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PLANT PATHOLOGY AND NEMATOLOGY

Nondestructive Evaluation of Cotton Genotypes for Resistance to Reniform Nematode

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ABSTRACT

Identification of new sources of reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) resistance in cotton is critical to expanding host plant resistance to manage this important pathogen. Phenotyping plants in early breeding generations without destructive sampling would be useful for introgression of nematode resistance from exotic germplasm resources; therefore, a rapid, nondestructive method was developed to assess host plant resistance to the reniform nematode based on the number of females infecting the roots. In one set of experiments, the root system was cut off at 0, 1, 2.5, or 5 cm below the soil line and used to assess the number of females infecting this portion of the root system. Resistance could be accurately determined while leaving up to 5 cm of roots with the shoot. In a second set of experiments, the rate of plant recovery and reproductive development was evaluated using a combination of root retention (0, 1, 2.5 cm, or all root) and shoot retention (leaves at top two nodes, leaves at bottom two nodes, no leaves, all leaves) treatments. Plants more rapidly recovered using a treatment combination in which the top leaves and 2.5 cm roots were kept. This combination performed similarly to plants with neither shoots nor roots modified and was harvested 20 days sooner compared to some other treatment combinations.

Reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) is an important pathogen of cotton (*Gossypium hirsutum* L.) in the southeastern U.S. associated with annual yield losses as high as 5% (Lawrence et al., 2018, 2019, 2020). Pest management practices to reduce seed cotton yield losses have focused mainly on the use of nematicides (Desaeger et al., 2020; Faske et al., 2021; Koenning et al., 2004, 2007; Robinson, 2007; Schumacher et al., 2020) and rotation to nonhost crops (Koenning et al., 2004; Robinson, 2007; Schumacher et al., 2020). Recently, cultivars with host plant resistance have been released, with a limited number of resistant cultivars such as PHY 332 W3FE, PHY 411 W3FE, and PHY 443 W3FE available commercially (https://phytogencottonseed.com/varieties; Turner et al., 2021). A single source of resistance has been used to develop these cultivars, and breeding programs are focused on identifying and deploying new sources of resistance to the reniform nematode that can be used to manage this pathogen. However, new sources of resistance will be derived mainly from diploid cotton species that will require extensive screening of breeding lines to successfully develop resistant cultivars.

Reniform nematode resistance can be determined by assessing nematode reproduction in greenhouse or growth chamber tests. Soil samples are collected to determine the nematode population size for each breeding line and compared to population sizes of known resistant and susceptible genotypes included as controls to classify the level of resistance. Although these assessments are nondestructive, allowing plants to be advanced to the next generation, the approach requires 60 to 90 days to allow sufficient reproduction of the nematode to quantify resistance (Araujo Filho et al., 2010; Jiao et al., 2015; Klepaldo et al., 2018; Rebois et al., 1968; Stetina and Erpelding, 2016; Weaver et al., 2007). Other researchers (Robinson et al., 2007) grew plants for 8 to 12 weeks until pot-bound, then placed the root ball into a fine mesh sleeve and transplanted into a larger pot containing soil infested with reniform nematodes. The roots that grew through the mesh were cut off 3 weeks after transplanting and the number of females infecting the roots was determined. These types of studies are more labor intensive, resulting in lower throughput.

Approaches to assess nematode infection 30 to 40 days after inoculation have been reported. Typically, the root system is removed from the plant and the shoot is discarded for these approaches with the level of resistance determined by either staining roots and counting females (Stetina and Erpelding, 2016; Stetina and Young, 2006; Thies et al., 2002), scoring egg masses on the roots (McCarty et al. 2013; Sharma and Ashokkumar, 1991; Silva et al., 2014; Williams et al., 1979), or extracting and counting eggs from the

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root system (Hussey and Barker, 1973; McCarty et al., 2017; Stetina and Young, 2006). Although these protocols for rapidly determining resistance have been effective, they are employed typically in later breeding generations when it is possible to destructively sample the plant population without losing germplasm lines with resistance. This has resulted in multiple populations of susceptible plants being unnecessarily maintained in breeding programs, with space and time devoted to advancing these materials prior to screening. An approach that would allow the rapid selection of resistant plants in early generation without destructive sampling will benefit breeding programs. Therefore, the objective of this research was to develop a rapid, nondestructive method to assess host plant resistance to the reniform nematode based on the number of females infecting the roots. This approach would permit larger populations to be evaluated and increase throughput by eliminating susceptible plants in early generations.

MATERIALS AND METHODS

Root Retention. This experiment evaluated whether resistance designations could be determined reliably if only a portion of the plant root system was examined. Three susceptible cotton genotypes (*G. hirsutum* 'Deltapine 16' and MD 25 and *G. arboreum* A2-101 [PI 529729]) and three resistant cotton genotypes (*G. hirsutum* LONREN 2, *G. arboreum* A2-190 [PI 615699], and *G. barbadense* TX 110) were evaluated in a repeated growth chamber test. One plant of each genotype was established in a plastic pot (Ray Leach SL-10 Cone-tainer™, Stuewe & Sons, Inc., Tangent, OR) filled with 120 cm^3 of a steam-sterilized soil mixture composed of two parts sand and one part sandy loam soil. Pots were placed in a growth chamber with a constant temperature of 27 °C and a photoperiod of 16 hr light and 8 hr dark. Plants were watered as needed to avoid moisture stress. At 7 d after planting, each plant was inoculated with 1,000 vermiform reniform nematodes suspended in 1 ml water. The nematode population used was MSRR03 (Arias et al., 2009), a single egg mass-derived population that was originally isolated from *G. hirsutum* and subsequently reared on tomato (*Solanum lycopersicon* L. cultivar Rutgers) in the greenhouse. Four root retention treatments were examined 4 wk after planting. Plants were removed from the pots, roots were gently rinsed free of soil using tap water, and the root system was removed at 0, 1, 2.5, or 5 cm below the soil line (Fig. 1). Roots were stained with red food coloring (Thies et al., 2002), and the number of female nematodes infecting the root system were counted on the portion of the root that was removed. Counts were made using a stereomicroscope at ×50 magnification.

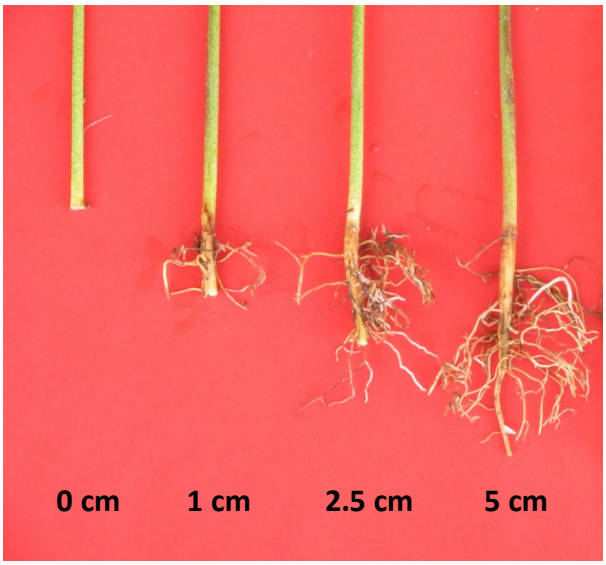

Figure 1. Cotton root portions retained with shoots for the root retention experiment.

The experiment was a completely randomized design with a factorial treatment arrangement and five replications. Data from trials planted on 9 March 2012 and 11 April 2012 were combined and transformed [$\log_{10}(x+1)$] prior to analysis of variance (ANOVA) using PROC MIXED in SAS 9.4 (SAS Institute, Cary, NC). Genotype, root retention, and their interactions were modeled as fixed effects, and trial was modeled as a random effect. Where significant effects were identified, post-ANOVA means separations were based on differences of least squares means at $p \leq 0.05$.

Plant Recovery. Two susceptible *G. hirsutum* genotypes (Deltapine 16 and MD 25) were evaluated to determine the rate of plant regrowth after root and shoot removal treatments. Eighty pots were planted for each genotype and placed in a growth chamber following the same procedure as described for the root retention experiment. Planting dates were 10 October 2012 and 9 April 2013. The experiment was a completely randomized design with a factorial treatment arrangement and five replications.

Five weeks after planting, each plant was removed from the pot and the root system was gently rinsed in water to expose the roots. Each plant then received a combination of shoot and root treatments (Table 1). The shoots were replanted into pots filled with 120 cm^3 of Metro Mix 360 potting medium (Sun Gro Horticulture Canada Ltd., Agawam, MA) and placed

back in a growth chamber with a constant temperature of 27 °C and a 16-hr photoperiod. Pots were placed in a container of water to provide sufficient moisture to reduce plant wilting after replanting and allow regrowth of the root system. Plants were maintained in the pots for 3 wk to simulate the amount of time frequently required to assess roots for experiments infected with nematodes. At the end of this interval (a total of 8 wk after planting), the number of leaves on all plants were recorded. Half of the plants were sacrificed to measure plant dry weights. These plants were removed from the potting mix, shoots were excised from roots at the soil line, and shoots and roots were placed in a drying oven at 60 °C for 48 h. At the end of this interval, dry weights were measured.

Table 1. Shoot and root treatments applied in the cotton plant recovery experiment

Treatment	Level
Shoot	No modification to shoot [control][z]
	Cut off lower leaves; leave top 2 fully expanded leaves [top]
	Cut off apical meristem; leave 2 lowest nodes and leaves if present [bottom]
	Cut off all leaves [stem]
Root	No modification to root [control]
	0 cm kept with shoot [0 cm]
	1 cm kept with shoot [1 cm]
	2.5 cm kept with shoot [2.5 cm]

[z] Value in bracket denotes the short descriptor for each treatment.

The other half of the plants were transplanted into greenhouse pots (Poly-tainer Can, Nursery Supplies, Inc., Orange, CA) filled with 5 L of Metro Mix 360 potting medium to evaluate reproductive development. These plants were placed in a greenhouse and grown at a temperature of approximately 27 °C with no artificial lighting. Plants were initially watered as needed and then watered daily starting 2 wk after transplanting. Plants were fertilized 4 wk after planting with 5 g of a slow-release formulation (Osmocote 14-14-14 Slow Release Plant Food, The Scotts Company, Marysville, OH). Reproductive development of the plants was assessed beginning 9 wk after they were established in the growth chamber. Parameters recorded were dates of first square, first flower, first boll set, and first open boll. When plants had at least four open bolls, all open bolls were harvested, and the following data recorded: date of harvest, total number of open bolls, and total number of seeds.

ANOVA was conducted on the combined data from both trials using PROC MIXED in SAS 9.4. Root treatment, shoot treatment, and their interactions were modeled as fixed effects, and genotype and trial were modeled as random effects. Where significant effects were identified, post-ANOVA means separations were based on differences of least squares means at $p \leq 0.05$.

RESULTS AND DISCUSSION

Root Retention. Significant differences were found between genotypes ($F = 113.55$, $p < 0.0001$) with respect to number of female nematodes infecting the root system, with each genotype displaying resistance or susceptibility as expected (Table 2). The number of females did not differ significantly based on the amount of root tissue retained with the stem ($F = 2.50$, $p = 0.0609$; Table 2), and no significant interactions between root retention and genotype ($F = 1.40$, $p = 0.1504$) were found. Female nematodes were sufficiently distributed along the root system to allow identification of susceptible and resistant genotypes under the test conditions employed in the experiment. Reniform nematodes infect feeder roots and are capable of penetrating root tissue at any point along the root (Robinson et al., 1997). Results of the present study demonstrated that resistance or susceptibility to reniform nematode could be reliably determined across a range of cotton genotypes while keeping up to 5 cm of root tissue with the plant to facilitate recovery of desirable individuals.

Table 2. Mean number of female reniform nematodes infecting roots for genotype and root retention treatments in the root retention experiment

Treatment	Level	Mean number of females	
Genotype	*Gossypium hirsutum* 'Deltapine 16'	113.5	a[z]
	Gossypium arboreum A_2-101	58.4	b
	Gossypium hirsutum MD25	57.4	b
	Gossypium barbadense TX 110	19.2	c
	Gossypium arboreum A_2-190	8.4	d
	Gossypium hirsutum LONREN 2	7.9	d
Root retention	0 cm	28.7	
	1 cm	27.5	
	2.5 cm	31.5	
	5 cm	22.7	

[z] Means followed by the same letter are not significantly different from each other at $p \leq 0.05$ based on differences of least squares means.

Plant Recovery. All plants for the two test genotypes survived the transplanting process and produced seed cotton. However, there were differences among the root and shoot removal treatment combinations for plant growth and reproductive development.

Number of Leaves and Plant Dry Weight. Significant differences were noted with respect to number of leaves and plant dry weight measured 8 wk after planting; at the time half of the recovered plants were sampled prior to transferring the remaining plants to the greenhouse for reproductive evaluation (Table 3). The bottom-shoot treatment resulted in a greater number of leaves on recovered plants, followed by control, top, and stem treatments. These results suggest removal of the apical meristem resulted in additional leaf development compared to plants where leaves were removed. Further, loss of lower leaves was less likely with the removal of the apical meristem compared to the control treatment. Greater leaf numbers were also associated with plants whose root system had not been modified (control), followed by 2.5, 1, and 0 cm root treatments, which indicated retaining a greater portion of the root system improved leaf development for the plants.

Table 3. Mean number of leaves, shoot dry weight, and root dry weight of cotton plants measured 8 wks after planting in the plant recovery experiment

Treatment	Mean number of leaves[y]	Mean shoot dry weight (g)[z]	Mean root dry weight (g)[z]
Shoot			
Control	6.8 b	1.38 a	0.18 a
Stem	3.3 d	0.35 c	0.04 c
Bottom	7.7 a	0.40 c	0.05 c
Top	4.6 c	0.85 b	0.11 b
Root			
Control	6.5 a	0.92 a	0.19 a
0 cm	4.0 d	0.55 c	0.02 d
1 cm	5.6 c	0.68 b	0.06 c
2.5 cm	6.2 b	0.83 a	0.10 b
***P* values**			
Shoot (S)	<0.0001	<0.0001	<0.0001
Root (R)	<0.0001	<0.0001	<0.0001
S x R	<0.0001	0.0124	<0.0001

Means followed by the same letter are not significantly different from each other at $p \leq 0.05$ based on differences of least squares means.

[y] **Analysis included 320 observations.**

[z] **Analysis included 160 observations.**

Shoot and root growth rates were also affected by shoot and root removal treatments. Greater shoot and root weights were recorded for plants with the shoot control treatment followed by top treatment, whereas significantly lower shoot and root weights were observed for the stem and bottom-shoot treatments (Table 3). These results suggest maintaining the majority of the leaves on the plants improved plant regrowth; however, removing some leaves aids in processing plants for nematode evaluation and replanting of the excised shoots. When processing a large number of plants, some leaf removal also helps reduce wilting before the plants can be watered. Retaining a greater portion of the root system also improved plant recovery with greater shoot weights recorded for the control treatment followed by the 2.5-cm root treatment (Table 3). The complete removal of the root system resulted in the lowest shoot weights. All plants for the root removal treatments showed further root development. Importantly, the complete removal of the root system did not hinder the redevelopment of roots from the shoot.

A significant interaction between shoot and root treatments was detected for total number of leaves, shoot dry weights, and root dry weights (Table 3). The following combinations of shoot/root treatments resulted in leaf numbers equal to or greater than the treatment in which neither shoots nor roots were modified (control/control): control/2.5 cm, bottom/control, bottom/2.5 cm, and bottom/1.0 cm (Fig. 2). All other treatment combinations had significantly fewer leaves than the control treatment combination. No combination of treatments resulted in shoot dry weights equal to or greater than the control treatment combination (Fig. 3). Among the treatments that included both shoot and root modification, the top/2.5 cm combination showed significantly greater shoot weights. For root dry weights, no combination of treatments resulted in weights equal to or greater than the control treatment combination (Fig. 4). Among the treatments that included both shoot and root modification, greater root weights were associated with the top/2.5 cm combination. These results indicate that keeping the top of the shoot and 2.5 cm of the root system treatment was superior to other treatment combinations for plant regrowth.

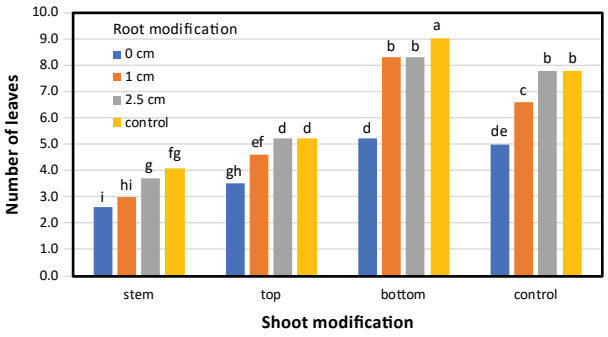

Figure 2. Shoot and root treatment interaction for number of leaves from 8-wk-old cotton plants in the plant recovery experiment. Data are means of 320 observations. Means designated with the same letter are not significantly different from each other at $p \leq 0.05$ based on differences of least squares means.

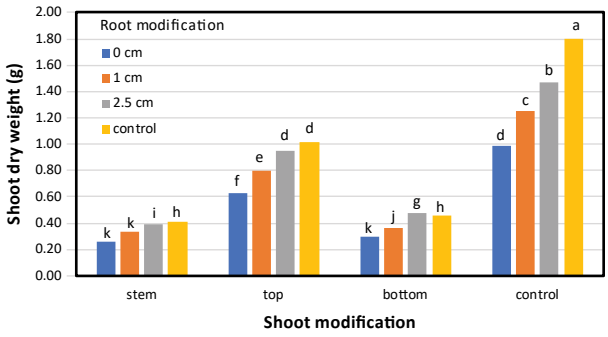

Figure 3. Shoot and root treatment interaction for shoot dry weights from 8-wk-old cotton plants in the plant recovery experiment. Data are means of 160 observations. Means designated with the same letter are not significantly different from each other at $p \leq 0.05$ based on differences of least squares means.

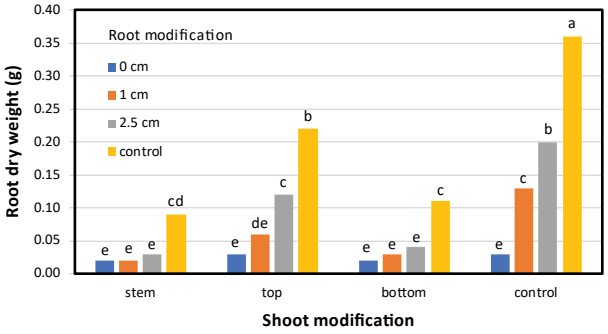

Figure 4. Shoot and root treatment interaction for root dry weights from 8-wk-old cotton plants in the plant recovery experiment. Data are means of 160 observations. Means designated with the same letter are not significantly different from each other at $p \leq 0.05$ based on differences of least squares means.

Reproductive Development. At harvest, significant differences were found in the number of open bolls and the number of seeds for some of the shoot and root modification treatments (Table 4). Shoot treatments significantly affected the number of open bolls, whereas root treatments affected the number of seeds produced. Modifying the shoot by keeping the bottom portion or just the stem resulted in more open bolls on these plants. The number of seeds produced was less affected by the root removal treatments with only the removal of the entire root system from the plants showing a significant reduction in seed numbers compared to the control treatment. These results indicate seed production was not greatly affected by the shoot or root treatments, and sufficient qualities of seed can be produced for further evaluation of selected breeding lines.

Table 4. Mean number of open bolls and number of seeds from cotton plants at harvest in the plant recovery experiment

Treatment	Mean number of open bolls	Mean number of seeds
Shoot		
Control	4.7 c[z]	103.0
Stem	5.3 a	99.7
Bottom	5.2 ab	101.8
Top	4.8 bc	106.0
Root		
Control	5.1	110.7 a
0 cm	4.9	93.1 b
1 cm	5.1	103.4 ab
2.5 cm	4.9	103.3 ab
***P* values**		
Shoot (S)	0.0229	0.6909
Root (R)	0.5192	0.0125
S x R	0.2301	0.5995

[z] **Means followed by the same letter are not significantly different from each other at $p \leq 0.05$ based on differences of least squares means.**

Analysis included 160 observations.

The rates at which flowers and bolls developed were affected by shoot and root treatments, alone and in combination (Table 5, Fig. 5). The time to reach each developmental stage was delayed when shoots or roots were modified. For the shoot treatments, the number of days to reach each developmental stage for the top treatment was similar to the control treatment, whereas the stem treatment in which all leaves were removed had the greatest delay in reproductive development. These results suggest that as the shoot was more severely modified the rate of reproductive development was significantly delayed. A similar response was also observed for the root modification treatments. The complete removal of the root system resulted in a significant delay in the number of days to reach each developmental stage compared to other treatments, whereas the 2.5-cm root treatment was not significantly different from the control treatment. A significant shoot-by-root treatment interaction (Fig.

5) identified five combinations of shoot/root modifications where the rate of reproductive development was equal to control plants with no shoot or root modification and included: top/control, control/1 cm, top/1 cm, control/2.5 cm, and top/2.5 cm. Four of these treatment combinations where a portion of the root system was removed would be useful when screening plants for reniform nematode resistance. The top/2.5 cm combination was harvested 20 d sooner compared to some other treatment combinations, which is beneficial to more rapidly generate seeds for further screening and selection in the next generation to increase throughput.

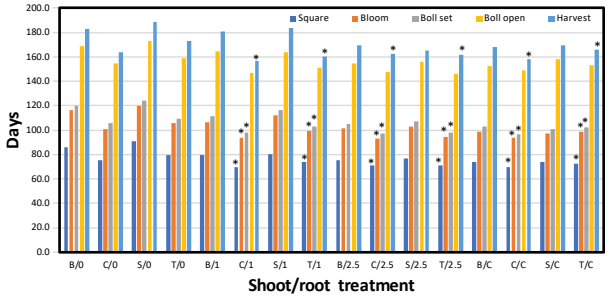

Figure 5. Shoot and root treatment interaction for days to first square, bloom, boll set, boll open, and harvest (four or more open bolls) from cotton plants in the plant recovery experiment. Root treatments were not modified (control; C) or 0, 1, or 2.5 cm kept with the shoot, and shoot treatments were not modified (control; C), stem (S) kept, top (T) leaves kept, or bottom (B) leaves kept. For each developmental stage with a significant ($p \leq 0.05$) shoot x root treatment interaction, bars marked with an * were in the group with the shortest time to reach that stage. Data are means of 160 observations.

This nondestructive method can be used by cotton breeding programs to rapidly identify reniform nematode resistant genotypes and recover those plants for further evaluation. This method can be used to screen successfully for reniform nematode resistance in early plant generations thereby reducing the number of plants advanced to the next generation, which would aid in the more rapid development of resistant cultivars in cotton breeding programs. The top leaves kept/2.5 cm roots kept combination has been used to successfully recover *G. arboreum* L. plants from F_2 populations to evaluate the inheritance of nematode resistance (Erpelding and Stetina, 2018, 2019). The method would be highly useful in backcross breeding programs to introgress reniform nematode resistance from exotic germplasm accessions where resistant plants can be identified for backcrossing to recover the *G. hirsutum* phenotype. Later generation testing for resistance to sedentary nematodes (Bourland and Jones, 2009; Creech et al., 2007; McCarty et al., 2013, 2017) is a typical protocol when releasing cotton germplasm with nematode resistance. The ability to conduct replicated tests is an advantage when testing at later generations, which can help identify escapes that would be misclassified as resistant. The nondestructive method would also be useful to confirm resistance, because selected genotypes could be screened over multiple generations.

Table 5. Mean number of days to first square, bloom, boll set, boll open, and harvest (4 or more open bolls) from cotton plants with shoot and root modifications in the plant recovery experiment.

Treatment	Square	Bloom	Boll set	Boll open	Harvest
Shoot					
Control	71.6 c[z]	95.4 c	99.3 c	149.5 b	160.3 b
Stem	80.6 a	108.2 a	112.0 a	162.9 a	176.8 a
Bottom	78.8 a	105.8 a	109.8 a	160.3 a	175.3 a
Top	74.3 b	99.7 b	103.2 b	152.4 b	165.3 b
Root					
Control	72.8 c	97.1 c	100.6 c	153.1 bc	165.4 bc
0 cm	82.9 a	110.9 a	114.8 a	163.9 a	177.2 a
1 cm	76.0 b	103.0 b	107.1 b	156.8 b	170.4 b
2.5 cm	73.5 c	98.0 c	101.9 c	151.4 c	164.7 c
***P* values**					
Shoot (S)	< 0.0001	< 0.0001	<0 .0001	< 0.0001	< 0.0001
Root (R)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
S x R	0.0432	0.0130	0.0332	0.1258	0.0159

[z] **Means followed by the same letter are not significantly different from each other at $p \leq 0.05$ based on differences of least squares means.**

Analysis included 160 observations.

The screening and plant recovery method that was developed could also be used with other sedentary nematodes or other host crops. The method could be applied to recover plants infected by root-knot nematode (*Meloidogyne* spp.), another economically important sedentary nematode that affects cotton. However, the distribution of root-knot nematodes along the root system can differ from that of reniform nematode. Root-knot nematodes invade the root tips so it is recommended that a root retention experiment be conducted to verify how much of the root system is needed to reliably distinguish between susceptible and resistant genotypes, because a greater portion of the root system could be maintained with the shoot for these evaluations. Davis et al. (2011) reported a similar concept to develop root-knot nematode resistance in cotton, where the least-galled plants were selected and repotted to advance the breeding lines to the next generation. It is possible to use this method to assess sedentary nematodes in other crops, but experiments need to be conducted to verify that resistance could be reliably determined in those crops with just a portion of the root system, and to identify shoot treatments that result in recovery of tested plants as was described in the present study for cotton. The method has been used successfully in support of an in-house program to breed soybean (*Glycine max* [L.] Merr.) for reniform nematode resistance.

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DISCLAIMER

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