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Experimental protocol for evaluating reniform nematode (Rotylenchulus reniformis) resistance in cotton

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Dear Dr. Mukherjee,

The invited manuscript entitled “Experimental protocol for evaluating reniform nematode (*Rotylenchulus reniformis*) resistance in cotton” has be submitted for your consideration.

Best regards,

John Erpelding

TITLE:

Screening Cotton Genotypes for Reniform Nematode Resistance

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KEYWORDS:

Cotton, germplasm, *Gossypium arboreum*, host-plant resistance, nematode screening, reniform nematode, resistance breeding, *Rotylenchulus reniformis*, vegetative propagation

SUMMARY:

Here, a protocol is presented for the rapid non-destructive screening of cotton genotypes for reniform nematode resistance. The protocol involves visually examining the roots of nematode-infected cotton seedlings to determine infection response. The vegetative shoot from each plant is then propagated to recover plants for seed production.

ABSTRACT:

A rapid non-destructive reniform nematode (*Rotylenchulus reniformis*) screening protocol is needed for the development of resistant cotton (*Gossypium hirsutum*) varieties to improve nematode management. Most protocols involve extracting vermiform nematodes or eggs from the cotton root system or potting soil to determine population density or reproduction rate. These approaches are generally time-consuming with a small number of genotypes evaluated. An alternative approach is described here in which the root system is visually examined for nematode infection. The protocol involves inoculating cotton seedling 7 days after planting with vermiform nematodes and determining the number of females attached to the root system 28 days after inoculation. Data are expressed as the number of females per gram of fresh root weight to adjust for variation in root growth. The protocol provides an excellent method for evaluating host-plant resistance associated with the ability of the nematode to establish an infection site; however, resistance that hinders nematode reproduction is not assessed. As with other screening protocols, variation is commonly observed in nematode infection among individual genotypes within and between experiments. Data are presented to illustrate the range of variation observed using the protocol. To adjust for this variation, control genotypes are included in experiments. Nonetheless, the protocol provides a simple and rapid method to evaluate host-plant resistance. The protocol has been successfully used to identify resistant accessions from the *G*. *arboreum* germplasm collection and evaluate segregating populations of more than 300 individuals to determine the genetics of resistance. A vegetative propagation

method for recovering plants for resistance breeding was also developed. After removal of the root system for nematode evaluation, the vegetative shoot is replanted to allow the development of a new root system. More than 95% of the shoots typically develop a new root system with plants reaching maturity.

INTRODUCTION:

Rotylenchulus reniformis (Linford and Oliveira), commonly referred to as the reniform nematode, is one of the major parasitic nematode species present in soils of the southeastern United States[1-3]. The nematode is an obligate, sedentary semi-endoparasite requiring a host plant to complete its life cycle[2,4]. Vermiform preadult female nematodes penetrate the host root system to establish a feeding site in the stele[2,3]. As the nematode feeds and matures, the posterior portion remaining outside of the host root will swell upon egg production, forming a characteristic kidney shape (**Figure 1**). *R. reniformis* is capable of feeding on the root system of more than 300 plant species, including cotton[4]. Upland cotton (*Gossypium hirsutum* L.) is widely cultivated in the southeastern United States, but the lack of *R*. *reniformis*-resistant varieties hinders nematode management[2,3]. Management strategies such as nematicide treatment and rotation with non-host crop species have been used to reduce soil *R*. *reniformis* population densities[5,6], but seed cotton yield losses can commonly range from 1 to 5%[2]. Symptoms of *R*. *reniformis* infection can include plant stunting, suppressed root growth, nutritional deficiencies, fruit abortion, and delayed maturity[2]. However, symptoms may not be apparent due to the uniformity of symptoms across the field; therefore, approaches to assess *R*. *reniformis* infection are needed to identify and develop resistant upland cotton varieties. Evaluation of *R*. *reniformis* resistance in cotton is considered difficult[7], because the infected root system may appear normal even though the plant may show symptoms of infection[8].

An effective nematode screening protocol is required for the identification of *R*. *reniformis*-resistant accessions from the cotton germplasm collection, and for the determination of the resistance genetics for these accessions. Such a protocol will aid in the transfer of resistance genes to upland cotton. Various bioassay methods have been used to assess *R*. *reniformis* infection in cotton[8-15]. In general, two major approaches have been used for the identification of *R*. *reniformis* resistant cotton genotypes. The most frequently used approach involves extracting eggs and/or vermiform nematodes from infected plants or soil[8,11,12,14,15]. The general methodology for this approach involves planting seeds for the individual cotton genotypes in separate pots, allowing the seedlings to develop for 7 to 14 days, inoculating the seedlings by adding a mixture of vermiform stages of *R*. *reniformis* to the soil, and allowing the nematodes to infect the root system for 30 to 60 days. Next, vermiform nematodes and/or eggs are extracted from the infected root system of each plant or from the potting soil. The number of extracted nematodes or eggs is then determined to estimate the population density and reproduction rate, which are compared to control genotypes in order to identify resistant genotypes.

An alternative approach, as described here, involves microscopically examining the cotton root system that has been infected with nematodes to determine the number of female nematodes parasitizing the roots[10,16]. Similar to other approaches, cotton genotypes are planted in separate pots and inoculated with vermiform nematodes approximately 7 days after planting. Within 30

days after inoculation, the root system is removed from individual plants and the soil is rinsed from the roots. Next, the nematodes attached to the root system are stained with red food coloring[17], and roots are microscopically examined to determine the number of infection sites with resistant cotton genotypes (identified based on the number of nematodes per gram of root) compared to a susceptible control[16]. This second approach has the advantage of increased throughput by reducing the number of days required for evaluation and increasing the number of individual genotypes evaluated in a single experiment. Screening methodologies that evaluate population density or reproduction rate are often more time-consuming than those based on visual observations of infection signs[7]. However, one limitation of this approach is that host-plant resistance that hinders nematode reproduction as determined by egg production is not assessed[13].

Screening protocols for *R. reniformis* resistance often destroy the root system during evaluation[7] and involve the vegetative shoot being discarded. To overcome this limitation, a method of vegetative propagation has been developed to allow the recovery of plants for seed production[18]. After removal of the root system for nematode evaluation, the vegetative shoot is planted in potting soil to allow the root system to regrow. This method has broad applications for most *R. reniformis* screening protocols. A simple and rapid method of vegetative propagation is of critical importance for breeding *R. reniformis* resistant upland cotton varieties, where the recovery of the progeny is required to advance resistant genotypes to the next generation.

A protocol is presented for the large-scale screening of cotton genotypes for reniform nematode resistance. The goal is to develop a simple and rapid non-destructive screening method to evaluate cotton breeding populations for nematode resistance in order to aid in the breeding of resistant upland cotton varieties. Using this protocol, data are typically obtained within 35 days, with more than 300 genotypes evaluated in a single experiment. Data are presented for resistant and susceptible genotypes to illustrate the variation commonly observed using these methods.

PROTOCOL:

1. Maintaining a Source of *R. reniformis* Inoculum

1.1. Fill terra cotta clay pots (15 cm in diameter, 13.5 cm in height) with a steam pasteurized mix of 1-part field soil and 2-parts sand. Plant a susceptible tomato (*Solanum lycopersicon*) variety in each pot and place the pots in a glasshouse.

Note: Other susceptible plant varieties such as cotton can be used instead of tomato.

1.2. Inoculate the tomato plants with vermiform reniform nematodes (see step 3.3). Maintain the plants in the glasshouse at a temperature of approximately 28 °C.

2. Planting Cotton Genotypes for *R. reniformis* Resistance Evaluation

2.1. Prepare soil by combining 2-parts fine sand with 1-part topsoil collected from the field.

2.2. Steam pasteurize the soil mixture to ensure that the soil is free of nematodes and soil-borne plant pathogens.

2.3. Add the soil mixture to conical plastic pots (4 cm in diameter, 21 cm in height). Prior to filling the pots, place a ball of cotton in the bottom of the pot to prevent soil loss. Partially fill the pots with soil approximately 2 cm from the top.

2.4. Prepare a plastic stake for each pot to designate the genotype to be planted.

2.5. Plant seeds of the cotton genotypes selected for evaluation.

2.5.1. Plant a single seed in each pot for the evaluation of segregating populations, in which each seed represents a unique genotype.

2.5.2. For cotton varieties or germplasm accessions, plant 2 to 3 seeds in a single pot to insure germination of at least one plant with the other seedlings removed from the pots prior to nematode inoculation.

Note**:** Alternatively, seeds can be germinated for 24 to 72 h prior to planting to minimize the number of pots with non-viable seeds.

2.6. Plant seeds of selected resistant and susceptible control genotypes.

Note**:** Control genotypes are replicated 5 to 10 times to assess natural variations inherent to the screening methodology.

2.7. Fill pots with additional soil to cover the seed in each pot.

2.8. Place the pots in the growth chamber. Maintain a constant temperature of 28 °C, an ambient air temperature, for the growth chamber. Provide artificial lighting with a mixture of fluorescent and incandescent lamps with a 16 h photoperiod.

2.9. Place water emitters in each pot, and water the pots twice per day using an automatic watering system. Adjust the watering system to supply additional water as the plant grows.

3. Nematode Inoculation of Cotton Plants and Preparation of Root Samples

3.1. Extract vermiform reniform nematodes maintained on susceptible tomato plants (see step 1) using elutriation[19] and centrifugal flotation[20] methodologies the day before inoculation. Store the extracted nematodes at 4 °C.

Note: Baermann funnel extraction[21] is an alternate method for nematode extraction.

3.2. Determine the number of nematodes extracted by counting the number of nematodes in a 100 µL subsample and prepare a suspension of 1,000 nematodes/mL in tap water for inoculations.

3.3. Inoculate the cotton seedlings 7 d after planting with the nematode suspension. Create a small depression in the soil next to the plant, and pipette 1 mL of the reniform nematode suspension into the depression.

3.4. Remove plants from the pots 28 d after inoculation for nematode evaluation.

Note: At this stage, plants are approximately 15 cm tall with 4 to 6 fully expanded leaves.

3.4.1. Remove most of the fully expanded leaves from the plants using scissors prior to removing the plants from the pots.

3.4.2. To remove plants from the pots, squeeze the pot and slide the soil out into the hand.

3.4.3. Gently remove the soil from the roots by agitating the root system in tap water in a 10 L container. Briefly rinse the root system in a container of clean tap water.

3.4.4. Remove the root system from the plant approximately 1 cm below the soil line using scissors.

3.5. Place the root system into a 120 mL plastic, non-sterile, disposable specimen container along with the plastic stake from the pot used for identification.

Note**:** Multiple samples are processed before proceeding to step 3.7. Proceed to step 4 for the vegetative propagation of the plant shoot.

3.6. Prepare a 12.5% (v/v) solution of red food coloring[17] in tap water to stain the nematodes attached to the root system.

3.7. Add approximately 30 mL of the red food coloring solution to the root sample in the specimen container to completely cover the root system.

3.8. Place the specimen container in a microwave oven and heat the root sample until the staining solution starts to boil. Remove the sample from the microwave oven and allow the sample to cool at room temperature.

3.9. Decant the red food coloring solution from the root sample and add approximately 100 mL of tap water to the specimen container to remove excess staining. Place the cover on the specimen container and store the sample in a refrigerator at 4 °C. Proceed to step 5 for evaluation of root infection.

Note**:** The protocol can be paused here.

4. Vegetative Propagation to Recover Plants for Seed Production

4.1. Place a ball of cotton in the bottom of a conical plastic pot (see step 2.3) and partially fill the pot with peat moss potting media. Then, place the vegetative shoot in the pot and firmly add potting media to fill the pot. Place a new labeled plastic stake in each pot to designate the cotton genotype.

4.2. Place the tray of pots in a plastic container (73.6 cm length x 45.7 cm width x 15.2 cm height) with water and briefly water the plants to moisten the potting media. Place the pots in a growth chamber with a constant temperature of 28 °C using a 16 h photoperiod. Add additional water to the plastic container as needed to maintain soil moisture.

4.3. Transplant the plants to larger pots for seed production after approximately 30 d. Partially fill a 6 L plastic pot with potting media (see step 4.1), remove the plant from the small pot, place the plant into the 6 L pot, and firmly add potting media to fill the pot.

4.4. Place the plants in a glasshouse and add water to moisten the potting media. Maintain the temperature in the glasshouse at approximately 28 °C (artificial lighting is required).

4.4.1. Water plants by hand as needed for approximately 30 d.

4.4.2. When approximately 75% of the plants require daily watering, water plants daily using an automatic watering system. Adjust the automatic watering system to water more frequently as needed for plant growth.

4.4.3. Add approximately 10 g of a slow-release fertilizer to each pot prior to the start of floral initiation.

4.5. Harvest plants at maturity and process the cotton seed samples to obtain seeds for further evaluation.

4.5.1 To harvest cotton seeds, remove the cotton from the open bolls on the plant by hand and place it in a labelled paper bag. The seeds are attached to the cotton fibers, which are removed in the following steps.

4.5.2. Remove lint fibers from the seed samples using a 10-saw laboratory gin.

4.5.3. Remove fuzz fibers from the seed samples using concentrated sulfuric acid. Neutralize seed samples in a 15% (v/v) solution of sodium carbonate, rinse the samples with tap water, and dry the samples in a forced air drier.

4.5.4. Place the seed samples in labelled envelopes for storage.

5. Evaluation of *R. reniformis* Root Infection

5.1. Remove the root sample from the specimen container and count the number of female nematodes attached to the root system using a stereomicroscope (20X magnification).

Note**:** Only female reniform nematodes are capable of infecting plant roots.

5.2. Place the root system on paper towels for approximately 10 min to remove excess moisture. Weigh the root system to determine the fresh root weight.

5.3. Enter the nematode count and fresh root weight data into a computer spreadsheet program and calculate the number of females per gram of root.

REPRESENTATIVE RESULTS:

Rotylenchulus reniformis infection of the root system for two varieties is presented in **Figure 1**. Relatively fewer female reniform nematodes are able to establish a feeding site for the resistant cotton genotype compared to the susceptible genotype. Variation in root growth is common between accessions, as illustrated in **Figure 2**. This variation as measured by fresh root weight can also be observed between plants of the same genotype (**Table 1**). *Gossypium arboreum* genotypes frequently show lower root growth rates than upland cotton genotypes. To compensate for this variation, data are collected on fresh root weights, which are used to calculate the number of female reniform nematode per gram root tissue for each genotype. The numbers of female reniform nematodes per gram of root for resistant genotypes are generally less than 10, whereas susceptible genotypes typically have greater than 30 nematodes per gram of root.

One challenge for screening cotton genotypes for *R. reniformis* resistance is the potential variation that can occur within and between experiments. To evaluate and adjust for this variation, resistant and susceptible cotton genotypes are included as controls and replicated in each experiment. **Table 1** presents data for two genotypes used as controls in two separate experiments using the protocol described above. The genotypes were replicated 10 times and inoculated 7 days after planting with 1,000 vermiform nematodes, then root systems were harvested 28 days after inoculation to count nematodes attached to the roots. Because the experiments were conducted at different times, the source of the nematodes used for inoculations was different; otherwise, all other parameters were similar. These data illustrate the variation that can be observed in reniform nematode evaluations. Female nematode counts and root weights were higher in experiment 1 for the two controls compared to experiment 2, resulting in higher numbers of females per gram of root for experiment 1. Because the female counts were considerably higher for experiment 1, the increases in root weights did not lower the numbers of females per gram of root to the levels observed for experiment 2. Considerable variation between replications of individual genotypes was also observed. However, the resistant *G. arboreum* genotype PI 615699 frequently showed substantially lower female counts and lower numbers of females per gram of root than the susceptible *G. hirsutum* genotype PI 529251. The

genotypes can be easy classified as resistant or susceptible when these means are used for comparison. Genotypes are classified as resistant when the numbers of females per gram of root are approximately 10% of the susceptible control.

A subset of data from a segregating F_2 population that was evaluated using the protocol is presented in **Table 2**. The population included 300 F_2 plants, and data for 50 plants representing the range in variation are presented. For the population of 300 plants, the number of observed nematodes infecting the root systems ranged from 0 to 50, with a mean of 9.4. Root weights ranged from 0.01-1.22 g, with a mean of 0.38 g. Female nematodes per gram of root ranged from 0-400, with a mean of 33.6. The parents were replicated in the evaluation. The resistant parent (PI 417895) showed a mean of 5.8 females per gram of root, with a mean root weight of 0.8 g; in contrast, the susceptible parent (PI 529729) showed a mean of 40.8 females per gram of root, with a mean root weight of 0.35 g. Twenty plants showed no nematode infection and were classified as resistant, but this may represent escapes. Both these plants and plants with poor root growth are typically removed from data analysis. This range in variation for root growth and nematode infection of the root systems are commonly observed for nematode evaluations; thus, the ability to screen a large number of plants in a single experiment can minimize this variation and allow for accurately assessing the genetics of resistance. The population showed quantitative variation for nematode infection, and plants were classified as resistant based on the data from the susceptible parent, which suggested that resistance was conferred by two recessive genes for this population. Additionally, the vegetative propagation protocol described above was successfully used to recover plants from this population. The rating of the F_3 progeny derived from individual F_2 plants frequently corresponded to the rating of the F_2 plant.

FIGURE AND TABLE LEGENDS:

Figure 1: *Rotylenchulus reniformis* infected root samples. The root sample from a resistant cotton genotype (lower left) shows a single female nematode attached to the root, whereas the susceptible genotype (upper right) shows multiple females attached to the root. The black bar represents a 0.1 mm scale.

Figure 2: Variation observed in root growth for two cotton genotypes. The root systems for two reniform nematode resistant *G. arboreum* accessions are presented to illustrate the variation that can be observed for root growth.

Table 1: Variation observed in reniform nematode infection response for two cotton genotypes included as controls. These data illustrate the variation that can occur within and between experiments for the susceptible *G. hirsutum* genotype PI 529251 and resistant *G*. *arboreum* genotype PI 615699; however, data means were significantly different, allowing the genotypes to be easily classified as resistant or susceptible.

Table 2: Reniform nematode infection response observed in a subset of 50 genotypes from a *G. arboreum* F_2 population. These data illustrate the range of variation that can be observed for segregating populations. Root weights, nematode counts, and number of females per gram of root are presented for each genotype, with plants classified as resistant, moderately resistant,

moderately susceptible, or susceptible.

DISCUSSION:

An effective screening protocol is required for 1) the identification of *R*. *reniformis* resistant cotton genotypes in order to evaluate the genetics of resistance and 2) the breeding of resistant varieties. Most protocols assess *R*. *reniformis* population densities or reproduction rates by extracting vermiform nematodes or eggs from the cotton root system or potting soil[8,11,12,14,15]. These approaches are often more time-consuming, and results tend to be more varied within and between experiments. Additionally, cotton genotypes may be more frequently misclassified in non-replicated glasshouse experiments using these protocols[11]. Nevertheless, similar results can be achieved for the various protocols or parameters evaluated[9,13].

An alternative screening protocol is presented in which the screening of cotton genotypes is conducted by assessing the number of female reniform nematodes parasitizing the root system. Resistant and susceptible cotton genotypes may initially show a similar number of female nematodes penetrating the root systems within 16 hours after inoculation, but within 36 hours, resistant genotypes start to show significantly fewer attached female nematodes and nematode development is hindered[9]. Thus, this screening protocol provides a more direct measure of nematode infection of the cotton root system compared to protocols that rely on the extraction of nematodes or eggs from the root system or potting soil. The method of cotton seedling inoculation with vermiform nematodes is similar among the approaches. Inoculations are typically conducted 7 to 14 days after planting, but the timing of the inoculation is less critical, as seeds can also be directly planted into reniform nematode-infested soil. Inoculations are conducted using 1,000 vermiform nematodes, but the protocol can be modified to increase or decrease the number used for inoculation, or two inoculations can be conducted at 7 and 14 days after planting to insure sufficient female nematodes are present for root infection. In soybean, the number of nematodes used for inoculation had no significant effect on egg mass ratings 21 days after planting; although, higher ratings were typically observed at the higher nematode population density[22]. The protocol can be optimized to determine the minimum nematode density for inoculation.

Nematode infection of the root system is assessed 28 days after inoculation for this protocol, which is generally earlier than in other protocols. This is a critical step in the protocol, as assessments are conducted prior to egg hatch. A significant delay in harvesting root samples can result in a second round of infection. However, this earlier evaluation has the advantage of increasing throughput. For the protocol presented, cotton genotypes are planted in a mixture of sand and soil, which is critical for the simple and quick removal of the root system from the pot. The use of an automatic watering system is essential when using small pots with a sand and soil mixture in order to prevent the pots from drying out. Red food coloring is used to stain the nematodes attached to the root system, which is a simple and safe method[17]. Once the root systems are stained, they can be stored in tap water at 4 °C before counting the number of nematodes attached to the root system; thus, a greater number of cotton genotypes can be evaluated in a single experiment, because no additional processing of the samples is required prior to the assessment of nematode counts. Also, it is advantageous to store the root samples

in tap water for several days, which allows roots to de-stain, making the counting easier.

The protocol described allows for the screening of larger populations in order to reduce environmental variation that will occur between experiments. Using a 900 m^2 plant growth chamber equipped with an automatic watering system, populations of 480 individual plants can be evaluated. The protocol has been successfully used to evaluate segregating populations of 300 or more individuals to characterize the genetics of resistance[10,18]. These populations showed that quantitative variation for nematode infection and resistance in *G*. *arboreum* may be more often associated with multiple recessive genes; thus, larger populations are required for genetic studies. Additionally, quantitative variation is observed in segregating populations, regardless of the protocol employed, to evaluate nematode infection response.

Host-plant resistance in cotton can hinder the ability of the nematode to infect the root system and establish a feeding site, but it may also affect the reproductive ability of the nematode. The screening protocol described evaluates the number of nematodes that are able to establish a feeding site on the cotton root system. Nematode reproduction as measured by egg production was not assessed in this protocol, which is an important limitation. Nonetheless, the protocol can be modified to collect this type of data. Alternately, other methodology can be used to collect this data after resistant genotypes have been identified, which reduces the need to screen a large number of individuals.

Data from nematode evaluations of individual genotypes can be variable within and between experiments, which is a common problem with all screening protocols used to assess *R*. *reniformis* resistance for cotton genotypes. The use of an experimental design with multiple replications for the screening of germplasm accessions will aid in assessing this variation for the identification of resistant genotypes. Additionally, including the same resistant and susceptible control genotypes between experiments is helpful in assessing this variation and comparing results from multiple experiments. These controls are also used to monitor the success of nematode inoculation. Additionally, data means from these controls are used to classify genotypes as resistant or susceptible[10,16]. Cotton genotypes are typically classified as resistant if they show less than 10% of the infection observed on the susceptible control[16,23]. Root growth is another factor contributing to the variation observed in the data using the protocol, because plants having a tap root with fewer lateral roots offer less sites for infection, which may result in fewer nematodes per gram of root.

The difficulty in developing new upland cotton varieties using other cotton species as a source of resistance genes requires a vegetative propagation protocol in order to advance the breeding lines to the next generation for further selection or additional breeding. A simple vegetative propagation protocol as described was developed to recover plants after nematode evaluation. The protocol has been successfully used to recover plants from large populations[18]. Typically, the root system is recovered within 30 days after the vegetative shoot is planted. Survival rates are frequently greater than 95%. Plants showing poor vigor can be lost when propagating a large number of plants. In general, less than 1% of the plants failed to show root or shoot growth. The protocol can be easily modified and used with other nematode screening protocols.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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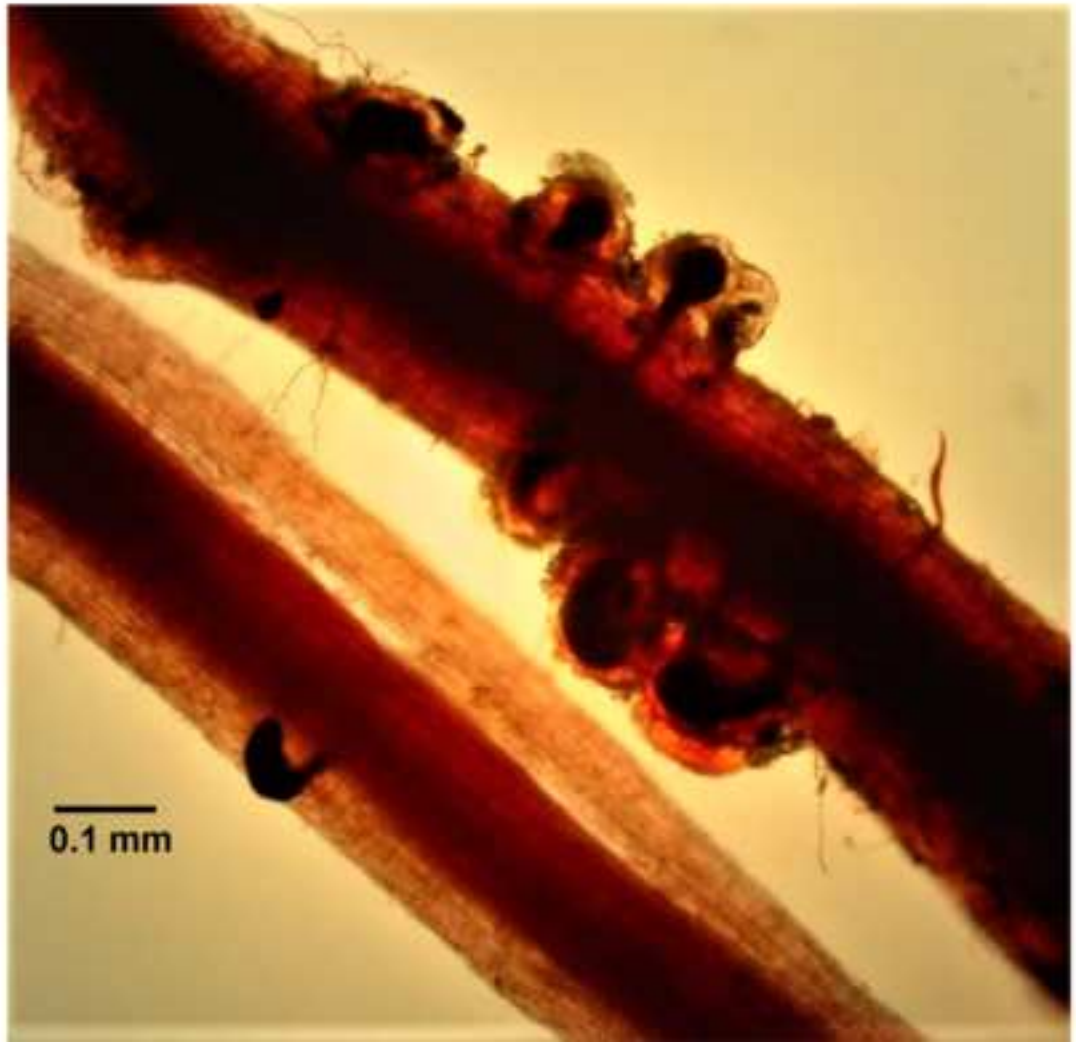

Figure 2

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Table 1

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		Experiment 1			Experiment 2		
Genotype	Replication	Females	Root Weight (g)	Females/g Root	Females	Root Weight (g)	Females/g Root
Susceptible Control							
PI 529251	1	61	1.45	42.1	39	0.81	48.1
	2	36	0.82	43.9	2	0.90	2.2
	3	44	0.32	137.5	18	0.84	21.4
	4	83	1.10	75.5	4	0.40	10.0
	5	no sample			8	0.41	19.5
	6	48	0.64	75.0	24	0.26	92.3
	7	72	0.85	84.7	21	0.69	30.4
	8	35	0.60	58.3	28	0.50	56.0
	9	61	0.92	66.3	11	0.37	29.7
	10	71	1.76	40.3	23	0.44	52.3
	mean	56.8	0.94	69.3	17.8	0.56	36.2
	SD	17.0	0.44	30.2	11.6	0.23	26.6
Resistant Control							
PI 615699	1	7	0.36	19.4	0	0.24	0.0
	2	4	0.46	8.7	0	0.14	0.0
	3	5	0.43	11.6	0	0.11	0.0
	4	2	0.49	4.1	0	0.20	0.0
	5	4	0.71	5.6	0	0.24	0.0
	6	2	0.79	2.5	2	0.33	6.1
	7	7	0.47	14.9	7	0.36	19.4
	8	6	0.22	27.3	1	0.31	3.2
	9	4	0.50	8.0	1	0.28	3.6
	10	5	0.17	29.4	1	0.25	4.0
	mean	4.6	0.46	13.2	1.2	0.25	3.6
	SD	1.8	0.19	9.5	2.1	0.08	6.0

Table 2

Click here to download Table Table 2.xlsx

Genotype Designation	Females	Root Weight (g)	Females/g Root	Classification
88	0	0.67	0.0	Resistant
156	0	0.10	0.0	Resistant
75	2	1.05	1.9	Resistant
298	2	0.58	3.4	Resistant
259	3	0.77	3.9	Resistant
208	1	0.21	4.8	Resistant
322	4	0.82	4.9	Resistant
189	2	0.35	5.7	Resistant
147	6	0.94	6.4	Resistant
267	2	0.18	11.1	Moderately Resistant
198	5	0.43	11.6	Moderately Resistant
251	2	0.17	11.8	Moderately Resistant
95	6	0.46	13.0	Moderately Resistant
248	3	0.23	13.0	Moderately Resistant
79	11	0.84	13.1	Moderately Resistant
340	4	0.29	13.8	Moderately Resistant
114	9	0.64	14.1	Moderately Resistant
168	6	0.40	15.0	Moderately Resistant
117	7	0.44	15.9	Moderately Resistant
77	10	0.57	17.5	Moderately Resistant
277	9	0.44	20.5	Moderately Resistant
47	8	0.34	23.5	Moderately Susceptible
96	20	0.85	23.5	Moderately Susceptible
139	15	0.60	25.0	Moderately Susceptible
253	2	0.08	25.0	Moderately Susceptible
247	15	0.53	28.3	Moderately Susceptible
308	8	0.28	28.6	Moderately Susceptible
152	9	0.31	29.0	Moderately Susceptible
123	8	0.26	30.8	Moderately Susceptible
296	18	0.58	31.0	Moderately Susceptible
138	10	0.31	32.3	Moderately Susceptible
151	5	0.15	33.3	Moderately Susceptible
102	31	0.77	40.3	Moderately Susceptible
67	5	0.12	41.7	Susceptible
51	18	0.43	41.9	Susceptible
311	21	0.48	43.8	Susceptible
334	4	0.09	44.4	Susceptible
266	33	0.74	44.6	Susceptible
260	7	0.14	50.0	Susceptible
49	16	0.32	50.0	Susceptible
149	20	0.39	51.3	Susceptible
104	22	0.34	64.7	Susceptible
238	39	0.57	68.4	Susceptible
144	24	0.33	72.7	Susceptible
225	24	0.30	80.0	Susceptible
87	38	0.43	88.4	Susceptible
126	50	0.51	98.0	Susceptible
272	3	0.03	100.0	Susceptible
154	24	0.12	200.0	Susceptible
286	3	0.01	300.0	Susceptible

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Ray Leach Cone-tainer	Stuewe and Sons Inc.	SC10U
Cone-tainer tray	Stuewe and Sons Inc.	RL98
Sand	various	
Cotton balls	various	
Pylon 4 inch plant labels (4 in L x 5/8 in W)	Pylon Platics	L-4-W
4 oz. specimen containers	Fisher Scientific	16-320-731
Red food coloring	McCormick & Co., Inc.	
1 mL Pipet tips	various	
10 L container	various	
6 L pots	Nursery Supplies Inc.	Poly-Tainer-Can No2A
Potting media	Sun Gro Horticulture	Metro-Mix 360
Fertilizer	Everris NA Inc.	Osmocote Plus
Plastic container (73.6 cm L x 45.7 cm W x 15.2 cm D)	Rubbermaid	3O29

Comments/Description

Any brand or vendor is acceptible.
Any brand or vendor is acceptible.

Inexpensive buckets work well.
Any brand or vendor is acceptible. Different size pots can be used
Any brand or vendor is acceptible.
Any brand or vendor is acceptible.
Any brand or vendor is acceptible.

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Author(s): John E. Erpelding, Salliana R. Stetina

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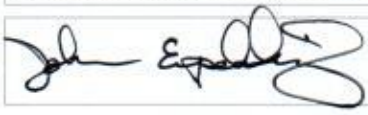
Signature:	 Date: 5/22/2018

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Based on the reviewers' and editorial comments the manuscript has be revised. Additions to the manuscript are in red type and deletions in blue type.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript was proofed to correct any typographical errors.

2. Figure 1: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

The scale has been added to the figure and the figure legend.

3. Please shorten the figure and table legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

All figure and table legends were shorten.

4. Please revise the title to be more concise if possible.

5. Please provide an email address for each author.

The email addresses for each author has been added.

6. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

The long abstract was revised with the goal of the protocol included as the first sentence.

7. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

A paragraph was added to the introduction stating the goal of the protocol.

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

The manuscript was edited to include SI abbreviations.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns were not used in the manuscript.

10. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The protocol section was extensively revised to include only action items.

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Additional details were added to the protocol steps.

12. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section. For example:

2.2: Please describe how to sterilize the soil mixture in the step itself, not in "Note".

2.3: Please specify the soil mixture needed and the size of the pots in the step itself, not in "Note".

Most of the notes were removed from the protocols and critical information was included in the step.

13. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

The protocol steps were revised to be less than 4 sentences and sub-steps were added as needed.

14. 4.1: Please provide the composition of potting media.

The composition of the potting media was added.

15. Line 273: Should step 5.1 be 4.1 instead?

This changed was made.

16. 4.5: Please describe how to process boll samples to obtain seeds.

This information was added as sub-steps.

17. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Approximately 2.25 pages of the protocol were highlight after editing.

18. Discussion: Please also discuss critical steps within the protocol and any limitations of the technique.

The discussion was modified to include this information.

19. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Journal titles were edited and the issue numbers were added.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript is well written and will be useful for researches who are searching for sources of needed resistance to the reniform nematode.

Minor Concerns:

The method does not provide a measure of nematode reproduction which can be important if the source of resistance is reducing eggs production and not nematode numbers. However, the authors point out that the protocol can be modified to collect this data if desired. In Table 1 need to spell out FGR (Females per gram of root) or use a footnote. It is spelled out in Table 2.

Table 1 was modified.

Reviewer #2:

Manuscript Summary:

This study provides a simple and safe protocol to evaluate reniform nematode resistance. In addition, it is more important to describe a vegetative propagation method which is a non-destructive screening protocol to obtain next generation seeds. From this point, it is a very valuable method for nematode resistance breeding program. Moreover, the method is reliable and repeatable. The paper is well written. The only concern is about the sections of introduction and discussion which should focus on the method itself. Therefore, it will be better to shorten the two sections. In conclusion, the manuscript can be accepted after the issue is dealt with.

Major Concerns:

Since the major purpose of this study is to provide the detail steps of evaluating nematode resistance, the introduction and discussion sections should be shortened a bit.

The introduction and discussion were shortened and revised to focus on the protocol.

Minor Concerns:

Line 57: Change "300 plants species" to "300 plant species".

This change was made.