A Quantitative Technique for Evaluating Cotton for Root-Knot Nematode Resistance

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ABSTRACT

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A technique was developed for evaluating cotton (*Gossypium* spp.) for resistance to the root-knot nematode (*Meloidogyne incognita acrita*). Resistance was based on egg production (a nematode response to the plant) and root galling (a plant response to the nematode). To assess root galling and egg production on test plants, individual plants were inoculated with 8,000 eggs in a greenhouse and evaluated 35–45 days later by counting egg masses and rating root galling. Plants almost free of egg masses and root galls were selected and their progeny tested. Final selection for resistance was based on actual numbers of eggs per plant. By using this technique, levels of resistance to egg production were differentiated among upland cultivars, F_3 lines, and *G. hirsutum* races. These levels could not be detected by rating root galling alone. Selection for resistance to root galling and egg production was necessary to develop cotton lines with high resistance to both processes. Cotton accessions exhibited higher levels of resistance and more levels of resistance (ranging from highly resistant to highly susceptible) than has been reported previously in cotton. Selecting highly resistant germplasm by this technique should result in development of agronomically desirable cotton cultivars capable of preventing economic loss from root-knot nematodes.

Additional key words: *Gossypium hirsutum* races, *G. barbadense* race, breeding for resistance.

For decades different variations of root-galling index (11) or root-knot index (13,15) has been the primary or only criterion for evaluating cotton (*Gossypium* spp.) for resistance to root-knot nematodes (*Meloidogyne incognita acritia* Chitwood and Oteifa). Rating schemes used by various workers for rating degrees of root galling or degrees of root galling combined with estimates of the amount of egg mass production on roots were reviewed (1). Little attention has been given to nematode response to the plant in evaluating cotton resistance. Egg production, an indicator of this response, has not been used as a resistance criterion.

During many years of breeding for resistance to root galling, considerable variation in egg production was observed among cotton lines that galled at similar rates. Because of this observation, quantitative methods were sought to permit selection against egg production.

Procedures that facilitate egg counting were reported by Loewenberg et al (8) and Wuest and Bloom (19) who picked egg masses from tobacco and tomato roots, respectively, and then separated the eggs from the egg masses with sodium hypochlorite (NaOCl). Hussey and Barker (7) used NaOCl to separate eggs from undisturbed egg masses on tomato roots to collect and count the eggs. No report was found in which egg counts were used for evaluating the resistance of large numbers of plants.

The purpose of this paper is to describe (i) a technique developed for evaluating large numbers of cotton plants for resistance to both egg production and root galling, and (ii) effectiveness of this technique for evaluating resistance of cotton.

MATERIALS AND METHODS

To produce root-knot nematode galls and eggs on test plants, plastic pots (7.6 × 7.0 cm) were filled with screened, methyl bromide-fumigated, dry, loamy fine sand of pH 6.0 to 6.5. The soil was wet 24 hr before use. Pots were recessed in soil in greenhouse benches to avoid rapid temperature fluctuations. A 4-ml aliquot of 8,000 root-knot nematode eggs in the single-strength Heller's solution described by Loewenberg et al (8) was deposited into holes 2 cm deep in the center of each pot. The eggs were then covered with screened, dry soil which was wetted immediately. Eggs for inoculum were produced on M-8, a doubled haploid of 'Deltapine 14' cotton, which was grown in every 10th row of each test.

To allow egg hatching, pots were covered for about 7 days with a layer of plastic film (100 μm thick) overlaid with a layer of paper, and 50% shading was placed about 30 cm above the paper. The plastic prevented excessive soil drying and the paper and shade prevented excessive soil heating.

Seeds were planted in sand that had been passed through a 1.5-mm mesh screen. After emergence and cotyledon expansion, but before lateral root formation, seedlings were lifted from the sand with straight radicles intact. Radicle tips were excised 5.5 cm below the hypocotyl-radicle junction to permit transplanting to normal depth into the pots of soil. A seedling was transplanted into the center of each pot and maintained under about 50% shade for 2–3 days after transplanting. For all tests, greenhouse temperatures were maintained at 25–35 C.

Thirty-five to 45 days after transplanting, root soil balls of one replicate at a time were gently removed from pots and transferred into 3.2-cm-mesh wire baskets that fit inside 18.9-L containers. Root-soil balls were then immersed in water. Gentle basket agitation quickly freed roots from soil. After washing, roots were held in water until processed.

An egg-mass count and root-knot index were determined concurrently on individual plant roots viewed under a binocular microscope with ×7 magnification. The root-knot index scale was the same as that previously reported (13). The egg-mass counts were determined when there were 15 or fewer large egg masses per plant in the first test and less than three per plant in all other tests.

To collect root-knot nematode eggs, infested roots were excised from stems, drained of excess water, pressed to uniform dryness, and weighed. Complete root systems from plants of one replicate were placed in a 0.47-L wide-mouth glass jar. NaOCl (1.05%) at 25 C was added to these roots at the rate of 35 ml/g root and the jar was sealed with a lid. Roots then were shaken at 180 cycles/min for 4 min with a laboratory shaker to disperse eggs on roots. This method of dispersing eggs with NaOCl is a modification of methods

previously reported (7,8,19). The NaOCl solution with eggs from roots and from two additional root washings was poured into a 75 μm-mesh sieve nested over a 25 μm-mesh sieve. Eggs were collected on the 25 μm-mesh sieve, washed in flowing tap water for about 20 sec to remove NaOCl and placed in about 50 ml of water in sample bottles.

To collect eggs for inoculum, procedures described above were used, except that 0.75% NaOCl was used for dispersing eggs and the eggs were washed in a 25 μm-mesh sieve in flowing tap water for 5 min, transferred to 4 L of water for 1–2 hr and washed again for 5 min. This method of collecting eggs is a modification of a method previously reported (7).

To facilitate egg counting, samples were diluted 1:10 serially until they contained between 10 and 50 eggs/ml. Eggs in three 1-ml aliquots per sample were counted under ×30 magnification with a binocular microscope, and the counts were averaged. This average was used to calculate total eggs per replicate, eggs per plant, and eggs per gram of root.

To determine the relationship between root-knot index and egg count, nine cotton accessions that varied widely in resistance to egg production were evaluated using the egg production, egg collection, and counting methods described above. Five of these accessions (Coker 100A and lines Auburn 623 RNR, Clevewilt 6-3-1, Auburn 56-1, and M-8) were American upland type and four (La. Mexico Wild, 1029, 1105 and *G. barbadense* RNR) were races of *G. hirsutum*, except the *G. barbadense* RNR race. All races were of foreign origin. Egg count and root-knot index ratings were made 40 days after transplanting seedlings into pots. The test was designed as a randomized complete block with six replicates. Replicates included 12 plants per entry.

Twenty F_3 breeding lines previously selected only on the basis of root-knot index from a cross between the resistant line Auburn 623 RNR (14) and susceptible line Auburn 56 were rated for both root-knot index and egg counts using the root-knot egg production and egg collection and counting methods described above. Parent lines and a susceptible check, M-8, were included. The test was designed as a randomized complete block with six replicates. Replicates included 12 plants per entry. Root-knot index and egg counts were determined 42 days after transplanting seedlings into pots.

To determine egg-mass count and root-knot index levels that provided most effective progress from individual plant selection, about 1,100 F_2 plants from crosses of a resistant and a susceptible cultivar were rated for root-knot index and number of egg masses. Regardless of root-knot index, plants with 15, or less, large egg masses were transplanted and grown to maturity in the greenhouse or field where F_3 seed were produced for testing the F_3 progeny. F_2 plants with highest resistance were identified based on egg counts in F_3 progeny tests.

To compare root-knot index versus egg-mass count as a criterion of resistance, *G. hirsutum* races 1029 and 1105, which are maintained in Texas under auspices of Regional Research Project S-77, were grown in five replicates in a test designed as a randomized complete block. Replicates included six plants per entry. Entries were rated for root-knot index and egg-mass count 37 days after transplanting into pots. After rating all plants for root-knot index, three plants per replicate were randomly chosen, and a 2.5-cm-length of lateral root next to the taproot was excised from the third lateral root, counted from the top lateral root on each plant. Egg-mass count per centimeter of root length was determined.

To compare root, root-knot nematode gall, egg mass diameters, and number of eggs per egg mass on resistant versus susceptible cotton roots, these assessments were made on 25 plants each of the resistant line Auburn 623 RNR and susceptible line M-8. Ten randomly selected egg masses per plant were measured and removed from roots. Eggs were dispersed with 1.05% NaOCl, washed free of NaOCl, and counted. One root gall with a single mature female inside and with an egg mass attached was selected at random from each plant. Gall diameter was measured at point of greatest girth along a line parallel to the root. Root diameter was measured 1 mm from the gall nearest the taproot.

RESULTS

Egg counts for nine accessions (five upland and four races) was a continuum with a low of 200 and a high of 122,000 eggs/g root. Root-knot index for the same nine accessions varied from about 1 to 5 (Table 1). There was a significant ($P = 0.05$) correlation ($r = 0.87$) between egg count and root-knot index among the five upland accessions, but egg count and root-knot index were not correlated among the four races of cotton. Two of these races, 1029 and 1105, had similar root-knot indexes but supported greatly different egg production levels. The *G. barbadense* RNR race had a root-knot index of 1.3, which indicated high resistance to root galling; however, it also had about 43,000 eggs/g root, indicating susceptibility to egg production. In comparison, the La. Mexico Wild race was more susceptible to root galling as indicated by a root-knot index of 2.3, but had much lower egg production, 12,000 eggs/g root.

The F_3 progenies from the cross between resistant Auburn 623 RNR and susceptible Auburn 56 all had the same resistance to root-galling based on root-knot index but they exhibited three different levels of resistance to egg production (Table 2). The F_3 progenies with lowest egg counts were equal to the Auburn 623 RNR parent in resistance to egg production. Among the 1,100 F_2 plants rated for resistance based on root-knot index and egg-mass

TABLE 1. Mean galling indexes of, numbers of nematode and eggs in plants of selected cotton accessions infected with *Meloidogyne incognita acrita*[w]

Entry no.	Cotton accession	Accession type[x]	Mean egg no./g root (× 10^3)	Mean root-knot index[y]
1	Auburn 623 RNR	U	0.2 a[z]	1.1 a
2	La. Mexico Wild	HR	12.0 b	2.3 b
3	1029	HR	12.1 b	3.7 cd
4	Clevewilt 6-3-1	U	15.0 b	3.3 c
5	*G. barbadense* RNR	BR	42.9 c	1.3 a
6	Auburn 56-1	U	55.6 d	3.9 cd
7	Coker 100A	U	66.6 d	4.3 de
8	1105	HR	113.0 e	3.5 c
9	M-8	U	122.0 e	4.9 e

[w] Determined 40 days after transplanting seedlings into pots with 8,000 eggs each.
[x] Abbreviations: U = upland, HR = *G. hirsutum* race, BR = *G. barbadense* race.
[y] Root-knot index is based on 1 = none or very light galling, 2 = light galling, 3 = moderate galling, 4 = heavy galling, and 5 = very heavy galling.
[z] Means with different letters are significantly different, $P = 0.05$, according to Duncan's multiple range test.

TABLE 2. Root galling index and egg production of *Meloidogyne incognita acrita* on the cotton parent plants used in a resistant × susceptible cross, on the F_3 lines selected for highest root galling resistance from this cross, and on the controls exposed to the nematode in greenhouse pots[w]

Entry	Mean egg no./plant (× 10^3)	Mean root-knot index[x]
Auburn 623 RNR (parent$_1$)	1.0 a[y]	1.1 a
15 F_3 progeny lines (group 1)[z]	1.0 a	1.2 a
3 F_3 progeny lines (group 2)	2.5 b	1.3 a
2 F_3 progeny lines (group 3)	5.0 c	1.2 a
Auburn 56 (parent$_2$)	45.0 d	3.8 b
M-8 (control)	120.0 e	4.7 c

[w] Plants were rated 42 days after being transplanted into soil infested with 8,000 nematodes per pot and grown in a greenhouse.
[x] Based on 1 = none or very light galling, 2 = light galling, 3 = moderate galling, 4 = heavy galling, 5 = very heavy galling.
[y] Means with different letters are significantly different at $P = 0.05$, according to Duncan's multiple range test.
[z] F_3 progeny lines not significantly different in this test were grouped and their means were averaged.

counts, no F_2 that had more than six large egg masses or a root-knot index greater than 3 was highly resistant when progeny tested in F_3 based on egg counts (Table 3). Of those highly resistant F_2 plants, only a small percentage had egg mass counts greater than three or a root-knot index greater than 1.

In the test of the rleationship between number of egg masses and root-knot index, root-knot indexes on races 1105 and 1029 were similar, 3.5 and 3.7, respectively, but number of egg masses per centimeter of lateral root was seven times greater on race 1105, 5.6 versus 0.8.

In the comparison of root, root-knot nematode gall and egg mass diameters, and number of eggs per egg mass on resistant versus susceptible upland cotton roots, gall diameters were 25 and 88% greater than root diameters on resistant and susceptible lines, respectively (Table 4). Egg masses from the susceptible line averaged twice the diameter and three times greater number of eggs per egg mass than those from the highly resistant line.

Most of the eggs laid by the nematode were extruded to the root surface where they could be removed by NaOCl. This was determined by shaking roots of both resistant and susceptible plants in 1.05% NaOCl for 8 min, in 2-min intervals, to remove all eggs. Roots were stained (10) and root galls were dissected. Less than 0.1% of total eggs laid remained partially inside roots; these were exposed but were inside small crevices made by egg laying in the root cortex.

DISCUSSION

By measuring egg production on the roots, cotton accessions and F_3 lines exhibited a range of levels of resistance to egg production from almost immune (< 500 eggs/g root) to highly susceptible (> 120,000 eggs/g root).

Egg counts were correlated with root-knot index among the upland accessions but not among the race accessions (Table 1). Differences in origins may account for differences between uplands and races in their response to root-knot nematodes. Many current upland cultivars, including upland accessions used in this study, probably descended with similar selection from common ancestors (18). In comparison, the race accessions are wild cottons from different foreign locations and are probably unrelated.

Independence between egg counts and root-knot index in the wild cotton races (Table 1) was similar to that between visually rated numbers of egg masses and galling reported in beans (6,20). This observation is also supported by previous reports that root-knot nematode growth and development were closely associated with syncytia formation (4,5,16,17). However, syncytia formation was observed to be independent of and without gall formation. This was attributed to syncytia and galls being different responses to root-knot infection (3). In addition, gall formation was observed in the absence of egg production, and even without the nematode entering the roots (9).

Independence between egg counts and root-knot index, failure of root-knot index technique alone to differentiate egg production levels in races 1029 and 1105, and failure of root-knot index to differentiate small differences in egg production levels among F_3 progenies (Table 2) show that selection against egg production and root galling both are necessary for developing highly resistant cotton cultivars.

Egg counts were more precise than egg-mass counts for assessing egg production. This was shown by variation between resistant and susceptible lines in egg-mass size and number of eggs per egg-mass. Egg masses from the susceptible line were twice as large and had three times greater number of eggs. However, egg-mass counts were more precise than root-knot index as an index of egg production. This was evident when egg-mass counts detected differences in egg production levels between races 1029 and 1105 that were not distinguished using root-knot index.

Selection of individual F_2 plants was based on egg-mass counts and root-knot index when selected plants were to be replanted because egg collection procedures destroyed roots. Even among F_2 selected with lowest root-knot index and egg-mass count levels, a high percentage were susceptible, based on egg counts in F_3 progeny tests (Table 3). This demonstrated the need for F_3 progeny testing based on egg counts for selecting lines with highest resistance.

Percentages of plants selected in F_2 showing high resistance based on egg counts in F_3 progeny tests may be substantially increased by selecting those with no more than 1 and 2 root-knot index and egg mass counts, respectively. This was indicated by the extremely low percentages of resistant F_2 with a root-knot index higher than 1 and an egg-mass count higher than 2 (Table 3).

Exceptionally high resistance to root-knot nematode reproduction was shown in cotton when only 12.5% as many eggs were obtained from resistant lines as were applied as inoculum (Tables 1 and 2). Based on egg counts, this is the highest level of resistance to root-knot nematodes ever reported in cotton. This is also the first report of the use of egg counts as a criterion for selecting cotton plants for root-knot nematode resistance. If cultivars can be bred with such a high level of resistance, they should reduce root-knot field populations below levels that cause economic damage. Data (R. Shepherd, *unpublished*) support this view.

In view of this high resistance, egg counts appear to be a much more appropriate selection criterion than root-knot indexes for root-knot resistance in cotton. Egg count is a measure of the nematode's response to the plant that indicates the nematode's ability to complete its life cycle. If this cycle can be broken, the nematode would cease to be a problem regardless of plant galling response. Egg count also is determined more precisely than root-knot index. Egg count is determined objectively and is quantitated, whereas root-knot index and other indexes reported (2,6,12) for

TABLE 3. Distribution among egg-mass count (EMC) and root-knot index (RKI) classes of F_2 plants from resistant × susceptible crosses infected with *Meloidogyne incognita acritia* and number and percent of these F_2 resistant in F_3 progeny tests based on egg counts (EC)

Rated classes[x]	Total F_2/class	Resistant F_2 class	
	(no.)	No.	Percent
EMC class[y]:			
0	12	3	25.0
1	32	4	12.5
2	36	5	13.9
3	26	2	7.7
4	44	2	4.5
5	34	0	0.0
6	48	1	2.1
RKI class[z]:			
1	87	7	8.0
2	302	9	3.0
3	557	1	0.2

[x]Only EMC and RKI classes containing F_2 with resistant F_3 progeny are given. Regardless of RKI, only F_2 with 15 or fewer EMC were selected and progeny tested based on EC.
[y]Numbers of large egg masses per plant.
[z]Root-knot index is based on 1 = none or very light galling, 2 = light galling, 3 = moderate galling, 4 = heavy galling, 5 = very heavy galling.

TABLE 4. Comparison of root, root-knot nematode gall, and egg-mass diameters and number of eggs per egg-mass on resistant versus susceptible cotton roots exposed to *Meloidogyne incognita acrita*

Cotton entry	Mean root diam[w] (mm)	Mean gall diam[x] (mm)	Gall diam as % of root diam[y] (%)	Mean egg-mass diam (mm)	Eggs per egg-mass (Mean no.)
Auburn 623 RNR	.60 a[z]	.76 a	125 a	.44 a	255 a
M-8	.56 a	1.05 b	188 b	.98 b	850 b

[w]Measured 1 mm away from gall toward taproot.
[x]Only galls with one egg mass were measured.
[y]Measured while on root at fullest point on line parallel with root.
[z]Means with different letters are significantly different, $P = 0.05$, according to Duncan's multiple range test.

assessing galling and egg-mass production are relative ratings determined subjectively by judging degrees of galling and estimating relative numbers of egg masses among entries or against standards.

The Auburn 623 RNR source of resistance and additional sources of resistance recently discovered (R. Shepherd, *unpublished*) and the described technique provide high potential for developing resistant cotton cultivars capable of preventing economic loss from root-knot nematodes.

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