

LONREN UPLAND COTTON GERMPLASM RESPONSE TO *ROTYLENCHULUS RENIFORMIS* INOCULUM LEVEL

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ABSTRACT

Sikkens, R. B., D. B. Weaver, K. S. Lawrence, S. R. Moore and E. van Santen. 2011. LONREN Upland Cotton Germplasm Response to *Rotylenchulus reniformis* inoculum level. *Nematropica* 41: 68-74.

Rotylenchulus reniformis (reniform nematode) can be a yield-limiting factor for upland cotton. Resistance to this nematode was discovered in a wild cotton relative, transferred to an upland background and released as the germplasm LONREN. The LONREN source of resistance to reniform nematode in upland cotton was evaluated for reaction to increasing levels of inoculum on root and shoot growth and nematode reproduction compared to susceptible genotypes. Genotypes tested were LONREN-1 and LONREN-2 (resistant); Fibermax 966 and Deltapine 555BR (susceptible); and one susceptible and one resistant F_{2,4} line from the cross LONREN-1 × Fibermax 966. Inoculum levels of 0, 500, 1000, 5000, 10,000, or 50,000 juveniles and vermiform of *R. reniformis* were used. Plant height and vigor ratings were collected weekly. Root and shoot fresh and dry mass, and nematode numbers were collected 60 days after inoculation. Based on inoculation rate, a reproduction factor (Rf) was calculated as the ratio Pf/Pi, where Pf = final nematode population and Pi = initial inoculum level. Root dry mass of genotypes with the LONREN resistance source was reduced at high inoculation density compared to low inoculum levels. An opposite trend was observed for root dry mass of reniform nematode susceptible genotypes, where root dry mass increased at higher inoculum levels. Shoot dry mass for all genotypes was largely unaffected except at the highest inoculum level. Shoot to root dry mass ratios for the LONREN resistance source genotypes were found to trend higher with increased inoculum levels, whereas the same ratios decreased in case of the susceptible genotypes. Examination of the roots of LONREN resistant source genotypes showed signs of necrosis. We conclude that the LONREN resistance source exhibits a type of hypersensitive response to reniform nematode. This reaction, while inhibiting reniform nematode reproduction, appears to be a factor in seedling stunting at higher nematode density levels.

Key Words: LONREN, reniform nematode, root necrosis, inoculum pressure

RESUMEN

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Rotylenchulus reniformis (nematodo reniforme) puede ser un factor limitante en la producción de algodón. Se descubrió resistencia a este nematodo en parientes silvestres del algodón y se transfirió dicha resistencia al algodón cultivado para crear el germoplasma LONREN. En este trabajo, evaluamos la reacción del germoplasma a diferentes niveles de inóculo de nematodo reniforme. La reacción se midió en términos de crecimiento de raíces y brotes y reproducción del nematodo. Se evaluaron los genotipos LONREN-1 y LONREN-2 (resistentes); Fibermax 966 y Deltapine 555BR (susceptibles); y una línea F_{2,4} susceptible y otra resistente del cruce LONREN-1 × Fibermax 966. Se usaron niveles de inóculo de 0, 500, 1000, 5000, 10,000 ó 50,000 juveniles y vermiformes de *R. reniformis*. Se midió la altura y el vigor de las plantas cada semana. El peso seco y fresco de raíces y brotes se midió 60 días después de la inoculación. El factor de reproducción (Rf) se calculó usando Pf/Pi, en donde Pf = población final del nematodo y Pi = nivel de inóculo inicial. Se observó una reducción en la masa seca de raíces de los genotipos con resistencia LONREN con los niveles de inóculo más altos. En los genotipos susceptibles, se observó la tendencia contraria, con un incremento de la masa de raíces con los niveles de inóculo más altos. No se observó efecto sobre la masa seca de los brotes, excepto con los niveles de inóculo más altos. Se encontró que la relación masa seca de brotes sobre masa seca de raíces aumentó con el nivel de inóculo para los genotipos LONREN, mientras que esta relación disminuyó en el caso de los genotipos susceptibles. Se encontró necrosis en las raíces de los materiales LONREN. Concluimos que

la fuente de resistencia LONREN muestra un tipo de resistencia hipersensible al nematodo reniforme. Esta reacción inhibe la reproducción del nematodo reniforme, pero también parece ser un factor en el pobre desarrollo de las plántulas a altos niveles de inóculo.

Palabras clave: LONREN, nematodo reniforme, necrosis de raíces, niveles de inóculo

INTRODUCTION

Upland cotton (*Gossypium hirsutum* L.) is attacked by a variety of plant-parasitic nematodes, including the reniform nematode (*Rotylenchulus reniformis* Linford and Oliveria). The reniform nematode was first identified as a cotton pathogen in 1940 (Smith, 1940), recognized as a serious problem a few years later (Jones *et al.*, 1959), and has been increasing in importance since that time (Lawrence and McLean, 1995). Losses to reniform nematode in U.S.- produced cotton have been estimated at approximately 204,000 bales, with AL, LA, and MS having the largest percent of total crop yield loss (Blasingame *et al.*, 2010). Reniform nematode is known to affect cotton primarily through a reduction in yield, boll size and lint percentage (Cook *et al.*, 1997; Jones *et al.*, 1959). Plants can be stunted, and may respond poorly to irrigation and fertilization (Birchfield and Jones, 1961). Management options, particularly commercially available sources of genetic resistance, are limited. Although reports of tolerance can be found in the literature (Cook *et al.*, 1997), no appreciable levels of resistance have been found in adapted upland cultivars (Robinson *et al.*, 1999; Usery *et al.*, 2005). Extensive searches of the cotton germplasm collection have revealed some accessions of *G. hirsutum* with moderate resistance, generally based on reduced reproduction of the nematode compared to a susceptible check (Robinson *et al.*, 2004; Weaver *et al.*, 2007), but no high levels of resistance have been reported. Apparent inconsistent resistance within *G. hirsutum* is also a problem. Yik and Birchfield (1984) reported three wild *G. hirsutum* accessions as resistant, but later Robinson and Percival (1997) reported the same accessions as not different from the susceptible 'Deltapine 16'. Similarly, Weaver *et al.*, (2007) found seven accessions to be moderately resistant, but resistance was not confirmed in later studies (Sürmelioglu *et al.*, 2010).

To find high levels of resistance to reniform nematode, researchers have turned to cultivated and noncultivated relatives of upland cotton. All four tested accessions of *G. longicalyx* (Hutch. & Lee) were reported to be immune to reniform nematode (zero egg production) (Yik and Birchfield, 1984). Female nematodes were observed to penetrate the roots of *G. longicalyx*, but remained vermiform during the test period without producing eggs (Agudelo *et al.*, 2005). Two other related species, *G. stocksii* Masters and *G. somalense* (Gürke) Hutchinson, were rated highly resistant based on very low nematode reproduction

in comparison to the susceptible check Deltapine 16. All of these species are of African origin. Yik and Birchfield (1984) also reported specific genotypes of *G. barbadense*, *G. arboreum*, *G. herbaceum*, and *G. raimondi* that had 20% or less egg production compared to Deltapine 16, and were rated as resistant. Later, work by Robinson and Percival (1997) and Robinson *et al.* (2004) found several accessions of *G. barbadense* to be resistant.

Using an unknown accession of *G. longicalyx*, Robinson *et al.* (2007) transferred the resistance to a *G. hirsutum* genetic background in an attempt to develop adapted *G. hirsutum* germplasm that could be used commercially to reduce losses associated with reniform nematode. However, not only are the ploidy levels different between the two species (*G. longicalyx* is diploid, $2n = 26$ and *G. hirsutum* is tetraploid, $2n = 52$), but they contain different genomes (*G. longicalyx* has the F genome, *G. hirsutum* the A and D genomes). Therefore tri-species hybrids were created, using either *G. armourianum* Kearny (diploid, D genome) or *G. herbaceum* L. (diploid, A genome) as "bridge" species to overcome the chromosome incompatibility issues. Six or more backcrosses were made to *G. hirsutum*, with selection during the backcross generations for nematode resistance along with cytogenetic analysis for ploidy level. Inheritance of resistance was found to be monogenic, with a single dominant gene conferring resistance (Robinson *et al.*, 2007). The gene for resistance was mapped to chromosome 11 (A genome) and was determined to be linked to the microsatellite marker BNL3279 114 and given the gene symbol REN^{lon} (Dighe *et al.*, 2009). REN^{lon} is also linked to a morphological marker, green seed fuzz, with gene symbol Fzg^{lon} . Two reniform-resistant BC7 germplasm lines were released by USDA in April of 2007, named LONREN-1 and LONREN-2 (Starr *et al.*, 2007). These germplasm lines were tested in both the greenhouse and field for reniform nematode resistance and agronomic traits, and were found to be acceptable (Starr *et al.*, 2007). After initial release of the germplasm, and during field testing over a wide range of environments, early-season stunting of the plants was observed in areas of high nematode population density (Nichols *et al.*, 2010). Two possible reasons given by Nichols were (1) the mechanism of resistance may have led to increased susceptibility to other pathogens or (2) linkage drag caused by segments of alien chromosome in the area around the REN^{lon} locus.

Our objectives were to characterize the LONREN source of resistance to reniform nematode in

early season plant growth and development at various levels of nematode population density.

MATERIALS AND METHODS

Genetic material

Six genotypes of *G. hirsutum* were studied. Two *G. longicalyx*-derived germplasm lines, LONREN-1 and LONREN-2 (resistant) were compared with susceptible 'Fibermax 966' (FM966) and 'Deltapine 555BR' (DP555BR). Also included were one susceptible and one resistant $F_{2,4}$ line from the cross LONREN-1 \times Fibermax 966, B104 (resistant) and B108 (susceptible). These lines were selected from a larger population of 100 random lines from the original cross, of which 21 were determined to be homogeneous for resistance by greenhouse inoculation tests (Sürmelioglu *et al.*, 2010). Lines were also evaluated for presence (or absence in susceptible lines) of the microsatellite marker BNL1066_156, one of the three markers closely linked to the REN^{lon} gene in LONREN-1 (Dighe *et al.*, 2009). Resistant lines were also screened for the closely-linked morphological marker gene for green seed fuzz, Fzg^{lon} . B104 (resistant) was found to be positive and homogeneous for the microsatellite marker and the Fzg^{lon} gene. B108 was negative and homogeneous for both traits.

Inoculation and isolation

Methods of inoculation and evaluation were similar to those of Weaver *et al.* (2007). Seeds were planted in the greenhouse in 150 ml cone-tainers (Ray Leach Cone-tainers™, 2.5 cm dia \times 20 cm depth) filled with soil (68% sand, 20% silt and 12% clay with 0.3% OM and pH of 6.4); one seed per cone-tainer. Each cone-tainer was surrounded by four unfilled spaces in the rack so that each plant had approximately 25 cm² of available area. The soil was sterilized by autoclaving twice at 121°F and 103.4 kPa for 2 hours on two consecutive days. Each genotype was inoculated with *R. reniformis* at population levels of 0, 500, 1,000, 5,000, 10,000, and 50,000 juveniles and vermiform adults per cone-tainer (150 ml of soil). Treatment combinations were replicated 10 times. Because of the possibility of cross-contamination between nematode inoculum levels (splash during watering), cone-tainers with similar inoculum levels were kept together and were not randomized. Racks were rotated weekly within the greenhouse zone to avoid location effects. Plants were watered and fertilized as needed using Peter's 20-10-20 (Buddies Plant Food, Ballinger, TX) water-soluble fertilizer. At 17, 31, and 45 days after planting, one plant of each treatment combination was harvested to observe root development. Data on root and shoot, fresh and dry mass, were also collected at those times. These data are not included because they did not provide further insight into the final results

and conclusions of the experiment. However, these observations reduced the number of replications from 10 to 7.

Nematode extraction

Sixty days after inoculation, nematodes were extracted from the soil of the remaining seven replicates using a modified Baermann funnel technique. Soil and plants were gently removed from their cone-tainers, encased in a 30 x 30 cm Kimwipe, and positioned on a 15 \times 15 cm pliable screen covering a 470 ml plastic funnel. Rubber tubing was attached to the stem of the funnel and pinched with a clamp. Water was added to the funnel covering the root system and evaporated water was replaced as needed. After 48 hours, 100 ml of water was drained from the tubing and sieved through a 25- μ m pore sieve to collect and count the vermiform nematodes. After carefully removing all soil from the roots, all plants were photographed before collecting the root and shoot fresh mass. Figure 1 presents an array of LONREN-2 plants showing representative growth characteristics for the group of reniform nematode resistance genotypes. A collection of FM966 plants is shown in Figure 2; it is a representative line-up for the group of reniform nematode susceptible genotypes. Roots and shoots were dried for 72 hours at 60°C prior to determining their dry mass and the shoot-to-root ratio determined. Based on final nematode counts, a reproduction factor (Rf) was also calculated as the ratio Pf/Pi, where Pf = final nematode population and Pi = initial inoculum level.

Statistical analysis

Data were analyzed using generalized linear models procedures as implemented in SAS® PROC GLIMMIX (SAS, 2010). Fixed effects consisted of genotype, population (= number of reniform nematode applied per 150 cc of soil) and their interaction. The student panel option was used to investigate the behavior of residuals. The normality assumption was confirmed for root and shoot dry mass. Final nematode population (number of nematodes per 150 cc after 60 days), number of nematodes per g fresh root mass, and reproductive factor (Rf) as well as the shoot-to-root-ratio required a lognormal distribution function. The most general model chosen was a full classification model because it does not assume a specific shape of a response. Whenever possible a covariance model was employed treating population levels as a quantitative independent variable. The P-values for pairwise contrasts among genotypes was adjusted for multiple comparisons using the simulation option for the LSMEANS command (SAS, 2010). Least squares means estimates from analyses using a log-normal distribution function were back-transformed to the original scale and presented with confidence intervals.

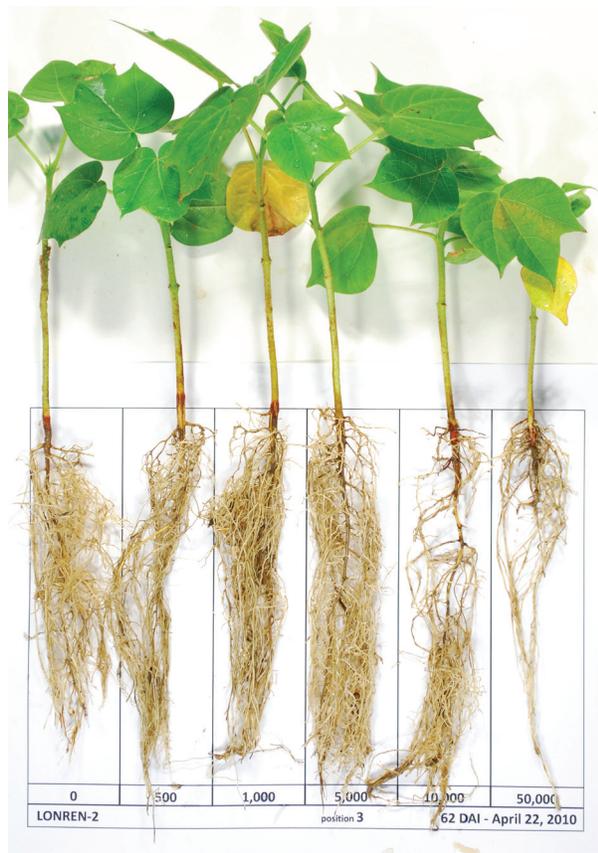


Fig. 1. Representative line-up of reniform nematode resistant LONREN-2 seedlings at various inoculum levels. Plants were photographed upon completion of two days of nematode extraction. Note the reduction in root volume and stunted shoots at higher inoculum levels.



Fig. 2. Representative line-up of reniform nematode susceptible FM966 seedlings at various inoculum levels. Plants were photographed after being positioned for two days on funnels for nematode extraction. Note the increased root biomass at higher inoculum levels.

RESULTS

Nematode reproduction

The genotype \times population interaction was not significant ($P > 0.35$) for nematode reproduction traits. Thus, inoculum level had no effect on genotype differences and rankings. Therefore, a main effects model was chosen. The response to increasing population levels could be modeled as a 2nd order polynomial. Population was used as a covariate to improve the precision of genotype mean estimates at a standard inoculation rate of 5,000 per 150 cc of soil. The reniform nematode-resistant $F_{2,4}$ genotype B104 could not be distinguished from the two LONREN lines for any of the three nematode reproduction traits. It was, however, clearly distinguishable from its non-resistant sister line B108 and the susceptible check FM966 (Fig. 3). Statistical significant differences between B104 and the commercial check DP555BR could not be ascertained ($P > 0.22$) as is evident from the overlapping confidence intervals.

Root and shoot mass

The genotype \times population interaction was a very important source of variation ($P < 0.0001$) for host plant biomass traits, hence the final model was a full interaction model. While there were similarities in the response of genotypes to increasing population levels – generally a drop in plant mass at lower populations, followed by a rise as population levels increased and another decline at very high population levels – the presence of rank and magnitude changes made it difficult to present a unified picture. Hence a tabular presentation of interaction means was chosen (Table 1). The major difference among genotypes was the dramatic decline in root mass for the reniform nematode-resistant lines at high population numbers. B104 root dry mass was reduced by 75% at the 50,000 inoculum level compared to non-inoculated control. Roots of several B104 individuals showed severe necrosis, where seriously stunted shoots were supported by very little remaining root biomass. The overall decline in dry root mass for all three LONREN genotypes was approximately 50%. This was in sharp contrast to the

Table 1. Genotype \times population interaction least squares means for root and shoot dry mass and shoot to root ratio on a dry matter basis. Shoot-to-root ratios were calculated on a per-cone-tainer basis, hence mean shoot-to-root ratio generally will not equal the shoot-to-root ratio calculated on the basis of the means in this table.

Genotype	Reniform Nematode inoculum density, per 150 cc soil						
	0	500	1,000	5,000	10,000	50,000	
Shoot dry mass, g cone⁻¹							
LONREN-1 (res.)	0.48	0.50	0.30	0.37	0.45	0.26	
LONREN-2 (res.)	0.67	0.60	0.61	0.66	0.73	0.42	
B104 (res.)	0.69	0.61	0.59	0.54	0.66	0.22	
B108 (sus.)	0.47	0.52	0.41	0.44	0.35	0.30	
FM966 (sus.)	0.60	0.46	0.31	0.32	0.41	0.30	
DP555BR (sus.)	0.36	0.27	0.26	0.36	0.17	0.25	
LSD _{0.05}							0.17
Root dry mass, g cone⁻¹							
LONREN-1	0.42	0.30	0.22	0.32	0.33	0.17	
LONREN-2	0.40	0.28	0.36	0.34	0.36	0.21	
B104 (res.)	0.36	0.45	0.36	0.30	0.42	0.09	
B108 (sus.)	0.20	0.25	0.31	0.53	0.43	0.57	
FM966	0.26	0.34	0.26	0.36	0.57	0.54	
DP555BR	0.25	0.36	0.39	0.44	0.18	0.36	
LSD _{0.05}							0.21
Shoot to root dry mass ratio							
LONREN-1	1.33	1.81	1.53	1.36	1.60	1.63	
LONREN-2	2.20	2.31	2.08	2.37	2.16	2.75	
B104 (res.)	2.11	1.62	1.75	1.85	1.59	2.31	
B108 (sus.)	2.51	2.53	1.56	1.76	0.91	0.68	
FM966	2.42	1.45	1.31	0.96	0.76	0.59	
DP555BR	1.58	0.78	0.73	1.02	1.35	1.05	
LSD _{0.05}							1.05

Table 2. Average shoot heights, measured 63 days after planting (56 days after inoculation)

Genotype	Reniform Nematode inoculum density, per 150 cc soil						
	0	500	1,000	5,000	10,000	50,000	
shoot height (cm)							
LONREN-1	7.6	6.4	8.1	7.7	6.4	5.8	
LONREN-2	9.7	8.7	11.1	10.1	9.2	7.8	
B104 (res.)	9.2	8.9	9.6	9.1	8.2	5.6	
B108 (sus.)	8.5	7.3	9.2	9.0	8.6	7.9	
FM966	9.0	8.6	8.4	9.6	9.4	10.4	
DP555BR	8.2	7.0	7.6	9.7	6.8	8.3	
LSD _{0.05}							1.61

susceptible entries that had increases in root dry mass ranging from 43% for DP555BR to 180% for B108 (Table 1).

B104 exhibited a similar decline in shoot dry mass at the 50,000 level compared to uninoculated (68%). All remaining genotypes also declined in shoot mass when exposed to extreme high levels of reniform nematodes in this experiment, with declines ranging from 36% (B108) to 50% (FM966).

The most interesting aspect of the results were the calculated dry mass shoot to root ratios in response to increasing nematode populations. Increases in root dry mass in response to increasing nematode population numbers, combined with declines in shoot dry mass, resulted in noticeable declines in shoot to root ratios

for susceptible entries B108 and FMR966 (Fig. 4). On the other hand, the resistant entries B104, LONREN-1, and LONREN-2 exhibited no significant change in dry mass shoot to root ratio in response to population levels. Check cultivar DP555BR exhibited a decline in response to inoculation but that response was not dependent on population level.

Figures 1 and 2 illustrate the changes in root mass under various reniform nematode inoculum levels. Figure 1 shows the decrease in root mass at high inoculum levels in reniform nematode resistant LONREN lines. Increase in root mass at high inoculum levels in reniform nematode susceptible lines is visible in the photograph of Figure 2.

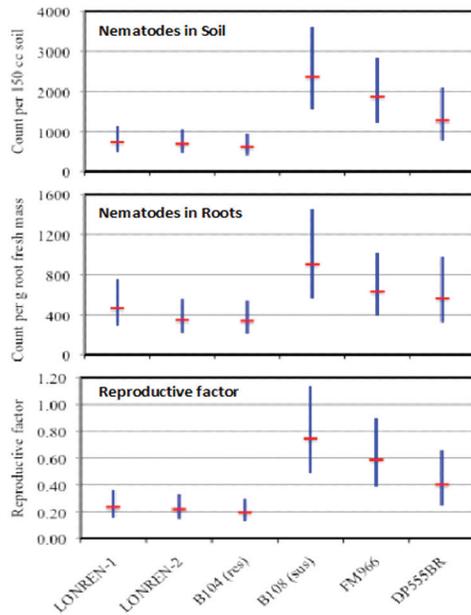


Fig. 3. Genotype least squares means for soil reniform level, reniform level per g root fresh mass, and reproductive factor (Rf) for six cotton genotypes inoculated with six reniform levels. The analysis was conducted using a lognormal distribution function. The horizontal red bars indicate the back-transformed genotype means and blue vertical lines the 95% confidence interval.

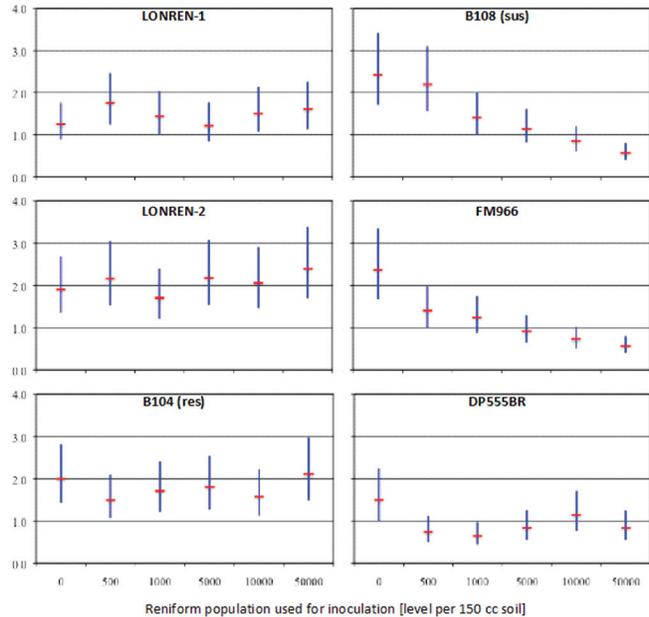


Fig. 4. Genotype x population interaction least squares means for shoot to root ratio of six cotton genotypes inoculated with six levels of reniform nematode. The analysis was conducted using a lognormal distribution function. The horizontal red bars indicate the back-transformed genotype means and blue vertical lines the 95% confidence interval.

Plant height

Table 2 presents average shoot heights, measured 63 days after planting (56 days after inoculation). Shoots of the two LONREN lines showed height decreases of about 20% at the 50,000 inoculum level compared to the non-inoculated controls. Nematode resistant line B104 experienced an even greater height reduction (40%). No reduction in seedling height was observed in the nematode-susceptible genotypes.

DISCUSSION

The reniform nematode-resistant LONREN lines showed a progressive decrease in root mass with increasing reniform nematode density levels. This decrease in root mass was accompanied by small decreases in shoot mass, resulting in shoot to root mass ratios largely unchanged at all inoculum levels. However, this relative stability of the shoot to root mass ratio should not hide the observation that, especially at the highest inoculum level, the shoots grew less and showed stunting. Similarly, at higher population levels roots had less mass and presented signs of necrosis. These observations were consistent with field observations; where, in the presence of high reniform

nematode numbers, LONREN lines were reported to show mild to severe stunting (Nichols *et al.*, 2010). The results of our research indicated that this stunting could be the result of a hypersensitive reaction in LONREN, similar to the earlier described reaction in *G. longicalyx* (Agudelo *et al.*, 2005). LONREN genotypes seem to grow well when reniform nematode populations are at a relatively low level. It appears that under such conditions any damage due to hypersensitivity is not immediately growth inhibiting. On the other hand, hypersensitivity of LONREN to reniform nematode becomes conspicuous in the presence of high nematode numbers, when root necrosis and plant stunting is observed.

In the case of the reniform nematode susceptible genotypes included in our study, the dramatic decline in the shoot to root mass ratios which accompanied increases in nematode population levels was primarily due to increases in root mass. Shoot heights and fresh shoot mass did not vary much for this group, although dry shoot mass was less at the highest nematode inoculum levels. Shoots of susceptible lines did not show easily detectable signs of stunting. Seedlings from these lines seem to be able to cope better with the onslaught of the reniform nematode, at least at the very early growth stages.

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