Resistance Quantitative Trait Loci *qMi-C11* and *qMi-C14* in Cotton Have Different Effects on the Development of *Meloidogyne incognita*, the Southern Root-Knot Nematode

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Abstract

Quantitative trait loci (QTLs) *qMi-C11* and *qMi-C14* impart a high level of resistance to *Meloidogyne incognita* in cotton. Breeders had previously backcrossed both QTLs into the susceptible Coker 201 to create the highly resistant M-120 RNR, and we crossed Coker 201 and M-120 RNR to create near-isogenic lines with either *qMi-C11* or *qMi-C14*. Previous work suggests different modes of action for *qMi-C11* and *qMi-C14*. To document individual and combined effects of the QTLs on nematode development and reproduction, Coker 201 (neither QTL), M-120 RNR (both QTLs), CH11 near isolate (*qMi-C11*), and CH14 near isolate (*qMi-C14*) were inoculated with *M. incognita*. At 4, 8, 12, 16, 20, 25, and 30 days after inoculation (DAI), roots were stained to observe nematode developmental stages (second-stage juvenile [J2], swollen second-stage juvenile [SJ2], third-stage juvenile [J3], fourth-stage juvenile [J4], and female), and the number of galls was counted. At 20, 25, 30, and 40 DAI, *M. incognita* eggs were harvested and counted. At 30 DAI,

80% of the nematodes on Coker 201 were female compared with 50, 40, and 33% females on CH14, CH11, and M-120 RNR, respectively, and greater proportions of nematodes remained in J2 in M-120 RNR (41%), CH11 (58%), and CH14 (27%) than in Coker 201 (9%). More nematodes progressed to J3 or J4 on Coker 201 and CH14 than on CH11 or M-120 RNR. Coker 201 had more eggs than the other genotypes at 30 DAI. Nematode development beyond J2 or SJ2 was significantly reduced by *qMi-C11*, and development beyond J3 or J4 was significantly reduced by *qMi-C14*. This study confirms that *qMi-C11* and *qMi-C14* act at different times and have different effects on the development of *M. incognita*, and therefore, they have different modes of action.

Keywords: causal agent, crop type, cultivar, disease management, field crops

The United States is the third largest producer of cotton in the world, with an average lint yield of 1,017 kg/ha (4,554 million hg total harvest) in 2017 (U.S. Department of Agriculture Foreign Agricultural Service 2018). *Meloidogyne incognita*, the southern root-knot nematode, is the most economically important pathogen of cotton, and it is estimated to inflict losses of 3.4% (107 million kg), valued at \$147 million across the U.S. cotton belt (Lawrence et al. 2015). The most commonly used management options to control *M. incognita* in cotton in the United States are nematicides, which may be applied as seed treatments, in-furrow applications at planting, or preplant fumigation (Burris et al. 2010; Davis and May 2005; Wheeler et al. 2013). Crop rotation with poor or nonhost crops is not a viable option for growers in some locations because of limited hectareage or profitability of effective rotation crops, and nematicides are typically expensive and do not provide season-long control. The best option for cost-effective, long-term management is the use of highly resistant varieties. Cotton varieties with a high level of resistance to *M. incognita* were first available to farmers in 2014; however, only 4% of the fields in the United States were planted with

resistant varieties in 2017 (U.S. Department of Agriculture Agricultural Marketing Service 2017).

The primary *M. incognita*-resistant cotton lines used in breeding programs derive their resistance from Auburn 623 RNR (Gutiérrez et al. 2010; Jenkins et al. 1995; Klump and Thomas 1987; Shen et al. 2006), which carries genes imparting a high level of resistance to *M. incognita* and *Fusarium* wilt (Shepherd 1982). Auburn 623 RNR was the progeny from a cross between Clevevilt-6 and Wild Mexican Jack Jones, two cotton genotypes with moderate resistance to *M. incognita* (Shepherd 1974). Auburn 623 RNR was the resistance source for Auburn 634 RNR, and Auburn 634 RNR was used to develop a number of *M. incognita*-resistant lines referred to as the M-line series. All of the M lines developed from Auburn 634 RNR are highly resistant to *M. incognita* (Shepherd et al. 1996). M lines, such as M-315 RNR and M-120 RNR, have been instrumental in studying *M. incognita* resistance in cotton. McPherson et al. (2004) first suggested that there were two resistance genes in the M lines, and two major quantitative trait loci (QTLs) controlling *M. incognita* resistance in the M lines were subsequently identified on chromosomes 11 and 14 (Shen et al. 2006; Ynturi et al. 2006). A resistance QTL on the same region of chromosome 11 as identified in the M lines was also identified in Acala NemX (Wang et al. 2006); however, it has not been determined whether the QTL in Acala NemX has the same resistance gene(s) as the M lines. The resistance QTLs in the M lines on chromosomes 11 (*qMi-C11*) and 14 (*qMi-C14*) are inherited from Clevevilt-6 and Wild Mexican Jack Jones, respectively (Gutiérrez et al. 2010; Shen et al. 2006). Cotton genotypes with both *qMi-C11* and *qMi-C14* are highly resistant to *M. incognita*.

Resistance to *M. incognita* in cotton in the germplasm line M-315 RNR is owing to postpenetration interference with nematode development (Jenkins et al. 1995). In the nematode lifecycle, *M. incognita* eggs hatch to release second-stage juveniles (J2), the infective stage of the nematode, which penetrate into roots and establish feeding sites. J2s that successfully establish feeding sites become sedentary, slightly swell (swollen second-stage juvenile [SJ2]), and then

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Funding: This research was conducted with financial support from Cotton Incorporated and the Georgia Cotton Commission.

The author(s) declare no conflict of interest.

Accepted for publication 4 October 2018.

develop through two additional juvenile stages (third-stage juvenile [J3] and fourth-stage juvenile [J4]) before becoming adults (Gheysen and Jones 2006; Taylor and Sasser 1978). Interference with *M. incognita* development in resistant cotton compared with a susceptible genotype has been reported at both 6 to 8 days after inoculation (DAI) and 18 to 24 DAI (Jenkins et al. 1995; McClure et al. 1974; McPherson et al. 2004; Mota et al. 2013), which approximately coincide with the transition from SJ2 to J3 and with egg production by females, respectively. In cotton populations with both resistance QTLs that were segregating for resistance to *M. incognita*, *qMi-C11* had a significant association with a reduction in root galling and egg production, whereas *qMi-C14* had little effect on galling but had a significant association with a reduction in egg production (Gutiérrez et al. 2010; He et al. 2014).

The development of near-isogenic lines differing for the resistance QTLs *qMi-C11* and *qMi-C14* would allow for the evaluation of the individual effects of each resistance QTL on *M. incognita* development and reproduction and on the plant's response to infection by the nematode. The genotype Coker 201 has neither QTL, but it was utilized as the *M. incognita*-susceptible recurrent parent to cross with the resistance source Auburn 634 RNR, and progeny from that cross that displayed high levels of resistance to *M. incognita* were backcrossed six times to Coker 201 before a plant was self-pollinated and released as M-120 RNR (Shepherd et al. 1996). In our study, M-120 RNR and Coker 201 were crossed to create Backcross BC₇F₁ seeds that were self-pollinated to obtain a BC₇F₂ population in which *qMi-C11* and *qMi-C14* were segregating. Simple sequence repeat (SSR) markers tightly linked to *qMi-C11* and *qMi-C14* (Kumar et al. 2016) were used to genotype the BC₇F₂ population and identify plants that carried only one of two QTLs. Plants homozygous for the resistance QTLs on either chromosome 11 or 14 were self-pollinated and advanced in the greenhouse to recover isogenic lines that carry either *qMi-C11* or *qMi-C14*. The objective of this study was to characterize the effects of *qMi-C11* and *qMi-C14* together and individually on *M. incognita* development and reproduction in cotton isogenic lines.

Materials and Methods

An experiment was conducted in the greenhouse to evaluate nematode reproduction and development on isogenic cotton lines that differed in QTLs for resistance to *M. incognita*. Four cotton genotypes were evaluated: an isogenic line with the resistance QTL *qMi-C11* (CH11), an isogenic line with the resistance QTL *qMi-C14* (CH14), the susceptible variety Coker 201 (with neither QTL), and the resistant line M-120 RNR (with both *qMi-C11* and *qMi-C14*). Cotton seeds were germinated in vermiculite and grown for 2 weeks. Seedlings of similar size then were transplanted, one per pot, into 10.6 × 10.6 × 12.4-cm pots filled with approximately 500 ml of steam-pasteurized soil (Tifton loamy sand). At transplant, seedlings were infected with 4,000 *M. incognita* J2 per plant. Nematode inoculum from greenhouse cultures was obtained from eggplant (*Solanum melongena*) roots that were washed free of soil and placed in a mist chamber for 5 days to obtain J2 from egg hatch. Pots were grouped by sampling date, and genotypes within a sampling date were arranged in a completely randomized design with six replications per genotype per sample date. The experiment was conducted twice, with one test initiated on 16 March 2015 and the other initiated on 22 June 2015.

The developmental progression of *M. incognita* was evaluated at 4, 8, 12, 16, 20, 25, and 30 DAI on each of the four genotypes. On each sample date, roots were stained using a slightly modified method of Byrd et al. (1983), and the numbers of J2, SJ2, J3, J4, and mature females inside the roots were counted. Roots were carefully cleaned by immersing the soil in water and gently washing them free of soil. Individual root systems were soaked in 30 ml of bleach solution (5.25% NaOCl) for 4 min and then rinsed under running tap water before soaking for 15 min in 30 ml of water. Roots were then placed in 30 ml of tap water plus 1 ml of cotton blue solution (0.35 g of cotton blue powder in 250 ml of lactic acid and 750 ml of distilled water), and the roots in stain solution were microwaved just until

the solution started boiling (approximately 10 s). Roots were destained by rinsing in tap water to remove excess stain and then soaking the roots in nonacidified glycerin. The roots and glycerin were microwaved just until the glycerin began to boil. After the glycerin cooled to room temperature, roots were observed with an inverted microscope, and the numbers of nematodes at each developmental stage were recorded. Before staining, each plant also was assessed for the number of root galls at 25, 30, and 40 DAI and total egg production at 20, 25, 30, and 40 DAI. Eggs were extracted by washing roots free of soil and shaking the root system in a beaker with 1.23% NaOCl for 4 min and collected eggs on a 500-mesh sieve.

The number of nematodes observed at each developmental stage (J2 + SJ2, J3 + J4, or female) also was expressed as a percentage of the total number of nematodes observed (J2 + SJ2 + J3 + J4 + female). Data for all variables were analyzed for differences among cotton genotypes using the PROC MIXED procedure in SAS 9.3 statistical software (SAS Institute Inc., Cary, NC). No interactions were seen between trial and genotype for the number or percentage of nematodes at each developmental classification (total number, J2 + SJ2, J3 + J4, and female). Therefore, the data from both trials were combined for analysis. Similarly, data from the two trials for egg counts and gall numbers were combined for analysis. Egg counts were log₁₀ transformed before analysis.

Results

The total number of nematodes observed increased greatly at 40 DAI, because a second generation of *M. incognita* had been produced, and the generations could not be distinguished. Therefore, the developmental stages of the nematodes at 40 DAI are not shown. The total number of nematodes (sum of all developmental stages) observed in the roots did not differ among the genotypes at 4 and 8 DAI (Fig. 1A). The total number of nematodes observed in the roots did not differ between Coker 201 and CH14 on any sampling date before 30 DAI. The total numbers of nematodes in the roots did not differ between M-120 RNR and CH11 on any sampling date (Fig. 1A). However, differences in total numbers of nematodes between Coker 201 and the genotypes carrying *qMi-C11* (CH11 and M-120 RNR) were observed. Coker 201 had more total nematode numbers in the roots than CH11 at 12, 16, 20, 25, and 30 DAI and more total nematode numbers in the roots than M-120 RNR at 12 DAI. Although M-120 RNR typically had numerically fewer total nematode numbers than other genotypes, statistical differences were not found between M-120 RNR and CH11.

The number of nematodes in the J2 + SJ2 did not differ among genotypes at either 4 or 8 DAI (Fig. 1B). The number of J2 + SJ2 in the roots of Coker 201 and CH14 did not differ on any sampling date. Coker 201 and CH14 had greater J2 + SJ2 counts than either CH11 or M-120 RNR only at 12 DAI. M-120 RNR had numerically fewer J2 + SJ2 than Coker 201 and CH14 on all sampling dates, and these two lines had statistically different numbers of J2 + SJ2 at 12, 16, and 20 DAI. The J2 + SJ2 counts in the roots differed between M-120 RNR and CH11 only at 20 DAI when CH11 had higher nematode counts (Fig. 1B).

Differences in the combined counts of J3 + J4 were first observed at 8 DAI (Fig. 1C). Coker 201 and CH14 had similar J3 + J4 levels on all sampling dates. However, Coker 201 had higher J3 + J4 levels at 8, 16, 20, 25, and 30 DAI than either CH11 or M-120 RNR, and CH14 had greater J3 + J4 counts than either CH11 or M-120 RNR from 12 to 30 DAI (Fig. 1C). M-120 RNR and CH11 had relatively low levels of J3 + J4, and they did not differ statistically from each other on any sampling date. J3 + J4 counts reached a high of 50 nematodes in Coker 201 and CH14 at 20 DAI, but counts did not exceed 20 nematodes for CH11 or M-120 RNR.

Females were first observed at 12 DAI. Coker 201 had numerically more females than the other genotypes on all sampling dates thereafter (Fig. 1D). Beginning 16 DAI, the number of females was statistically greater on Coker 201 than on CH11 or M-120 RNR, whereas the number of females on CH14 was similar to that on Coker 201 on all sampling dates except at 30 DAI, when CH14 had fewer females than Coker 201. The numbers of females on M-120 RNR

and CH11 did not differ statistically from each other on any sampling date, with relatively low levels (<40 females) found on both genotypes. CH14 was similar to Coker 201 and CH11 was similar to M-120 RNR in the number of females, and although CH14 had more females numerically than CH11 from 16 to 30 DAI, CH11 only differed statistically from CH14 at 30 DAI.

The number of J2 + SJ2 in CH14 as a percentage of the total number of all developmental stages was statistically similar to Coker 201 on all sample dates, and the percentage of nematodes in J2 + SJ2 in CH11 was similar to M-120 RNR on all sampling dates. The percentage of nematodes at J2 + SJ2 was smaller for Coker 201 than CH11 and M-120 RNR after 12 DAI. At 30 DAI, the percentage of nematodes in the J2 + SJ2 was the least in Coker 201 at only 8% compared

with 45, 18, and 37% for CH11, CH14, and M-120 RNR, respectively (Fig. 2A). The minimum percentages of nematodes in the J2 + SJ2 on any sampling date were 8% for Coker 201, 44% for CH11, 18% for CH14, and 37% for M-120 RNR (Fig. 2A). CH11 had a greater percentage of J2 + SJ2 than CH14 at 20, 25, and 30 DAI.

The number of J3 + J4 as a percentage of the total number of nematodes for Coker 201 only differed from the percentage in CH14 at 30 DAI when CH14 had a greater percentage of J3 + J4 than Coker 201 (Fig. 2B). The percentage of nematodes at J3 + J4 on M-120 RNR was lower than that on Coker 201 at 20 and 25 DAI. CH11 and CH14 did not differ statistically except at 30 DAI, when the percentage of nematodes at J3 + J4 on CH14 was greater than that on CH11.

Fig. 1. Number of *Meloidogyne incognita* at different developmental stages in the roots of cotton near isolines that differ in *M. incognita* resistance genes. **A**, Total numbers are the sum of all development stages. **B**, Second-stage juvenile (J2) + swollen second-stage juvenile (SJ2). **C**, Third-stage juvenile (J3) + fourth-stage juvenile (J4). **D**, Females. Different letters over bars within a sampling date indicate significant differences at $\alpha = 0.05$ according to Tukey's test. DAI = day after inoculation, NS = not significant.

Females were a greater percentage of the total number of nematodes for Coker 201 than for CH11 and M-120 RNR beginning at 16 DAI and continuing thereafter. At 30 DAI, 80% of the nematodes on Coker 201 were female, whereas CH14, CH11, and M-120 RNR

had just 50, 40, and 33% females, respectively. The percentages of nematodes that were females did not differ among CH14, CH11, and M-120 RNR except at 25 DAI (Fig. 2C). The evaluation at 40 DAI had the developmental stages confounded by a second *M. incognita* generation resulting from first generation eggs that hatched; therefore, those data are not presented.

Total egg production and the total number of galls per root system also were evaluated. Coker 201 and CH14 had similar numbers of galls per root system at 25, 30, and 40 DAI, and M-120 RNR and CH11 also had similar levels of galling (Fig. 3). Both CH11 and M-120 RNR had fewer galls than Coker 201 at 30 and 40 DAI. Coker 201 supported greater egg production than the other genotypes at both 30 and 40 DAI (Fig. 4). CH11 and CH14 had statistically similar levels of egg production at both 30 and 40 DAI, despite large numerical differences at 30 DAI.

Discussion

Previous research documented that resistance to *M. incognita* in cotton from the Auburn 623 RNR source is expressed at two stages of nematode development and that there is an epistatic interaction between the two QTLs (He et al. 2014; Jenkins et al. 1995). Both of those findings suggest the QTLs have different modes of action. Independently, the two QTLs, *qMi-C11* and *qMi-C14*, each confer partial resistance to *M. incognita*. Our study was designed to allow evaluation of the individual effects of the QTLs without the confounding effects of different genetic backgrounds.

The data in our study for J2 + SJ2 and J3 + J4 were combined for analysis because of the amount of variability observed in the individual stages: combining the stages made trends more evident and increased our ability to show that numerical differences were statistically significant. An increase in total nematode numbers was observed between 4 and 20 DAI on all genotypes, and then, nematode levels remained steady or declined until a second generation of *M. incognita* became evident at 40 DAI. Previous studies also showed a decrease in total nematode numbers after 20 DAI for some resistant cotton lines and resistant melon (*Cucumis melo*) accessions (Faske 2013; Faske and Starr 2009; Jenkins et al. 1995; Stetina 2015).

Although *M. incognita* stages were easily identifiable inside the roots after staining, the total nematode numbers and J2 + SJ2 percentage that we observed may have been affected by mechanical handling during our extraction procedure. Nematodes may have been lost on the early sampling dates, because root tips and surrounding tissues of young seedlings, where nematodes would be soon after

Fig. 2. *Meloidogyne incognita* at different developmental stages (expressed as percentage of the total number of nematodes) in the roots of cotton near isolines that differ in *M. incognita* resistance genes. A, Second-stage juvenile (J2) + swollen second-stage juvenile (SJ2). B, Third-stage juvenile (J3) + fourth-stage juvenile (J4). C, Females. Different letters over bars within a sampling date indicate significant differences at $\alpha = 0.05$ according to Tukey's test. DAI = days after inoculation, NS = not significant.

Fig. 3. Number of galls presented on the roots of cotton near isolines that differ in *Meloidogyne incognita* resistance genes at 25, 30, and 40 days after inoculation (DAI). Different letters over bars within a sampling date indicate significant differences at $\alpha = 0.05$ according to Tukey's test.

penetration, are delicate and prone to being broken off when roots are removed from soil. As seedlings age, the sections of the root containing nematodes strengthen and do not break off as readily, resulting in less loss of infected root tissue and an apparent increase in the total number of nematodes. Another possibility is that new infections may occur many DAI.

The numbers of J2 + SJ2 were similar for all genotypes at 4 and 8 DAI, which suggests that the resistance QTLs do not affect the entrance of *M. incognita* into the roots. Previous research also found that penetration of *M. incognita* is not affected by the resistance genes from the Auburn 623 RNR source (Creech et al. 1995; Faske and Starr 2009; McClure et al. 1974). Although the total number of nematodes in the roots in our study increased by a relatively large amount after inoculation until 20 DAI, the number of J2 + SJ2 increased only modestly after 8 DAI, because the increased ability to observe J2s as young roots matured was countered by nematodes maturing into J3s and later stages. The actual counts of J2s + SJ2s increased slightly through 20 DAI; however, the J2 + SJ2 as a percentage of the total declined for all genotypes as some nematodes matured. The decline in percentage was more rapid for Coker 201 and CH14, resulting in a lower percentage of J2 + SJ2 on those genotypes. The greater percentage of J2 + SJ2 in M-120 RNR and CH11 indicates that fewer nematodes progress to J3 in plants with *qMi-C11*, which confirms the conclusions from previous studies of the Auburn 623 resistance source (Faske and Starr 2009; Jenkins et al. 1995). The lower numbers of J3 + J4 on M-120 RNR and CH11 may indicate that some J2s leave the roots or die after penetration instead of progressing to further stages, whereas they continue to develop on CH14 or Coker 201. Emigration of *Meloidogyne* spp. from the roots of resistant plants when J2s fail to establish feeding sites has been reported for melon and soybean accessions (Faske 2013; Pedrosa et al. 1996). Nematodes that remain in the roots but fail to successfully establish a feeding site will die, and they will not be observed and counted. Egression and death are the likely causes for the lower total number of nematodes observed on M-120 RNR and CH11.

The J3 + J4 counts increased through 20 DAI and then decreased for all genotypes, but the changes were greater for Coker 201 and CH14, because more nematodes progressed from SJ2 to J3. Although the numbers of J3 + J4 were greater for Coker 201 and CH14 than for M-120 RNR and CH11, J3 + J4 as a percentage of the total did not differ consistently among the genotypes. However, at 30 DAI, both cotton genotypes containing *qMi-C14* had a larger percentage (statistically for CH14 and numerically for M-120 RNR) of J3 + J4 than

the genotypes that did not have that QTL, because apparently, *qMi-C14* prevents some nematodes from developing into females. From the time that plants were inoculated until 25 DAI, CH14 was indistinguishable from the susceptible Coker 201 in the total number of nematodes, the number of nematodes at each developmental stage, or the proportion of nematodes at the various developmental stages. However, by 30 DAI, Coker 201 had approximately twice as many females as CH14, resulting in a greater proportion of nematodes on Coker 201 reaching the female stage, because *qMi-C14* stopped many nematodes from developing into females on CH14.

In a normal *M. incognita* lifecycle, a J2 penetrates near a root tip and establishes a feeding site by causing giant cells to form that ultimately result in a gall on the root (Miyashita et al. 2014). After a feeding site is successfully established, the nematode can progress through the developmental stages and produce eggs. Resistance genes reduce nematode reproduction by interfering with some element of that process. In our study, cotton genotypes containing *qMi-C11* had less galling than genotypes without that QTL. Therefore, we conclude that *qMi-C11* interferes with the successful establishment of a feeding site, thereby ultimately resulting in fewer nematodes maturing and producing eggs. We also observed on genotypes containing the *qMi-C11* locus a few galls in which no nematodes were observed, a phenomenon previously observed and labeled “empty galls” (McClure et al. 1974). It is not known whether the nematodes that initiate the empty galls die and disintegrate, move to establish a feeding site elsewhere, or leave the root.

Both QTLs inhibit the development of *M. incognita*: *qMi-C11* retards early nematode development, and *qMi-C14* retards J4 to female development. Subsequently, both QTLs reduce egg production compared with that measured in a susceptible line. Jenkins et al. (1995) had also observed reduced progression of nematodes in both the early and late stages of the *M. incognita* lifecycle. Egg production in genotypes containing *qMi-C11* and *qMi-C14* shows that, although those lines have significant resistance to *M. incognita*, they are not immune.

Creech et al. (1995) and Jenkins et al. (1995) documented inhibited nematode development, egg production, and gall production in M-315 RNR, which contains both resistance genes from the Auburn 623 RNR source of resistance, and they speculated that the resistance gene derived from Cleve wilt (now known to be associated with *qMi-C11*) affected nematode development at around 6 DAI and that the gene derived from Wild Mexican Jack Jones (now known to be associated with *qMi-C14*) was responsible for affecting development around 24 DAI. Later research documented positive epistatic effects between *qMi-C11* and *qMi-C14*, resulting in greater than additive effects (He et al. 2014), which means that the individual effects of each resistance QTL are best studied without the influence of the other QTL being present. Our study utilized near isolines that separated the QTLs and proved that *qMi-C11* and *qMi-C14* act at different times and have different effects on the development of *M. incognita* in cotton. Therefore, we conclude that *qMi-C11* and *qMi-C14* have different modes of action. Combining (pyramiding) resistance genes with different modes of action into a single genotype is likely the best method of improving disease control and prolonging the usefulness of the resistance genes by minimizing selection pressure on the pathogen (Djjan-Caporalino et al. 2014).

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Fig. 4. Number of eggs harvested from the roots of cotton near isolines that differ in *Meloidogyne incognita* resistance genes at 20, 25, 30, and 40 days after inoculation (DAI). Different letters over bars within a sampling date indicate significant differences at $\alpha = 0.05$ according to Tukey's test.

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