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Comparisons of Female and Egg Assays to Identify *Rotylenchulus reniformis* Resistance in Cotton

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Abstract: More plants can be screened for reniform nematode resistance each year if the time involved can be shortened. In this study, the hypothesis that female counts are as efficient as egg counts in identifying resistant genotypes was tested. In two greenhouse experiments *Gossypium* genotypes which varied from resistant to susceptible to reniform nematode (*Rotylenchulus reniformis*) were compared to a susceptible control cultivar. Infested field soil served as the inoculum source for the first experiment, and vermiform stages extracted from greenhouse cultures were used to infest soil in the second experiment. Six replicates of each genotype were harvested 25 d after planting and swollen females were counted. The remaining plants were harvested 35 d after planting and eggs extracted from the roots were counted. Processing and counting times recorded in the first experiment were similar for both assessment methods, but 10 additional days were required for egg-based assessment. Contrast analyses showed that assessments based on females per gram of root were equivalent to assessments based on eggs per gram of root for the five genotypes tested in the first experiment and for an expanded set of 13 genotypes tested in the second experiment. The results indicated that either life stage can be used to screen for resistance.

Key words: cotton, *Gossypium barbadense*, *G. hirsutum*, reniform nematode, resistance, *Rotylenchulus reniformis*.

The reniform nematode (*Rotylenchulus reniformis*) has become the predominant phytoparasitic nematode on Upland cotton (*Gossypium hirsutum*) in the mid south area of the United States. Losses to this pathogen in 2003 and 2004 averaged 8.25%, 4.25%, and 8.25% in Alabama, Louisiana, and Mississippi, respectively (Blasingame and Patel, 2004, 2005), though losses in individual fields can be considerably higher. This translated to a total loss of approximately 670,000 bales of cotton in these three states during this period (Blasingame and Patel, 2004, 2005). Damage by the reniform nematode on cotton has been implicated as a factor in cotton yield stagnation in the past two decades (Blasingame, 2002).

Currently, reniform nematode management in cotton relies on a combination of chemical and cultural control tactics. The nematicides aldicarb and 1,3-dichloropropene are widely used for reniform nematode suppression in cotton (Kirkpatrick, 2004). In the southern United States, rotation of cotton with corn, sorghum, peanut, or resistant soybean cultivars has been reported to suppress reniform nematode populations (Lawrence et al., 1992; Gazaway et al., 1998; Davis et al., 2003; Plunkett et al., 2003; Davis and Webster, 2005; Royal and Hammes, 2005; Westphal and Scott, 2005).

Unfortunately, no cotton cultivars are commercially available that have resistance to reniform nematode (Lawrence and McLean, 2001). Development of resistant cultivars has been slow partly because limited sources of useful levels of resistance are available in Upland cotton and related *Gossypium* species (Stewart and Robbins, 1996; Lawrence and McLean, 2001; Young, 2002; Bell and Robinson, 2004) and partly because of the lack of efficient screening methods for large numbers of genotypes. Ultimately, large-scale screening may rely on molecular markers, but rapid phenotype determination will be critical in marker development. A rapid screening method is also needed to confirm resistant phenotypes before selected germplasm is released.

Various life stages of reniform nematode have been measured to evaluate resistance of *Gossypium* species to this pathogen. Overstreet and McGawley (1994) and Cook et al. (2001) evaluated cotton cultivars for resistance to reniform nematode in season-long field studies by comparing soil nematode densities at harvest with initial densities at planting. Resistance has been identified by measuring number of eggs per gram of root 30 to 35 d after inoculation (Carter, 1981; Yik and Birchfield, 1981, 1984), vermiform stages extracted from soil 49 d after initial inoculation (Robinson et al., 2004), or a combined number of eggs and vermiform nematodes extracted from soil in tests ranging from 49 to 60 d in duration (Stewart and Robbins, 1996; Robinson and Percival, 1997; Robinson et al., 1999; Robinson et al., 2000). In one case eggs were recovered from the root systems of 30-d-old plants and incubated in a zinc sulfate solution for 4 d to induce hatching, and the newly-hatched juveniles were counted (Bowman and Green, 1991). Four germplasm lines have been released using eggs per gram of root as the measure of resistance (Jones et al., 1988). The underlying mechanism of resistance to the reniform nematode is the failure of the female to establish or maintain a specialized feeding site or syncytium and develop to the egg-laying stage (Carter, 1981; Agudelo et al., 2005). Measuring the number of swollen females after 20 to 30 d would be a measure of successful feeding by the female and could decrease the time needed for the assay. Birchfield and

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Brister (1963) reported differences in resistance among cotton and its relatives based on the number of reniform nematodes infecting the roots, although they did not specify if all individuals or only swollen females were included in their counts. Numbers of swollen females were the basis for evaluating reniform nematode resistance in *Gossypium* species (Carter, 1981; Young, 2002; Young et al., 2004) and were considered efficient for screening soybean cultivars (Williams et al., 1979).

For the effective assessment of the large number of genotypes evaluated in commercial screening programs, shorter-duration evaluations are preferred, provided their results are at least equivalent to findings from longer-duration tests. To date, there are no published reports of direct comparisons of reniform nematode resistance determinations based on different life stages of the nematode. Therefore, the research reported here was designed to test the hypothesis that female counts are as efficient as egg counts for identification of resistant genotypes. A preliminary report of this work has been published (Stetina and Young, 2005).

Materials and Methods

General procedures: Cotton plants were grown in a greenhouse in 7.6-cm-diam. clay pots containing approximately 300 g of the soil mixture described for each experiment. Three cotton seeds were sown in each pot and plants were thinned to 1/pot 7 d after emergence. Plants were watered daily as needed with deionized water and were not fertilized due to the short duration of the experiments.

Swollen female nematodes were counted 25 d after planting (experiment 1) or inoculation (experiment 2). At harvest, plant tops were removed and discarded. Roots were removed from the soil by gentle agitation in tap water. Roots were drained on paper towels for up to 10 min before fresh weights were determined. Next, roots were stained with red food coloring (Thies et al., 2002) by microwaving batches of five root systems simultaneously, then allowing the stain to cool to room temperature before draining. Swollen females attached to the roots were counted using a stereomicroscope (×20). Results were expressed as females per gram of root to compensate for differences in the size of root systems.

Roots were separated from soil 35 d after planting as described for swollen females and nematode eggs were counted. Individual root systems were cut into 2.5-cm segments and placed in a beaker with a stir bar. Eggs were extracted by stirring in 0.6% NaOCl for 10 min (Hussey and Barker, 1973). Two stir plates were used for this step so that two samples could be processed simultaneously. The contents of the beaker were poured over nested 710-µm-pore and 25-µm-pore sieves, and eggs collected on the 25-µm-pore sieve were counted using a microscope. Results were expressed as eggs per gram of root to compensate for differences in the size of root systems.

Data analysis for these experiments used contrasts (Gomez and Gomez, 1984; Schork and Remington, 2000) to determine if the difference between a given variety and the susceptible control (Deltapine 33 B) based on the number of females was equivalent to the difference between the same varieties based on the number of eggs. No contrast results are presented for the susceptible control because it was not possible to compare the control variety with itself. Prior to analysis using the mixed models procedure of SAS software (SAS Institute, Cary, NC), nematodes per gram of root values were transformed by $x^1 = \log_{10}(x + 1)$ to normalize the data and to compensate for differences in scale between female and egg counts. The equation tested with the contrast analysis was:

$$(\log_{10}[F_{genX} + 1] - \log_{10}[F_{genC} + 1]) - (\log_{10}[E_{genX} + 1] - \log_{10}[E_{genC} + 1]) = 0$$

where F = number of females, E = number of eggs, genX = genotype being tested, and genC = control genotype defined by the researcher. For this research the control genotype was Deltapine 33 B, a cotton cultivar susceptible to reniform nematode. If the F value for the contrast was significant at $P \leq 0.05$, the relationship between the test and control genotypes based on the number of females was not the same as that based on the number of eggs, so the two life stages could not be used interchangeably to assess relative resistance of the host plant. If the *F* value for the contrast was not significant at this level, either life stage of the nematode could be used to evaluate the relative resistance of a genotype.

Experiment 1: The objective of this experiment was to determine if the level of infection on a given variety could be assessed comparably using either female or egg counts. The experiment was conducted four times, from March through December 2004. Ambient greenhouse temperatures averaged 35.5 °C, 35.3 °C, 29.6 °C, and 29.4 °C for runs 1, 2, 3, and 4, respectively. The soil mixture for this experiment consisted of one part field soil naturally infested with high numbers of reniform nematodes and two parts steam-sterilized sand. Soil for both experiments 1 and 2 was a Bosket very fine sandy loam collected from research plots in Elizabeth, Mississippi, where reniform nematode was the only phytoparasitic nematode found. Soil was collected from the field at the initiation of each run, so fluctuations in nematode population density were due to natural seasonal variation. Vermiform stages of reniform nematode were extracted from a subsample of the soil mix by elutriation (Byrd et al., 1976) and centrifugal flotation (Jenkins, 1964) to determine the initial infestation levels in test pots, which were 23,614 vermiform stages/200 cm^3 soil in runs 1 and 2, 9,971 vermiform stages/

200 cm^3 soil in run 3, and 4,425 vermiform stages/200 cm^3 soil in run 4, respectively. Two treatments were combined in a factorial arrangement and assigned in a completely randomized design. One treatment was life stage assessed: female or egg. The second treatment was one of six cotton genotypes, which ranged from moderately resistant to susceptible to reniform nematode: LA RN 4–4, LA RN 910, LA RN 1032, Deltapine 16, Stoneville 4892 BR, or Deltapine 33 B (susceptible control). The LA RN lines were reported to have resistance to reniform nematode (Jones et al., 1988). Each of the 12 treatment combinations was replicated six times. Due to differences in seasons and initial inoculum levels, data from each run were analyzed independently. The time spent processing samples and counting nematodes was recorded on a per person basis for each run. Results for female and egg processing times were compared using the mixed models procedure of SAS for analysis of variance.

Experiment 2: The objective of this experiment was to confirm that female- and egg-based assessments would provide comparable results across a broader set of genotypes than those on which the procedures were tested initially. The experiment was conducted twice, in June and July 2005. Ambient greenhouse temperatures averaged 32.4 °C and 32.6 °C for runs 1 and 2, respectively. The soil mixture for this experiment consisted of one part steam-sterilized field soil and two parts steam-sterilized sand. One week after planting, plants were thinned to one per pot and the soil in each pot was infested by pipetting approximately 4,000 vermiform reniform nematodes suspended in 4 ml of tap water into a depression (9-mm-diam. and 3-cm deep) made near the base of the plant. The nematodes for this study were reared on tomato (*Solanum lycopersicon* 'Rutgers') plants in a greenhouse and were extracted from the stock pots using the elutriation and centrifugal flotation procedures described for experiment 1. Two treatments were combined in a factorial arrangement and assigned in a randomized complete block design. One treatment was life stage assessed: female or egg. The second treatment was one of 14 cotton genotypes, which ranged from resistant to susceptible to reniform nematode: LA RN 4–4, LA RN 910, LA RN 1032, Texas 110, Deltapine 16, Stoneville 4892 BR, 19–16–3-1, 21–13–3-5, 22–23–2-1, 23–21–3-5, 24–07–2-1, 24–07–2-5, 25–03–2-5, or Deltapine 33 B (susceptible control). All genotypes were *G. hirsutum*, except Texas 110 which is a resistant *G. barbadense* line (Yik and Birchfield, 1984). Genotypes with number designations are lines from day-neutral Texas race stock accessions T19 (designations beginning with 19 or 21), T1347 (designations beginning with 22 or 23), and T1348 (designations beginning with 24 or 25) that were selected for moderate levels of resistance to reniform nematode (Young et al., 2004). Each of the 28 treatment combinations was replicated six times. Preliminary analysis indicated no significant run x genotype interactions, so data from both runs of the experiment were combined for final analysis.

Results

Experiment 1: All cotton roots in this study supported development and reproduction of reniform nematode. The number of females per gram of root ranged from 26 to 3,870 across all four runs of the experiment and across all genotypes. The number of eggs per gram of root ranged from 513 to 127,433 across all four runs of the experiment and across all genotypes. Root weights averaged 0.34, 0.30, 0.40, and 0.39 g for plants used to count females in runs 1, 2, 3, and 4, respectively. Root weights averaged 0.37, 0.31, 0.49, and 0.51 g for plants used to count eggs in runs 1, 2, 3, and 4, respectively. Mean numbers of females and eggs produced on each genotype in each run of the experiment are shown in Table 1.

Female- and egg-based assessments did not differ in their ability to assess the relative resistance of five tested genotypes of cotton (Table 1). However, the two assessments did differ with respect to the time required to complete the screening. Both assessments required similar time commitments in the laboratory to separate nematodes from soil or roots and count them (Figure 1). The only laboratory processing step that differed was counting, with less time needed to count eggs than to count females (Figure 1). However, the total laboratory processing time was small compared to the time needed to raise the specified life stage of the nematode on plants in the greenhouse. Plants used to measure egg production were held in the greenhouse 10 d longer than plants used to assess females. When greenhouse time was combined with laboratory processing time, measuring females took less time than did measuring eggs (mean time for females = 36,493 min; mean time for eggs = 50,776 min; $F = 57{,}278$, $P < 0.0001$).

Experiment 2: Most cotton roots in this study supported growth and reproduction of reniform nematode. The number of females per gram of root ranged from 0 (six plants) to 122 across all genotypes. The number of eggs per gram of root ranged from 0 (five plants) to 11,581 across all genotypes. The average root weights were 0.97 g for plants used to count females and 1.16 g for plants used to count eggs. Mean numbers of females and eggs produced on each genotype are shown in Table 2. Female- and egg-based assessments did not differ in their ability to assess the relative resistance of tested genotypes of cotton on an expanded set of 13 genotypes (Table 2), confirming the results from experiment 1.

Discussion

Resistance assessments based on females provided results that were equivalent to those based on eggs. Be-

TABLE 1. Genotype means and contrast[a] results comparing female- and egg-based assessments of infection by *Rotylenchulus reniformis* on five cotton (*Gossypium hirsutum*) genotypes and a susceptible control cultivar (Deltapine 33 B).

Cotton genotype	Run 1		Run 2		Run 3		Run 4	
Means:	Female[b]	Egg[b]	Female	Egg	Female	Egg	Female	Egg
Deltapine 33 B	467	11,324	1,349	15,871	337	9,112	407	23,741
Deltapine 16	551	4,564	1,683	15,018	517	9,739	574	12,829
LA RN 4-4	294	6,242	696	9,856	317	7,261	349	16,904
LA RN 910	381	3,620	751	11,896	395	11,516	252	28,860
LA RN 1032	123	7,823	918	7,927	509	18,205	472	21,043
Stoneville 4892 BR	300	4,807	965	11,156	348	7,713	193	24,843
Contrasts:	*F*	*P*[c]	*F*	*P*	*F*	*P*	*F*	*P*
Deltapine 16	3.89	0.053	0.14	0.707	0.19	0.668	2.07	0.155
LA RN 4-4	0.06	0.808	0.06	0.800	0.04	0.845	0.08	0.782
LA RN 910	2.96	0.091	0.17	0.686	0.01	0.928	1.03	0.314
LA RN 1032	3.12	0.082	0.18	0.674	0.11	0.739	0.16	0.690
Stoneville 4892 BR	0.58	0.448	0.00	0.981	0.05	0.818	1.41	0.241

Statistics in each run of the experiment were calculated based on six replications.

[a] $(\log_{10}[F_{genX}+1]-\log_{10}[F_{genC}+1])-(\log_{10}[E_{genX}+1]-\log_{10}[E_{genC}+1])=0$, where F = number of females, E = number of eggs, genX = genotype being tested, and genC = control cultivar Deltapine 33 B.

[b] Female and egg values are geometric means and are expressed as number per gram of root.

[c] *P* values greater than 0.05 indicate that either life stage of the nematode can be used to evaluate the relative resistance of that genotype with equivalent results.

cause the reported resistance mechanism in cotton is the failure of female nematodes to successfully establish or maintain a feeding site (Carter, 1981; Agudelo et al., 2005), a screening system that targets this life stage should allow accurate phenotypic assessments. The equivalency of results from the screening methods based on female counts and egg counts is probably not an artifact of the small set of lines originally tested, which varied from moderately resistant to susceptible in their response to the reniform nematode. A wide range of inoculum levels was used, yet equivalent results were demonstrated in all four runs of the first experiment. The second set of lines tested not only included eight additional genotypes, but also broadened the range of phenotypes to include more representatives with moderate to high levels of resistance, and the assessments still provided equivalent results. Although other re-

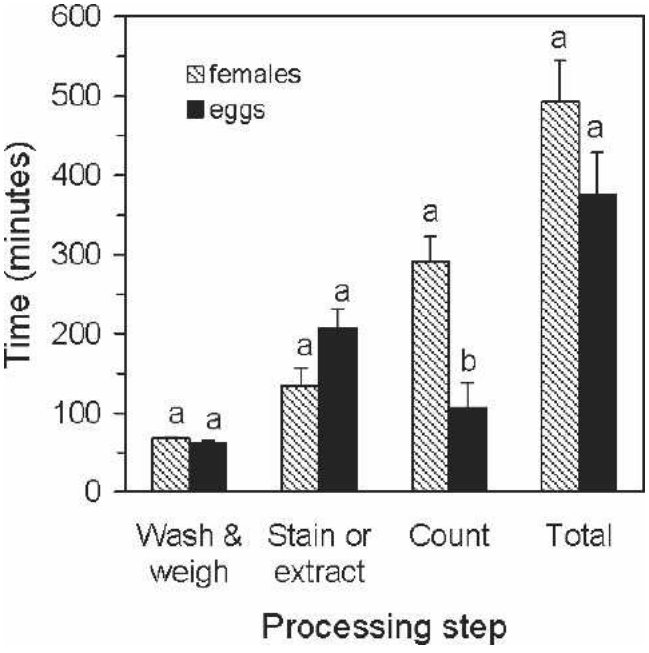

FIG. 1. Time required for one person to process samples in the laboratory and determine the number of *Rotylenchulus reniformis* females and eggs on the roots of 36 cotton plants. Values are means of four replications. For bars (± SE) within each grouping, means with the same letter do not differ ($P \leq 0.05$).

TABLE 2. Genotype means and contrast[a] results comparing female- and egg-based assessments of infection by *Rotylenchulus reniformis* on 13 cotton genotypes and a susceptible control cultivar (Deltapine 33 B).

	Means		Contrasts	
Cotton genotype[b,c]	Female[d]	Egg[d]	F	*P*[e]
Deltapine 33 B	19	471	—	—
Deltapine 16	7	532	1.62	0.214
LA RN 4-4	17	502	0.04	0.841
LA RN 910	10	421	0.36	0.556
LA RN 1032	16	272	0.20	0.657
Stoneville 4892 BR	20	816	0.34	0.564
Texas 110	10	78	1.64	0.211
19-16-3-1	14	357	0.00	0.996
21-13-3-5	10	177	0.17	0.684
22-23-2-1	10	243	0.00	0.951
23-21-3-5	28	253	1.34	0.257
24-07-2-1	10	516	0.68	0.418
24-07-2-5	18	343	0.10	0.760
25-03-2-5	13	160	0.60	0.445

Statistics were calculated based on 12 replications from two combined runs of the experiment.

[a] $(\log_{10}[F_{genX}+1]-\log_{10}[F_{genC}+1])-(\log_{10}[E_{genX}+1]-\log_{10}[E_{genC}+1])=0$, where F = number of females, E = number of eggs, genX = genotype being tested, and genC = control cultivar Deltapine 33 B.

[b] All genotypes are *Gossypium hirsutum,* with the exception of Texas 110 which is *G. barbadense.*

[c] Genotypes with number designations are lines from day-neutral Texas race stock accessions T19 (designations beginning with 19 or 21), T1347 (designations beginning with 22 or 23), and T1348 (designations beginning with 24 or 25) selected for moderate levels of resistance to reniform nematode.

[d] Female and egg values are geometric means and are expressed as number per gram of root.

[e] *P* values greater than 0.05 indicate that either life stage of the nematode can be used to evaluate the relative resistance of that genotype with equivalent results.

searchers have reported the use of females (Carter, 1981; Young, 2002; Young et al., 2004) or eggs (Yik and Birchfield, 1981; Yik and Birchfield, 1984; Jones et al., 1988) to evaluate cotton for resistance to the reniform nematode, this is the first report in which these life stages were compared statistically to determine if they can be used interchangeably. Carter (1981) tested accessions of *G. arboreum* for resistance to reniform nematode and the resulting means separations for females per gram of root or egg masses per gram of root identified similar groupings of varieties. While egg production was the primary basis for evaluating *Gossypium* species and other members of the Malvaceae for resistance to reniform nematode, Yik and Birchfield (1984) noted that a correlation existed between female development and egg production. However, in both of these studies, numbers of females and eggs were determined from the same plants harvested on the same date. Resistance determinations based on eggs or females were considered to be equally valid in a study evaluating soybean accessions for resistance to reniform nematode (Lim and Castillo, 1979), who reported strong positive correlations between these parameters and a resistance rating calculated from combined indices of necrosis, nematode counts, egg mass counts, and number of eggs per egg mass. Again, these conclusions were made based on parameters measured on the same plants harvested on the same date. While the results of these studies agree with the findings of the current study regarding interchangeability of life stages, the research reported herein differs in that the results were found to be comparable for counts made on different plants harvested at different times.

Researchers also have used vermiform stages of reniform nematode extracted from soil to assess responses to this pest (Overstreet and McGawley, 1994; Stewart and Robbins, 1996; Robinson and Percival, 1997; Robinson et al., 1999, Robinson et al., 2000; Cook et al., 2001; Robinson et al., 2004). These types of evaluations rely on successful feeding site establishment, but they also rely on the production, hatch, and survival of at least one additional nematode generation. Factors other than host resistance can impact all of these phases in the nematode life cycle. Thus, assays relying on reproduction and development of two or more generations may allow more chance for environmental factors other than host resistance to influence screening results.

Female nematodes have been used successfully and routinely to screen for resistance to other sedentary phytoparasitic species, including soybean cyst nematode (*Heterodera glycines*) and root-knot nematodes (*Meloidogyne* spp.). Host plant response to soybean cyst nematode typically is evaluated based on the number of white or yellow females present on plant roots at the end of one infection cycle (Cook and Noel, 2002; Shannon et al., 2004). Numbers of female soybean cyst nematodes also are the basis for making HG Type determinations (Niblack et al., 2002), which recently replaced race designations (Golden et al., 1970; Riggs and Schmitt, 1988). Work by Palmateer et al. (2000) demonstrated that numbers of eggs or juveniles of soybean cyst nematode resulted in the same race designations derived from counting females. For practical evaluation of a large number of genotypes, female-based rating schemes have been developed for both soybean cyst nematode and root-knot nematode. A 0 to 5 rating scale based on the number of cysts on the roots (soybean cyst nematode) or the incidence of female-containing galls on the roots (root-knot nematode) allows rapid identification of the most resistant genotypes (Young, 1998). Although female counts have proven reliable for plant species where resistance functions by limiting successful development of adult females, they may not be appropriate for evaluating species with resistance expressed as reduced fecundity.

Assessments based on females provide results in less greenhouse time than those based on eggs. The results reported here indicated that laboratory processing and counting times for female- and egg-based assessments were comparable, though these times should not be considered absolute. It is likely that processing times will change with the equipment used. For example, more root systems could be stained simultaneously in a larger capacity microwave, and using additional stir plates or a shaker to extract eggs would shorten the processing time considerably. Likewise, the time required to collect the data would be determined by the level of experience of personnel counting females and eggs. However, even if these or similar modifications could cut processing time in half, the time savings for sample processing would be minimal compared to the time saved in the greenhouse for the different nematode life stages to develop (10 fewer d for females). Processing times for female- and egg-based assessments were not reported by Lim and Castillo (1979), Carter (1981), or Yik and Birchfield (1984). A report by Robinson et al. (2000) concluded that resistance assessments based on soil stages of the reniform nematode required less processing time and were associated with less experimental error than assessments based on eggs from roots of the same cotton plants in a growth chamber environment.

The significance of the time savings associated with female-based assessments becomes evident when considered in the context of the large number of genotypes that would be evaluated in commercial screening programs. Evaluating the number of females increases the number of samples that can be screened in a finite amount of greenhouse space in a defined period of time. Female counts were taken 25 d after inoculation, which translates to 14.6 sets of plants screened in a year (365 d/yr divided by 25 d/test = 14.6 tests/yr). Egg counts were taken 35 d after inoculation, which trans-

lates to 10.4 sets of plants screened in a year (365 d/yr divided by 35 d/test = 10.4 tests/yr). Thus, approximately 40% more samples can be assessed per year if females are counted instead of eggs.

In summary, these experiments demonstrated that both females and eggs of the reniform nematode can be used with equal efficacy to screen for resistance in cotton and its relatives. Further, the time needed to determine reliably if a plant is resistant to reniform nematode can be shortened by using females for phenotype determination.

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