Reproduction of *Meloidogyne incognita* Race 3 on Flue-cured Tobacco Homozygous for *Rk1* and/or *Rk2* Resistance Genes

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Abstract: Most commercial tobacco cultivars possess the *Rk1* resistance gene to races 1 and 3 of *Meloidogyne incognita* and race 1 of *Meloidogyne arenaria*, which has caused a shift in population prevalence in Virginia tobacco fields toward other species and races. A number of cultivars now also possess the *Rk2* gene for root-knot resistance. Experiments were conducted in 2013 to 2014 to examine whether possessing both *Rk1* and *Rk2* increases resistance to a variant of *M. incognita* race 3 compared to either gene alone. Greenhouse trials were arranged in a completely randomized design with Coker 371-Gold (C371G; susceptible), NC 95 and SC 72 (*Rk1Rk1*), T-15-1-1 (*Rk2Rk2*), and STNCB-2-28 and NOD 8 (*Rk1Rk1* and *Rk2Rk2*). Each plant was inoculated with 5,000 root-knot nematode eggs; data were collected 60 d postinoculation. Percent galling and numbers of egg masses and eggs were counted, the latter being used to calculate the reproductive index on each host. Despite variability, entries with both *Rk1* and *Rk2* conferred greater resistance to a variant of *M. incognita* race 3 than plants with *Rk1* or *Rk2* alone. Entries with *Rk1* alone were successful in reducing root galling and nematode reproduction compared to the susceptible control. Entry T-15-1-1 did not reduce galling compared to the susceptible control but often suppressed reproduction.

Key words: genetics, *Meloidogyne incognita*, *Nicotiana tabacum*, reproductive index, Virginia.

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

In plant nematology, host resistance is defined as the inhibition of reproduction on a host (Roberts, 2002). Conversely, hosts with tolerance do not necessarily inhibit nematode reproduction, but plant growth and yield are generally not affected (Roberts, 2002). The first root-knot resistance gene for tobacco was successfully introduced from *Nicotiana tomentosa* Ruiz and Pav., was called *Rk*, and was released in the commercial cv. NC 95 in 1961 (Yi et al., 1998). Most commercial tobacco cultivars currently planted in the United States are homozygous for this single dominant gene, now known as *Rk1* (Koenning et al., 1999). *Rk1* imparts resistance to *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 host races 1 and 3 and *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949 host race 1 (Schneider, 1991; Ng'ambi et al., 1999b). According to Ng'ambi et al. (1999b), the effect of

Rk1 on *M. incognita* races 2 and 4, *M. arenaria* race 2, *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949, and *Meloidogyne hapla* Chitwood, 1949 is minimal or nonexistent. However, Ternouth et al. (1986) noted the *Rk1* gene conferred "some resistance to *M. javanica*."

The first account of commercial tobacco in Zimbabwe containing a second tobacco root-knot resistance gene, along with *Rk1*, was in 1993 (Way, 1994; Jack and Lyle, 1999; Jack, 2001). This gene was identified in Zimbabwe in 1950 and labeled as "*T*" (Schweppenhauser, 1975). It was discovered in local *N. tabacum* plants that had been grown along the Zambezi River in Zimbabwe since the 1700s in soil heavily infested with *M. javanica* (Schweppenhauser, 1975; Mackenzie et al., 1986; Ternouth et al., 1986). Plant selections were determined to be "partially resistant" to *M. javanica* after experimental inoculation resulted in only one or two females and no egg production (Schweppenhauser, 1975). Ternouth et al. (1986) observed that resistance to *M. javanica* conferred by the "*T*" gene was greater than that provided by "*S*" (or *Rk1*). The "*T*" gene is now often referred to as *Rk2* in the United States. Smeeton further observed very high resistance to *M. javanica* when both *Rk1* and the "*T*" (or *Rk2*) gene were present together (Ternouth et al., 1986). Additionally, Shepherd (1982) reported results from two trials in which *M. javanica* juvenile root invasion on the "better breeding lines" was only 20% of that on susceptible cultivars, although subsequent nematode development was only slightly lower. If this is the case, the mode of action of "*T*" (or *Rk2*) would be very different from that observed for *Rk1*, which was determined to inhibit successful giant cell formation, but not penetration (Schneider, 1991). Although early reports on *Rk2* stated tentatively that the mechanism of the high resistance to *M. javanica* was apparently controlled by "multiple factors," it was later concluded that the resistance was inherited as a monogenic dominant trait, probably also involving one or more modifying genes (Schweppenhauser, 1975).

Plant selections from the local Zimbabwe tobacco were crossed with cultivated tobacco entries to improve leaf morphology and agronomic traits, resulting in the breeding line RKT15-1-1 (Mackenzie et al., 1986). In 1979, Smeeton crossed RKT15-1-1 with flue-cured tobacco cv. SC 72, and then NC 89, to create the STNC breeding lines: STNCA and STNCB, which thus possessed both *Rk1* and *Rk2* (Ternouth et al., 1986). The STNC breeding lines were subsequently crossed with other commercial cultivars to improve their flue-cured tobacco characteristics (Ternouth et al., 1986). This resistance was incorporated into multiple flue-cured tobacco cultivars developed in Zimbabwe, beginning with "*RK1*" (STNCB 2-28 × ms Kutsaga E1) released in 1993 (Way, 1994; Jack and Lyle, 1999; Jack, 2001). Beginning in 2007, resistance or tolerance arising from combinations of *Rk1* and *Rk2* have been introduced into flue-cured tobacco cultivars released in the United States, such as CC 13, CC 33, CC 35, CC 37, CC 65, and PVH 2275 (Reed, 2007; Johnson, 2015).

Although *M. incognita* has traditionally been considered the most common root-knot nematode species found on tobacco in Virginia (Johnson, 1989), a 2004 survey of 170 flue-cured tobacco fields in Virginia revealed that of the 43.5% of tobacco fields infested with root-knot nematodes, 56.7% were infested with *M. arenaria*, 25.0% with *M. hapla*, 16.7% with *M. incognita*, 11.7% with *M. javanica*, and 8.3% with unknown *Meloidogyne* species (Eisenback, 2012). A 2010 follow-up survey of 276 Virginia flue-cured tobacco fields identified a similar percentage of fields infested with root-knot nematodes (44.9%), with *M. arenaria* present in 58.8% of the infested fields, *M. hapla* in 22.3%, *M. incognita* in 11.1%, *M. javanica* in 11.1%, and unknown *Meloidogyne* species in 6.3% (Eisenback, 2012). *Meloidogyne arenaria* was the most commonly detected root-knot nematode species in these surveys, and the prevalence of *M. arenaria* increased from 56.7% in 2004 to 58.8% in 2010, whereas that of *M. incognita* decreased from 16.7% in 2004 to 11.1% in 2010. With this apparent shift in root-knot nematode populations in Virginia's tobacco fields, cultivars with only the *Rk1* gene may no longer adequately limit nematode reproduction, depending on the root-knot nematode species present. The research in Zimbabwe alleged that *Rk1* and *Rk2* confer resistance to *M. javanica*, but effects of these genes on other *Meloidogyne* species and races are largely undocumented. The objective of this work was to investigate whether or not possessing both *Rk1* and *Rk2* resistance genes in tobacco increased resistance to a variant of *M. incognita* race 3 compared to possessing either gene alone.

Materials and Methods

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

To reverify the population identity morphologically, eight female stylets were excised following the procedure outlined by Eisenback (1985) and viewed using a scanning electron microscope. Perineal patterns were cut from 10 mature females following the technique of Eisenback (1985) and viewed using a compound microscope at ×630. To additionally clarify the species identification, gel electrophoresis of EST isozymes was performed on three females. Species-specific sequence-characterized amplified region (SCAR) primers (MiF/MiR, IncK14F/R, Rinc/Rinc) and PCR-DNA sequences on ribosomal RNA (rRNA) 18S, internal transcribed spacer (ITS), 28S, D2/D3, histone, and mitochondrial DNA COII-16S gene were then examined (W. M. Ye, pers. comm.). Additionally, three trials of a greenhouse differential host test were performed in 2014 to 2015 to potentially identify the population to a host race level (Taylor and Sasser, 1978).

Greenhouse trials evaluating resistance genes: Five greenhouse experiments were conducted in 2013 to 2014 to investigate the resistance efficacy of *Rk1* and/or *Rk2* genes in tobacco. Three experiments were carried out at the Virginia Tech campus in Blacksburg, VA, and two at the Virginia Tech Southern Piedmont Agricultural Research and Extension Center in Blackstone, VA. Each experiment was arranged in a completely randomized design with six replications, except for the April to June 2013 trial in Blacksburg, VA, which had seven replications. Six plant entries were evaluated: Coker 371-Gold (C371G; susceptible to the four most common *Meloidogyne* species: *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla*); NC 95 and SC 72 (homozygous for *Rk1*); T-15-1-1 (homozygous for *Rk2*); and STNCB-2-28 and NOD 8 (homozygous for both *Rk1* and *Rk2*). Seedlings with four to six true leaves (~5–10 cm tall) were planted in 15-cm-diam. clay pots with a 2:1 mixture of topsoil (53% sand, 40% silt, 7% clay, pH 5.5) to Profile Greens Grade porous ceramic material (Profile Products LLC, Buffalo Grove, IL). Plants were each inoculated with 5,000 root-knot nematode eggs 1 wk after transplant by pipetting or pouring the egg suspension into two 4-cm deep holes on either side of the plant. Plants were kept in a greenhouse at approximately 20°C to 35°C and grown without supplemental lighting.

Approximately 60 d after inoculation, trials were taken down. Root galling and numbers of egg masses and eggs from roots were compared among entries. Roots were rinsed free of soil and the whole root system was weighed. Galled roots were separated from

nongalled roots and root percent galling was calculated based on the fresh weight of galled roots versus the fresh weight of the entire root system. Roots were recombined, mixed, and divided in half by weight. Half were stained with 0.15 g/liter Phloxine B for 5 min to define egg masses (Dickson and Struble, 1965). Numbers of egg masses from three 1-g subsamples per plant were counted using a dissecting microscope at ×10 to estimate number of females per gram root. Eggs were bleach-extracted from the surface of roots in the second half of each root system following the procedure by Hussey and Barker (1973). Extracted eggs were suspended in 500-ml water and counted in two 10-ml aliquots from each extraction using a compound microscope at ×40. To assess nematode reproductive capability on each entry, the reproductive index (P_f/P_i) was calculated by dividing the final number of eggs extracted per plant (P_f) by the initial number of egg inoculum (P_i) (Sasser et al., 1984).

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

Fig. 1. A–F. Perineal pattern morphology of the *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 race 3 isolate used in this study on resistance in tobacco. G–L. Excised female root-knot nematode stylets, photographed using a scanning electron microscope.

Results

Species identification: The nematode population received had been identified as *M. arenaria* race 2; however, low reproduction on tobacco entry NC 95 suggested another identity. The morphology of the female stylets and perineal patterns (Fig. 1) were not consistent with either *M. arenaria* or *M. incognita,* but more similar to these two species than any other root-knot nematode species.

Results from gel electrophoresis of EST isozymes suggested the population was *M. incognita,* as did PCR-DNA sequences on rRNA 18S, ITS, 28S, D2/D3, histone, and mitochondrial DNA COII-16S genes (W. M. Ye, pers. comm.). Results using the *M. incognita*-specific SCAR primer set MiF/MiR and results from the differential host tests tentatively identified the population as *M. incognita* race 3 (Fig. 2). However, results from the *M. incognita*-specific primer sets IncK14F/R and Finc/Rinc suggested the population was another biotype of *Meloidogyne* (W. M. Ye, pers. comm.).

Evaluation of resistance genes: There were no significant differences in total mean root weights among entries in three of five trials evaluating the effects of resistance genes, and no meaningful data were recovered for total mean root weight in any trials (data not shown).

Significant ($P < 0.001$) differences were observed among entries in mean percent root galling in every trial (Table 1). Percent root galling for the entry with *Rk2* alone (T-15-1-1) was always between 17.0% and

73.1%, whereas galling of the susceptible entry (C371G) was always between 38.5% and 77.1%. Mean percent root galling for T-15-1-1 was never significantly different from that of C371G. Entries containing *Rk1* alone (SC 72 and NC 95) displayed significantly lower galling than susceptible C371G in all trials, and galling was significantly lower than plants with *Rk2* alone in four of five trials ($P \leq 0.05$). Mean percent root galling on entries with the *Rk1* gene was always less than or equal to 7.3%. Plant entries containing both *Rk1* and *Rk2* resistance genes together (NOD 8 and STNCB) always exhibited significantly less galling than the susceptible entry and the *Rk2* entry ($P \leq 0.05$). Differences in galling between entries with *Rk1* and *Rk2* versus those with *Rk1* alone were only statistically significant ($P \leq 0.05$) in Blacksburg April to June 2013, but galling was always numerically lower for entries with both *Rk1* and *Rk2* compared to those with only *Rk1*. Root galling was always less than 1.0% in entries with *Rk1* and *Rk2* together.

Mean egg mass counts were significantly different among entries in every trial ($P < 0.001$). T-15-1-1, with *Rk2* alone, had significantly fewer egg masses per gram root than susceptible C371G in three of five trials, and egg mass numbers were always numerically lower for T-15-1-1 than for C371G ($P \leq 0.05$) (Table 2). Numbers of egg masses for T-15-1-1 ranged from 13 and 74 per gram root across all trials, while between 22 and 137 egg masses per gram root were found on susceptible C371G. Egg masses per gram root were always significantly lower
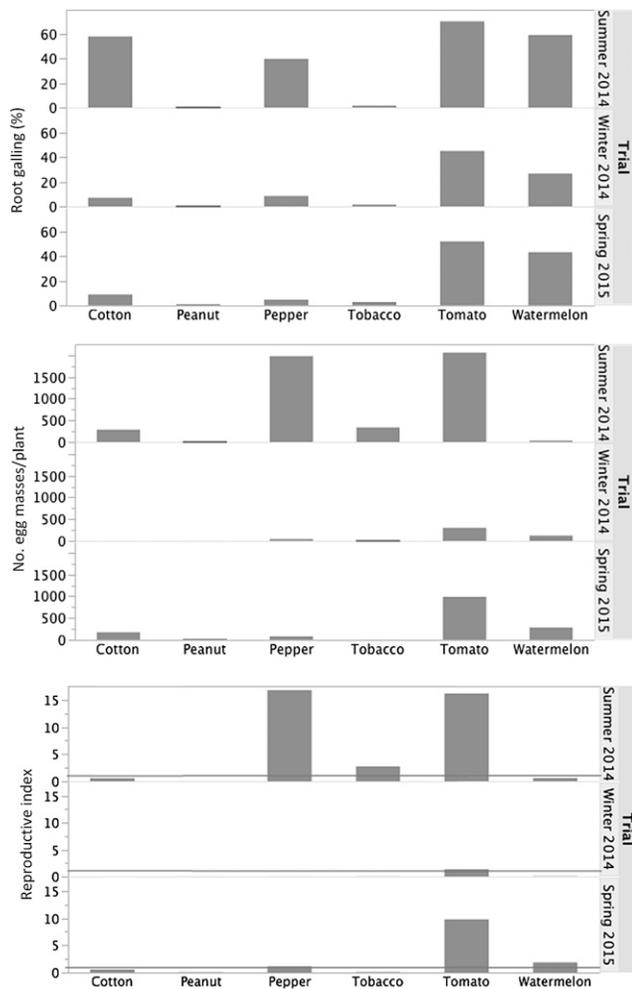
FIG. 2.   Percent root galling, number of egg masses/plant, and the reproductive index of *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 race 3 on differential host plants, across three trials in 2014 to 2015. The horizontal line marks a reproductive index of 1. Tobacco (*Nicotiana tabacum* L.), cotton (*Gossypium hirsutum* L.), pepper (*Capsicum annuum* L.), watermelon [*Citrullus lanatus* var. *lanatus* (Thunb.) Matsum. and Nakai], peanut (*Arachis hypogaea* L.), and tomato (*Solanum lycopersicum* L.).

on entries containing *Rk1* alone (NC 95 and SC 72) compared to susceptible C371G, while significantly fewer egg masses per gram root were observed on NC 95 and SC 72 than on T-15-1-1 (*Rk2*) in four of five trials ($P \leq$ 0.05). Numbers of egg masses per gram root for NC 95 and SC 72 were always between zero and eight throughout the trials. NOD 8 and STNCB 2-28, with both *Rk1* and *Rk2,* always had significantly fewer egg masses per gram root than susceptible C371G, and fewer than the *Rk2* entry T-15-1-1 in all trials except Blacksburg April to June 2013 ($P \leq$ 0.05). *Rk1Rk2* entries exhibited significantly fewer egg masses per gram root than entries possessing *Rk1* alone in Blackstone September to November 2013 and Blackstone May to July 2014 ($P \leq$ 0.05).

Significant differences were observed in mean egg count per gram root among entries in every trial ($P < 0.001$).

Although eggs per gram root were always numerically lower on T-15-1-1, containing the *Rk2* gene alone, compared to susceptible C371G, this trend was statistically significant only in Blacksburg April to June 2013 ($P \leq 0.05$) (Table 2). Significantly fewer eggs per gram of root were always noted on entries possessing *Rk1* gene alone compared to susceptible C371G, and significantly fewer than for T-15-1-1 in three of five trials ($P \leq 0.05$). In Blacksburg April to June 2013, fewer eggs per gram root were extracted from T-15-1-1 than from roots of entries possessing only *Rk1*. Significantly fewer eggs per gram root were enumerated from the *Rk1Rk2* entries NOD 8 and STNCB 2-28 than from T-15-1-1 in three of five trials ($P \leq 0.05$). Significantly fewer eggs were also counted per gram root on NOD 8 and STNCB 2-28 compared to the *Rk1* entries NC 95 and SC 72 in the Blacksburg April to Jun 2014 and Blackstone May to July 2014 trials ($P \leq 0.05$).

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

DISCUSSION

Despite variability in our results, entries with both *Rk1* and *Rk2* (*Rk1Rk2*) conferred greater resistance to root-knot nematodes than entries with *Rk1* or *Rk2* alone, corroborating Smeeton's observations that *Rk1* and *Rk2* together conferred higher resistance to *M. javanica* than either gene alone (Ternouth et al., 1986). Results of experiments performed at Virginia Tech in 2010 to 2011 examining reproduction of *M. javanica* on the same flue-cured tobacco entries used in this experiment (C371G, T-15-1-1, SC 72, NC 95, NOD 8, and STNCB-2-28) support these results (Johnson et al., 2012). In those experiments, as in our current investigations, root galling, egg masses per gram root, and eggs per gram root on NOD8 and STNCB 2-28 (with *Rk1Rk2*) were significantly reduced compared to those

TABLE 1.   Mean percent root galling of six tobacco (*Nicotiana tabacum* L.) entries inoculated with a variant of *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 race 3 from five greenhouse trials conducted in 2013 to 2014.

Plant entry	Resistance genes	Blacksburg April–June 2014	Blackstone May–July 2014	Blackstone September–November 2013	Blacksburg April–June 2013	Blacksburg November 2013–January 2014
		Root galling (%)[a] by trial				
C371G	None	77.1 a	67.0 a	46.1 a	38.5 a	53.3 a
T-15-1-1	*Rk2*	73.1 a	71.6 a	48.0 a	17.0 ab	50.1 a
SC 72	*Rk1*	0.2 b	0.4 b	2.4 b	7.3 bc	2.0 b
NC 95	*Rk1*	0.6 b	0.4 b	1.5 b	6.4 c	0.6 b
NOD 8	*Rk1Rk2*	0.0 b	0.1 b	0.3 b	0.0 d	0.2 b
STNCB-2-28	*Rk1Rk2*	0.0 b	0.0 b	0.2 b	0.7 d	0.0 b

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

on NC 95 and SC 72 (with *Rk1* alone) or T-15-1-1 (with *Rk2* alone), in two of three greenhouse trials. Similar results were also observed in a 2014 field study in a flue-cured tobacco field infested with *M. arenaria* in Mecklenburg County, VA, in which root galling was compared among cultivars and breeding lines varying in *Rk1* and/or *Rk2*. Cultivars used in the Mecklenburg County trial were the same as those used in our experiment. Galling was significantly lower on cultivars possessing both *Rk1Rk2* than on the susceptible control, C371G; cultivars with both *Rk1* and *Rk2* had the lowest percent galling of any entries in the experiment, which included entries possessing *Rk1* alone and *Rk2* alone (Pollok et al., 2015).

The role of *Rk2* in suppressing root-knot nematode reproduction may be quite different than that of *Rk1*. Ternouth et al. (1986) concluded that resistance to

M. javanica conferred by *Rk2* was greater than that provided by the *Rk1* gene. Results from a 2010 to 2011 Virginia Tech greenhouse study support this to an extent, where *M. javanica* egg masses and eggs per gram root were significantly reduced in *Rk2* plants versus *Rk1* plants in one of three greenhouse trials (Johnson et al., 2012). In our results, *Rk2* alone often reduced parasitism and reproduction by a variant of *M. incognita* race 3 compared to the susceptible entry, but reductions were generally less than those associated with *Rk1* alone. Ng'ambi et al. (1999b) noted that the extent of nematode reproduction was generally consistent with that of root galling, but we always observed similar galling on *Rk2* compared to the susceptible control. Galling of *Rk2* plants was also similar to that on the susceptible entry in one of three *M. javanica* greenhouse trials in 2010 to 2011 (Johnson et al., 2012). T-15-1-1 (*Rk2*) did not

TABLE 2.   Egg masses per gram root, eggs per gram root, and reproduction of a variant of *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 race 3 on six tobacco (*Nicotiana tabacum* L.) entries grown in five greenhouse trials conducted in 2013 to 2014.

Plant entry	Resistance genes	Blacksburg April–June 2014	Blackstone May–July 2014	Blackstone September–November 2013	Blacksburg April–June 2013	Blacksburg November 2013–January 2014
		No. of egg masses per gram root[a] by trial				
C371G	None	137 a	22 a	55 a	59 a	47 a
T-15-1-1	*Rk2*	74 b	17 a	48 a	13 b	15 b
SC 72	*Rk1*	1 c	2 b	7 b	8 bc	0 c
NC 95	*Rk1*	1 c	1 b	5 b	4 cd	1 c
NOD 8	*Rk1Rk2*	0 d	0 c	0 c	2 de	0 c
STNCB-2-28	*Rk1Rk2*	0 cd	0 c	0 c	1 e	0 c
		No. of eggs per gram root[a] by trial				
C371G	None	8,541 a	2,630 a	1,899 a	1,821 a	452 a
T-15-1-1	*Rk2*	5,372 a	1,690 a	751 a	140 bcd	54 ab
SC 72	*Rk1*	188 b	285 b	37 b	366 b	2 c
NC 95	*Rk1*	157 b	203 b	33 b	356 bc	6 bc
NOD 8	*Rk1Rk2*	22 d	42 c	9 b	59 cd	5 c
STNCB-2-28	*Rk1Rk2*	60 c	55 c	5 b	31 c	18 bc
		Reproductive index[a,b] by trial				
C371G	None	88.5 a	48.3 a	15.6 a	18.9 a	2.1 a
T-15-1-1	*Rk2*	63.8 a	34.0 a	7.1 a	2.6 b	**0.2 b**
SC 72	*Rk1*	1.9 b	4.8 b	**0.3 b**	4.4 b	**0.0 b**
NC 95	*Rk1*	1.6 b	3.4 b	**0.3 b**	4.6 b	**0.0 b**
NOD 8	*Rk1Rk2*	**0.2 c**	**0.7 c**	**0.1 b**	**0.5 c**	**0.0 b**
STNCB-2-28	*Rk1Rk2*	**0.4 c**	**0.9 c**	**0.0 b**	**0.3 b**	**0.1 b**

[a] Means followed by the same letter(s) are not significantly different according to statistical analysis of transformed ($\log_{10}$ [x + 1]) data and the Tukey–Kramer honest significant difference test ($P = 0.05$).
[b] Reproductive index = final population/initial population (P_F/P_i). Values less than 1.0 are accented with bold text.

significantly suppress galling by *M. arenaria* compared to the susceptible control in a 2014 field experiment (Pollok et al., 2015). Considerable root galling on *Rk2* plants, yet reduced reproduction, suggests that some nematodes are able to enter root tips, feed and develop, but not reproduce.

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

Two quantitative trait loci (QTLs) recently discovered in cotton each confer only moderate resistance to *M. incognita* when present alone, but when present together confer near immunity (Gutiérrez et al., 2010; He et al., 2014; Batista da Silva et al., 2015). Both QTLs reduced nematode egg production, but *qMi-C11* reduced galling, while *qMi114* did not (He et al., 2014). The authors hypothesized that *qMi-C11* may confer an early hypersensitive reaction that prevents giant cell and gall formation, while *qMi114* may confer a later response in developing giant cells that does not stop gall formation but does block subsequent nematode development and reproduction (He et al., 2014). Resistance genes in soybean to reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) have also shown positive epistatic effects when combined (Ha et al., 2007). These recent studies into epistatic nematode resistance gene effects in cotton and soybean suggest the need for additional research into epistatic effects in nematode resistance in tobacco. Additional research to compare mechanisms involved in how *Rk1* inhibits galling and reproduction of races 1 and 3 of *M. incognita*, to how *Rk2* reduces reproduction of other *Meloidogyne* species, but not galling, could reveal important and useful aspects of host resistance to root-knot nematodes in tobacco.

Alternatively, feeding and/or reproduction may be simply slowed, which might explain why a number of nematodes were still able to produce egg masses and eggs. In tomato, a change in root exudates significantly reduced root penetration by *M. incognita* juveniles (Vos et al., 2012). However, the exudate change was attributed to root colonization by arbuscular mycorrhizal fungi, not a resistance gene. Conversely, Ye et al. (2009) observed a change in root structure in resistant rootstocks of *Prunus* spp. that prevented *M. incognita* from penetrating roots. Elucidating specific mechanism(s) of resistance conferred by *Rk1Rk2* as a hypersensitive response, modification of root exudates, or possibly an alteration of root composition itself could have significant implications for improving nematode management on tobacco.

The population used in these experiments was identified as a variant of *M. incognita* race 3 despite some inconsistent results. Our population reacted negatively to IncK14F/R and Finc/Rinc *M. incognita*-specific primers, but Adam et al. (2007) noted primers IncK14F/R and Finc/Rinc did not always produce consistent results in their study. Similarly, variable results were also obtained from three host specificity assays to identify the species to race. Reproduction during the Winter 2014 trial was low for all plant hosts, presumably due to winter low-light conditions (Witzenberger et al., 1988; Gislerød et al., 1989; Meng et al., 2015). Results between Summer 2014 and Spring 2015 trials varied considerably, and reproduction was very low on pepper, cotton, tobacco, and peanut in the Spring 2015 trial. Despite the reproductive index of cotton and watermelon being less than one in at least two trials, those hosts were designated as susceptible based on egg mass numbers and the amount of root galling. Variation in host specificity exemplifies the difficulty in identifying root-knot nematode populations to race. For example, Robertson et al. (2009) analyzed 140 root-knot nematode populations from Spain and noted six were *M. incognita* and able to reproduce on tomato, but not pepper, cotton, tobacco, or peanut, and labeled it as *M. incognita* race 5. Others have also noted a great deal of host variation between populations (Eisenback et al., 1981; Kirkpatrick and Sasser, 1983; Hartman and Sasser, 1985; Barker and Melton, 1990; Noe, 1992). Similarly, 2004 and 2010 nematode surveys of tobacco fields in Southside Virginia resulted in 8.3% and 6.3% of unidentifiable *Meloidogyne* populations, respectively (Eisenback, 2012). Perineal pattern morphology can differ between and among populations, which might explain the variable results in the survey (Netscher, 1978; Eisenback et al., 1980).

Rk1 is effective in providing resistance to *M. incognita* races 1 and 3 and *M. arenaria* race 1 (Barker and Melton, 1990; Ng'ambi et al., 1999a, 1999b). The drastic reduction of reproduction on plants with *Rk1* compared to the susceptible entry confirms these results, since our population was established to be a variant of *M. incognita* race 3. Varying results of resistance to *M. javanica* caused by the *Rk1* gene have been reported.

Ng'ambi et al. (1999b) noted that *M. incognita* races 2 and 4, *M. arenaria* race 2, and *M. javanica* caused significant galling on flue-cured tobacco cv. Speight G 28 (*Rk1*), similar to Barker and Melton's (1990) observation of a "slight level of resistance (in cultivars with *Rk1*) to *M. javanica* compared to susceptible cultivars." Conversely, a significant reduction in *M. javanica* egg masses, eggs, and the reproductive index on plants with *Rk1* alone were observed in the experiments performed at Virginia Tech in 2010 to 2011 (Johnson et al., 2012).

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

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

Nematode management can be critical to producing a satisfactory flue-cured tobacco crop. Shifts in nematode population structure due to deployment of species-specific host resistance are increasing the need for cultivars with resistance to multiple nematodes, especially species and races of *Meloidogyne*. Flue-cured tobacco cultivars that possess both *Rk1* and *Rk2* will provide broader and greater resistance to root-knot nematodes than either gene alone, providing growers with a valuable tool for managing root-knot nematode populations.

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