine accumulation in conventional and low-alkaloid flue-cured tobacco 79

Abstract 79

Introduction 80

Materials and Methods 83

Results 84

Discussion 112

Literature cited 118

List of Figures

Figure 1.1	Flue-cured tobacco is typically germinated in greenhouses employing a hydroponic “float bay” system prior to transplant to the field.	2
Figure 1.2	A typical example of the impact of root-knot nematode parasitism on flue-cured tobacco; the sporadic distribution of impacted plants and the lack of apparent chlorosis and leaf necrosis in the aerial portion of the affected plants is characteristic of these nematodes.	3
Figure 1.3	A simple depiction of the lifecycle of a root-knot nematode resulting in the formation of a female, and the induced changes in root morphology caused by successful parasitism. “N” and “GC” refer to the nematode and the feeding site, the giant cell, in the figure; hypertrophy of the cells selected for giant cell formation results in the characteristic galls observed on plant roots.	5
Figure 1.4	An example of a healthy root system (left) and a root system exhibiting severe root-knot induced root galling (right).	6

Figure 4.1 Total root nicotine content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019. 102

Figure 4.2 Total leaf nicotine content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019. 103

Figure 4.3 Root total carbohydrate content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019. 104

Figure 4.4 Leaf total carbohydrate content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019. 105

List of Tables

Table 2.1	Root galling of flue-cured tobacco entries by *M. arenaria* race 2 in greenhouse pot tests in 2018 and 2019.	35
Table 2.2	Numbers of inoculated *M. arenaria* race 2 nematodes observed in roots of flue-cured tobacco entries in greenhouse pot tests in 2018 and 2019.	37
Table 2.3	Egg masses, eggs per gram of root, and reproductive indices of *M. arenaria* race 2 on flue-cured tobacco entries from greenhouse pot tests in 2018 and 2019.	39
Table 2.4	Root galling by *M. arenaria* race 2 on flue-cured tobacco entries in field trials in Palmer Springs, Virginia in 2018 and 2019.	43
Table 2.5	Numbers of *M. arenaria* race 2 nematodes observed in roots on flue-cured tobacco entries in field trials in Palmer Springs, Virginia in 2018 and 2019.	44
Table 2.6	Egg mass and eggs production by *M. arenaria* race 2 on flue-cured tobacco entries in field trials in Palmer Springs, Virginia in 2018 and 2019.	46

Table 2.7 Initial and finial soil nematode counts for flue-cured tobacco entries in field trials in Palmer Springs, Virginia in 2018 and 2019. 48

Table 3.1 Root galling of flue-cured tobacco entries by *M. arenaria* race 2 in greenhouse pot tests in 2017. 65

Table 3.2 Egg masses, eggs per gram of root, and reproductive indices of *M. arenaria* race 2 on flue-cured tobacco entries from greenhouse pot tests in 2017. 66

Table 4.1 Root galling of flue-cured tobacco entries by *M. arenaria* race 2 from greenhouse pot tests in 2019. 90

Table 4.2 Numbers of inoculated *M. arenaria* race 2 nematodes observed in roots of flue-cured tobacco entries from greenhouse pot tests in 2019. 91

Table 4.3 Egg masses, eggs per gram of root, and reproductive indices of *M. arenaria* race 2 on flue-cured tobacco entries from greenhouse pot tests in 2019. 94

Table 4.4 Root mass of flue-cured tobacco entries inoculated with *M. arenaria* race 2 from greenhouse pot tests in 2019. 96

Table 4.5 Root galling and *M. arenaria* race 2 present in roots flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019. 98

Table 4.6 Egg mass and egg production by *M. arenaria* race 2 on flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019. 99

Table 4.7 Total nicotine content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019. 101

Table 4.8 Total carbohydrate content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019. 106

Table 4.9 Total nicotine content of flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019. 109

Table 4.10 Total carbohydrate content of flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019. 111

Chapter 1

Literature Review and Research Objectives

Tobacco (*Nicotiana tabacum* L.) is a valuable agricultural commodity, cultivated around the world (FAO, 2016). While tobacco production in the United States has decreased over the past two decades, the United States is still the fourth largest tobacco producer in the world, after China, Brazil, and India (FAO, 2018). Tobacco contributed over 1.2 billion dollars to the United States' economy in 2016 (FAO, 2016). Flue-cured tobacco accounts for the majority of tobacco plantings in the United States, with over 14,000 acres planted in Virginia alone in 2020 (USDA, 2020).

Root-knot nematodes (*Meloidogyne* spp.) first appeared in the literature without any specified nomenclature in 1855 (Berkley). Over a century of taxonomic revision, the genus was first referred to as *Meloidogyne* in 1887 (Göldi, 1892). These nematodes were variously classified as members of the genus *Heterodera* (Müller, 1884; Treub, 1885) prior the acknowledgement of the genus as *Meloidogyne* by Chitwood (1949; Hunt and Handoo, 2009). Root-knot nematodes may account for as much as 15% of global yield losses in tobacco annually (Schneider, 1991a). Root-knot nematodes may decrease yields for flue-cured tobacco growers by 1 to 5% in Virginia, and present similar problems throughout the Southeastern United States (Fortnum et al., 2001; Koenning et al., 1999). Root-knot nematode parasitism of tobacco typically results in chlorosis and stunting of the aerial portion of the plant which may be confused with symptoms of drought stress, nutrient deficiency and other plant stressors related to the inhibition of vascular function (Taylor and Sasser, 1978). Successful root-knot nematode parasitism induces the formation of a hyperplasic, multinucleated feeding site, called a giant cell, that functions a sink for host nutrients, and which results in a characteristic root galling symptom in the case of most species (Jones and Goto, 2011).

Figure 1.1. Flue-cured tobacco is typically germinated in greenhouses employing a hydroponic "float bay" system prior to transplant to the field.

Figure 1.2. A typical example of the impact of root-knot nematode parasitism on flue-cured tobacco; the sporadic distribution of impacted plants and the lack of apparent chlorosis and leaf necrosis in the aerial portion of the affected plants is characteristic of these nematodes.

Root-knot nematodes comprise a highly polyphagous genus of plant parasitic nematodes with over 90 distinct species (Jones and Goto, 2011; Jones et al., 2013). The root-knot nematode lifecycle varies in length depending on a number of environmental and ecological factors, but typically takes about 28 days (Agrios, 2005; Ravichandra, 2008). Motile, infective, vermiform second stage juveniles (J2s) (the first molt having occurred within the egg) follow chemical gradients established by host root exudates through soil to locate root tips (Teillet et al., 2013). Once a susceptible root has been located, the J2 utilizes a combination of mechanical stylet thrusting and secreted cell wall degrading enzymes and effectors to bypass host defenses (Perry and Moens, 2011; Escobar et al., 2015). J2s ideally enter roots anterior to zone of elongation and migrate intercellularly towards the root tip in order to bypass the Casparian strip, which presents a barrier to entry to the vascular cylinder (Escobar et al., 2015). Upon successfully navigating to the vascular cylinder, J2s, ultimately become sedentary after inducing giant cell (described briefly above) formation in selected vascular cells (Bird et al., 2009; Perry and Moens, 2011), typically on the order of "five to eight cells" (Escobar et al., 2015). These cells undergo extensive hyperplasia, resulting in the so called giant-cell and in distinct and characteristic galling (Escobar et al., 2015), although in some species and hosts galling is minimal to nonexistent (Volvas et al., 2005; Elling, 2013) or much more similar in structure to *Rhizobium* nodules than prototypical root-knot galls (Eisenback and Dodge, 2012). The J2 eventually molts into an immobile J3 and subsequently J4, and feeding is suspended during these lifestages (Manzanilla-Lopez and Bridge, 2004; Escobar et al., 2015). At this point, if environmental and ecological conditions are appropriate, the J4 will undergo a final molt to become an adult, typically a female.

Figure 1.3. A simple depiction of the lifecycle of a root-knot nematode resulting in the formation of a female, and the induced changes in root morphology caused by successful parasitism. “N” and “GC” refer to the nematode and the feeding site, the giant cell, in the figure; hypertrophy of the cells selected for giant cell formation results in the characteristic galls observed on plant roots (image from Bartlem et al., 2013).

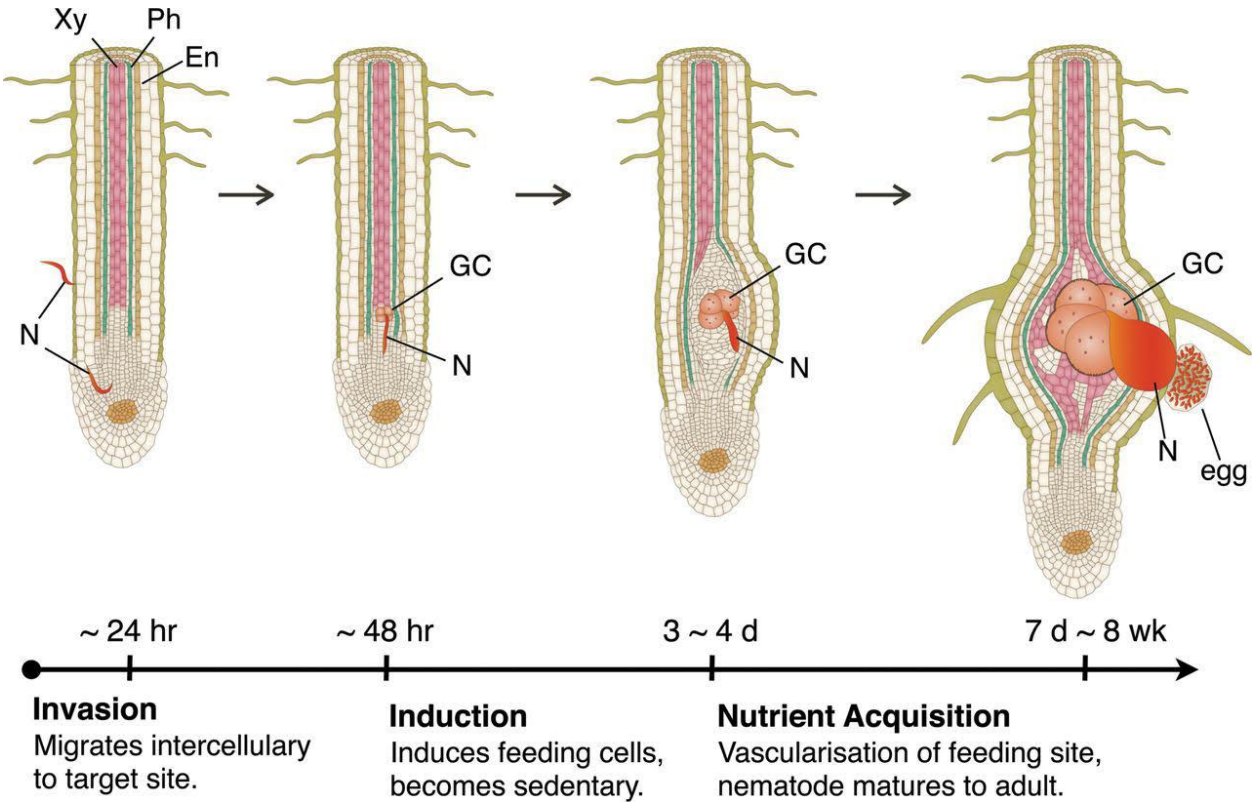


Figure 1.4. An example of a healthy root system (left) and a root system exhibiting severe root-knot induced root galling (right).

Root-knot nematodes typically reproduce via mitotic parthenogenesis, so males are typically rare although exceptions to this rule do exist (Eisenback and Triantaphyllou, 1991; Berg et al., 2008). If conditions do not favor reproduction at the point of initiating the giant cell (i.e. overcrowding, poor host nutritional status), the J2 will undergo a different molting program, resulting in a migratory, vermiform (but significantly larger than the J2) nematode with distinctively male morphology which will evacuate the root (Triantaphyllou, 1973; Eisenback and Triantaphyllou, 1991). When females are fully formed, they typically exhibit a a pyrifom shape and the body cavity is dominated by two large ovaries (Escobar et al., 2015). Eggs are deposited in a gelatinous matrix of consisting of glycoproteins secreted by the rectal glands (Sharon and Spiegel, 1993; Escobar et al., 2015) and which acts as a physical barrier preventing the eggs from dehydrating (Wallace, 1968) and which may also play some role in suppressing microbial activity on and around the egg mass, which is extruded from root tissue and is exposed to the rhizosphere (Sharon and Spiegel, 1993; Orion et al., 2001). The first molt occurs within the egg, and the lifecycle can begin again in the presence of a suitable host (Escobar et al., 2015).

Historically, control of root-knot nematodes has been predicated largely on the use of soil fumigants and non-fumigant nematicides. Fumigants are broad-spectrum, nematicidal (Spurr, 1985), volatile substances that vaporize and move throughout soil. For example, 1,3 dichloropropene and chloropicrin are still important fumigant tools for nematode control (Johnson et al., 2005). Nonfumigant nematicides are more often nematistatic rather than lethal upon contact (Spurr, 1985) and can be broadly classified as organophosphates and carbamates. However, historically effective chemical interventions have undergone significant regulatory scrutiny over the past several decades and are no longer readily available to growers. For example, methyl bromide is a highly effective soil fumigant and nematicide that has traditionally

played an important role in suppressing root-knot and other soil born nematodes. Significant regulatory scrutiny resulted in bans on use of methyl bromide throughout the European Union in 2010 (Kearn et al., 2014), and ultimately resulted in a cascade of similar prohibitions in other regions. Similar issues threaten the ongoing use of the carbamate and organophospate non-fumigant nematicides (Lamondia, 2008; USEPA, 2008; Escobar et al., 2015; USEPA, 2020). Better management practices, such as crop rotation, proper field cultivation and the destruction of crop reidues are also integral parts of root-knot nematode management in tobacco (Johnson et al., 2005). Biocontrols, particularly nematode antagonists (Escobar et al., 2015) have also shown some potential to contribute to the control of root-knot nematodes (Johnson et al., 2005), but most have not proven to be effective as standalone management strategies (Viaene et al., 2006) and effectively applying these controls under field conditions presents a number of challenges, including the biological and ecological constraints demanded by the introduction of another organism to dynamic soil environments.

Host resistance has been historically, and is increasingly, an important aspect of mitigating root-knot nematode parasitism in flue-cured tobacco (Koenning et al., 1999). Host resistance in plant nematology is seen as the near or complete inhibition of reproduction, in contrast to tolerance, in which nematode reproduction is not necessarily inhibited, but quality of the crop or commodity of interest is retained despite nematode increase (Roberts, 2002). Root-knot nematode resistance was first introduced to a commercial tobacco cultivar in 1961 in the form of the gene *Rk*, know referred to as *Rk1,* which was originally discovered in *N. tomentosa* Ruis and Pav. (Yi et al., 1998). This gene is now widely available in flue-cured tobacco cultivars grown commercially in the United States (Koenning et al., 1999). *Rk1* has been reported to impart 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, 1991b; Ng'ambi et al., 1999b). Ternouth et al. (1986) suggested that the gene imparts some level of resistance or tolerance to *M. javanica* (Treub 1885). However, Ng'ambi et al. (1999b) reported that *Rk1* imparts little or no resistance to *M. javanica*, *M. incognita* host races 2 and 4, *M. arenaria* race 2, and *M. hapla* Chitwood 1949, observing that these species/races caused considerable galling on *Rk1* resistant varieties relative to susceptible controls.

Another gene, originally known as "*T*," was discovered in Zimbabwe in 1950 (Schweppenhauser, 1975). Schweppenhauser et al. (1975) had originally suggested that "*T*" was in effect a quantitative trait locus, but ultimately concluded that "*T*" or *Rk2* is a monogenic, dominant gene with effects augmented by one or two additional genes. This gene was present in *N. tabacum* plants in subsistence gardens along the Zambezi River which had been planted continuously for over 250 years in soils heavily infested with *M. javanica* (Schweppenhauser, 1975; Mackenzie et al., 1986; Ternouth et al., 1986). Individual plants exhibiting what was termed a partial resistance to *M. javanica* did not support reproduction and only limited development of adult female nematodes in preliminary experiments (Schweppenhauser, 1975). Subsequent research suggested that "*T*", or *Rk2* as it is also known, conferred a higher level of resistance to *M. javanica* than *Rk1*, also known in Zimbabwe and South Africa as "*S*" (Ternouth et al., 1986). This research also demonstrated that "stacking" both genes in a plant selection induced a very high level of resistance to *M. javanica* (Ternouth et al., 1986).

Shepherd (1982) referred to significant reductions in successful root penetration by juveniles of *M. javanica* on "better breeding lines" relative to susceptible entries. This report is vague about the identity of these breeding lines, but context suggests these so-called better breeding lines could have been selections carrying the *Rk2*, or "*T*" gene. If this is the case, the

implied mechanism of resistance associated with *Rk2* or "*T*" would be considerably different from that associated with *Rk1*. The mechanism of resistance conferred by *Rk1* has been described as a hypersensitive response that inhibits the formation of the feeding site, thus inhibiting gall formation and subsequent nematode reproduction (Schneider, 1991b; Ng'ambi et al., 1999b). The hypersensitive response is also the mechanism of resistance associated with the *Mi* gene family in tomatoes (Dropkin, 1969; Milligan et al., 1998; El-Sappah et al., 2019), which imparts resistance to root-knot nematodes, and the *Php* gene in tobacco, which imparts resistance to the tobacco cyst nematode, *Globodera tabacum* (Miller and Gray, 1972) Behrens, 1975 (Johnson et al., 2009).

In the 1980's, Zimbabwean researchers crossed local plant selections possessing the "T" gene with established entries to improve agronomic traits that were lacking in the landrace tobacco selections (Mackenzie et al., 1986). The resulting line, RKT15-1-1, was crossed subsequently with the established flue-cured tobacco cultivars SC 72 and NC 89 (Ternouth et al., 1986). This work resulted in two breeding lines, STNCA and STNCB, which possessed both *Rk1* and *Rk2*, and which were also subsequently crossed with a number of established flue-cured cultivars to refine and define the agronomic and flue-cured characteristics of the lines (Ternouth et al., 1986). The *Rk2* gene first became commercially available in Zimbabwe in 1993, when a cross between STNCB 2-28 and ms Kutsaga E1 was released, which incorporated *Rk2* into a flue-cured tobacco cultivar along with *Rk1* (Way, 1994; Jack and Lyle, 1999; Jack, 2001). Flue-cured tobacco cultivars possessing both *Rk* genes became commercially available in the United States in 2007, when a number of cultivars were released by Cross Creek Seed Company and ProfiGen do Brasil (Reed, 2007).

The *Rk1/Rk2* resistance trait has been incorporated into commercial flue-cured tobacco varieties, starting in 2007 (Johnson, 2020) but does not approach the ubiquity of *Rk1* as a standalone form of resistance. Surveys of flue-cured tobacco fields in Virginia conducted over the past two decades have demonstrated that *M. incognita,* which has historically been the most widespread root-knot nematode species in Virginia tobacco fields (Johnson, 1989), has been superseded in prevalence by *M. arenaria* (Eisenback, 2012). In 2004, root-knot nematodes were present in 43.5% of 170 surveyed flue-cured tobacco fields, with *M. arenaria* infesting 56.7% of the fields surveyed, while *M. hapla*, *M. incognita*, and *M. javanica* infested 25.0, 16.7, and 11.7% of surveyed fields, respectively (Eisenback, 2012). As of 2010, the proportion of infested fields had not changed meaningfully (44.9%), with similar trends in species distribution observed in 276 surveyed Virginia tobacco fields. *Meloidogyne arenaria* continued to predominate, infesting 58.8% of surveyed fields, while *M. incognita* was less abundant, infesting 11.1% of surveyed fields (Eisenback, 2012); unidentified *Meloidogyne* species accounted for between 6.3 and 8.3% of the collected specimens in both years (Eisenback, 2012). While root-knot nematode resistant varieties possessing the *Rk1* gene have been effective in controlling *M. incognita* races 1 and 3 and *M. arenaria* race 1, other root-knot nematodes that are not mitigated by *Rk1*, most notably *M. arenaria* race 2, have become more of a threat to flue-cured tobacco crop security (Fortnum et al., 1984; Barker, 1989).

Nicotiana repanda has long been seen as a potential source of disease resistance for cultivated tobacco, including root-knot nematode resistance (Burk and Heggestad, 1966; Stavely et al., 1973). For example, Schweppenhauser et al. (1975) observed that *N. repanda* was the only species evaluated out of 64 *Nicotiana* species that was completely resistant to *M. javanica*. However, due to chromosomal incompatibilities between *N. repanda* and *N. tabacum,* the

manifestation of lethal genes, and other factors, attempts to hybridize or incorporate genes from *N. repanda* with commercially viable *N. tabacum* selections were not successful for many years (Bui et al., 1992). For example, in the late 1960's, and again in the mid 1980's, attempts were made to integrate resistance to tobacco mosaic virus (TMV), tobacco cyst nematode (*Globodera tabacum solanacearum,* Miller and Gray) and *M. javanica* and *M. arenaria* from *N. repanda* into two commercial tobacco cultivars using an interspecific bridge hybridization procedure (Burk, 1967; Gwynn et al., 1986). These crosses were ultimately successful in transferring TMV and cyst nematode resistance to some progeny lines, but root-knot nematode resistance was lost after backcrossing to amplify agronomic traits (Davis et al. 1988a, 1998b). Cell culturing techniques were also employed with little success (Bui et al., 1992). However, resistance to *M. arenaria* and TMV was successfully transferred from *N. repanda* to *N. tabacum* in 1992 using *N. sylvestris* as a bridge species, along with a form of protoplast fusion (Bui et al., 1992).

Nicotine is present in most tobacco cultivars at levels between one and five percent on a dry weight basis (Lewis et al., 2020), and in combustible cigarettes at levels between one and two percent of total weight (Proctor, 2017). New FDA regulatory standards for nicotine content could push growers to produce tobacco that would contain nicotine at "sub-addictive" levels, which could be significantly lower than the levels achievable with current low-alkaloid tobacco cultivars and genetic technologies (USFDA, 2017; Lewis, 2018).

A number of different factors govern the alkaloid content of tobacco, including environmental conditions, planting and cultivation practices, water and nutrient management, variety selection (Henry et al., 2019), and disease pressure (Pressier et al., 2007). All of these factors affect the complex genetic and metabolic regulatory networks that impact the synthesis, accumulation, and translocation of tobacco alkaloids, including nicotine. Nicotine is primarily

synthesized in growing root tips, and translocated to leaves (Zenkner et al., 2019), where it performs functions for the plant such as aboveground herbivory by some insects and mammals (Pressier et al., 2007).

Nicotine inhibits the motility and penetrative ability of *M. incognita* infective juveniles in laboratory tests (Davis and Rich, 1987), but there is no definitive evidence that nicotine is involved in tobacco resistance to root-knot parasitism. However, some research suggests that root-knot parasitism is associated with alterations in nicotine synthesis and translocation (Hanounik and Osborne, 1975, 1977; Hanounik et al., 1975). Nicotine synthesis and accumulation in tobacco roots, and subsequent translocation to and accumulation in leaves, may be up or down regulated under nematode attack (Zacheo et al., 1974; Barker and Weeks 1991), which primarily occurs at the nicotine synthesis sites located in root tips. Parasitism by *M. incognita* has been shown to increase nicotine synthesis in plant roots to a greater extent in nematode-resistant tobacco than in nematode-susceptible tobacco, although nicotine synthesis increased in both relative to healthy controls (Davis and Rich, 1987). Importantly, the nicotine content of leaves increased significantly in resistant varieties under nematode pressure relative to healthy plants or susceptible cultivars (Hanounik and Osborne, 1975). However, more recent research suggests no correlation between nicotine concentration in plant tissues and resistance to root-knot nematodes (Pressier et al., 2007).

Cultivated tobacco, including flue-cured tobacco, contains numerous carbohydrates. These carbohydrates play an important role in the aroma and flavor of combustible tobacco products and are balanced with nicotine in tobacco blends to produce the desired user experience, which must consider aroma, flavor, appearance, and perceived effect (Weeks, 1985; Roemer et al., 2012; Banožic et al., 2020). Climatic factors, nutrient availability and agronomic practices such as the

timing of the removal of the terminal inflorescence may all influence the accumulation of carbohydrates and augment the ratio of sugars to alkaloids in harvested leaf (Weybrew and Woltz, 1975; Campbell et al., 1982; Grisic and Čavlek, 2019). However, relatively little research has investigated the impact of root-knot nematode parasitism on the accumulation of carbohydrates in flue-cured tobacco. Barker and Weeks (1991) demonstrated that plants experiencing low levels of parasitism by *M. incognita* accumulated slightly higher levels of reducing sugars (a form of carbohydrates formed during the process of curing flue-cured tobacco) than healthy plants, but that reducing sugars were significantly lower from plants under higher root-knot pressure than in healthy plants.

Research Objectives

The objectives of this study were to investigate the impact of resistance genes *Rk1* and *Rk2* alone and in combination on the parasitism and reproduction of *M. arenaria* race 2 on flue-cured tobacco, and to determine whether a form of resistance derived from *N. repanda* imparts resistanceto this nematode consistent or superior to that conferred by the *Rk1*/*Rk2* system. Furthermore, we sought to determine how root-knot nematode parasitism impacts the synthesis, translocation, and accumulation of nicotine and carbohydrates from root and to leaves, and to what extent root-knot nematode resistance interacts with nematode parasitism to influence changes in these agronomic factors.

Literature Cited

Agrios, G. N. 2005. Plant diseases caused by nematodes. Pp. 825-874 in G. N. Agrios (ed) Plant Pathology (5th edition). Academic Press, Cambridge.

Banožić, M., Jokić, S., Ačkar, D., Blažić, M., and Šubarić, D. 2020. Carbohydrates-key players in tobacco aroma formation and quality determination. Molecules 25: 1734-1747.

Barker, K. R. 1989. Yield relationships and population dynamics of *Meloidogyne* spp. on flue-cured tobacco. Journal of Nematology 21:597-603.

Barker, K. R., and Weeks, W. W. 1991. Relationships between soils and levels of *Meloidogyne incognita* and tobacco yield and quality. Journal of Nematology 23: 82-90.

Bartlem, D., Jones, M., and Hammes, U. 2013. Vascularization and nutrient delivery at root-knot nematode feeding sites in host roots. Journal of experimental botany 65:10.

Berg, R. H., Fester, T., and Taylor, C. G. 2008. Development of the root-knot nematode feeding cell. Pp. 115-152 in R. H. Berg, and C. G. Taylor (eds.), Cell biology of plant nematode parasitism. Berlin: Springer Berlin Heidelberg.

Berkeley, M. J. 1855. Vibrio forming cysts on the roots of cucumbers. Gardener's Chronicle and Agricultural Gazette 14:220.

Bird, D., Opperman, C., and Williamson, V. 2009. Plant infection by root-knot nematode. Pp. 1-13 in R. H. Berg, and C. G. Taylor (eds.), Cell biology of plant nematode parasitism. Springer Berlin Heidelberg.

Bui, P. T., Jenns, A. E., Schneider, S. M., and Daub, M. E. 1992. Resistance to tobacco mosaic virus and *Meloidogyne arenaria* in fusion hybrids between *Nicotiana tabacum* and an *N. repanda* X *N. sylvestris* hybrid. Phytopathology 82: 1305-1310.

Burk, L. G. 1967. An interspecific bridge-cross. J. Hered. 58: 215-218.

Burk, L. G., and Heggestad, H. E. 1966. The genus *Nicotiana*: A source of resistance to diseases of cultivated tobacco. Econ. Bot. 20: 76-88.

Campbell, C. R., Chaplin, J. F., and Johnson, W. H. 1982. Cultural factors affecting yield, alkaloids and sugars of close-grown tobacco. Agronomy Journal 74 (2): 279–283.

Chitwood, B. G. 1949. "Root-Knot Nematodes"-Part I. A Revision of the Genus Meloidogyne Goeldi, 1887. Proceedings of the Helminthological Society of Washington 16:92-97.

Davis, E. L., and Rich, J. R. 1987. Nicotine content of tobacco roots and toxicity to *Meloidogyne incognita*. Journal of Nematology 19 (1): 23-29.

Davis, E. L., Rich, J. R., and Gwynn, G. R. 1988a. Reaction of selected *Nicotiana* spp. X *N. tabacum* crosses grown in microplots to three *Meloidogyne* spp. Nematropica 18: 109-115.

Davis, E. L., Rich, J. R., Gwynn, G. R, and Sisson, V. 1988b. Greenhouse evaluation of *Nicotiana* spp. for resistance to root-knot nematodes. Nematropica 18: 99-107.

Dropkin, V. H. 1969. The necrotic reaction of tomatoes and other hosts resistant to *Meloidogyne*: Reversal by temperature. Phytopathology 59:1632-1637.

Eisenback, J. D. 2012. Effects of resistant tobacco on population dynamics of root-knot nematode species in Virginia. 45th Tobacco Workers Conference, 16–19 January 2012, Williamsburg, VA, Paper 89 (Abstr.).

Eisenback, J. D., and Dodge, D. J. 2012. Description of a unique, complex feeding socket caused by the putative primitive root-knot nematode, *Meloidogyne kikuyensis*. Journal of Nematology 44:148-152.

Eisenback, J. D. and Triantaphyllou, H. H. 1991. Root-knot nematodes: *Meloidogyne* species and races. Pp 191-274 in W. R. Nickle (ed) Manual of Agricultural Nematology. Marcell Dekker, New York.

Elling, A. A. 2013. Major emerging problems with minor *Meloidogyne* species. Phytopathology 103:1092-1102.

El-Sappah, A. H., M M, I., H El-Awady, H., Yan, S., Qi, S., Liu, J., Cheng, G. T., & Liang, Y. 2019. Tomato Natural Resistance Genes in Controlling the Root-Knot Nematode. Genes, 10: 925-944.

Escobar, C., Barcala, M., Cabrera, J., and Fenoll, C. 2015. Overview of Root-Knot Nematodes and Giant Cells. Pp. 1-32 in C. Escobar and C. Fenoll (eds) Plant Nematode Interactions: A View on Compatible Relationships. Amsterdam: Elsevier.

FAO. 2016. FaoStat Database Collections. http://www.fao.org/faostat/en/?#data/QV. Accessed September 15, 2020.

FAO. 2018. FaoStat Database Collections. http://www.fao.org/faostat/en/#rankings/countries_by_commodity. Accessed September 22, 2020.

Fortnum, B. A., Krausz, J. P., and Conrad, N. 1984. Increasing incidence of *Meloidogyne arenaria* on flue-cured tobacco in South Carolina. Plant Disease 68:244-245.

Fortnum B. A., Lewis, S. A., and Johnson, A. W. 2001 Crop rotation and nematicides for management of mixed populations of *Meloidogyne* spp. on tobacco. Journal of Nematology 33: 318–324.

Göldi, E. A. 1892. Relatório sôbre a moléstia do cafeeiro na provincia do Rio de Janeiro. Archivos do Museu Nacional Rio de Janeiro 8:1-121.

Gršić, K., and Čavlek, M. 2019. Effect of topping height and ripeness on quality of flue-cured tobacco varieties. Journal of Central European Agriculture 20 (3):841-851.

Gwynn, G. R., Barker, K. R., Reilly, J. J., Komn, D. A., Burk, L. G., and Reed, S. M. 1986. Genetic resistance to tobacco mosaic virus, cyst nematodes, root-knot nematodes, and wildfire from *Nicotiana repanda* incorporated into *N. tabacum*. Plant Disease 70: 958-962.

Hanounik, S.B., and Osborne, W.W., 1975. Influence of *Meloidogyne incognita* on the content of amino acids and nicotine in tobacco grown under gnotobiotic conditions. Journal of Nematology 7: 332–336.

Hanounik, S.B., Osborne, W.W., and Pirie, W.R., 1975. Relationships between the population density of *Meloidogyne incognita* and growth of tobacco. Journal of Nematology 7: 352–356.

Hanounik, S.B., and Osborne, W.W., 1977. The relationships between population density of *Meloidogyne incognita* and nicotine content of tobacco. Nematologica 23: 147–152.

Henry, J. B., Vann, M. C., and Lewis, R. S. 2019. Agronomic practices affecting nicotine concentration in flue-cured tobacco: a review. Agron. J. 111 (6): 3067-3075.

Hunt, D. J., and Handoo, Z. A. 2009. Taxonomy, identification and principal species. Pp. 55-88 *in* R. N. Perry, M. Moens, and J. L. Starr, eds. Root-knot nematodes. London: CABI.

Jack, A. M. 2001. Kutsaga RK26 - A new root-knot, Granville wilt and black shank resistant variety. Zimbabwe Tobacco 10: 25–31.

Jack, A. M, Lyle, J. 1999. Kutsaga RK26 - A new root-knot resistant variety. Zimbabwe Tobacco 8: 28–31.

Johnson, C. S. 1989. Managing root-knot on tobacco in the southeastern United States. Journal of Nematology 21:604.

Johnson, C.S. 2020. Disease control. Pp. 47-59 in T. D. Reed, C. S. Johnson, C. A. Wilkinson, and S. Barts, eds. 2020 Flue-Cured Tobacco Production Guide 436-048. Blacksburg, VA: VA Cooperative Extension Publication.

Johnson, C. S., Way, J., and Barker, K. R. 2005. Nematode parasites of tobacco. Pp. 675-708 in M. Luc, R. A. Sikora, and J. Bridge, eds. Plant parasitic nematodes in subtropical and tropical agriculture, 2nd ed. Wallingford, UK: CAB International.

Johnson, C. S., Wernsman, E. A., and Lamondia, J. A. 2009. Effect of a chromosome segment marked by the *Php* gene for resistance to *Phytophthora nicotianae* on reproduction of tobacco cyst nematodes. Plant Disease 93:309-315.

Jones, M. G. K. and Goto, D. B. 2011. Root-knot Nematodes and Giant Cells. Pp. 83-100 in J. Jones, G. Gheysen and C. Fenoll (eds) Genomics and Molecular Genetics of Plant-Nematode Interactions. New York, New York: Springer.

Jones, J. T., Haegeman, A., Danchin, E. G. J., Gaur, H. S., Helder, J., Jones, M. G. K., Kikuchi, T., Manzanilla-López, R., Palomares-Ruis, J., Wesemael, W. M. L., and Perry, R. N. 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular Plant Pathology 14:946-961.

Kearn, J., Ludlow, E., Dillon, J., O'Connor, V., & Holden-Dye, L. 2014. Fluensulfone is a nematicide with a mode of action distinct from anticholinesterases and macrocyclic lactones. Pesticide Biochemistry and Physiology 109: 44-57.

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O., and Fortnum, B. A. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. Journal of Nematology 31: 587-618.

LaMondia, J. A. 2008. Early crop root destruction for management of tobacco cyst nematodes. Journal of Nematology 40: 26–29.

Lewis, R. S. 2018. Potential mandated lowering of nicotine levels in cigarettes: a plant perspective. Nic. And Tob. Res. 21 (7): 991-995.

Lewis, R. S., Drake-Stowe, K. E., Heim, C., Steede, T., Smith, W., and Dewey, R. E. 2020. Genetic and Agronomic Analysis of Tobacco Genotypes Exhibiting Reduced Nicotine Accumulation Due to Induced Mutations in Berberine Bridge Like (BBL) Genes. Frontiers in plant science 11 (368).

Mackenzie, J., Smeeton, B. W., Jack, A. M., and Ternouth, R. A. F. 1986. Review on breeding for resistance to root-knot, *Meloidogyne javanica*, in flue-cured tobacco in Zimbabwe. Proc. CORESTA Symposium, 1986: 26–30 October 1986, Taormina, Sicily, Italy: 272–276.

Manzanilla-Lopez, R. H. E. K. and Bridge, J. 2004. Pp 636-716 in Z. X. Chen, S. Y. Chen and D. W. Dickson (Eds.), Plant diseases caused by nematodes. Wallingford, UK: CABI Publishing.

Milligan, S. B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V. M. 1998. The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell 10:1307-1319.

Müller, C. 1884. Mitteilungen über die unseren Kulturpflanzen schädlichen, das Geschlecht *Heterodera* bildenden Wurmer. Landwirtschaftliche Jahrbücher, Berlin 13:1-42.

Ng'ambi, T. B. S., Rufty, R. C., Barker, K. R., and Melton, T. A. 1999. Identification of sources of resistance to four species of root-knot nematodes in tobacco. Journal of Nematology 31: 272–282.

Orion, D., Kritzman, G., Meyer, S. l. F., Erbe, E. F., and Chitwood, D. J. 2001. A role of the gelatinous matrix in the resistance of root-knot nematode (*Meloidogyne* spp.) eggs to microorganisms. Journal of Nematology 33:203-207.

Perry, R. and Moens, M. 2011. Introduction to plant-parasitic nematodes; modes of parasitism. Pp. 3-20 in J. Jones, G. Gheysen, and C. Fenoll (Eds.), Genomics and molecular genetics of plant-nematode interactions. Springer; Netherlands.

Preisser, E. L., Gibson, S. E., Adler, L. S., and Lewis, E. E. 2007. Underground herbivory and the costs of constitutive defense tobacco. Acta Oecologica 31: 210-215.

Proctor, R. N. 2017. FDA's new plan to reduce the nicotine in cigarettes to sub-addictive levels could be a game-changer. Tob. Control 26 (5): 487-488.

Ravichandra, N. G. 2008. Plant nematology. New Delhi, India: I. K. International Pvt Ltd.

Reed, T.D. 2007. Flue-cured tobacco disease control. Pp. 5–36 in T. D. Reed, C. S. Johnson, P. J. Semtner, and C. A. Wilkinson, eds. 2007 Flue-Cured Tobacco Production Guide 436-048. Blacksburg, VA: VA Cooperative Extension Publication.

Roberts PA. 2002. Concepts and consequences of resistance. Pp. 23–41 in J. L. Starr, R. Cook, and J. Bridge, eds. Plant resistance to parasitic nematodes. Wallingford, UK: CAB International.

Roemer, E., Schorp, M. K., Piadé, J.-J., Seeman, J. I., Leyden, D. E., and Haussmann, H. J. 2012. Scientific assessment of the use of sugars as cigarette tobacco ingredients: A review of published and other publicly available studies. Crit. Rev. Toxicol. 42: 244–278.

Schneider, S. M. 1991a. Nematode diseases. Pp. 37-40 in G. Lucas, and H. D. Shew, eds. Compendium of tobacco diseases. St. Paul, MN: APS Press.

Schneider, S. M. 1991b. Penetration of susceptible and resistant tobacco cultivars by *Meloidogyne* juveniles. Journal of Nematology 23:225-228.

Schweppenhauser, M. A. 1975. A source of *Nicotiana tabacum* resistant to *Meloidogyne javanica*. Tobacco Science 19: 42–45.

Sharon, E., and Spiegel, Y. 1993. Glycoprotein characterization of the gelatinous matrix in the root-knot nematode *Meloidogyne javanica*. Journal of Nematology 25:585-589.

Shepherd, J. A. 1982. Report to the third regional conference on root-knot nematode research held at the international institute of tropical agriculture, Ibidan, Nigeria. Proceedings of

the 3rd Research Planning Conference on root-knot nematodes, *Meloidogyne* spp., Regions IV and V. 16-20 November, 1981.

Spurr, H. W., Jr. 1985. Mode of action of nematicides. Pp. 269-276 in J. N. Sasser, and C. C. Carter, eds. An advanced treatise on *Meloidogyne*, vol. 1, Biology and control. Raleigh, NC:North Carolina State University Graphics.

Stavely, J. R., Pittarelli, G. W., and Burk, L. G. 1973. *Nicotiana repanda* as a potential source for disease resistance in *N. tabacum*. The Journal of Heredity 64: 265-271.

Taylor, A. L., and Sasser, J. N. 1978. Identification and control of root-knot nematodes (*Meloidogyne* spp). Crop Publ. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C pp. 111.

Teillet, A., Dybal, K., Kerry, B. R., Miller, A. J., Curtis, R. H. C., and Hedden, P. 2013. Transcriptional changes of the root-knot nematode *Meloidogyne incognita* in response to *Arabidopsis thaliana* root signals. PLoS One 8: e61259.

Ternouth, R. A. F., Mackenzie, J., and Shepherd, J. A. 1986. Introduction of *Meloidogyne javanica* resistance into flue-cured tobacco in Zimbabwe. Proc. CORESTA Symposium, 1986: 26–30 October 1986, Taormina, Sicily, Italy: 277–280.

Treub, M. 1885. Onderzoekingen over Sereh-Ziek Suikkeriet gedaan in s'Lands Plantentium te Buitenzorg. Mededeelingen uit's Lands Plantentuin, Batavia 2:1-39.

Triantaphyllou, A. C. 1973. Environmental sex differentiation of nematodes in relation to pest management. Annual Review of Phytopathology 11:441-462.

USDA. 2020. United States Department of Agriculture, National Agricultural Statistics Service, Acreage, June 2020. www.nass.usda.gov/publications/reports/acrg0620. Accessed November 16, 2020.

USEPA. 2008. United States Environmental Protection Agency, implementation of risk mitigation measures for soil fumigant pesticides. http://www.epa.gov/pesticides/reregistration/soil_fumigants/. Accessed November 22, 2020.

USFDA. 2017. FDA News Release: FDA announces comprehensive regulatory plan to shift trajectory of tobacco-related disease, death. https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ ucm568923. Accessed June 17, 2020.

Viaene, N., Coyne, D. L., & Kerry, B. R. 2006. Biological and cultural management. Pp. 346-369 in R. Perry and M. Moens (Eds.), Plant nematology. Wallingford, UK: CABI.

Vovlas, N., Rapoport, H. F., Díaz, R. M. J., Castillo, P., Nematologia, B., Nazionale, C., et al. 2005. Differences in feeding sites induced by root-knot nematodes, *Meloidogyne* spp., in Chickpea. Phytopathology 95:368-375.

Wallace, H. R. (1968). The influence of soil moisture on survival and hatch of *Meloidogyne javanica*. Nematologica, 14:231-242.

Way, J. I. 1994. Susceptibility of four tobacco cultivars to *Meloidogyne* species. CORESTA Information Bulletin, Congress Issue, 9–24 October 1994, Harare, Zimbabwe: 110 (Abstr.).

Weeks, W. W. 1985. Chemistry of tobacco constituents influences flavor and aroma. Rec. Adv. Tob. Sci. 11: 175–200.

Weybrew, J. A., and Woltz, W. G. 1975. Production factors affecting chemical properties of the flue-cured leaf. IV. Influence of management and weather. Tobacco International 177 (6): 46–48.

Yi, H. Y., Rufty, R. C., Wernsman, E. A., and Conkling, M. C. 1998. Mapping the root-knot nematode resistance gene (Rk) in tobacco with RAPD markers. Plant Disease 82: 1319–1322.

Zacheo, G., Lamberti, F., and Durbin, R. D. 1974. Effect of *Meloidogyne incognita* (Kofoid and White) Chitwood on the nicotine content of tobacco (*Nicotiana tabacum* L.). Nematologia Mediterranea 2(2): 165-170.

Zenkner, F. F., Margis-Pinhiero, M., and Cagliari, A. 2019. Nicotine biosynthesis in *Nicotiana*: a metabolic overview. Tob. Sci. 56 (1): 1-9.

CHAPTER 2

Reproduction of *Meloidogyne arenaria* race 2 on flue-cured tobacco possessing root-knot nematode resistance genes *Rk1* and/or *Rk2*

ABSTRACT

The use of host resistance has become a critical aspect of root-knot nematode control in flue-cured tobacco production. While resistance to *M. incognita* races 1 and 3, as well as race 1 of *M. arenaria* has become widespread in commercial flue-cured tobacco varieties due to the introduction of resistance gene *Rk1*, this has created a shift in nematode pest pressure, leading to increasing prevalence of *M. arenaria* race 2, which is not controlled by *Rk1*. As a second form of resistance, *Rk2*, becomes increasingly available commercially, it is important to clarify the efficacy of this gene, alone or in combination with *Rk1*, against *M. arenaria* race 2. Greenhouse pot tests and field trials were conducted in 2018 and 2019 to investigate the effect of *Rk1* and *Rk2* in combination on parasitism and reproduction of *M. arenaria* race 2 on flue-cured tobacco. Greenhouse and field trials included Hicks (a root-knot susceptible standard cultivar), K326 (homozygous for *Rk1*), T-15-1-1 (homozygous for *Rk2*), STNCB 2-28 (homozygous for both *Rk1* and *Rk2*), and CC 13 (Homozygous for *Rk1* and heterozygous for *Rk2*). Plants were inoculated with 5,000 *M. arenaria* race 2 eggs in the greenhouse or infested under natural, variable pressure by the nematode in the field and data were collected after 28 days (greenhouse trials) or every three weeks following transplant until 18 weeks at the field level, simulating a full growing season. Data collected include root galling index, nematodes present in roots, and egg counts, the latter of which were used to determine reproductive indices for different entries in greenhouse trials. While our results varied considerably, we present evidence that the combination of both *Rk1* and *Rk2* provides greater resistance to *M. arenaria* race 2 than either

gene alone. While the effect of either gene alone was inconsistent, we did observe some significant reductions in galling and subsequent reproduction associated with each gene relative to the susceptible control.

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a valuable agricultural commodity, cultivated around the world (FAO, 2016). While tobacco production in the United States has decreased over the past two decades, the crop still contributed over 1.2 billion dollars to the economy in 2016 (FAO, 2016). Flue-cured tobacco accounts for the majority of tobacco plantings in the United States, with over 14,000 acres planted in Virginia in 2020 (USDA, 2020). Root-knot nematodes (*Meloidogyne* spp.) can cause serious issues for flue-cured tobacco growers in the Southeastern United States, and may reduce yields by 1 to 5% in Virginia (Fortnum et al., 2001; Koenning et al., 1999). In the past several decades, some of the most effective chemical controls for root-knot nematodes have become unavailable to tobacco growers (Lamondia, 2008; USEPA, 2008). As such, the use of root-knot resistant or tolerant cultivars is an essential tool for root-knot nematode management in flue-cured tobacco (Johnson et al., 2005).

Nematode resistance is defined as the complete inhibition of reproduction by a nematode on a given host (Roberts, 2002). On nematode tolerant hosts, nematode reproduction is not necessarily inhibited, but tolerant hosts do not exhibit adverse responses to nematode parasitism in relevant agronomic aspects such as vigor and yield (Roberts, 2002). Root-knot nematode resistance was first introduced to a commercial tobacco cultivar in 1961 in the form of the gene *Rk*, know referred to as *Rk1,* which was originally discovered in *N. tomentosa* Ruis and Pav. (Yi et al., 1998). This gene has been widely incorporated into flue-cured tobacco cultivars grown commercially in the United States (Koenning et al., 1999). *Rk1* has been reported to impart

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). Ternouth et al. (1986) suggested that the gene imparts some level of resistance or tolerance to *M. javanica* (Treub 1885). However, Ng'ambi et al. (1999b) reported that *Rk1* imparts little or no resistance to *M. javanica*, *M. incognita* host races 2 and 4, *M. arenaria* race 2, and *M. hapla* Chitwood 1949.

Another gene, originally known as "*T*," was discovered in Zimbabwe in 1950 (Schweppenhauser, 1975). This gene was present in *N. tabacum* plants in subsistence gardens along the Zambezi River which had been planted continuously for over 250 years in soils heavily infested with *M. javanica* (Schweppenhauser, 1975; Mackenzie et al., 1986; Ternouth et al., 1986). Individual plants exhibiting what was termed a partial resistance to *M. javanica* did not support reproduction and only limited development of adult female nematodes in preliminary experiments (Schweppenhauser, 1975). Subsequent research suggested that "*T*", or *Rk2* as it is also known, conferred a higher level of resistance to *M. javanica* than *Rk1*, also known in Zimbabwe and South Africa as "*S*" (Ternouth et al., 1986). This research also demonstrated that "stacking" both genes in a plant selection induced a very high level of resistance to *M. javanica* (Ternouth et al., 1986). Additionally, an ambiguously worded report from 1982 (Shepherd) referred to significant reductions in successful root penetration by juveniles of *M. javanica* on "better breeding lines" relative to susceptible entries, and it may be reasonably inferred that these so-called better breeding lines were selections carrying the *Rk2,* or "*T*" gene. If this is the case, the implied mechanism of resistance associated with *Rk2* or "*T*" would be considerably different from that associated with *Rk1*, which doesn't reduce root penetration by juveniles, but inhibits formation of giant cells (Schneider, 1991). Schweppenhauser et al. (1975) had originally

suggested that "*T*" was in effect a quantitative trait locus, but ultimately concluded that "*T*" or *Rk2* is a monogenic, dominant gene with effects augmented by one or two additional genes.

In the 1980's, Zimbabwean researchers crossed local plant selections possessing the "*T*" gene with established entries to improve agronomic traits that were lacking in the landrace tobacco selections (Mackenzie et al., 1986). The resulting line, RKT15-1-1, was crossed subsequently with the established flue-cured tobacco cultivars SC 72 and NC 89 (Ternouth et al., 1986). This work resulted in two breeding lines, STNCA and STNCB, which possessed both *Rk1* and *Rk2*, and which were also subsequently crossed with a number of established flue-cured cultivars to refine and define the agronomic and flue-cured characteristics of the lines (Ternouth et al., 1986). The *Rk2* gene first became commercially available in Zimbabwe in 1993, when a cross between STNCB 2-28 and ms Kutsaga E1 was released, which incorporated *Rk2* into a flue-cured tobacco cultivar along with *Rk1* (Way, 1994; Jack and Lyle, 1999; Jack, 2001). Flue-cured tobacco cultivars possessing both *Rk* genes became commercially available in the United States in 2007, when a number of cultivars were released by Cross Creek Seed Company and ProfiGen do Brasil (Reed, 2007). This combination of root-knot nematode resistance has become increasingly available to growers since 2007 (Johnson, 2020)

Surveys of flue-cured tobacco fields in Virginia conducted over the past two decades have demonstrated that *Meloidogyne incognita,* which has historically been the most widespread root-knot nematode species in Virginia tobacco fields (Johnson, 1989), has been superseded in prevalence by *M. arenaria* (Eisenback, 2012). In 2004, root-knot nematodes were present in 43.5% of 170 surveyed flue-cured tobacco fields, with *M. arenaria* infesting 56.7% of the fields surveyed, while *M. hapla*, *M. incognita*, and *M. javanica* infested 25.0, 16.7, and 11.7% of surveyed fields, respectively (Eisenback, 2012). As of 2010, the proportion of infested fields had

not changed meaningfully (44.9%), with similar trends in species distribution observed in 276 surveyed Virginia tobacco fields. *Meloidogyne arenaria* continued to predominate, infesting 58.8% of surveyed fields, while *M. incognita* was less abundant, infesting 11.1% of surveyed fields (Eisenback, 2012); unidentified *Meloidogyne* species accounted for between 6.3 and 8.3% of the collected specimens in both years (Eisenback, 2012). The increased prevalence of *M. arenaria* presents a potential challenge to growers, as the *Rk1* gene may not be effective in managing this species, and the resistance or tolerance conferred to flue-cured tobacco against *M. arenaria* by *Rk1* in combination with *Rk2* are unclear. While previous work in Virginia has confirmed that *Rk1* in combination with *Rk2* confers resistance to *M. javanica* (Ma and Johnson, unpublished data) and *M. incognita* race three (Pollock et al., 2016), it is crucial to better understand the impact of these genes on *M. arenaria* race 2. The presented work investigated the effect of *Rk1* and *Rk2,* alone and in combination, on the penetrative and reproductive capacity of a population of *M. arenaria* race 2 on flue-cured tobacco.

MATERIALS AND METHODS

Greenhouse trials

A population of *M. arenaria* race 2 collected from a flue-cured tobacco field in Halifax County, VA was maintained on susceptible tomato (*Solanum lycopersicum* L.) variety 'Rutgers' in greenhouses at the Virginia Tech campus in Blacksburg, VA and at the Southern Piedmont Agricultural Research and Extension Center near Blackstone, VA (SPAREC). Egg inoculum for greenhouse trials was collected from infested roots using the method of Hussey and Barker (1973). Eggs were collected in 1 L of tap water, counted with a compound microscope at X 20 – to 40, and an egg suspension calibrated to contain 125 eggs/ml of suspension immediately prior to inoculation. Three greenhouse experiments were conducted in 2018 at SPAREC with an

additional trial in 2019 at the Virginia Tech Campus in Blacksburg, VA to evaluate the impact of resistance genes *Rk1* and/or *Rk2* on parasitism of flue-cured tobacco by *M. arenaria*. Experiments were arranged in randomized complete block designs with seven replications, except for the test in Blackstone in September, 2018, which had six replications. These experiments assessed resistance to *M. arenaria* in a panel of five flue-cured tobacco entries: Hicks (susceptible to the four major tropical root-knot nematode species, *M. incognita, M. javanica, M. arenaria,* and *M. hapla*); K326 (homozygous for *Rk1*); T-15-1-1 (homozygous for *Rk2*); CC 13 (homozygous for *Rk1* and heterozygous for *Rk2*); and STNCB-2-28 (homozygous for both *Rk1* and *Rk2*). Seed of K326 and CC 13 was donated by Cross Creek Seed, Raeford, NC, while seed of the remaining four entries was produced in the SPAREC flue-cured tobacco nursery in 2016 and 2017. Seed were germinated in organic vermiculite (The Epsoma Company, Millville, NJ) and four to five week old seedlings were transplanted to individual 7.6 cm clay pots containing a 2:1 mixture of steam sterilized sandy loam field soil with Profile Greens-Grade Porous Ceramic soil amendment (Profile Products, Buffalo Grove, Il). Seedlings with 4 to 6 true leaves were transplanted into 15 cm clay pots containing the same soil substrate. Plants were transplanted into a 5 to 6 cm deep hole in the substrate and inoculated with a 40 ml aliquot containing 5,000 nematode eggs applied directly to the root mass during transplanting. Plants were maintained in greenhouses at ambient air temperatures of 20 to 33 ^{o}C, with natural lighting.

Plant roots were washed free of soil substrate 28 days following inoculation and blotted dry; the aerial portion of the plants discarded. Fresh root weights were recorded and the whole root system was evaluated for galling according to the gall count index developed by Taylor and Sasser (1978), in which gall counts are ranked as follows: 0 galls present = 0; 1-2 galls present = 1; 3-10 galls present = 2; 11-30 galls present = 3; 31- 100 galls present = 4; and more than 100

galls present = 5. Roots were cut into 4 to 6 cm long sections and thoroughly mixed. Root-knot presence in roots was assessed in three 1 g subsamples from each plant. Roots were cleared in 1% sodium hypochlorite and stained with 0.005% acid fuchsin (Byrd et al., 1983). Roots were examined and nematodes were counted with a stereomicroscope at X10 to 40. For egg mass counts, two 1 g subsamples were stained in 0.15 g/L Phloxine-B (Daykin and Hussey, 1985) for approximately five minutes and were counted with a stereomicroscope at X10 to 40. Eggs were extracted from the remaining root system in the manner described above and counted at X40 using an inverted compound microscope. The reproductive index for each plant was calculated by dividing the final egg count (P_f) by the initial egg inoculum number (P_i).

Field Trials

A field trial was conducted in 2018 and in 2019 to evaluate the above described panel of flue-cured tobacco entries in a flue-cured tobacco field in Palmer Springs, Virginia infested with a population of *M. arenaria* race 2. Trials were arranged in randomized complete block designs with 11 replications in 2018 and 10 replications in 2019. Plots consisted of 16.1 m long rows spaced 1.2 m apart. Plots were mechanically transplanted, fertilized, and maintained in accordance with the recommendations of Virginia Cooperative Extension (Reed et al., 2018).

Initial soil nematode population densities were determined after bed formation and prior to transplant each year, while final population densities were determined after the final sampling at 18 weeks after transplant. From each plot, 24 2 cm by 16 cm soil samples were collected and bulked. Nematode counts were performed at the Virginia Tech Nematode Diagnostic and Assay Lab on the Virginia Tech Blacksburg Campus. Bulked samples were initially hand mixed to reduce aggregates, sifted, and a 250 cubic centimeter subsample from each plot was subjected to nematode extraction using a mechanical elutriator, sugar flotation, and decantation sieving

(Barker, 1985). Two plants were destructively sampled from each plot beginning three weeks after transplant, and every three weeks until 18 weeks after transplanting. Soil was washed free of the root systems of the sampled plants and galling was assessed as described above. Fresh weights of the entire root systems were recorded and fibrous feeder roots were separated from lignified structural roots. The number of nematodes present in roots, egg masses on roots, and eggs per gram of root were determined for the fibrous feeder root portion of the root system of each plant. Egg mass production was evaluated by counting Phloxine-B stained egg masses (Daykin and Hussey, 1985) on three 1 g subsamples at X10 to 40. Numbers of nematodes present in roots were determined by clearing two 1 g subsamples of feeder roots with sodium hypochlorite and staining the roots with acid fuchsin (Byrd et al., 1983) which were examined with a stereomicroscope at X10 – 40. Eggs were extracted from the remaining feeder roots by agitation in 1% sodium hypochlorite (Hussey and Barker, 1973). Eggs were suspended in 1 L of tap water and two 10 ml aliquots were counted at X40 with a compound microscope. These counts were used to calculated the approximate number of nematodes extracted from the known mass of feeder roots, which was then used to calculate the number of eggs per gram of feeder root for the entire root system.

Statistical Analysis

Data were transformed ($\log_{10}(x + 1)$) prior to statistical analysis. Data from all trials were subjected to analysis of variance (ANOVA) using PROC GLM in SAS (version 9.4; SAS Institute, Cary, NC). Differences among treatment means were identified using Fisher's protected Least Significant Difference test ($P \leq 0.05$).

RESULTS

Greenhouse Trials

Results varied across the four greenhouse trials conducted in 2018 and 2019, so each trial was analyzed independently. Significant differences in root galling were found in all four trials. Galling was lowest on entries CC 13 and STNCB 2-28 in every experiment, and highest on the susceptible entry Hicks in all but the April-May 2018 trial (Table 2.1). Galling of CC 13 and STNCB 2-28 was significantly less than that of other entries in all tests except the April-May 2018 trial, and in the trial conducted in 2019, in which galling of CC 13 was similar to that of K326 (Table 2.1). Galling was significantly lower on CC 13 and STNCB 2-28 compared to entries K326 and T-15-1-1, which respectively possess *Rk1* and *Rk1* alone, in all studies but the April-May 2018 trial (Table 2.1); in this trial, galling of K326 was similar to that of CC 13 and STNCB 2-28. Galling was significantly lower on entry K326 than T-15-1-1 in two of the four trials (Table 2.1), and galling of both of these entries was significantly lower than that of Hicks in two trials. However, in the trial conducted in Blackstone from April to May of 2018, galling of Hicks was significantly less than that of T-15-1-1.

Table 2.1. Root galling of flue-cured tobacco entries by *M. arenaria* race 2 in greenhouse pot tests in 2018 and 2019.[z]

		Root Galling (0-5)[y]			
		Blackstone			Blacksburg
		2019	2018		2018
Genotype	Entry	April-May	April-May	September	September
r1krk2	Hicks	4.7 a	3.6 b	5.0 a	4.7 a
RK1rk2	K326	2.8 c	3.1 b	3.4 b	4.4 a
rk1RK2	T-15-1-1	3.5 b	4.4 a	3.9 b	4.6 a
RK1RK2	CC 13	2.3 cd	2.6 b	2.1 c	3.0 b
RK1RK2	STNCB 2-28	1.8 d	3.1 b	2.1 c	2.6 b

[z]Data presented are non-transformed means from seven, seven, six and seven replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] Taylor and Sassers' Indexed Scale of Gall Count-0 = 0; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100; and 5 = > 100 galls per root system.

Nematodes per gram of root varied considerably among trials, but were typically lowest on CC 13 and/or STNCB 2-28 (Table 2.2). Roots of CC 13 and STNCB 2-28 always contained significantly fewer nematodes than susceptible entry Hicks, except in the April-May trial conducted in 2018, in which CC 13 was the only entry with significantly fewer nematodes in roots relative to the other four entries (Table 2.2). Significantly fewer nematodes were present in the roots of both K326 and T-15-1-1 than Hicks in the trial conducted in Blackstone in September of 2018, and K326 had fewer nematodes in roots than Hicks in the trial conducted in 2019 (Table 2.2). Significantly fewer nematodes were present in the roots of entries CC 13 and STNCB 2-28 than entries K326 and T-15-1-1 in the trials conducted in both locations in September of 2018 (Table 2.2).

Table 2.2. Numbers of inoculated *M. arenaria* race 2 nematodes observed in roots of flue-cured tobacco entries at the conclusion of greenhouse pot tests in 2018 and 2019.[z]

		Nematodes/g feeder root			
		Blackstone			Blacksburg
		2019	2018		2018
Genotype	Entry	April-May	April-May	September	September
r1krk2	Hicks	84 a	55 a	26 a	144 a
RK1rk2	K326	25 bc	28 a	10 b	169 a
rk1RK2	T-15-1-1	50 ab	47 a	9 b	158 a
RK1RK2	CC 13	13 cd	14 b	6 c	69 b
RK1RK2	STNCB 2-28	11 d	26 a	3 c	45 b

[z]Data presented are non-transformed means from seven, seven, six and seven replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Egg mass counts differed significantly in all trials. CC 13 and STNCB 2-28 always had significantly fewer egg masses compared to Hicks, and less than K326 in three trials (Table 2.3). Egg mass production was lower on both CC 13 and STNCB 2-28 than T-15-1-1 in one trial, although in another trial, CC 13 had significantly fewer egg masses than T-15-1-1, while egg mass production on STNCB 2-28 was intermediate (Table 2.3). Significantly fewer egg masses were present on T-15-1-1 relative to Hicks in two trials, and in only one trial did K326 exhibit significantly lower egg mass production than Hicks (Table 2.3)

Reproduction varied dramatically across trials. Egg production was significantly lower on CC 13 and STNCB 2-28 than on all other entries in the trial conducted in September of 2018 in Blacksburg, and along with K326, was less than that of Hicks in the trial conducted from April to May in 2018 in Blackstone (Table 2.3). Egg counts were low (not exceeding 22 eggs per gram of root) across all entries in the trial conducted in September of 2018 in Blackstone, but both Hicks and T-15-1-1 had significantly fewer eggs per gram of root than CC 13 in this trial. There was not a consistent trend in relative egg production among the susceptible entry and those possessing either *Rk1* or *Rk2* alone in the three trials where significant differences were present (Table 2.3). No differences in egg production were found among entries in the trial conducted from April to May of 2019 in Blackstone, in which egg counts were also relatively low (Table 2.3).

Table 2.3. Egg masses, eggs per gram of root, and reproductive indices of *M. arenaria* race 2 on flue-cured tobacco entries from greenhouse pot tests in 2018 and 2019.[z]

		Egg masses per gram of root			
		Blackstone			Blacksburg
		2019	2018		2018
Genotype	Entry	April-May	April-May	September	September
r1krk2	Hicks	7 a	25 a	2 a	52 ab
RK1rk2	K326	4 a	6 bc	1 a	60 a
rk1RK2	T-15-1-1	1 b	11 ab	0 b	32 b
RK1RK2	CC 13	1 b	3 c	0 b	20 c
RK1RK2	STNCB 2-28	1 b	6 bc	0 b	13 c

		Eggs per gram of root			
		Blackstone			Blacksburg
		2019	2018		2018
Genotype	Entry	April-May	April-May	September	September
r1krk2	Hicks	30 a	754 a	1 b	1,038 a
RK1rk2	K326	42 a	60 b	10 ab	1,182 a
rk1RK2	T-15-1-1	8 a	215 ab	2 b	795 a
RK1RK2	CC 13	37 a	53 b	22 a	241 b
RK1RK2	STNCB 2-28	10 a	96 b	4 ab	264 b

		Reproductive index[y]			
		Blackstone			Blacksburg
		2019	2018		2018
Genotype	Entry	April-May	April-May	September	September
r1krk2	Hicks	0.1 a	2.2 a	0.0 a	2.4 a
RK1rk2	K326	0.1 a	0.3 b	0.0 a	2.3 a
rk1RK2	T-15-1-1	0.0 a	0.8 b	0.0 a	1.4 b
RK1RK2	CC 13	0.0 a	0.2 b	0.0 a	0.5 c
RK1RK2	STNCB 2-28	0.1 a	0.2 b	0.0 a	0.4 c

[z] Data presented are non-transformed means from seven, seven, six and seven replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] Reproductive index = final population/initial population (P_f/P_i).

Reproductive indices for all four entries possessing *Rk1* and/or *Rk2* were significantly lower than that of susceptible Hicks in the trial conducted April to May of 2018 in Blackstone (Table 2.3). In the trial conducted in Blacksburg in September of 2018, the reproductive indices of T-15-1-1, CC 13, and STNCB 2-28 were significantly lower than those of Hicks and K326, while CC 13 and STNCB 2-28 were also significantly lower than T-15-1-1 (Table 2.3). In the remaining trials, reproduction was low and significant differences in reproductive indices were not detected (Table 2.3).

Field Trials

No root galling was observed on the roots of plants sampled at 3 and 6 weeks after transplanting in 2018 (Table 2.4). Limited galling was observed on the root systems (less than 10 galls per root system) of all entries at 9 and 12 weeks, but no significant differences were found (Table 2.4). Galling of STNCB 2-28 was significantly less than that of Hicks, K326, and T-15-1-1 at 15 weeks, and was significantly less than that of K326 at 18 weeks (Table 2.4)

In 2019, galling was observed on the roots of plants sampled at 3 and 6 weeks after transplanting, but no significant differences were found among entries; no more than one or two galls were observed on root systems (Table 2.4). CC 13 and STNCB 2-28 both had significantly less galling than T-15-1-1 at 9 weeks, and at this timepoint galling of CC 13 was also significantly less than that of entry K326, while galling of susceptible Hicks was intermediate (Table 2.4). Galling was significantly less on both CC 13 and STNCB 2-28 relative to K326 and Hicks at 12 weeks after transplanting, while at 18 weeks, STNCB 2-28 had significantly less root-galling than Hicks, K326 and T-15-1-1; no significant differences were observed at 15 weeks (Table 2.4).

Significantly fewer nematodes were present in the roots of T-15-1-1 than CC 13 3 weeks after transplanting in 2018, whereas in 2019, fewer nematodes were present in the roots of CC 13 than Hicks, K326, and T-15-1-1 (Table 2.5). No differences in nematodes abundance were found at 6 weeks in either year, and in 2018, no significant differences were present at 9 weeks (Table 2.5). In 2019, CC 13 had significantly fewer nematodes present in roots than K326. In both years, starting at 12 weeks, STNCB 2-28 had the fewest nematodes in roots; with the exception of 15 weeks in 2018, STNCB 2-28 always had significantly fewer nematodes present in roots than Hicks, and often relative to K326 as well, while other entries were typically intermediate in nematode abundance (Table 2.5). The number of nematodes present in the roots of entry CC 13 (which is homozygous for *Rk1* and heterozygous for *Rk2*) was never significantly different from that of Hicks (Table 2.5).

Egg masses and/or eggs were not observed on any entry at 3 or 6 weeks after transplanting in either year, and in both years no significant differences were observed at 9 weeks after transplanting, when reproduction was first observed (Table 2.6). In both years, STNCB 2-28 typically had the fewest egg masses present, although there were notable exceptions to this trend, particularly at 12 weeks in 2018, when K326 had significantly fewer egg masses present than Hicks, while all other entries experienced intermediate egg mass production (Table 2.6). No single entry consistently exhibited the highest egg mass numbers in either year (Table 2.6). In 2019, egg production on STNCB 2-28 was significantly less than on K326, while no significant differences were found at this timepoint in 2018 (Table 2.6). At 15 weeks in 2018, egg production was significantly lower on CC 13, STNCB 2-28, and susceptible Hicks relative to T-15-1-1, whereas at the same timepoint in 2019, egg counts were significantly lower for CC 13

and STNCB 2-28 relative to Hicks (Table 2.6). No significant differences in egg production were found at 18 weeks in either year (Table 2.6).

Reproductive indices were not calculated for field trials because the amount of egg inoculum present in the soil at the planting can only be speculated upon based on pre-plant root-knot second-stage juvenile abundance. Mean initial and final soil *M. arenaria* counts are presented in Table 2.7. In our 2018 study, *M. arenaria* juveniles were not present at detectable levels in pre-plant soil samples from 71% of the plots, while following termination of the study, *M. arenaria* juveniles were not present at detectable levels in soil nematode extracts from 10% of the studied plots. In 2019, *M. arenaria* juveniles were not present at detectable levels in pre-plant soil nematode extracts from 53% of plots, while following termination of the 2019 study, *M. arenaria* juveniles were not present at detectable levels in soil nematode extracts from 56% of the studied plots.

Table 2.4. Root galling by *M. arenaria* race 2 on flue-cured tobacco entries in field trials in Palmer Springs, Virginia in 2018 and 2019.[z]

		Root galling (0-5)[y]					
		2018					
		Weeks after transplanting					
Genotype	Entry	3	6	9	12	15	18
r1krk2	Hicks	0.0 a	0.0 a	0.8 a	0.9 a	1.9 a	2.7 ab
RK1rk2	K326	0.0 a	0.0 a	0.9 a	1.2 a	1.8 a	3.4 a
rk1RK2	T-15-1-1	0.0 a	0.0 a	0.6 a	1.2 a	1.9 a	2.1 ab
RK1RK2	CC 13	0.0 a	0.0 a	0.9 a	0.8 a	1.1 ab	2.5 ab
RK1RK2	STNCB 2-28	0.0 a	0.0 a	0.8 a	0.4 a	0.2 b	1.5 b
		2019					
		Weeks after transplanting					
Genotype	Entry	3	6	9	12	15	18
r1krk2	Hicks	0.1 a	0.4 a	2.0 abc	2.0 a	2.0 a	2.4 a
RK1rk2	K326	0.1 a	0.4 a	2.6 ab	2.3 a	2.0 a	2.6 a
rk1RK2	T-15-1-1	0.1 a	0.6 a	3.6 a	1.3 ab	0.7 a	2.6 a
RK1RK2	CC 13	0.2 a	0.0 a	0.4 c	0.5 b	1.3 a	1.8 ab
RK1RK2	STNCB 2-28	0.1 a	0.3 a	0.8 bc	0.3 b	2.0 a	0.8 b

[z]Data presented are non-transformed means from eleven, eleven, eight, ten, seven, and eleven replications respectively in 2018, and ten, seven, four, six, three, and four replications respectively in 2019. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's HSD test ($P \leq 0.05$).

[y] Taylor and Sassers' Indexed Scale of Gall Count-0 = 0; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100; and 5 = > 100 galls per root system.

Table 2.5. Numbers of *M. arenaria* race 2 nematodes observed in roots of flue-cured tobacco entries at six timepoints from field trials in Palmer Springs, Virginia in 2018 and 2019.[z]

		Nematodes/g root					
		2018					
		Weeks after transplanting					
Genotype	Entry	3	6	9	12	15	18
r1krk2	Hicks	1 ab	5 a	47 a	84 a	35 ab	38 a
RK1rk2	K326	2 ab	3 a	25 a	73 ab	32 ab	62 a
rk1RK2	T-15-1-1	1 b	7 a	27 a	49 ab	40 a	31 ab
RK1RK2	CC 13	3 a	5 a	7 a	63 a	11 ab	46 a
RK1RK2	STNCB 2-28	1 ab	3 a	31 a	33 b	8 b	16 b
		2019					
		Weeks after transplanting					
Genotype	Entry	3	6	9	12	15	18
r1krk2	Hicks	5 a	9 a	20 ab	55 a	35 a	37 a
RK1rk2	K326	7 a	4 a	26 a	79 a	41 a	32 ab
rk1RK2	T-15-1-1	5 a	14 a	13 ab	20 ab	8 ab	14 ab
RK1RK2	CC 13	2 b	4 a	5 b	39 a	18 ab	16 ab
RK1RK2	STNCB 2-28	5 ab	4 a	13 ab	6 b	5 b	6 b

[z]Data presented are non-transformed means from eleven, eleven, seven, nine, seven, and ten replications respectively in 2018, and ten, seven, four, six, three, and four replications respectively in 2019. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

DISCUSSION

Results of greenhouse trials suggest that the presence of both resistance genes *Rk1* and *Rk2* increases resistance to *M. arenaria* race 2 in flue-cured tobacco relative to susceptible entries and those possessing either resistance gene alone. These results confirm findings of previous studies demonstrating that a combination of both *Rk1* and *Rk2* increases resistance to root-knot nematodes more than either gene alone, effective against *M. javanica* (Ternouth et al., 1986; Ma, W., Johnson, C. S., Eisenback, J. D., and Reed, T. D., unpublished data), *M. incognita* races 1 and 3 (Barker and Melton, 1990; Ng'ambi et al., 1999a, b; Pollock et al., 2016) and *M. arenaria* races 1 and 2 (Ng'ambi et al., 1999a, 1992b; Pollok et al., 2015).

Our results also suggest that the zygosity of *Rk2* when present in combination with the homozygous *Rk1* gene does not have a significant effect on root galling and root-knot nematode reproduction. In our greenhouse trials, we only observed one case in which entry CC 13, which is homozygous for *Rk1* and heterozygous for *Rk2*, and STNCB 2-28, which is homozygous for both genes differed significantly in a metric of root-knot parasitism, and in that one case, CC 13 actually exhibited fewer nematodes in roots than STNCB 2-28 (in the trial conducted from April to May in Blackstone). At three timepoint in our field trials (12 weeks after transplanting in both 2018 and 2019, and 18 weeks in 2018), significantly fewer nematodes were present in the roots of STNCB 2-28 than CC 13. However, we never found significant differences between these two entries in root galling or nematode reproduction, again suggesting that the heterozygosity of *Rk2* when in combination with *Rk1* may not adversely impact the nematode resistance of flue-cured tobacco under field conditions.

Table 2.6. Egg mass and egg production by *M. arenaria* race 2 on flue-cured tobacco entries in field trials in Palmer Springs, Virginia in 2018 and 2019.[z]

		Weeks after transplanting			
Genotype	Entry	9	12	15	18
		Egg masses per gram of root			
		2018			
r1krk2	Hicks	2 a	38 a	8 ab	16 ab
RK1rk2	K326	0 a	1 b	1 b	21 a
rk1RK2	T-15-1-1	1 a	11 ab	16 a	19 ab
RK1RK2	CC 13	0 a	24 ab	4 b	21 ab
RK1RK2	STNCB 2-28	0 a	28 ab	1 b	6 b
		2019			
r1krk2	Hicks	3 a	14 a	31 a	27 a
RK1rk2	K326	5 a	34 a	39 a	26 ab
rk1RK2	T-15-1-1	4 a	18 a	5 a	4 b
RK1RK2	CC 13	2 a	14 ab	11 a	9 ab
RK1RK2	STNCB 2-28	2 a	1 b	0 a	5 b
		Eggs per gram of root			
		2018			
r1krk2	Hicks	28 a	212 a	38 b	80 a
RK1rk2	K326	50 a	225 a	45 ab	133 a
rk1RK2	T-15-1-1	27 a	105 a	168 a	79 a
RK1RK2	CC 13	22 a	266 a	43 b	95 a
RK1RK2	STNCB 2-28	48 a	445 a	39 b	50 a
		2019			
r1krk2	Hicks	684 a	3,885 ab	12,457 a	2,328 a
RK1rk2	K326	473 a	7,686 a	4,673 ab	2,276 a
rk1RK2	T-15-1-1	1,284 a	3,830 ab	2,223 ab	995 a
RK1RK2	CC 13	643 a	3,120 ab	989 b	1,347 a
RK1RK2	STNCB 2-28	1,129 a	1,296 b	1,231 b	793 a

[z]Data presented are non-transformed means from eleven, eleven, seven, nine, seven, and ten replications respectively in 2018, and ten, seven, four, six, three, and four replications respectively in 2019. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's HSD test ($P \leq 0.05$).

The relationship between the presence of either resistance gene alone and relative inhibition of nematode parasitism by *M. arenaria* race 2 was somewhat less clear in our greenhouse data, but suggests that *Rk2* may be somewhat more effective against *M. arenaria* race 2 than *Rk1*. The number of nematodes present in roots was never different on entries K326 (which possesses *Rk1* only) and T-15-1-1 (possessing only *Rk2*), but root galling was significantly lower on T-15-1-1 than K326 in two of four trials. While egg mass counts were significantly lower for T-15-1-1 than K326 in three of our trials, egg counts and reproductive indices were similar on these entries in all trials, except in the trials conducted in September of 2018 in Blackstone, in which the reproductive index of T-15-1-1 was lower than that of K326. However, under field conditions, the number of nematodes present in roots, root galling, and egg production were not significantly different at any timepoint in either year.

Table 2.7. Initial and finial soil nematode counts for flue-cured tobacco entries in field trials in Palmer Springs, Virginia in 2018 and 2019.[z]

		M. arenaria juveniles/500 cc of soil	
		2018	
Genotype	Entry	Before planting	After final harvest
r1krk2	Hicks	11	2,744
RK1rk2	K326	13	2,753
rk1RK2	T-15-1-1	22	1,858
RK1RK2	CC 13	29	1,382
RK1RK2	STNCB 2-28	11	753
		2019	
Genotype	Entry	Before planting	After final harvest
r1krk2	Hicks	64	8
RK1rk2	K326	12	96
rk1RK2	T-15-1-1	60	24
RK1RK2	CC 13	26	44
RK1RK2	STNCB 2-28	50	12

[z]Data presented are non-transformed means from eleven and ten replications respectively in 2018, and ten and four replications respectively in 2019.

In two of our four trials, nematode inoculum was clearly viable, as assessed by penetration and gall index, but reproduction was very low (reproductive indices not exceeding 0.1) and no significant differences could be found among any entries. This is not entirely surprising given that the root-knot nematode lifecycle typically takes about 25 days at 27 ^{o}C (Agrios, 2005), varying around this average based on a number of factors including host plant, root-knot species and environmental conditions, in particular temperature (Eisenback and Triantaphyllou, 1991). These trials were both conducted in the Spring and Fall of 2019 and 2018, respectively, in the same greenhouse in Blackstone. However, a concurrent study was conducted with inoculum from the same population of *M. arenaria* race 2 in Blacksburg in the Fall of 2018, in which reproduction was relatively high (reproductive indices ranged from 0.5 to 2.4). We speculate that some difference in temperature or the timing of the removal of the shade cloths from the two greenhouse facilities may account for this discrepancy between the two trials conducted in the Fall of 2018, and the generally low reproduction in the two relevant trials.

The results from our field trials were unclear, with few significant differences found among entries in either year. Root-knot nematode pressure in the field was highly variable in both years and probably accounted for substantial variability in our results. Additionally, sampling resolution remains an ongoing issue; pre- and post-season soil nematode counts found few or no root-knot nematode juveniles in many plots where plants did in fact experience severe nematode parasitism; the opposite was also true for many plots in which many root-knot nematodes were found in pre- and post-season counts. Novel sampling methods, randomization philosophies and analytical techniques have been developed recently to address similar issues in field trials assessing host resistance and tolerance to root lesion nematodes (*Pratylenchus* spp.) in Australia (Reeves et al., 2020) which may offer solutions in future field trials assessing host

resistance to root-knot nematodes in tobacco. In a field trial conducted at the same location in 2014, galling of entries possessing both *Rk1* and *Rk2* was significantly less than entries possessing either gene alone and a susceptible check (Pollok et al., 2015).

The results of our study suggest *Rk1* reduces nematode penetration and feeding site initiation by *M. arenaria* race 2, but subsequent reductions in feeding site establishment and fecundity were not significant relative to a susceptible host. We observed significant reductions in galling on K 326, which possesses *Rk1* only, relative to the susceptible control Hicks in two of four trials, but significant reductions in nematode reproduction on K 326 versus Hicks were only observed in one trial.

The mechanism of resistance associated with *Rk2* is not clear, nor is that afforded by the combination of *Rk1* with *Rk2* (Pollok et al., 2016). In a 1982 report, Shepherd described significant reductions in root penetration by *M. javanica* juveniles on so called "better breeding lines" which were not identified specifically. However, subsequent development of the successful juveniles was not impacted by the trait possessed by these lines (Shepherd, 1982). Based on subsequent reports from this research group (Ternouth et al., 1986), it may be inferred that these "better breeding lines" could have possessed the "*T*" or *Rk2* trait, in which case the implied mechanism of resistance would be different from the hypersensitive response conferred by *Rk1*. Pollok et al. (2015) reported that *Rk2* did not significantly reduce galling by *M. arenaria* race 2 relative to a susceptible entry under field conditions, but reductions in galling were significant when *Rk2* was combined with *Rk1*. Pollok et al. (2016) also observed limited reductions in galling caused by *M. incognita* race 3 on plants possessing only *Rk2* in greenhouse trials. They also observed that subsequent nematode development was inhibited by *Rk2*, while the presence of both resistance genes reduced all metrics of root-knot nematode parasitism. In

our study, *Rk2* alone reduced root galling relative to the susceptible control in two of four trials, and the number of penetrated nematodes present in roots was also significantly lower than in the susceptible entry in one of these trials. We observed significant reductions in reproduction associated with *Rk2*, as expressed by reproductive indices, in two trials; in no case were actual egg counts significantly reduced relative to the susceptible entry. Thus, taken along with the results presented by other authors, our results do not necessarily clarify the mode of action associated with *Rk2*. Similarly, while our results confirm that the presence of both genes imparts increased resistance to root-knot nematodes, the specific mechanism of inhibition associated with this stacked resistance is still somewhat unclear.

Despite some variability in our trials, our results suggest that commercially available tobacco cultivars may now possess partial resistance to all of the most widely distributed, historically important root-knot nematode parasites of flue-cured tobacco. However, the increasing abundance of *M. enterolobii* in flue-cured tobacco production regions in the southeastern United States presents a new root-knot nematode pest challenge that cannot be mitigated by currently available forms of host resistance including genes of the *Rk* and *Mi* families (Ye, 2018). Further research is necessary to identify sources of resistance to this nematode, which could displace other species and present tobacco growers with a pest that cannot be managed with commercially available host resistance.

Literature Cited

Agrios, G. N. 2005. Plant diseases caused by nematodes. Pp. 825-874 in G. N. Agrios (ed) Plant Pathology (5th edition). Academic Press, Cambridge.

Barker, K.R. 1985. Nematode extraction and bioassays. Pp. 19-35 in K.R. Barker, C.C. Carter and J.N. Sasser (eds) An Advanced Treatise on *Meloidogyne* Vol. II: Methodology. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C.

Byrd, D. W., Jr., Kirkpatrick, T., and Barker, K. R. 1983. An improved technique for clearing and staining plant tissues for detection of nematodes. J. Nematol. 15:142-143.

Daykin, M. E., and Hussey, R. S. 1985. Staining and histopathological techniques in nematology. Pp. 41 in K.R. Barker, C.C. Carter and J.N. Sasser (eds) An Advanced Treatise on *Meloidogyne* Vol. II: Methodology. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C.

Dropkin, V. H. 1969. The necrotic reaction of tomatoes and other hosts resistant to *Meloidogyne*: Reversal by temperature. Phytopathology 59:1632-1637.

Eisenback, J. D. 2012. Effects of resistant tobacco on population dynamics of root-knot nematode species in Virginia. 45th Tobacco Workers Conference, 16–19 January 2012, Williamsburg, VA, Paper 89 (Abstr.).

Eisenback, J. D. and Triantaphyllou, H. H. 1991. Root-knot nematodes: *Meloidogyne* species and races. Pp 191-274 in W. R. Nickle (ed) Manual of Agricultural Nematology. Marcell Dekker, New York.

El-Sappah, A. H., M M, I., H El-Awady, H., Yan, S., Qi, S., Liu, J., Cheng, G. T., & Liang, Y. 2019. Tomato Natural Resistance Genes in Controlling the Root-Knot Nematode. Genes, 10: 925-944.

FAO. 2016. FaoStat Database Collections. http://www.fao.org/faostat/en/?#data/QV. Accessed September 15, 2020.

Fortnum BA, Lewis SA, Johnson AW. 2001 Crop rotation and nematicides for management of mixed populations of Meloidogyne spp. on tobacco. Journal of Nematology 33: 318–324.

Hussey, R. S., and Barker, K. R. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp. including a new technique. Plant Dis. Rep. 57:1025-1028.

Jack, A. M. 2001. Kutsaga RK26 - A new root-knot, Granville wilt and black shank resistant variety. Zimbabwe Tobacco 10: 25–31.

Jack, A. M, Lyle, J. 1999. Kutsaga RK26 - A new root-knot resistant variety. Zimbabwe Tobacco 8: 28–31.

Johnson, C. S. 1989. Managing root-knot on tobacco in the southeastern United States. Journal of Nematology 21:604.

Johnson, C. S., Way, J., Barker, K. R. 2005. Nematode parasites of tobacco. Pp. 675–708 in M. Luc, R. A. Sikora, and J. Bridge, eds. Plant parasitic nematodes in subtropical and tropical agriculture, 2nd ed. Wallingford, UK: CAB International.

Johnson, C. S., Wernsman, E. A., and Lamondia, J. A. 2009. Effect of a chromosome segment marked by the *Php* gene for resistance to *Phytophthora nicotianae* on reproduction

of tobacco cyst nematodes. Plant Disease 93:309-315.

Johnson, C.S. 2020. Disease control. Pp. 47-59 in T. D. Reed, C. S. Johnson, C. A. Wilkinson, and S. Barts, eds. 2020 Flue-Cured Tobacco Production Guide 436-048. Blacksburg, VA: VA Cooperative Extension Publication.

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O., and Fortnum, B. A. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. Journal of Nematology 31: 587-618.

LaMondia, J. A. 2008. Early crop root destruction for management of tobacco cyst nematodes. Journal of Nematology 40: 26–29.

Mackenzie, J., Smeeton, B. W., Jack, A. M., and Ternouth, R. A. F. 1986. Review on breeding for resistance to root-knot, *Meloidogyne javanica*, in flue-cured tobacco in Zimbabwe. Proc. CORESTA Symposium, 1986: 26–30 October 1986, Taormina, Sicily, Italy: 272–276.

Milligan, S. B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V. M. 1998. The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell 10:1307-1319.

Ng'ambi, T. B. S., Rufty, R. C., and Barker, K. R. 1999a. Genetic analysis of *Meloidogyne arenaria* Race 1 resistance in tobacco. Plant Dis 83:810-813.

Ng'ambi, T. B. S., Rufty, R. C., Barker, K. R., and Melton, T. A. 1999b. Identification of sources of resistance to four species of root-knot nematodes in tobacco. Journal of Nematology 31: 272–282.

Pollok, J. R., Darnell, L. A., Johnson, C. S., and Reed, T. D. 2015. Resistance to root-knot nematode in flue-cured tobacco cultivars in Virginia, 2014. Plant Disease Management Report, 9:N015.

Pollok, J. R., Johnson, C. S., Eisenback, J. D., and Reed, T. D. 2016. Reproduction of *Meloidogyne incognita* Race 3 on Flue-cured Tobacco Homozygous for Rk1 and/or Rk2 Resistance Genes. J Nematol. 48 (2): 79-86.

Reed, T. D., Johnson, C. S., Semtner, P. J., and Wilkinson, C. A. 2018. 2018 Flue-cured tobacco production guide. Va. Coop. Ext. Publ. No. 436-048. Blacksburg, VA.

Reed, T.D. 2007. Flue-cured tobacco disease control. Pp. 5–36 in T. D. Reed, C. S. Johnson, P. J. Semtner, and C. A. Wilkinson, eds. 2007 Flue-Cured Tobacco Production Guide 436-048. Blacksburg, VA: VA Cooperative Extension Publication.

Reeves, K. L., Forknall, C. R., Kelly, A. M., Owen, K. J., Fanning, J., Hollaway, G. J., and Loughman, R. 2020. A novel approach to the design and analysis of field experiments to study variation in the tolerance and resistance of cultivars to root lesion nematodes (*Pratylenchus* spp.). Phytopathology 110:1623-1631**.**

Roberts PA. 2002. Concepts and consequences of resistance. Pp. 23–41 in J. L. Starr, R. Cook, and J. Bridge, eds. Plant resistance to parasitic nematodes. Wallingford, UK: CAB International.

Schneider, S. M. 1991. Penetration of susceptible and resistant tobacco cultivars by *Meloidogyne* juveniles. Journal of Nematology 23: 225-228.

Schweppenhauser, M. A. 1975. A source of *Nicotiana tabacum* resistant to *Meloidogyne javanica*. Tobacco Science 19: 42–45.

Shepherd, J. A. 1982. Report to the third regional conference on root-knot nematode research held at the international institute of tropical agriculture, Ibidan, Nigeria. Proceedings of the 3rd Research Planning Conference on root-knot nematodes, *Meloidogyne* spp., Regions IV and V. 16-20 November, 1981.

Taylor, A. L., and Sasser, J. N. 1978. Identification and control of root-knot nematodes (Meloidogyne spp). Crop Publ. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C pp. 111.

Ternouth, R. A. F., Mackenzie, J., and Shepherd, J. A. 1986. Introduction of *Meloidogyne javanica* resistance into flue-cured tobacco in Zimbabwe. Proc. CORESTA Symposium, 1986: 26–30 October 1986, Taormina, Sicily, Italy: 277–280.

USDA. 2020. United States Department of Agriculture, National Agricultural Statistics Service, Acreage, June 2020. www.nass.usda.gov/publications/reports/acrg0620. November 16, 2020.

USEPA. 2008. United States Environmental Protection Agency, implementation of risk mitigation measures for soil fumigant pesticides. http://www.epa.gov/pesticides/reregistration/soil_fumigants/. Accessed November 22, 2020.

Way, J. I. 1994. Susceptibility of four tobacco cultivars to *Meloidogyne* species. CORESTA Information Bulletin, Congress Issue, 9–24 October 1994, Harare, Zimbabwe: 110 (Abstr.).

Ye, W. 2018. Nematodes of agricultural importance in North and South Carolina. Pp 247-276 in John J. Chitambar and Sergei A. Subbotin (eds) Plant Parasitic Nematodes in Sustainable Agriculture of North America. Springer, New York.

Yi, H. Y., Rufty, R. C., Wernsman, E. A., and Conkling, M. C. 1998. Mapping the root-knot nematode resistance gene (Rk) in tobacco with RAPD markers. Plant Disease 82: 1319–1322.

CHAPTER 3

Reproduction of *Meloidogyne arenaria* race 2 on flue-cured tobacco incorporating a resistance trait introgressed from *Nicotiana repanda*

ABSTRACT

As efficacious chemical controls for root-knot nematode become increasingly restricted due to environmental and human health concerns, host resistance to this group of pathogens has become an increasingly critical aspect of flue-cured tobacco production. While resistance to *M. incognita* races 1 and 3, as well as race 1 of *M. arenaria*, imparted by the gene *Rk1*, is widely available in commercially available flue-cured tobacco varieties, *Rk2*, which imparts increased resistance to *M. javanica* alone and when in combination with *Rk1*, is only available in select commercially available varieties. Furthermore, the efficacy of this gene against the increasingly prominent *M. arenaria* race 2 is unclear, and a lack of diversity in flue-cured tobacco germplasm suggests the need to develop new sources of host resistance to root-knot nematodes. Greenhouse trials were conducted in 2017 to evaluate root-knot nematode resistance in a line possessing root-knot resistance traits derived from *N. repanda*, 81-R-617A, to determine how this potential source of resistance compares to the commercially available root-knot nematode resistance afforded by *Rk1* and *Rk2* alone, and in combination. Trials were arranged in completely randomized block design and compared 81-R-617A with a group of flue-cured tobacco entries Hicks (a root-knot susceptible standard cultivar), K326 (homozygous for *Rk1*), T-15-1-1 (homozygous for *Rk2*), STNCB 2-28 and BAG 29-15-3-32-1 (homozygous for both *Rk1* and *Rk2*), and CC 13 (homozygous for *Rk1* and heterozygous for *Rk2*). Data collected after trials of 60 days included percent root galling, egg mass and egg counts, the latter of which were used to determine reproductive indices for entries. Despite some variability in our results, entry 81-R-617A

exhibited significantly lower root galling, egg mass production and egg counts relative to the susceptible control, entries possessing either *Rk1* or *Rk2*, and three entries possessing both resistance genes, suggesting that the *N. repanda* species may hold a novel source of root-knot nematode resistance which may be incorporated into commercial flue-cured tobacco varieties.

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a valuable agricultural commodity cultivated around the world, with the value of unprocessed tobacco globally estimated at over 16 billion dollars in 2016 (FAO, 2016). Tobacco production has declined considerably in the United States over the past two decades, but unprocessed tobacco sales still generated over 1.2 billion dollars nationally as of 2016 (FAO, 2016). In the United States, flue-cured tobacco accounts for the majority of the crop, with over 14,000 acres planted in Virginia alone in 2020 (USDA, 2020). Root-knot nematodes (*Meloidogyne* spp.) can seriously complicate production for tobacco growers in the southeastern United States, causing crop losses of 1 to 5% in Virginia in affected fields (Fortnum et al., 2001; Koenning et al., 1999). The use of resistant cultivars for root-knot nematode management is a fundamental tool for flue-cured tobacco growers (Johnson, 2005), particularly in light of the increasing restrictions on some of the most historically effective chemical management options, including soil fumigants (USEPA, 2008) and non-fumigant nematicides in the organophosphate and carbamate classes (USEPA, 2020).

Resistance is defined in plant nematology as the near or complete inhibition of reproduction by the nematode, whereas tolerance is defined as a response in which nematode reproduction is not necessarily inhibited, but in which the host does not suffer serious deleterious consequences as a result of nematode parasitism and reproduction (Roberts, 2002). In flue-cured tobacco, resistance to root-knot nematodes is mediated by one or both of two genes. Root-knot

nematode resistance first became commercially available in Zimbabwe in 1961 with the introduction of a cultivar possessing a gene, *Rk*, originally discovered in *N. tomentosa* Ruis and Pav. (Yi et al., 1998). This gene, now known as *Rk1*, is widely available in commercial flue-cured tobacco cultivars and imparts what has been described as some level of resistance to *M. incognita* (Kofoid and White 1919) Chitwood (1949) host races 1 and 3 and *M. arenia* (Neal 1889) Chitwood (1949) host race 1 (Schneider 1991; Ng'ambi et al. 1999). *Rk1* may have some inhibitory effect on the reproduction of *M. javanica* on flue-cured tobacco (Ternouth et al., 1986) but contradicting research suggests that *Rk1* imparts minimal or no resistance to *M. javanica*, *M. incognita* host races 2 and 4, *M. arenaria* race 2, and *M. hapla* Chitwood 1949 (Ng'ambi et al., 1999).

In 1993, another root-knot nematode resistance gene, now known as *Rk2*, was introduced into a cultivar commercially available in Zimbabwe, Kutsaga RK26, stacked with *Rk1* (Way, 1994; Jack and Lyle, 1999; Jack, 2001). This gene, originally known as "*T*," was discovered in 1950 in *N. tabacum* plants in subsistence gardens along the Zambezi river in Zimbabwe. These gardens had been maintained for over 250 years in soils heavily infested with *M. javanica* (Schweppenhauser, 1975; Mackenzie et al., 1986; Ternouth et al., 1986). This gene imparts resistance to *M. javanica* and imparts far greater resistance to this nematode than *Rk1*, although combining both genes resulted in an even higher level of resistance (Schweppenhauser, 1975; Ternouth et al., 1986). Tobacco cultivars possessing both *Rk1* and *Rk2* became commercially available in the United States from Cross Creek Seed Company and ProfiGen do Brasil in 2007 (Reed, 2007).

Recent research has confirmed the high level of resistance conferred by the combination of both *Rk1* and *Rk2* to populations of *M. javanica* (Johnson, unpublished data) and *M. incognita*

race 3 (Pollock et al., 2016) from Virginia flue-cured tobacco fields. *M. incognita* had historically been regarded as the greatest nematode threat to tobacco in Virginia (Johnson, 1989), but over the past several decades, *M. arenaria* has superseded *M. incognita* in abundance (Eisenback, 2012). A 2004 survey of 170 Virginia tobacco fields found 43.5% of surveyed fields were infested with root-knot nematodes, with *M. arenaria* present in 56.7% of infested fields and *M. incognita* present in 16.7% of infested fields, while *M. hapla, M. javanica* and unidentified root-knot species were present in 25%, 11.7% and 8.3% of infested fields, respectively (Eisenback, 2012). As of a 2010 survey of 276 Virginia flue-cured tobacco fields, root-knot nematodes were present in 44.9% of surveyed fields, with *M. arenaria* present in 58.8% of those infested fields, while *M. incognita* was present in only 11.1% of infested fields, with other species present at similar levels to 2004 (Eisenback, 2012).

N. repanda has long been seen as a potential source of disease resistance for cultivated tobacco, including root-knot nematode resistance (Burk and Heggestad, 1966; Stavely et al., 1973). For example, Schweppenhauser et al. (1975) observed that *N. repanda* was the only species evaluated out of 64 *Nicotiana* species that was completely resistant to *M. javanica*. However, due to chromosomal incompatibilities between *N. repanda* and *N. tabacum*, the manifestation of lethal genes, and other factors, attempts to hybridize or incorporate genes from *N. repanda* with commercially viable *N. tabacum* selections were not successful for many years (Bui et al., 1992). For example, in the late 1960's, and again in the mid 1980's, attempts were made to integrate resistance to tobacco mosaic virus (TMV), tobacco cyst nematode (*Globodera tabacum solanacearum*, Miller and Gray) and *M. javanica* and *M. arenaria* from *N. repanda* into two commercial tobacco cultivars using an interspecific bridge hybridization procedure (Burk, 1967; Gwynn et al., 1986). These crosses were ultimately successful in transferring TMV and

cyst nematode resistance to some progeny lines, but root-knot nematode resistance was lost after necessary backcrosses (Davis et al., 1988a, 1988b). Cell culturing techniques were also employed with little success (Bui et al., 1992). However, resistance to *M. arenaria* and TMV was successfully transferred from *N. repanda* to *N. tabacum* in 1992 using *N. sylvestris* as a bridge species, along with a form of protoplast fusion (Bui et al., 1992). The objective of this work was to evaluate resistance to a population of *M. arenaria* race 2 in a flue-cured tobacco entry possessing traits from *N. repanda*, and to compare that possible resistance to resistance in flue-cured tobacco entries possessing the root-knot nematode resistance genes *Rk1* and/or *Rk2* alone or in combination.

MATERIALS AND METHODS

Greenhouse Trials

A nematode population originally obtained from a flue-cured tobacco field in Halifax County, Virginia provided inoculum in these trials. The population was maintained on susceptible tomato (*Solanum lycopersicum* L.) variety 'Rutgers' in greenhouse facilities on the Virginia Tech campus in Blacksburg, Virginia and at the Virginia Tech Southern Piedmont AREC (SPAREC) in Blackstone, Virginia. Egg inoculum for greenhouse trials wwas extracted from infested roots following the method of Hussey and Barker (1973). Four greenhouse experiments were conducted in 2017 to evaluate the impact of a putative source of resistance to root-knot nematodes derived from *N. repanda* on the reproductive capacity of a population of *M. arenaria* race 2 on a panel of seven flue-cured tobacco entries: Hicks (susceptible to the four major tropical root-knot nematode species, *M. incognita, M. javanica, M. arenaria,* and *M. hapla*); K326 (homozygous for *Rk1*); T-15-1-1 (homozygous for *Rk2*); CC 13 (homozygous for *Rk1* and heterozygous for *Rk2*); STNCB-2-28 (homozygous for both *Rk1* and *Rk2*); BAG 29-15-

3-32-1 (also homozygous for both *Rk1* and *Rk2*); and 81-R-617A (contains traits derived from *N. repanda* which may influence resistance to root-knot nematodes). Seed of K326 and CC 13 was obtained from Cross Creek Seed (Raeford, NC). The seed of BAG 29-15-3-32-1 and 81-R-617A was provided by Dr. Ramsey Lewis at North Carolina State University (Raleigh, NC). Seed of the remaining four entries was produced in the flue-cured tobacco nursery at SPAREC. Seed was germinated in organic vermiculite (The Epsoma Company, Millville, NJ). Four to five-week-old seedlings were transplanted to individual 7.6 cm clay pots containing a 2:1 mixture of steam sterilized sandy loam field soil to Profile Greens Grade Pourous Ceramic soil amendment (Profile Products, Buffalo Grove, Il). Seedlings with 4 to 6 true leaves were transplanted into 15 cm clay pots containing the same soil substrate. Plants were inoculated with a 40 ml aliquot containing 5,000 nematode eggs applied directly to the root mass during transplanting. Plants were maintained in greenhouses in Blacksburg at ambient temperature (20 to 33 °C) with natural lighting for the duration of the experiments. In trials conducted in Blackstone, plants were grown in open-top root zone growth chambers (Environmental Growth Chambers, Chagrin, OH) maintaining a soil temperature of ~27 ± 2 °C (day/night cycle fluctuation) with ambient lighting.

Trials were terminated 60 days after inoculation and plants were evaluated for percent root galling, egg mass deposition, and egg production. The percent galled roots were estimated visually on the entire root mass of the plant. Root systems were subsequently cut into 4 to 6 cm sections and mixed thoroughly. Three 1 g subsamples were stained with 0.15 g/L Phloxine-B (Daykin and Hussey, 1985) for approximately five minutes to define egg masses and subsequently counted at x10-x20 with a dissecting microscope. Eggs were extracted using the bleach agitation method of Hussey and Barker (1973) as described above. Eggs were suspended in 1 L of tap water and counted in two 10 ml aliquots counted at X40 using an inverted

compound microscope. Additionally, the reproductive index (P_f/P_i) was calculated for each plant by dividing the final egg count (P_f) by the initial egg inoculum number (P_i).

Statistical Analysis

Data from all trials were transformed ($\log_{10}(x + 1)$) for analysis of variance (ANOVA) using PROC GLM in SAS (version 9.4; SAS Institute, Cary, NC). Differences among treatment means were identified using Fisher's protected Least Significant Difference test ($P \leq 0.05$).

RESULTS

Both year and location of tests, and the interaction between test and genotype, had significant effects on results, so root-knot parasitism data from each trial were analyzed independently ($P \leq 0.0001$ year and location; $P \leq 0.0181$ interaction between test and genotype).

Significant differences in root galling were found among entries in all trials (Table 3.1). 81-R617A had significantly lower root galling than all other entries in the June to August trial conducted in Blackstone (Table 3.1). In the trial in Blacksburg from September to November, 81-R-617A exhibited significantly less root galling than all entries with the exception of STNCB 2-28 and BAG 29-15-3-32-1, both of which hare homozygous for resistance genes *Rk1* and *Rk2* (Table 3.1). Similarly, in the trial in Blacksburg from June to August, galling was lowest on 81-R-617A and BAG 29-15-3-32-1 relative to T-15-1-1, which in this trial experienced the most root galling observed across all four trials (Table 3.1). No entry consistently exhibited the highest levels of root galling in these trials (Table 3.1).

Table 3.1. Root galling of flue-cured tobacco entries by *M. arenaria* race 2 in greenhouse pot tests in 2017.[z]

		Root % galling			
		June-August		September-November	
Genotype	Entry	Blackstone	Blacksburg	Blackstone	Blacksburg
r1krk2	Hicks	42.5 bc	42.5 ab	45.0 a	54.0 a
RK1rk2	K326	45.0 bc	56.3 ab	51.7 a	21.0 b
rk1RK2	T-15-1-1	53.8 abc	71.3 a	36.7 ab	21.0 b
RK1RK2	CC 13	60.0 ab	41.3 ab	20.0 ab	27.0 b
RK1RK2	STNCB 2-28	67.5 a	55.0 ab	36.7 ab	16.0 bc
RK1RK2	BAG 29-15-3-32-1	40.0 c	36.3 b	20.0 ab	15.0 bc
N. repanda	81-R-617A	20.0 d	37.5 b	3.7 b	6.2 c

[z] Data presented are non-transformed means from three, five, four and four replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 3.2. Egg masses and eggs per gram of root, as well as reproductive indices of *M. arenaria* race 2 on flue-cured tobacco entries in greenhouse pot tests in 2017.[z]

		Egg masses per gram of root			
		June-August		September-November	
Genotype	Entry	Blackstone	Blacksburg	Blackstone	Blacksburg
r1krk2	Hicks	64 ab	38 b	51 a	21 a
RK1rk2	K326	90 a	41 b	45 a	22 a
rk1RK2	T-15-1-1	83 a	86 ab	17 a	15 ab
RK1RK2	CC 13	89 a	98 ab	38 a	9 bc
RK1RK2	STNCB 2-28	65 a	126 a	24 a	10 bc
RK1RK2	BAG 29-15-3-32-1	67 a	60 ab	16 ab	15 ab
N. repanda	81-R-617A	30 b	60 ab	6 b	5 c

		Eggs per gram of root			
		June-August		September-November	
Genotype	Entry	Blackstone	Blacksburg	Blackstone	Blacksburg
r1krk2	Hicks	5,697 ab	9,938 ab	2,907 a	439 ab
RK1rk2	K326	11,612 a	2,728 c	1,488 a	596 a
rk1RK2	T-15-1-1	6,585 ab	4,613 c	965 a	596 a
RK1RK2	CC 13	9,071 a	8,311 ab	1,197 a	170 b
RK1RK2	STNCB 2-28	6,656 ab	13,568 a	904 a	294 ab
RK1RK2	BAG 29-15-3-32-1	5,215 ab	7,354 ab	946 a	268 ab
N. repanda	81-R-617A	3,988 b	5,381 abc	17 b	54 c

		Reproductive index[y]			
		June-August		September-November	
Genotype	Entry	Blackstone	Blacksburg	Blackstone	Blacksburg
r1krk2	Hicks	37.9 a	3.9 c	6.9 a	2.1 ab
RK1rk2	K326	40.5 a	2.3 c	4.6 a	3.0 a
rk1RK2	T-15-1-1	33.4 a	6.9 bc	3.8 a	2.7 a
RK1RK2	CC 13	64.0 a	22.9 a	5.5 a	0.9 c
RK1RK2	STNCB 2-28	45.1 a	26.7 ab	2.9 ab	1.2 bc
RK1RK2	BAG 29-15-3-32-1	36.3 a	15.4 abc	3.6 a	1.1 bc
N. repanda	81-R-617A	29.3 a	6.3 bc	0.0 b	0.3 d

[z] Data presented are non-transformed means from three, five, four and four replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] Reproductive index = final population/initial population (P_f/Pi).

Significant differences in egg masses were found among entries in all trials (Table 3.2).81-R-617A had significantly fewer egg masses than all entries except for susceptible Hicks in the June-August trial in Blackstone, and fewer egg masses than all entries with the exception of BAG 29-15-3-32-1 in the test conducted in Blackstone from September to November (Table 3.2). In the trial conducted concurrently in Blacksburg, 81-R-617A had significantly fewer egg masses present than all entries with the exception of CC 13 and STNCB 2-28 (Table 3.2). In contrast to these trials, egg mass production was significantly less onHicks and K326 than on STNCB 2-28, while egg production on 81-R-617A and BAG 29-15-3-32-1 was intermediate, along with T-15-1-1 and CC 13 (Table 3.2).

Significantly fewer eggs were recovered from 81-R-617A than K326 and CC 13 in the trial from June-August in Blackstone, while in the trials in Blackstone and Blacksburg from September to November, 81-R-617A experienced significantly less egg production than all other entries (Table 3.2). In the trial conducted from June to August in Blacksburg, significantly fewer eggs were recovered from K326 and T-15-1-1 than all entries except for 81-R-617A, which was intermediate in egg production in this trial (Table 3.2). Entries possessing both resistance genes almost never differed significantly in egg production from whatever entry experienced the greatest egg production in all four trials (Table 3.2).

Trends in reproductive indices were similar to those in egg production. Entry 81-R-617A had a reproductive index significantly less than all other entries except for STNCB 2-28 in the trial conducted in Blackstone from September to November, while in the concurrent trial conducted in Blacksburg, the reproductive index of 81-R-617A was significantly less than all other entries (Table 3.2). Hicks and K326 had reproductive indices significantly lower than entries CC 13 and STNCB 2-28 in the trial conducted in Blacksburg from June to August, and T-

15-1-1 had a lower reproductive index than CC 13 (Table 3.2). No significant differences in reproductive indices were observed among entries in the trial in Blackstone from June to August (Table 3.2).

Discussion

The results of these trials suggest that entry 81-R-617A, which possesses traits derived from *N. repanda*, may exhibit resistance to *M. arenaria* race 2 that is greater than that imparted by resistance genes *Rk1* and *Rk2* alone or in combination. Despite some inconsistency in our results, galling was akmost always numerically lowest on 81-R-617A, and in two trials this trend was significant relative to all other entries. Significantly fewer egg masses were observed on the roots of 81-R-617A than all entries except for the susceptible check in one trial conducted in from June to August, while in the two trials conducted from September to November in Blackstone and Blacksburg, 81-R-617A had significantly lower egg mass counts than all entries except for BAG 29-15-3-32-1, and CC 13 and STNCB 2-28. In contrast to the observation of Ng'ambi et al. (1999a), we did not see root necrosis associated with reduced egg production in the highly susceptible entry, Hicks, or any other entry; in fact, egg numbers were high on all entries in all four tests. Egg production on 81-R-617A was lowest in three of our four trials, with the exception of the trial conducted from June to August in Blacksburg, in which it was intermediate with other entries. The reproductive index of entry 81-R-617A was numerically and significantly lowest in two of our trials. However, in the other trial in which reproductive indices differed (June-August, conducted in Blacksburg), susceptible entry Hicks and K326, which possesses *Rk1* only, had a significantly lower reproductive index than that of entries CC 13 (homozygous for *Rk1* and heterozygous for *Rk2)* and STNCB 2-28 (homozygous for both genes), while reproductive indices of the remaining entries, including 81-R-617A, were intermediate.

Way (1994b) demonstrated that a male sterile hybrid, RK3, which is homozygous for *Rk1* and heterozygous for *Rk2,* showed significant reductions in parasitism relative to a susceptible control. This is similar to our observation that entry CC 13, which has the same root-knot nematode resistance genotype as RK3, experienced significantly less root-knot nematode parasitism and reproduction than the susceptible entry, but did not differ significantly from the two entries homozygous for both genes, STNCB 2-28 and BAG 29-15-3-32-1, with the exception of one trial in which BAG 29-15-3-32-1 exhibited significantly lower root galling that both CC 13 and STNCB 2-28. However, our results appear to contrast with Way's (1994a) observation that BAG line plants, of which STNCB 2-28 is a parent, are significantly more resistant than STNCB 2-28, and that BAG lines are more resistant than RK3, which is heterozygous for *Rk2* but homozygous for *Rk1*. We did not detect significant differences in any metrics of reproduction among any of the entries possessing both resistance genes, regardless of zygosity, except in the aforementioned case of root galling.

Resistance to *M. javanica* in male sterile hybrids between *N. repanda* and *N. longiflora* or *N. palmeri* was reported by Schweppenhauser et al. in 1963. Davis et al. (1988a) observed a high level of resistance to *M. javanica* and *M. arenaria* in a *N. repanda* line called 46-G, but did not indicate what race of *M. arenaria* was used in their trials. While we saw significant reductions in galling and reproduction on 81-R-617A relative to a susceptible entry and other entries lacking the *N. repanda* derived traits, considerable galling and egg production still occurred in several trials. Davis et al. (1988a) reported that the *N. repanda* entry in their study, 46-G, exhibited little or no gall development relative to standard cultivars; reproduction was inhibited completely, as inferred by the absence of juvenile nematodes in soil at the end of trials (Davis et al., 1988a). The authors also identified sources of resistance to both *M. javanica* and *M.*

arenaria in breeding lines from other *Nicotiana* species, but noted that lines with favorable agronomic traits relative to standard cultivars lost this resistance, or tolerance in many cases, upon backcrossing with *N. tabacum* (Davis et al., 1988a). Bui et al. (1992) demonstrated that resistance to *M. arenaria* and tobacco mosaic virus could be integrated into *N. tabacum* from *N. repanda* using a form of protoplast fusion and *N. sylvestris* as a bridge species, but found that nematode resistance was considerably more difficult to retain in backcrosses than resistance to the virus, being present in only 25 of 270 backcrosses. Again, the authors of this study do not indicate what race of *M. arenaria* was used to evaluate root-knot nematode resistance (Bui et al., 1992). Ng'ambi et al. (1999b) observed a "moderate" level of resistance to *M. arenaria* race 2 in 81-R-617A and a related breeding line, 81-RL-2K, in contrast to a "high level" of resistance observed in a breeding line from South Africa, SA1214. In their trials, an average of over 7,000 *M. arenaria* race 2 eggs were present per gram of root for entry 81-R-617A, compared with fewer than 3,000 egg per gram of root on SA 1214, a considerable difference, but one that is not clearly identified as significant. Importantly, the authors note that these lines are the first *N. repanda* entries to be identified with resistance to *M. arenaria* race 2 that could be compatible with existing *N. tabacum* accessions for breeding and crop improvement (Ng'ambi et al., 1999b).

Ng'ambi et al. (1999a) demonstrated that resistance to *M. arenaria* race 1 in a breeding line related to 81-R-617A, 81-RL-2K, is conditioned by the same single dominant gene which confers resistance to *M. incognita* races 1 and 3 in the commercial flue-cured tobacco cultivar Speight G 28. This gene, *Rk1,* is widely available in commercial flue-cured tobacco in the United States (Koenning et al., 1999). *Rk1* confers resistance to root-knot nematodes via a hypersensitive response that inhibits nematode feeding site development and subsequent galling and reproduction (Schneider, 1991; Ng'ambi et al., 1999b) The presence of this gene explains

the resistance to "*M. arenaria*" populations not identified to race observed by other authors, which can be inferred were race 1 (Davis et al., 1988a; Bui et al., 1992). However, *Rk1* does not impart resistance to *M. arenaria* race 2, and appears to have relatively little impact on *M. javanica* (Ng'ambi et al., 1999b), suggesting that a different system is responsible for the resistance to these nematodes observed in *N. repanda* and related breeding lines.

Another resistance gene, known as *Rk2* provides considerably greater resistance to *M. javanica* than *Rk1,* and if the two genes are "stacked" an even greater level of resistance is conferred (Ternouth et al., 1986; Ma, W., Johnson, C. S., Eisenback, J. D., and Reed, T. D. unpublished data). There is also evidence that the combination of both genes confers a significantly greater degree of resistance to a variant of *M. incognita* race 3 than either resistance gene alone, although in the study in question, *Rk1*, rather than *Rk2*, was associated with greater reductions in nematode parasitism and reproduction (Pollok et al., 2016). However, the mechanism of resistance associated with *Rk2* is unknown, although it has been speculated to be different than the hypersensitive response associated with *Rk1* because *Rk2* does not appear to consistently inhibit gall formation, but does reduce reproduction (Pollok et al., 2016).

It is unclear what gene or genes interact with *Rk1* to confer the relatively high level of resistance to *M. arenaria* race 2 we observed in the line with *N. repanda* traits in our trials, which was equivalent to or greater than that exhibited by entries possessing both *Rk1* and *Rk2*. The relatively drastic reductions in root galling we observed on 81-R-617A suggests the possibility of a hypersensitive response. Alternatively, in other systems, resistance is associated with the inhibition of penetration, whether by alterations in the composition of root exudates perceived by infective juveniles due to the presence of arbuscular mycorrhizal fungi, which has been observed in tomato (Vos et al., 2012) or by augmentation of root morphology, which has

been observed in nematode resistant *Prunus* spp., wherein resistant varieties feature a different arrangement of epidermal cells in root tips compared with susceptible varieties (Ye et al., 2009). Pollok et al. (2016) speculated that the *Rk2* may simply slow down nematode feeding and development, or is involved in inhibition of a specific stage of egg mass development. It is possible that some combination of the aforementioned mechanisms is involved in resistance to root-knot nematodes in flue-cured tobacco entries like 81-R-617A that possess traits derived from *N. repanda*.

It would be of great value to determine what gene or genes are implicated in nematode resistance in *N. repanda*. As Ng'ambi et al. (1999a) observe, the genetic basis of resistance associated with *Rk1* is "narrow" and they caution that "repeated use of this resistance may select resistance-breaking biotypes." As Murphy et al. (1987) observed, tobacco varieties within similar market classes are highly related. This presents potential challenges for breeders attempting to improve disease resistance and agronomic traits in varieties in the future because of a limited genetic diversity within available germplasm (Lewis and Nicholson, 2007). Introducing new genetic sources of disease resistance to this pool of germ plasm from sources like *N. repanda* could help remedy some of these challenges. Additionally, moving forward, tobacco growers in the southeastern United States face a new, highly destructive, polyphagous nematode threat in the form of *M. enterolobii*. This nematode, which has recently been found on root-knot resistant sweet potato in South Carolina (Rutter et al., 2018) and is present in at least 8 counties in North Carolina (Schwartz et al., 2019) is a quarantine level pathogen (Thiessen, 2018) that, while not yet known to be directly impacting tobacco growers, presents a potentially serious threat, as this nematode is not controlled by available forms of resistance including *Mi* and *Rk* mediated resistance (Ye, 2018). Perhaps exploiting *N. repanda* germplasm, which has long been a source

of novel traits for cultivated tobaccos (Stavely et al., 1973) could present solutions to these and other issues facing tobacco breeders and growers alike.

Literature Cited

Bui, P. T., Jenns, A. E., Schneider, S. M., and Daub, M. E. 1992. Resistance to tobacco mosaic virus and *Meloidogyne arenaria* in fusion hybrids between *Nicotiana tabacum* and an *N. repanda* X *N. sylvestris* hybrid. Phytopathology 82: 1305-1310.

Burk, L. G. 1967. An interspecific bridge-cross. J. Hered. 58: 215-218.

Burk, L. G., and Heggestad, H. E. 1966. The genus *Nicotiana*: A source of resistance to diseases of cultivated tobacco. Econ. Bot. 20: 76-88.

Davis, E. L., Rich, J. R., and Gwynn, G. R. 1988a. Reaction of selected *Nicotiana* spp. X *N. tabacum* crosses grown in microplots to three *Meloidogyne* spp. Nematropica 18: 109-115.

Davis, E. L., Rich, J. R., Gwynn, G. R, and Sisson, V. 1988b. Greenhouse evaluation of *Nicotiana* spp. for resistance to root-knot nematodes. Nematropica 18: 99-107.

Daykin, M. E., and Hussey, R. S. 1985. Staining and histopathological techniques in nematology. Pp. 41 in K.R. Barker, C.C. Carter and J.N. Sasser (eds) An Advanced Treatise on *Meloidogyne* Vol. II: Methodology. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C.

Eisenback, J. D. 2012. Effects of resistant tobacco on population dynamics of root-knot nematode species in Virginia. 45th Tobacco Workers Conference, 16–19 January 2012, Williamsburg, VA, Paper 89 (Abstr.).

FAO. 2016. FaoStat Database Collections. http://www.fao.org/faostat/en/?#data/QV. Accessed September 15, 2020.

Fortnum, B. A., Lewis, S. A., and Johnson, A. W. 2001 Crop rotation and nematicides for management of mixed populations of *Meloidogyne* spp. on tobacco. Journal of Nematology 33: 318–324.

Gwynn, G. R., Barker, K. R., Reilly, J. J., Komn, D. A., Burk, L. G., and Reed, S. M. 1986. Genetic resistance to tobacco mosaic virus, cyst nematodes, root-knot nematodes, and wildfire from *Nicotiana repanda* incorporated into *N. tabacum*. Plant Disease 70: 958-962.

Hussey, R. S., and Barker, K. R. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp. including a new technique. Plant Dis. Rep. 57:1025-1028.

Johnson, C. S. 1989. Managing root-knot on tobacco in the southeastern United States. Journal of Nematology 21:604.

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O., and Fortnum, B. A. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. Journal of Nematology 31: 587-618.

Lewis, R. S. and Nicholson, J. S. 2007. Aspects of the evolution of *Nicotiana tabacum* L. and the status of the United States *Nicotiana* germplasm collection. Genet. Resour. Crop. Ev. 54:727—740.

Mackenzie, J., Smeeton, B. W., Jack, A. M., and Ternouth, R. A. F. 1986. Review on breeding for resistance to root-knot, *Meloidogyne javanica*, in flue-cured tobacco in Zimbabwe. Proc. CORESTA Symposium, 1986: 26–30 October 1986, Taormina, Sicily, Italy: 272–276.

Murphy, J. P., Cox, T. S., Rufty, R. C., and Rodgers, D.M. 1987. A representation of the pedigree relationships among flue-cured tobacco cultivars. Tob. Sci. 31:70–75.

Ng'ambi, T. B. S., Rufty, R. C., and Barker, K. R. 1999a. Genetic analysis of *Meloidogyne arenaria* Race 1 resistance in tobacco. Plant Dis 83:810-813.

Ng'ambi, T. B. S., Rufty, R. C., Barker, K. R., and Melton, T. A. 1999b. Identification of sources of resistance to four species of root-knot nematodes in tobacco. Journal of Nematology 31: 272–282.

Reed, T. D. 2007. Flue-cured tobacco disease control. Pp. 5–36 in T. D. Reed, C. S. Johnson, P. J. Semtner, and C. A. Wilkinson, eds. 2007 Flue-Cured Tobacco Production Guide 436-048. Blacksburg, VA: VA Cooperative Extension Publication.

Roberts, P. A. 2002. Concepts and consequences of resistance. Pp. 23–41 in J. L. Starr, R. Cook, and J. Bridge, eds. Plant resistance to parasitic nematodes. Wallingford, UK: CAB International.

Rutter, W. B., Skantar, A. M., Handoo, Z. A., Mueller, J. D., Aultman, S. P. and Agudelo, P. 2019. *Meloidogyne enterolobii* found infecting root-knot nematode resistant sweetpotato in South Carolina, United States. Plant Disease 103:775-775.

Schneider, S. M. 1991. Penetration of susceptible and resistant tobacco cultivars by *Meloidogyne* juveniles. Journal of Nematology 23: 225-228.

Schwartz, T. (Advised by Davis, E., Ye, W., Quesada, L., and Thiessen, L.). 2019. Distribution, virulence, and sweetpotato resistance to *Meloidogyne enterolobii* in North Carolina. Thesis, Master of Science, NC State Dept. of Plant Pathology.

Schweppenhauser, M. A. 1975. A source of *Nicotiana tabacum* resistant to *Meloidogyne javanica*. Tobacco Science 19: 42–45.

Schweppenhauser, M. A., Raeber, J. G., and Daulton, R. A. C. 1963. Resistance to the root-knot nematode, *Meloidogyne javanica,* in the genus *Nicotiana*. Pp. 222-229 in: Proc. World Tob. Sci. COngr., 3rd. Mardon Printers Ltd., Salisbury, S. Rhodesia.

Stavely, J. R., Pittarelli, G. W., and Burk, L. G. 1973. *Nicotiana repanda* as a potential source for disease resistance in *N. tabacum*. The Journal of Heredity 64: 265-271.

Ternouth, R. A. F., Mackenzie, J., and Shepherd, J. A. 1986. Introduction of *Meloidogyne javanica* resistance into flue-cured tobacco in Zimbabwe. Proc. CORESTA Symposium, 1986: 26–30 October 1986, Taormina, Sicily, Italy: 277–280.

Thiessen, L. 2018. Impact of Guava Root Knot Nematode Internal Quarantine on Tobacco and Field Crops in NC. NC State Extension Statement. go.ncsu.edu/readext?557138.

USDA. 2020. United States Department of Agriculture, National Agricultural Statistics Service, Acreage, June 2020. www.nass.usda.gov/publications/reports/acrg0620. Accessed November 16, 2020.

USEPA. 2008. United States Environmental Protection Agency, implementation of risk mitigation measures for soil fumigant pesticides. http://www.epa.gov/pesticides/reregistration/soil_fumigants/. Accessed November 22, 2020.

USEPA 2020. Groups of pesticides in registration review. https://www.epa.gov/pesticide-reevaluation/groups-pesticides-registration-review. Accessed November 22, 2020.

Way, J. I. 1994a. Testing breeding lines for root-knot nematode resistance. Presented at CORESTA Congress, Harare. CORESTA Information Bulletin, Congress Issue. Pp. 146 [Abstract].

Way, J. I. 1994b. Susceptibility of four tobacco cultivars to *Meloidogyne* species. Presented at CORESTA Congress, Harare. CORESTA Information Bulletin, Congress Issue. Pp. 110 [Abstract].

Vos, C., Claerhout, S., Mkandawire, R., Panis, B., De Waele, D., and Elsen, A. 2012. Arbuscular mycorrhizal fungi reduce root-knot nematode penetration through altered root exudation of their host. Plant and Soil 354:335-345.

Ye, H., Wang, W.-J., Liu, G.-J., Zhu, L.-X., and Jia, K.-G. 2009. Resistance mechanisms of *Prunus* rootstocks to root-knot nematode, *Meloidogyne incognita*. Fruits 64:295-303.

Ye, W. 2018. Nematodes of agricultural importance in North and South Carolina. Pp 247-276 in John J. Chitambar and Sergei A. Subbotin (eds) Plant Parasitic Nematodes in Sustainable Agriculture of North America. Springer, New York.

Yi, H. Y., Rufty, R. C., Wernsman, E. A., and Conkling, M. C. 1998. Mapping the root-knot nematode resistance gene (Rk) in tobacco with RAPD markers. Plant Disease 82: 1319–1322.

CHAPTER 4

Influence of *Meloidogyne arenaria* race 2 on accumulation of nicotine and carbohydrates in conventional and low-alkaloid flue-cured tobacco

ABSTRACT

Nicotine, the principal alkaloid in flue-cured tobacco, is synthesized in root tips and translocated to leaves. It has been shown to play an important role in defense against some plant pathogens, and may be involved in resistance to root-knot nematodes. The FDA has released a proposal that suggests that the nicotine content of consumer tobacco products should be reduced considerably. As such, research efforts have been directed to the development of low-alkaloid tobacco lines. Root-knot nematode parasitism may inhibit translocation of root-derived plant products to foliage because parasitism includes induction of specialized feeding sites (giant cells) that can significantly diminish vascular function. Similarly, root-knot nematode parasitism could inhibit the synthesis and translocation of other agronomically important constituents of flue-cured tobacco, including carbohydrates. One 28-day and two 45-day greenhouse tests conducted in 2019 assessed the impact of *Meloidogyne arenaria* on the accumulation of nicotine, as well as total carbohydrates, in leaves, as well as roots, of conventional and low-alkaloid tobacco lines. Relative resistance to root-knot nematodes in low-alkaloid entries was also evaluated by assessing root galling, nematode presence in roots and egg mass and egg production. A field trial conducted in 2019 investigated the same questions under naturally infested conditions. Alkaloid levels varied among conventional and low-alkaloid lines in all trials. Inoculation with *M. arenaria* increased alkaloid accumulation in roots in the 28-day trial and reduced accumulation in leaves in one 45-day trial. Conversely, inoculation led to decreased carbohydrate

concentration in roots. Significant differences in resistance to root-knot nematode penetration, gall formation and egg mass production were observed among entries in the 28-day trial. Alkaloid status did not have a significant effect on root-knot nematode parasitism in any of the trials. While the results of our field trial varied considerably, they confirm that nicotine content does not appear to have a direct impact on root-knot nematode parasitism, but also suggest that under natural conditions, low-alkaloid flue-cured tobacco entries still accumulate appreciable leaf nicotine, regardless of root-knot nematode parasitism.

INTRODUCTION

Nicotine is present in most tobacco cultivars at levels between 1 and 5 percent on a dry weight basis (Lewis et al., 2020). Nicotine is present in combustible cigarettes at levels between one and two percent of total weight (Proctor, 2017; Lewis et al., 2020). While some low alkaloid tobacco cultivars have been developed, a proposal by the FDA suggesting reductions in nicotine content of consumer products could push growers to produce tobacco that would contain nicotine at "sub-addictive" levels, which could be significantly lower than the levels achievable with current low-alkaloid tobacco cultivars and genetic technologies (USFDA, 2017; Lewis, 2018).

A number of different factors govern the alkaloid content of tobacco, including environmental conditions, planting and cultivation practices, water and nutrient management, (Henry et al., 2019), and disease pressure (Pressier et al., 2007). All of these factors affect the complex metabolic regulatory networks that impact the synthesis, accumulation, and translocation of tobacco alkaloids, including nicotine. Nicotine is primarily synthesized in growing root tips, and translocated to leaves (Zenkner et al., 2019), where it performs functions for the plant such as reducing above ground herbivory by some insects and mammals (Pressier et al., 2007).

While the value of flue-cured tobacco production in the United States has decreased by over a billion dollars over the past two decades (FAO, 2016), flue-cured tobacco remains an important crop for many growers, with over 14,000 acres planted in Virginia in 2020 (USDA, 2020). Root-knot nematodes (*Meloidogyne* spp.) are responsible for losses of 1 to 5 percent of tobacco yields in Virginia (Koenning et al., 1999) and were present in 44% of surveyed Virginia tobacco fields in our most recent survey (Eisenback, 2012). Within affected fields, the majority of root-knot populations were identified as *Meloidogyne arenaria*, with some populations of *M. incognita*, *M. javanica*, and *M. hapla*, as well as some populations which could not be identified (Eisenback, 2012). Significantly, the relative abundance of *M. incognita* has trended down in the past decade or so, while abundance of *M. arenaria* has trended up (Eisenback, 2012). This presents cause for concern because resistance to *M. incognita* is virtually ubiquitous in commercial tobacco varieties, while resistance to *M. arenaria* is relatively poorly understood and only sporadically incorporated into commercial flue-cured tobacco varieties (Johnson et al., 2005; Pollock et al., 2016; Johnson, 2018).

Nicotine inhibits the motility and penetrative ability of *M. incognita* infective juveniles in laboratory tests (Davis and Rich, 1987), but there is no definitive evidence that nicotine is involved in tobacco resistance to root-knot parasitism. There is, however, abundant evidence that root-knot parasitism is associated with alterations in nicotine synthesis and translocation (Hanounik and Osborne, 1975, 1977; Hanounik et al., 1975). Nicotine synthesis and accumulation in tobacco roots, and subsequent translocation to and accumulation in leaves, may be up or down regulated under nematode attack (Zacheo et al., 1974; Barker and Weeks, 1991), which primarily occurs at the nicotine synthesis sites located in root tips. Parasitism by *M. incognita* has been shown to increase nicotine synthesis in plant roots to a greater extent in

nematode-resistant tobacco than in nematode-susceptible tobacco, although nicotine synthesis increased in both relative to healthy controls (Davis and Rich, 1987). Importantly, the nicotine content of leaves increased significantly in resistant varieties under nematode pressure relative to healthy plants or susceptible cultivars (Hanounik and Osborne, 1975). However, more recent research suggests no correlation between nicotine concentration in plant tissues and resistance to root-knot nematodes (Pressier et al., 2007).

Tobacco contains numerous carbohydrates which play an important role in the aroma and flavor of combustible tobacco products and are balanced with nicotine in tobacco blends to produce the desired user experience (Weeks, 1985; Roemer et al., 2012; Banožic et al., 2020). Climatic factors, nutrient availability and agronomic practices such as the timing of the removal of the terminal inflorescence may all influence the accumulation of carbohydrates and augment the ratio of sugars to alkaloids in harvested leaf (Weybrew and Woltz, 1975; Campbell et al., 1982; Grisic and Čavlek, 2019). However, relatively little research has investigated the impact of root-knot nematode parasitism on the accumulation of carbohydrates in flue-cured tobacco. Barker and Weeks (1991) demonstrated that plants experiencing low levels of parasitism by *M. incognita* accumulated slightly higher levels of reducing sugars (a type of carbohydrate formed during the curing process) than healthy plants, but that reducing sugars were significantly lower from plants under higher root-knot pressure than in healthy plants. This appears to be the only specific reference to experimental evidence of the impact of root-knot nematodes on the accumulation of carbohydrates in flue-cured tobacco in the literature.

The use of host resistance to plant parasitic nematodes has become an integral part of flue-cured tobacco production in Virginia, as tobacco growers consistently see their options for chemical control of nematodes restricted (Johnson et al., 2005; Johnson, 2018). Confronted with

an abundance of *M. arenaria*, growers will increasingly need to plant cultivars with resistance to this nematode in order to continue to produce sustainable harvests of flue-cured tobacco. Based on the evidence in published literature, the interaction between tobacco plants and populations of root-knot nematodes could lead to increased and inconsistent nicotine content in harvested leaf, presenting a potential challenge to growers. Published research on this interaction focused on *Meloidogyne incognita*, and while the mechanism of resistance to *M. arenaria* is not well understood, it is known to be different than that associated with resistance to *M. incognita* (Schneider, 1991; Pollock et al., 2016). The goals of this study were to evaluate the impact of *M. arenaria* parasitism on the nicotine and carbohydrates content of tobacco resistant and susceptible to this increasingly prevalent nematode, as well as whether low-alkaloid tobacco is more susceptible to root-knot nematode parasitism than tobacco with conventional alkaloid content.

MATERIALS AND METHODS

Greenhouse Trials

The nematode population used as inoculum in these trials was originally sampled from a flue-cured tobacco field in Halifax County, Virginia, and was maintained on susceptible tomato (*Solanum lycopersicum* L.) variety 'Rutgers' in greenhouse facilities on the Virginia Tech campus in Blacksburg, Virginia and at the Virginia Tech Southern Piedmont AREC (SPAREC) in Blackstone, Virginia. Three greenhouse experiments were conducted in 2019 to evaluate the impact of a population of *M. arenaria* race 2, as well as the impact of the presence of different root-knot nematode resistance genes, on the accumulation of total alkaloids and carbohydrates in conventional and low alkaloid tobacco entries. Trials were arranged in a split-plot design to assess the impacts of both factors on parameters of interest. In a preliminary trial conducted in

Blackstone, six entries were evaluated in six replications: Hicks (susceptible to the four major tropical root-knot nematode species, *M. incognita, M. javanica, M. arenaria,* and *M. hapla*); K326 (homozygous for *Rk1*); LAFC 53 (low-alkaloid entry homozygous for *Rk1*; derived from NC 95); T-15-1-1 (homozygous for *Rk2*); CC 13 (homozygous for *Rk1* and heterozygous for *Rk2*); and STNCB-2-28 (homozygous for both *Rk1* and *Rk2*). Seed of K326 and CC 13 was donated by Cross Creek Seed (Raeford, NC), while seed of the other five entries was generated in the flue-cured tobacco nursery at the Blackstone facility in 2017 and 2018. A different group of six entries was evaluated in two and four replications, respectively, in the two subsequent trials conducted in Blackstone and in Blacksburg: Hicks; NC95 (homozygous for *Rk1*); LAFC 53; LAFC Exp1 (low-alkaloid entry homozygous for *Rk1*, derived from K326); LAFC Exp2 (low-alkaloid entry homozygous for *Rk1*, derived from K326); and STNCB-2-28. Seed of entries LAFC Exp1 and 2 was provided by Altria Client Services (Richmond, VA). Seed of the remaining entries was generated in the flue-cured tobacco nursery in Blackstone. Seed was germinated in 5x5 cell (17 cc/cell) expanded polystyrene tobacco float trays containing Carolina's Choice Tobacco Mix plant growth medium (Carolina Soil Company, Kinston, NC). Five to six week old seedlings were manually clipped once prior to being transplanted to individual 7.6 cm clay pots containing a 2:1 mixture of steam sterilized sandy loam field soil to Profile Greens Grade Porous Ceramic soil amendment (Profile Products, Buffalo Grove, Il). Seedlings with 4 to 6 true leaves were transplanted into 15 cm clay pots containing the same soil substrate. Treated plants were inoculated with a 40 ml aliquot containing 5,000 eggs of *M. arenaria*, while control plants received 40 ml of clean water upon transplantation. Plants were maintained on benchtops in greenhouses at ambient temperature (20-33 °C) without supplemental lighting.

The preliminary trial conducted in Blackstone was terminated 28 days after inoculation; subsequent trials were terminated 45 days after inoculation. Roots were washed free of soil and the fresh weights of the roots of all plants were recorded; whole root systems of inoculated plants were graded for galling according to the gall count index of Taylor and Sasser (1978). A leaf from each stalk position of the aerial portion of each plant was retained in an 8 x 14 cm paper coin envelope and placed in a forced air dryer at 45 C. The root portion of each plant was cut into 4 to 6 cm sections, mixed thoroughly, and 2 to 4 g subsample was retained in an 8 x 14 cm paper coin envelope and placed in a forced air dryer at 45 C. After four to six days, dried samples were ground in a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO) and retained in 60-ml polypropylene specimen cups prior to alkaloid and carbohydrates analysis.

The remaining roots of inoculated plants were subsampled and assessed for egg mass deposition, nematode penetration by life stage, and egg production. To count egg masses, two 1 g subsamples were stained with 0.15 g/L Phloxine-B (Daykin and Hussey, 1985) for five minutes, then washed free of residual stain with tap water and examined with a stereomicroscope at X10 to 20. Nematodes present in roots were determined for three 1 g subsamples cleared with sodium hypochlorite for two minutes and stained with 0.005% acid fuchsin (Byrd et al., 1983). Nematodes within the roots were counted using a stereomicroscope at X10 to 25. Eggs were extracted from the portion of the root system remaining after sub-sampling by agitating the roots in 1% sodium hypochlorite for two minutes after the method of Hussey and Barker (1973). Eggs were collected in 1 L of tap water, and counted in two 10 ml aliquots with a compound microscope at X20-40. Reproductive indices (P_f/P_i) were calculated by dividing the final total egg count (P_f) of each root system by the initial egg inoculum number (P_i).

Field Trial

A field trial was conducted in Palmer Springs, Virginia in 2019 to evaluate the effects of an endemic population of *M. arenaria* race 2, in the presence or absence of two different root-knot nematode resistance genes, on the accumulation of total alkaloids and carbohydrates in conventional and low alkaloid tobacco entries. Entries evaluated in this study were: Hicks (susceptible to the four major tropical root-knot nematode species, *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*); K326 (homozygous for *Rk1*); NC 95 (homozygous for *Rk1*); LAFC 53 (low-alkaloid entry homozygous for *Rk1*; derived from NC 95); LAFC Exp1 (low-alkaloid entry homozygous for *Rk1*, derived from K326); LAFC Exp2 (another low-alkaloid entry homozygous for *Rk1*, of similar origin to LAFC Exp1); T-15-1-1 (homozygous for *Rk2*); CC 13 (homozygous for *Rk1* and heterozygous for *Rk2*); and STNCB-2-28 (homozygous for both *Rk1* and *Rk2*). Seed of K325, NC 95, and CC 13 was donated by Cross Creek Seed (Raeford, NC), while seed of entries LAFC Exp1 and 2 was provided by Altria Client Services (Richmond, VA). Seed of the remaining five entries was generated in the flue-cured tobacco nursery at SPAREC in 2017 and 2018. The trial was arranged in randomized complete block design with ten replications, with the exception of entries KAFC Exp1 and 2, for which plant availability limited replication to six per entry. Plots consisted of 16.1 m long rows spaced 1.2 m apart. Plots were mechanically transplanted, fertilized and maintained as closely as possible to recommendations of Virginia Cooperative Extension (Reed et al., 2019).

Initial soil nematode population densities were determined after bed formation and prior to transplant each year, while final population densities were determined after final sampling at 18 weeks. From each plot, 24 2 cm by 16 cm soil samples were collected and bulked. Nematode counts were performed at the Virginia Tech Nematode Diagnostic and Assay Lab on the Virginia

Tech Blacksburg campus. Bulked samples were initially hand mixed or hammered to reduce aggregates, sifted, and a 250 cubic centimeter subsample from each plot was subjected to nematode extraction using a mechanical elutriator and a combination of sugar flotation and decantation sieving and counted at X40 with compound microscopes (Barker, 1985). Nematode parasitism was assessed at 12, 15, and 18 weeks after transplant. Two plants were destructively sampled from each plot at each timepoint. Soil was washed free of the root system and galling was assessed on the indexed scale of Taylor and Sasser (1978). The fresh weights of the entire root systems were recorded and fibrous feeder roots were separated from structural roots. Nematode penetration by life stage, egg mass deposition, and eggs per gram of feeder root were determined the fibrous feeder root portion of the root system. Egg masses were stained on two 1 g subsamples with 0.15 g/L Phloxine-B (Daykin and Hussey, 1985) for five minutes and counted with a stereomicroscope at X10 to 20. Nematodes present in roots were counted in three 1 g subsamples cleared with sodium hypochlorite for two minutes and stained with 0.005% acid fuchsin (Byrd et al., 1983) using a stereomicroscope at X10-25. Eggs were extracted from the portion of the root system remaining after sub-sampling by agitating the roots in 1% sodium hypochlorite for two minutes after the method of Hussey and Barker (1973). Eggs were collected in 1000 ml of tap water, and counted in two 10 ml aliquots with a compound microscope at X20-40.

Alkaloid and carbohydrates accumulation were evaluated in roots and leaves of the same sampled plants beginning 12 weeks after transplanting and subsequently at 15 and 18 weeks. At these three timepoints, 4 g subsamples were removed from the feeder root portion of each recovered root system during processing and placed in 8 x 14 cm paper coin envelopes organized by replication in forced air dryers at 45 C along with the leaves. After four to six days, dried

samples were processed in a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO) and retained in 40 ml polypropylene specimen cups prior to total alkaloid and carbohydrates analysis as described below. From each sampled plant, one leaf from each stalk position was also removed by hand and retained as a bulked sample prior to the destructive sampling of the root system.

Determination of Total Alkaloid and Carbohydrates Content in Roots and Leaves

Total alkaloids and carbohydrates in leaves and roots were quantified via continuous flow analysis according to CORESTA Recommended Methods Nos. 85 (2017) and 89 (2019), respectively, as described briefly below. Milled leaf and root tissue samples were further homogenized by hand grinding in liquid nitrogen with mortar and pestle. A 250 mg subsample of ground tissue was placed in a 50 ml flask with 25 ml of 5% acetic acid and agitated on a platform shaker for 30 minutes. The resulting suspension was gravity filtered through 8 µm pore quantitative filter paper. Total alkaloids and carbohydrates were determined using a SEAL AutoAnalyzer 3 AA3 HR continuous segmented flow analyzer (Seal Analytical Inc., Mequon, WI). Samples were run through the instrument according to the manufacturers' recommendations for determination of total alkaloids as nicotine or determination of total carbohydrates. A modular digital photometric colorimeter (Seal Analytical Inc., Mequon, WI) was paired with the AA3 platform to quantify the results of the analyses. Colorimetric measurements were captured at 460 nm and 420 nm for total alkaloid and total carbohydrates analyses, respectively.

Statistical Analysis

Data from all trials were subjected to analysis of variance (ANOVA) using PROC GLM in SAS (version 9.4; SAS Institute, Cary, NC). Data for nematode parasitism data were transformed ($\log_{10}$ (x + 1) prior to data analysis and means separation. Percent total nicotine present in leaves and roots on a dry weight basis was calculated using the formula described in CORESTA Recommended Method No. 85 (2017). Percent total carbohydrates present in leaves and roots on a dry weight basis was calculated using the formula described in CORESTA Recommended Method No. 89 (2019). Means for percent total alkaloids and carbohydrates were transformed (arcsine) prior to means separation. Differences among treatment means were identified using Fisher's protected Least Significant Difference test ($P \leq 0.05$ unless otherwise noted).

RESULTS

Root-knot parasitism in greenhouse trials

Significant differences in root galling were found among entries in the 28-day test conducted in Blackstone and the 45-day trial in Blacksburg. Root gall index was significantly lower on all entries relative to susceptible Hicks in the 28-day trial (Table 4.1). In this trial, galling was significantly lower on CC 13 and STNCB 2-28 than LAFC 53 and T-15-1-1, while STNCB 2-28 also had a lower gall index than K326 (Table 4.1). In the 45-day trial in Blacksburg, galling was significantly lower on STNCB 2-28 than on NC 95 and LAFC 53, while the remaining entries experienced intermediate galling (Table 4.1). Differences in galling in the 45-day trial conducted concurrently in Blackstone were not significant.

Table 4.1. Root galling of flue-cured tobacco entries by *M. arenaria* race 2 from greenhouse pot tests in 2019.[z]

			Root galling (0-5)[y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	4.7 a	4.5 a	4.8 ab
K 326	*RK1RK1rk2rk2*	conventional	2.8 bc	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	4.0 a	5.0 a
LAFC 53	*RK1RK1rk2rk2*	low	3.3 b	4.5 a	5.0 a
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	4.0 a	4.8 ab
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	3.5 a	4.8 ab
T-15-1-1	*rk1rk1RK2RK2*	conventional	3.5 b	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	2.3 cd	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	1.8 d	3.0 a	4.3 b

[z] Data presented are non-transformed means from six, two, four and three replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] Taylor and Sassers' Indexed Scale of Gall Count-0 = 0; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100; and 5 = > 100 galls per root system; nt=not tested.

Table 4.2. Numbers of inoculated *M. arenaria* race 2 nematodes observed in roots of flue-cured tobacco entries from greenhouse pot tests in 2019.[z]

			Nematodes/g feeder root[y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	84 a	70 a	124 a
K 326	*RK1RK1rk2rk2*	conventional	25 bc	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	58 a	110 a
LAFC 53	*RK1RK1rk2rk2*	low	45 ab	71 a	136 a
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	43 a	107 a
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	28 a	135 a
T-15-1-1	*rk1rk1RK2RK2*	conventional	50 ab	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	13 cd	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	11 d	43 a	65 b

[z] Data presented are non-transformed means from six, two, four and three replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] nt=not tested.

Significantly fewer nematodes were present in the roots of K326, CC 13, and STNCB 2-28 relative to the susceptible entry, Hicks, in the 28-day trial, while CC 13 and STNCB 2-28 also had significantly fewer nematodes in roots compared to LAFC 53 and T-15-1-1 (Table 4.2). Additionally, STNCB 2-28 had significantly fewer nematode in roots relative to K326 in this trial (Table 4.2). In the 45-day trial conducted in Blacksburg, STNCB 2-28 had significantly fewer nematodes in roots than all other entries (Table 4.2). Differences among entries in the 45-day test conducted in Blackstone were not significant.

Significant differences in egg mass production were found in all three trials. In the 28-day trial, T-15-1-1, CC 13, and STNCB 2-28 had significantly fewer egg masses per gram of root than Hicks and K326, while CC 13 and STNCB 2-28 also had significantly fewer egg masses than LAFC 53 (Table 4.3). Significantly fewer egg masses were observed on STNCB 2-28 than Hicks in the 45-day trial conducted in Blackstone (Table 4.3). In the concurrent trial conducted in Blacksburg, LAFC Exp1 and STNCB 2-28 both had significantly fewer egg masses per gram of root than Hicks (Table 4.3).

Significant differences in egg production and reproductive indices were only detected in the 45-day trial in Blacksburg. Significantly fewer eggs were recovered from NC 95, LAFC 53, LAFC Exp2, and STNCB 2-28 than Hicks, while the egg count for LAFC Exp1 was intermediate (Table 4.3). The reproductive indices of all entries possessing resistance genes were lower than that of Hicks in this trial (Table 4.3).

Root masses varied significantly in all three trials. In the 28-day trial in Blackstone, the mean root mass of LAFC 53 was significantly lower than that of K326, while in the 45-day trial at the same location, Hicks had a mean root mass that was significantly less than that of LAFC Exp2 (Table 4.4). In the 45-day trial conducted concurrently in Blacksburg, LAFC Exp1 and

STNCB 2-28 had significantly lower mean root masses than LAFC 53, while other entries were intermediate (Table 4.4).

Table 4.3. Egg masses, eggs per gram of root, and reproductive indices of *M. arenaria* race 2 on flue-cured tobacco entries from greenhouse pot tests in 2019.[z]

			Egg masses per gram of root[y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	7 a	40 a	62 a
K 326	*RK1RK1rk2rk2*	conventional	4 a	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	27 ab	44 ab
LAFC 53	*RK1RK1rk2rk2*	low	3 ab	21 ab	35 ab
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	23 ab	32 b
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	26 ab	36 ab
T-15-1-1	*rk1rk1RK2RK2*	conventional	1 bc	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	1 c	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	1 c	14 b	32 b

			Eggs per gram of root[y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	29 a	10,533 a	22,363 a
K 326	*RK1RK1rk2rk2*	conventional	42 a	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	5,859 a	11,003 b
LAFC 53	*RK1RK1rk2rk2*	low	23 a	9,396 a	8,967 b
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	6,454 a	13,371 ab
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	5,860 a	6,570 b
T-15-1-1	*rk1rk1RK2RK2*	conventional	8 a	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	37 a	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	10 a	4,984 a	11,417 b

			Reproductive index[y,x]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	0.1 a	59.2 a	118.5 a
K 326	*RK1RK1rk2rk2*	conventional	0.1 a	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	37.2 a	57.0 b
LAFC 53	*RK1RK1rk2rk2*	low	<0.0 a	61.1 a	47.5 b
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	35.6 a	62.0 b
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	40.1 a	33.4 b
T-15-1-1	*rk1rk1RK2RK2*	conventional	0.1 a	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	<0.0 a	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	0.1 a	27.4 a	43.5 b

[z] Data presented are non-transformed means from six, two and four replications respectively, inoculated with 5,000 *M. arenaria* eggs. Data were transformed ($\log_{10} + 1$) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] nt=not tested.

[x] Reproductive index = final population/initial population (P_f/P_i).

Table 4.4. Root mass of flue-cured tobacco entries inoculated with *M. arenaria* race 2 from greenhouse pot tests in 2019.[z]

			Root mass (g)[y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	11.1 ab	25.9 b	24.5 abc
K 326	*RK1RK1rk2rk2*	conventional	12.8 a	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	30.1 ab	24.9 ab
LAFC 53	*RK1RK1rk2rk2*	low	7.9 b	30.7 ab	29.2 a
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	29.3 ab	23.1 bc
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	32.0 a	25.9 ab
T-15-1-1	*rk1rk1RK2RK2*	conventional	9.9 ab	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	10.4 ab	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	9.6 ab	29.4 ab	18.8 c

[z] Data presented are non-transformed means from six, four and two replications respectively, inoculated with 5,000 *M. arenaria* eggs or uninoculated. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] nt=not tested.

Root-knot parasitism in field trials

Significant differences in root galling were observed at 12 and 18 weeks after transplanting in the field trial conducted in Palmer Springs in 2019, while differences in nematodes in roots were found at all three timepoints (Table 4.5). Root galling was significantly lower on NC 95, LAFC Exp1, T-15-1-1, CC 13, and STNCB 2-28 than on LAFC Exp2 at 12 weeks. At 15 weeks, CC 13 had a significantly lower root gall index than LAFC 53, LAFC Exp1, and LAFC Exp2 (Table 4.5). Significantly fewer nematodes were present in the roots of NC 95, T-15-1-1, and STNCB 2-28 than LAFC Exp2; additionally, T-15-1-1 and STNCB 2-28 had significantly fewer nematodes in roots than K326, LAFC 53, and LAFC Exp1 (Table 4.5). At 15 weeks, T-15-1-1 and CC 13 had significantly fewer nematodes present in roots than LAFC Exp2 (Table 4.5). Significantly fewer nematodes were present in the roots of CC 13 and STNCB 2-28 relative to LAFC 53 and LAFC Exp1 (Table 4.5).

Differences in egg mass production were found among entries at all three timepoints, while egg counts differed significantly at 12 and 18 weeks (Table 4.6). Significantly fewer egg masses were observed on entries NC 95, T-15-1-1, and STNCB 2-28 compared to LAFC Exp2 at 12 weeks, and STNCB2-28 also had significantly fewer egg masses than K326, LAFC 53, and CC 13 at this timepoint (Table 4.6). At 15 weeks, CC 13 and STNCB 2-28 had significantly fewer egg masses per gram of root than LAFC Exp1, while at 18 weeks, CC 13 and STNCB 2-28 exhibited fewer egg masses than all of the LAFC entries (Table 4.6). Egg production was significantly lower on STNCB 2-28 than LAFC Exp2 at both 12 and 18 weeks, while all other entries were intermediate, despite varying considerably numerically (Table 4.6).

Table 4.5. Root galling and numbers of *M. arenaria* race 2 nematodes on roots of flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019.[z]

			Root galling (0-5) [y]		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	2.3 abc	2.0 a	3.0 ab
K 326	*rk1rk1rk2rk2*	conventional	2.7 abc	2.0 a	2.5 ab
NC 95	*RK1RK1rk2rk2*	conventional	1.7 bc	2.0 a	3.0 ab
LAFC 53	*RK1RK1rk2rk2*	low	3.3 ab	2.7 a	4.5 a
LAFC Exp1	*RK1RK1rk2rk2*	low	1.3 bc	1.3 a	5.0 a
LAFC Exp2	*RK1RK1rk2rk2*	low	5.0 a	2.7 a	5.0 a
T-15-1-1	*rk1rk1RK2RK2*	conventional	2.0 bc	0.3 a	4.0 ab
CC 13	*RK1RK1RK2rk2*	conventional	1.0 bc	0.7 a	0.5 b
STNCB 2-28	*RK1RK1RK2RK2*	conventional	0.3 c	0.7 a	1.0 ab

			Nematodes per gram of root		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	29 abc	32 ab	64 ab
K 326	*RK1RK1rk2rk2*	conventional	77 ab	44 ab	50 ab
NC 95	*RK1RK1rk2rk2*	conventional	10 bcd	31 ab	76 ab
LAFC 53	*RK1RK1rk2rk2*	low	80 ab	40 ab	113 a
LAFC Exp1	*RK1RK1rk2rk2*	low	47 ab	35 ab	136 a
LAFC Exp2	*RK1RK1rk2rk2*	low	194 a	163 a	63 ab
T-15-1-1	*rk1rk1RK2RK2*	conventional	8 cd	9 b	36 ab
CC 13	*RK1RK1RK2rk2*	conventional	65 abc	14 b	9 b
STNCB 2-28	*RK1RK1RK2RK2*	conventional	4 d	15 ab	8 b

[z] Data presented are non-transformed means from three, three and two replications respectively, under natural, variable pressure by a population of *M. arenaria* race 2. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] Taylor and Sassers' Indexed Scale of Gall Count-0 = 0; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100; and 5 = > 100 galls per root system.

Table 4.6. Egg mass and egg production by *M. arenaria* race 2 on flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019.[z]

			Egg masses per gram of root		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	14 abc	34 ab	36 ab
K 326	*RK1RK1rk2rk2*	conventional	37 ab	19 ab	49 ab
NC 95	*RK1RK1rk2rk2*	conventional	10 bc	22 ab	30 ab
LAFC 53	*RK1RK1rk2rk2*	low	45 ab	29 ab	59 a
LAFC Exp1	*RK1RK1rk2rk2*	low	20 abc	76 a	132 a
LAFC Exp2	*RK1RK1rk2rk2*	low	95 a	68 ab	89 a
T-15-1-1	*rk1rk1RK2RK2*	conventional	3 bc	10 ab	17 ab
CC 13	*RK1RK1RK2rk2*	conventional	22 ab	9 b	2 b
STNCB 2-28	*RK1RK1RK2RK2*	conventional	0 c	3 b	4 b

			Eggs per gram of root		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	3,862 ab	4,872 a	2,488 ab
K 326	*RK1RK1rk2rk2*	conventional	6,553 ab	2,958 a	3,170 ab
NC 95	*RK1RK1rk2rk2*	conventional	2,559 ab	2,873 a	3,115 ab
LAFC 53	*RK1RK1rk2rk2*	low	7,845 ab	1,937 a	8,293 ab
LAFC Exp1	*RK1RK1rk2rk2*	low	6,978 ab	6,945 a	8,596 ab
LAFC Exp2	*RK1RK1rk2rk2*	low	12,652 a	6,595 a	10,407 a
T-15-1-1	*rk1rk1RK2RK2*	conventional	2,796 ab	1,209 a	1,696 ab
CC 13	*RK1RK1RK2rk2*	conventional	5,284 ab	1,444 a	650 ab
STNCB 2-28	*RK1RK1RK2RK2*	conventional	2,043 b	3,815 a	610 b

[z] Data presented are non-transformed means from three, three and two replications respectively, under natural, variable pressure by a population of *M. arenaria* race 2. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Nicotine and carbohydrate content in greenhouse trials

No significant interactions between entry and inoculation impacted root or leaf nicotine content in the 28-day trial conducted in Blackstone ($P \geq 0.3920$). Inoculation increased root nicotine accumulation in the roots of entries in the test but did not impact translocation and accumulation of nicotine in leaves (Figure 4.1). LAFC 53 and CC 13 accumulated significantly lower concentrations of nicotine in roots than Hicks, while LAFC 53 also accumulated significantly less root nicotine than the remaining entries in this trial, whereas LAFC 53 alone accumulated significantly less translocated nicotine in leaves than all entries excepting CC 13 (Table 4.7). Interactions between entry and inoculation did not influence total carbohydrate concentrations in roots or leaves in this trial ($P \geq 0.1382$). Inoculation significantly reduced mean carbohydrates content in roots but did not have a significant effect on the accumulation of leaf carbohydrates in this trial (Figures 4.3, 4.4). Root carbohydrates were significantly lower for LAFC 53 and CC 13 than Hicks, while LAFC 53 also had significantly lower root carbohydrate concentrations than T-15-1-1 and STNCB 2-28 (Table 4.8). Leaf total carbohydrates were significantly lower for entry T-15-1-1 relative to Hicks, K326, and STNCB 2-28 (Table 4.8).

Table 4.7. Total nicotine content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019.[z]

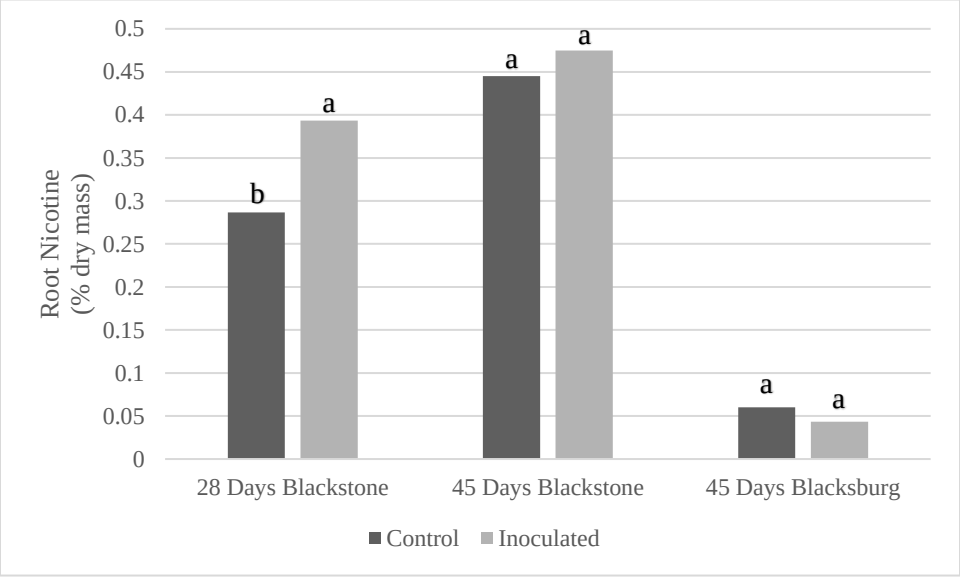
			Root nicotine (% of dry root mass) [y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	0.47 a	0.64 a	0.16 a
K 326	*RK1RK1rk2rk2*	conventional	0.41 ab	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	0.73 a	0.03 bc
LAFC 53	*RK1RK1rk2rk2*	low	0.15 c	0.26 c	0.00 c
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	0.31 c	0.00 c
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	0.29 c	0.00 c
T-15-1-1	*rk1rk1RK2RK2*	conventional	0.37 ab	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	0.27 bc	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	0.35 ab	0.53 b	0.12 ab

			Leaf nicotine (% of dry leaf mass) [y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	0.26 a	0.50 a	0.48 a
K 326	*RK1RK1rk2rk2*	conventional	0.25 a	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	0.65 a	0.32 a
LAFC 53	*RK1RK1rk2rk2*	low	0.13 b	0.18 b	0.00 b
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	0.20 b	0.00 b
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	0.21 b	0.00 b
T-15-1-1	*rk1rk1RK2RK2*	conventional	0.28 a	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	0.22 ab	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	0.29 a	0.68 a	0.45 a

[z] Data presented are non-transformed means from six, four and two replications respectively, inoculated with 5,000 *M. arenaria* eggs or uninoculated. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

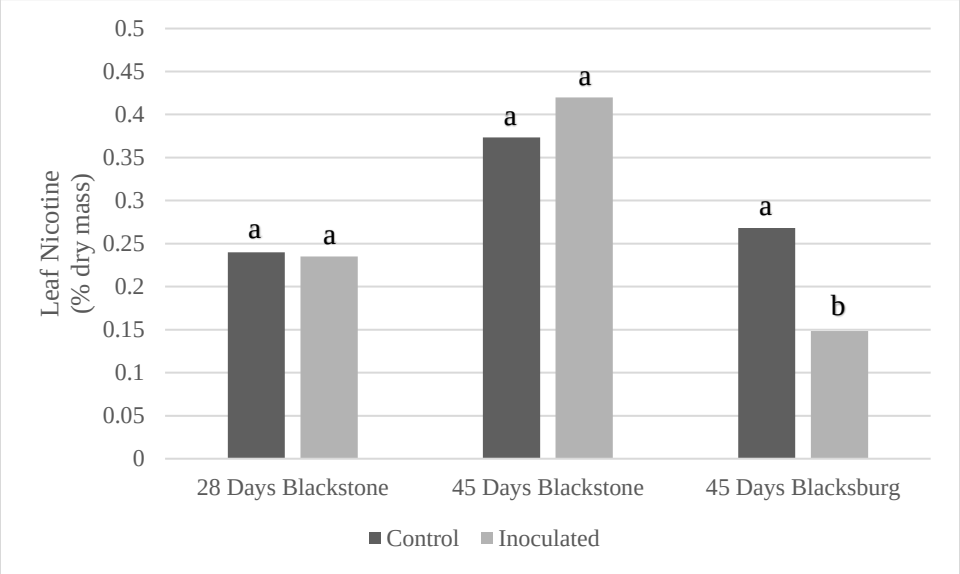
[y] nt=not tested.

Figure 4.1. Total root nicotine content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019.[z]



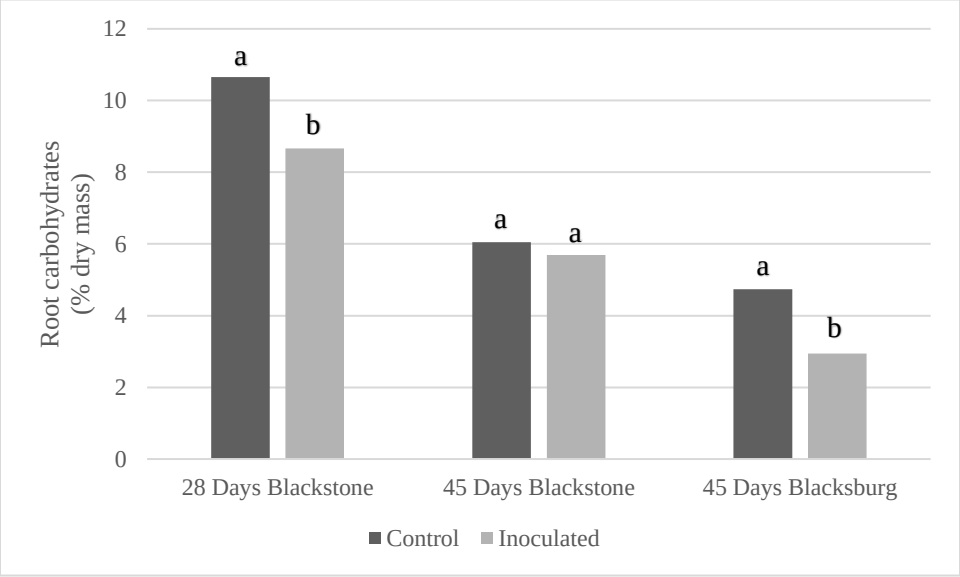
[z] Data presented are non-transformed means from six, four and two replications respectively, inoculated with 5,000 *M. arenaria* eggs or uninoculated. Data were transformed (arcsine) prior to analysis of variance. Means within each test followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Figure 4.2. Total leaf nicotine content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019.[z]



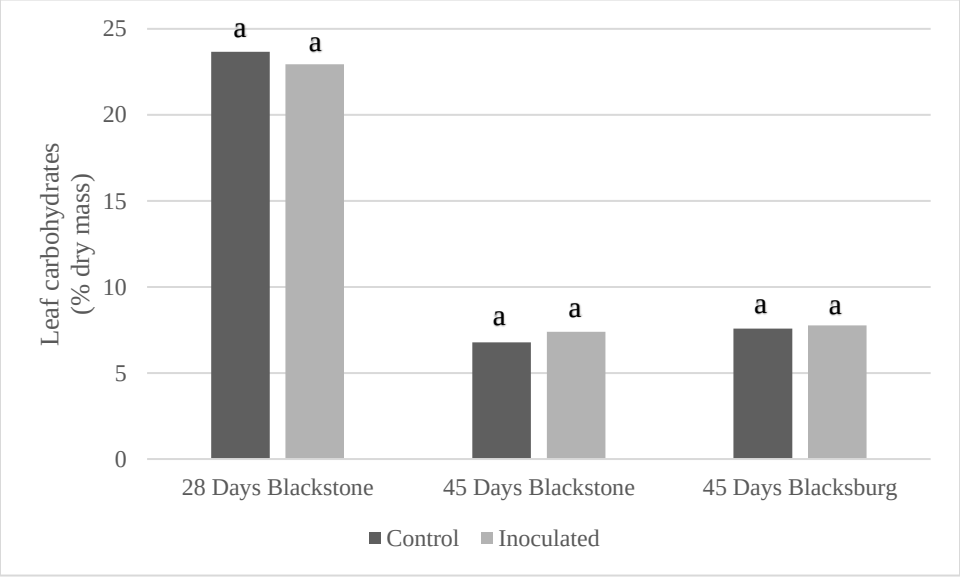
[z] Data presented are non-transformed means from six, four and two replications respectively, inoculated with 5,000 *M. arenaria* eggs or uninoculated. Data were transformed (arcsine) prior to analysis of variance. Means within each test followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.10$).

Figure 4.3. Root total carbohydrate content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019.[z]



[z] Data presented are non-transformed means from six, four and two replications respectively, inoculated with 5,000 *M. arenaria* eggs or uninoculated. Data were transformed (arcsine) prior to analysis of variance. Means within each test followed by the same letter(s) are not significantly different according to Fisher's HSD test ($P \leq 0.05$).

Figure 4.4. Leaf total carbohydrate content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019.[z]



[z] Data presented are non-transformed means from six, four and two replications respectively, inoculated with 5,000 *M. arenaria* eggs or uninoculated. Data were transformed (arcsine) prior to analysis of variance. Means within each test followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 4.8. Total carbohydrate content of flue-cured tobacco entries inoculated or uninoculated with a population of *M. arenaria* race 2 from greenhouse pot tests in 2019. [z]

			Root sugars (% of dry root mass) [y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	11.2 a	7.1 a	3.6 bc
K 326	*RK1RK1rk2rk2*	conventional	9.7 abc	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	7.1 a	3.3 c
LAFC 53	*RK1RK1rk2rk2*	low	7.7 c	3.9 b	3.1 c
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	6.4 a	5.2 a
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	5.2 ab	3.3 c
T-15-1-1	*rk1rk1RK2RK2*	conventional	10.4 ab	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	8.7 bc	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	10.3 ab	5.5 ab	4.5 ab

			Leaf sugars (% of dry leaf mass) [y]		
			28-Day	45-Day	
Entry	Genotype	Alkaloids	Blackstone	Blackstone	Blacksburg
Hicks	*rk1rk1rk2rk2*	conventional	26.0 a	8.3 a	8.6 a
K 326	*RK1RK1rk2rk2*	conventional	24.0 a	*nt*	*nt*
NC 95	*RK1RK1rk2rk2*	conventional	*nt*	7.3 a	8.6 a
LAFC 53	*RK1RK1rk2rk2*	low	23.5 ab	5.6 a	7.3 ab
LAFC Exp1	*RK1RK1rk2rk2*	low	*nt*	7.1 a	6.8 b
LAFC Exp2	*RK1RK1rk2rk2*	low	*nt*	6.1 a	6.1 b
T-15-1-1	*rk1rk1RK2RK2*	conventional	19.6 b	*nt*	*nt*
CC 13	*RK1RK1RK2rk2*	conventional	22.8 ab	*nt*	*nt*
STNCB 2-28	*RK1RK1RK2RK2*	conventional	25.0 a	8.2 a	8.7 a

[z] Data presented are non-transformed means from six, four and two replications respectively, inoculated with 5,000 *M. arenaria* eggs or uninoculated. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[y] nt=not tested.

Interactions between entry and treatment did not have significant effects ($P \geq 0.5629$) on nicotine accumulation in roots or leaves in either 45-day study. Inoculation did not have a significant effect on nicotine accumulation in roots or leaves in either of the 45-day trials at the $P \leq 0.05$ significance level (Figures 4.1, 4.2); however, at the $P \leq 0.10$ significance level, inoculation significantly reduce translocation of nicotine to leaves in the 45-day trial in Blacksburg (Figure 4.2).

In the Blackstone trial, root nicotine content was significantly lower for the three LAFC entries relative to all other entries, while STNCB 2-28 also accumulated significantly less root nicotine than Hicks and NC 95, while leaf nicotine content was less in the LAFC entries relative to all other evaluated entries (Table 4.7). Trends in significant differences in root carbohydrates among entries were relatively similar in the concurrent trial in Blacksburg, and in the Blacksburg trial were the same for leaves as those observed in the concurrent 45-day trial in Blackstone (Table 4.7).

No significant interactions between entry and treatment impacted total carbohydrate levels in either 45-day trial ($P \geq 0.2425$). In 45-day trial conducted in Blacksburg, inoculation reduced carbohydrate content of roots, but this was not true for the concurrent trial in Blackstone (Figure 4.3). Inoculation was not a significant factor in the accumulation of carbohydrates in leaves in either trial (Figure 4.4). In the Blackstone trial, root total carbohydrates were significantly lower for LAFC 53 than LAFC Exp1, while other entries were intermediate; no significant differences in leaf carbohydrates were observed in this study (Table 4.8). In the Blacksburg trial conducted concurrently, root nicotine content was significantly lower for Hicks, NC 95, LAFC 53, and LAFC Exp2 relative to LAFC Exp1, while root carbohydrate accumulation in NC 95, LAFC 53 and LAFC Exp2 was also significantly less than that observed

for STNCB 2-28 (Table 4.8). Leaf carbohydrate contents were significantly lower for entries LAFC Exp1 and LAFC Exp2 relative to Hicks, NC 95, and STNCB 2-28 in this trial (Table 4.8).

Nicotine and carbohydrate content in field trials

Root and nicotine content varied significantly among entries at all three timepoints. Entry LAFC Exp2 had not accumulated a detectable level of nicotine in roots at 12 weeks after transplanting, a significantly lower level than all other entries with the exception of LAFC 53 and LAFC Exp1 (Table 4.9). At this point, leaf nicotine content of the three LAFC entries was significantly less than that of all other entries (Table 4.9). At 15 weeks, the three LAFC entries accumulated significantly less nicotine in roots than NC 95, while all other entries were intermediate (Table 4.9). The three LAFC entries accumulated significantly less nicotine in roots than all of the other entries at the 18-week timepoint (Table 4.9). Entries LAFC 53 and LAFC Exp2 had significantly less nicotine in leaves than T-15-1-1 at this timepoint, and LAFC 53 also had significantly less leaf nicotine than Hicks (Table 4.9).

Table 4.9. Total nicotine content of flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019.[z]

			Root alkaloids (% of dry root mass)		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	1.11 a	1.48 ab	1.24 a
K 326	*RK1RK1rk2rk2*	conventional	0.83 ab	1.10 a-d	1.62 a
NC 95	*RK1RK1rk2rk2*	conventional	1.16 a	1.69 a	1.37 a
LAFC 53	*RK1RK1rk2rk2*	low	0.24 bc	0.17 cd	0.02 b
LAFC Exp1	*RK1RK1rk2rk2*	low	0.55 abc	0.44 bcd	<0.00 b
LAFC Exp2	*RK1RK1rk2rk2*	low	<0.00 c	0.09 d	<0.00 b
T-15-1-1	*rk1rk1RK2RK2*	conventional	0.98 ab	1.30 abc	1.89 a
CC 13	*RK1RK1RK2rk2*	conventional	1.26 a	0.96 a-d	1.88 a
STNCB 2-28	*RK1RK1RK2RK2*	conventional	1.07 a	0.90 a-d	1.28 a

			Leaf alkaloids (% of dry leaf mass)		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	1.48 a	2.88 a	3.30 ab
K 326	*RK1RK1rk2rk2*	conventional	1.30 a	1.50 b	2.05 abc
NC 95	*RK1RK1rk2rk2*	conventional	1.56 a	2.64 a	1.48 abc
LAFC 53	*RK1RK1rk2rk2*	low	0.06 b	0.01 c	0.36 c
LAFC Exp1	*RK1RK1rk2rk2*	low	<0.00 b	<0.00 c	1.41 abc
LAFC Exp2	*RK1RK1rk2rk2*	low	0.01 b	0.01 c	1.28 bc
T-15-1-1	*rk1rk1RK2RK2*	conventional	1.46 a	2.53 ab	3.69 a
CC 13	*RK1RK1RK2rk2*	conventional	1.65 a	1.97 ab	2.09 abc
STNCB 2-28	*RK1RK1RK2RK2*	conventional	1.91 a	1.53 b	2.43 abc

[z] Data presented are non-transformed means from three, three and two replications respectively, under natural, variable pressure by a population of *M. arenaria* race 2. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Significant differences in total carbohydrate content of roots were observed among entries at all three timepoints, while differences in leaf carbohydrate content were only significant at 12 weeks. Total carbohydrates in roots were significantly lower for entries K326, LAFC 53, and LAFC Exp2 compared to Hicks and T-15-1-1, while LAFC 53 and LAFC Exp2 also accumulated lower carbohydrate levels relative to NC 95, LAFC Exp1, and STNCB 2-28 (Table 4.10). At this timepoint, carbohydrate content of leaves was significantly lower for entries NC 95 and LAFC 53 compared to STNCB 2-28, while other entries were intermediate (Table 4.10). The root carbohydrate content of entry LAFC 53 was significantly less than that of entries Hicks and NC 95 at 15 weeks (Table 4.10). At 18 weeks, carbohydrate contents were lower for entries LAFC Exp1 and LAFC Exp2 relative to entries K326, T-15-1-1, and STNCB 2-28 (Table 4.10).

Table 4.10. Total carbohydrate content of flue-cured tobacco entries from a field trial in Palmer Springs, Virginia in 2019.[z]

			Total root sugars (% of dry root mass)		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	3.5 a	3.0 a	3.7 abc
K 326	*RK1RK1rk2rk2*	conventional	2.3 bc	2.0 ab	4.6 a
NC 95	*RK1RK1rk2rk2*	conventional	2.7 ab	3.0 a	3.4 a-d
LAFC 53	*RK1RK1rk2rk2*	low	1.3 c	0.8 b	2.2 bcd
LAFC Exp1	*RK1RK1rk2rk2*	low	3.1 ab	1.4 ab	1.5 d
LAFC Exp2	*RK1RK1rk2rk2*	low	1.3 c	1.2 ab	1.8 cd
T-15-1-1	*rk1rk1RK2RK2*	conventional	3.4 a	2.0 ab	5.1 a
CC 13	*RK1RK1RK2rk2*	conventional	2.2 bc	2.2 ab	3.1 a-d
STNCB 2-28	*RK1RK1RK2RK2*	conventional	2.4 ab	2.1 ab	4.2 ab

			Total leaf sugars (% of dry leaf mass)		
Entry	Genotype	Alkaloids	12 weeks	15 weeks	18 weeks
Hicks	*rk1rk1rk2rk2*	conventional	7.5 ab	4.3 a	8.0 a
K 326	*RK1RK1rk2rk2*	conventional	7.1 ab	5.2 a	8.1 a
NC 95	*RK1RK1rk2rk2*	conventional	4.8 b	3.7 a	8.0 a
LAFC 53	*RK1RK1rk2rk2*	low	5.0 b	5.8 a	10.1 a
LAFC Exp1	*RK1RK1rk2rk2*	low	5.6 ab	3.5 a	9.0 a
LAFC Exp2	*RK1RK1rk2rk2*	low	6.0 ab	5.1 a	10.0 a
T-15-1-1	*rk1rk1RK2RK2*	conventional	6.0 ab	4.8 a	8.0 a
CC 13	*RK1RK1RK2rk2*	conventional	6.1 ab	3.7 a	6.6 a
STNCB 2-28	*RK1RK1RK2RK2*	conventional	8.8 a	6.9 a	8.1 a

[z] Data presented are non-transformed means from three, three and two replications respectively, under natural, variable pressure by a population of *M. arenaria* race 2. Data were transformed (arcsine) prior to analysis of variance. Means within a column followed by the same letter(s) are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

DISCUSSION

The results of our greenhouse trials varied considerably, but suggest, as have those of other authors (Hanounik and Osborne, 1974; Davis and Rich, 1987), that the presence of root-knot nematodes leads to increased accumulation of nicotine in the roots of flue-cured tobacco, whereas translocation to the leaves of plants is reduced by the nematode. Conversely, the only significant effects of inoculation on the carbohydrate content of the evaluated entries in our greenhouse trials were reductions in roots. These results appear to confirm the observations of Barker and Weeks (1991), although their study did not address the influence of root-knot nematode resistance in the host, and specifically investigated reducing sugars, which are formed during curing, whereas we evaluated total carbohydrate content. We never found significant differences in root-knot nematode parasitism or reproduction between low-alkaloid entries possessing *Rk1* and conventional entries of the same resistance genotype in any of our greenhouse trials, and with the exception of 12 weeks after transplanting, the same was true in the field. At that timepoint, entry LAFC Exp2 exhibited significantly higher levels of nematodes in roots, galling, and egg mass production masses relative to NC 95; however, it did not differ significantly from K326, which shares the same resistance genotype as the two aforementioned entries. Importantly, we never saw interactions between entry (and for our purposes, root-knot nematode resistance genotype) and the presence of the nematode influencing alkaloid or carbohydrates levels in any trials.

No differences in metrics of nematode parasitism with the exception of egg mass production were seen among entries in the 45-day trial conducted in Blackstone, and these differences did not appear to impact overall reproduction. In the 28-day trial, STNCB 2-28 and CC 13 exhibited significantly less nematodes in roots than Hicks, LAFC 53, and T-15-1-1, while

galling was significantly lower on all evaluated entries relative to the susceptible entry, Hicks, and was significantly lower on STNCB 2-28 than on all other entries with the exception of CC 13. In the 45-day trial in Blacksburg, fewer nematodes were present in roots of STNCB 2-28 relative to all entries, but galling of STNCB 2-28 was only lower than that of LAFC 53 ad LAFC Exp1.

In our 45-day trial in Blacksburg, reproduction (as measured by reproductive index) was significantly lower on all entries possessing resistance genes, regardless of alkaloid content, relative to susceptible Hicks. Reproduction was low across all entries in our 28-day trial, which is unsurprising as the lifecycle of root-knot nematodes is on average about 25 days at 27 °C (Agrios, 2005); our 28-day trial was conducted from April through May of 2019, during which the average temperature in the greenhouse was approximately 26 °C. In this trial, egg mass counts were significantly lower for entry STNCB 2-28, CC 13, and T-15-1-1 relative to K326 and susceptible entry Hicks, but many egg masses were clearly still developing upon observation, so subsequent egg counts were correspondingly low (not exceeding 42 eggs per gram of root, which was the mean count for K 326) and not significantly different among entries.

In a study evaluating tobacco response to *M. incognita* parasitism, Lamberti et al. (1971) observed that leaf nicotine content of tobacco planted in plots treated with 1,3 dichloropropene (a preplant soil fumigant and nematicide) was lower than that of leaves from untreated plots. They also observed severe root galling in plants from the untreated plots, while galling was much less on the roots of plants from the treated plots (Lamberti et al., 1971). Similarly, Zacheo et al. (1974) found that treatment with nematicides was correlated with “a reduction in nicotine content” of plant leaves, and that while leaf nicotine content “was increased by nematode attack,” there was “on the other hand…a progressive decrease in the nicotine content of the roots

as the inoculum was increased." They also observed that the ratio of nicotine in leaves relative to roots was "significantly higher" in plants infested with *M. incognita* than in controls, and suggest that nematode infestation "may stimulate nicotine translocation" (Zacheo et al., 1974).

In contrast to the observations of Zacheo et al (1974), Hanounik and Osborne (1974) demonstrated significant increases in root nicotine content in the roots of tobacco cultivars both resistant and susceptible to *M. incognita* upon inoculation with the nematode, while leaf nicotine content increased by over 100% in the resistant cultivar upon inoculation, whereas the susceptible cultivar saw a significant reduction in leaf nicotine content. They suggest that this difference is likely due to the root damage caused by root-knot nematode parasitism on the susceptible cultivar, while "nicotine movement into leaves apparently was not adversely affected due to lack of significant root damage" in the resistant cultivar (Hanounik and Osborne, 1974).

Davis and Rich (1987) demonstrated that nicotine has a definite nematistatic, but not nematicidal, effect on *M. incognita* in experiments with infective second-stage juveniles treated with nicotine and used to inoculate susceptible tomatoes. While motility and host seeking behavior were negatively correlated with exposure to nicotine, root galling still occurred at relatively high concentrations of nicotine (Davis and Rich, 1987). The authors then examined the effect of nicotine *in situ* by confronting resistant and susceptible flue-cured tobacco cultivars with *M. incognita.* Their results were similar to those of Hanounik and Osborne (1974), and demonstrated that root nicotine content increased in both susceptible and resistant cultivars over a period of four to sixteen days (Davis and Rich, 1987). However, while Hanounik and Osborne (1974) observed greater accumulations of nicotine in the roots of susceptible plants relative to resistant plants, Davis and Rich (1987) found the opposite to be true. This discrepancy is probably attributable to the relatively long duration of the former study, which was conducted for

55 days, and allowed the nematodes to destroy more of the root system of the susceptible plants, sequestering nicotine in the roots to a far greater degree than observed over a four to sixteen day trial. This is somewhat in contrast to our observation that differences in root carbohydrates among inoculated and control plants were only significant, and were numerically greater, in our 28-day trial. Root nicotine levels were dramatically lower in one 45-day trial, and just slightly greater in another trial, relative to the 28-day duration. While Davis and Rich (1987) observed that "concentrations of nicotine were apparently sufficient to affect *M. incognita* in both susceptible and resistant tobacco roots," they indicate that this evidence does not, in and of itself, implicate nicotine directly in resistance to root-knot nematodes in tobacco.

Barker and Weeks (1991) demonstrated that the presence of *M. incognita* reduced leaf nicotine content in root-knot susceptible flue-cured tobacco, but also noted that the effect of inoculum level on nicotine concentration was "inconsistent." Their study focused heavily on the relationship between different soil types and yield and quality of tobacco leaf, so many of the observed inconsistencies were associated with differential rates of reproduction and host seeking in different soils (Barker and Weeks, 1991). However, they ultimately concluded that their results confirmed the observation of Davis and Rich (1987) that nicotine is likely not directly implicated in host resistance to root-knot nematodes in tobacco (Barker and Weeks, 1991). The results of Preisser et al. (2007) suggest that this is correct. They investigated the influence of "constitutive" nicotine content in different tobacco lines on the population density of *M. incognita*. In other words, they used the available literature, much of it reviewed above, to determine a threshold at which nematode parasitism induced nicotine biosynthesis. They then inoculated several tobacco entries possessing different innate nicotine levels with sub-threshold numbers of *M. incognita* and determined whether nematode parasitism differed among lines

(Preisser et al., 2007). Finding no statistical differences in nematode population density among lines at the conclusion of their experiment, they concluded that, “constitutive nicotine content did not affect nematode survival or reproduction”, and based on related work, actually demonstrated that high nicotine content may require a “tradeoff” cost in attenuated flowering relative to other lines, and that high nicotine actually reduced plant tolerance to “low levels of nematode infection” (Preisser et al., 2007).

Literature addressing the relationship between nematode parasitism, and root-knot nematode parasitism in particular, and carbohydrate content in tobacco is relatively sparse. Barker and Weeks (1991) demonstrated that “moderate-to-high levels of [*M. incognita*] resulted in lower sugar content in the cured tobacco leaf,” but that sugars actually increased at lower nematode population densities, a phenomenon that could be an example of a “compensatory response” to the nematode, which has been shown to actually increase plant growth under certain circumstances (Barker and Olthof, 1976).

In their study, Barker and Weeks (1991) utilized four levels of nematode inoculum, ranging from zero to 25,000 eggs per 500 cubic centimeters of soil; in our greenhouse trials, we inoculated approximately 1000 cubic centimeters of soil with 5,000 *M. arenaria* eggs, which is intermediate to the two highest levels of inoculum used in the aforementioned study, and thus analogous to the “moderate-to-high” level of inoculum Barker and Weeks described. Unfortunately, due to the cultivars employed in their study, both of which were susceptible to root-knot nematodes, it is difficult to draw parallels between some of our results and those of Barker and Weeks. However, in our greenhouse trials, the only significant effects of inoculation on carbohydrate content were reductions in root concentrations in our 28-day trial and in one of our 45-day trials; no observed differences in leaf carbohydrate content were attributable to the

presence of the nematode in our greenhouse test results. Significant differences in carbohydrate content were found among entries in cases where the presence of the nematode was not a significant effect, but there was not a consistent pattern among entries, and nicotine levels did not appear to be correlated with sugars accumulation.

In the field, the only significant difference in carbohydrate content of leaves was observed at 12 weeks, but differences in roots were found at all timepoints. While low-alkaloid entries LAFC 53 and LAFC Exp2 exhibited the lowest levels of carbohydrates in roots and leaves in most cases, another low-alkaloid entry of similar pedigree and with the same resistance genotype, LAFC Exp1, often exhibited high levels of carbohydrates, comparable with standard susceptible cultivar Hicks. While nematode pressure varied across the physical space in which our field trial was conducted, we did not observe a correlation between preplant and post-harvest soil nematode counts and observed parasitism, so it is difficult to calibrate the results of our field trial to the observations of Barker and Weeks (1991). The results of our field trial suggest that some low-alkaloid entries (in our case, entries LAFC Exp2) may be more susceptible to parasitism by root-knot nematodes than conventional root-knot resistant entries under field conditions, but this was not true for the other low-alkaloid entries we evaluated.

Literature Cited

Banožić, M., Jokić, S., Ačkar, D., Blažić, M., and Šubarić, D. 2020. Carbohydrates-key players in tobacco aroma formation and quality determination. Molecules 25: 1734-1747.

Barker, K.R. 1985. Nematode extraction and bioassays. Pp. 19-35 in K.R. Barker, C.C. Carter and J.N. Sasser (eds) An Advanced Treatise on *Meloidogyne* Vol. II: Methodology. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C.

Barker, K. R. and Olthof, T. H. A. 1976. Relationships between nematode population densities and crop responses. Annual Review of Phytopathology 14:327-353.

Barker, K. R., and Weeks, W. W. 1991. Relationships between soils and levels of *Meloidogyne incognita* and tobacco yield and quality. Journal of Nematology 23: 82-90.

Byrd, D. W., Jr., Kirkpatrick, T., and Barker, K. R. 1983. An improved technique for clearing and staining plant tissues for detection of nematodes. J. Nematol. 15:142-143.

Campbell, C. R., Chaplin, J. F., and Johnson, W. H. 1982. Cultural factors affecting yield, alkaloids and sugars of close-grown tobacco. Agronomy Journal 74 (2): 279–283.

CORESTA. 2017. Recommended Method No. 85—Tobacco—Determination of the content of total alkaloids as nicotine—continuous-flow analysis method using KSCN/DCIC. Cooperation Centre for Scientific Research Relative to Tobacco. Ref. RAC-052-2-CRM-85.

CORESTA. 2019. Recommended Method No. 89—Tobacco— Determination of the content of total sugars—continuous-flow analysis method using Hydrochloric Acid / P-Hydroxy Benzoic Acid Hydrazide (PAHBAH). Cooperation Centre for Scientific Research Relative to Tobacco. Ref. RAC-054-2-CRM-89.

Davis, E. L., and Rich, J. R. 1987. Nicotine content of tobacco roots and toxicity to *Meloidogyne incognita*. Journal of Nematology 19 (1): 23-29.

Daykin, M. E., and Hussey, R. S. 1985. Staining and histopathological techniques in nematology. Pp. 41 in K.R. Barker, C.C. Carter and J.N. Sasser (eds) An Advanced Treatise on *Meloidogyne* Vol. II: Methodology. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C.

Eisenback, J. D. 2012. Effects of resistant tobacco on population dynamics of root-knot nematode species in Virginia. 45th Tobacco Workers Conference, 16–19 January 2012, Williamsburg, VA, Paper 89 (Abstr.).

FAO. 2016. FaoStat Database Collections. http://www.fao.org/faostat/en/?#data/QV. Accessed September 15, 2020.

Gršić, K., and Čavlek, M. 2019. Effect of topping height and ripeness on quality of flue-cured tobacco varieties. Journal of Central European Agriculture 20 (3):841-851.

Hanounik, S.B., and Osborne, W.W., 1975. Influence of *Meloidogyne incognita* on the content of amino acids and nicotine in tobacco grown under gnotobiotic conditions. Journal of Nematology 7: 332–336.

Hanounik, S.B., and Osborne, W.W., 1977. The relationships between population density of *Meloidogyne incognita* and nicotine content of tobacco. Nematologica 23: 147–152.

Hanounik, S.B., Osborne, W.W., and Pirie, W.R., 1975. Relationships between the population density of *Meloidogyne incognita* and growth of tobacco. Journal of Nematology 7: 352–356.

Henry, J. B., Vann, M. C., and Lewis, R. S. 2019. Agronomic practices affecting nicotine concentration in flue-cured tobacco: a review. Agron. J. 111 (6): 3067-3075.

Hussey, R. S., and Barker, K. R. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp. including a new technique. Plant Dis. Rep. 57:1025-1028.

Johnson, C. S., Way, J., and Barker, K. R. 2005. Nematode parasites of tobacco. Pp. 675–708 in M. Luc, R. A. Sikora, and J. Bridge, eds. Plant parasitic nematodes in subtropical and tropical agriculture, 2nd ed. Wallingford, UK: CAB International.

Johnson, C. S. Flue-cured tobacco disease control. 2018. Pp. 47-64 in Reed, T. D., Johnson, C. S., Semtner, P. J., and Wilkinson, C. A. 2018. 2018 Flue-cured tobacco production guide. Va. Coop. Ext. Publ. No. 436-048. Blacksburg, VA.

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O., and Fortnum, B. A. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. Journal of Nematology 31: 587-618.

Lewis, R. S. 2018. Potential mandated lowering of nicotine levels in cigarettes: a plant perspective. Nic. And Tob. Res. 21 (7): 991-995.

Lewis, R. S., Drake-Stowe, K. E., Heim, C., Steede, T., Smith, W., and Dewey, R. E. 2020. Genetic and Agronomic Analysis of Tobacco Genotypes Exhibiting Reduced Nicotine Accumulation Due to Induced Mutations in Berberine Bridge Like (BBL) Genes. Frontiers in plant science 11 (368).

Pollok, J. R., Johnson, C. S., Eisenback, J. D., Reed, T. D. 2016. Reproduction of *Meloidogyne incognita* Race 3 on Flue-cured Tobacco Homozygous for Rk1 and/or Rk2 Resistance Genes. J Nematol. 48(2):79-86.

Preisser, E. L., Gibson, S. E., Adler, L. S., and Lewis, E. E. 2007. Underground herbivory and the costs of constitutive defense tobacco. Acta Oecologica 31: 210-215.

Proctor, R. N. 2017. FDA's new plan to reduce the nicotine in cigarettes to sub-addictive levels could be a game-changer. Tob. Control 26 (5): 487-488.

Reed, T. D., Johnson, C. S., Semtner, P. J., and Wilkinson, C. A. 2019. 2019 Flue-cured tobacco production guide. Va. Coop. Ext. Publ. No. 436-048. Blacksburg, VA.

Roemer, E., Schorp, M. K., Piadé, J.-J., Seeman, J. I., Leyden, D. E., and Haussmann, H. J. 2012. Scientific assessment of the use of sugars as cigarette tobacco ingredients: A review of published and other publicly available studies. Crit. Rev. Toxicol. 42: 244–278.

Schneider, S. M. 1991. Penetration of susceptible and resistant tobacco cultivars by *Meloidogyne* juveniles. Journal of Nematology 23: 225–228.

Taylor, A. L., and Sasser, J. N. 1978. Identification and control of root-knot nematodes (Meloidogyne spp). Crop Publ. Dept. Plant Pathology, North Carolina State Univ. and U.S Agency Int. Dev. Raleigh, N.C pp. 111.

USFDA. 2017. FDA News Release: FDA announces comprehensive regulatory plan to shift trajectory of tobacco-related disease, death. https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ ucm568923. Accessed June 17, 2020.

USDA. 2020. United States Department of Agriculture, National Agricultural Statistics Service, Acreage, June 2020. www.nass.usda.gov/publications/reports/acrg0620. Accessed November 16, 2020.

Weeks, W. W. 1985. Chemistry of tobacco constituents influences flavor and aroma. Rec. Adv. Tob. Sci. 11: 175–200.

Weybrew, J. A., and Woltz, W. G. 1975. Production factors affecting chemical properties of the flue-cured leaf. IV. Influence of management and weather. Tobacco International 177 (6): 46–48.

Zacheo, G., Lamberti, F., and Durbin, R. D. 1974. Effect of *Meloidogyne incognita* (Kofoid et White) Chitwood on the nicotine content of tobacco (*Nicotiana tabacum* L.). Nematologia Mediterranea 2(2): 165-170.

Zenkner, F. F., Margis-Pinhiero, M., and Cagliari, A. 2019. Nicotine biosynthesis in *Nicotiana*: a metabolic overview. Tob. Sci. 56 (1): 1-9.