A PROTOCOL FOR ISOLATING AND ENUMERATING *THIELAVIOPSIS BASICOLA* FROM COTTON ROOTS: APPLICATIONS TO LONREN STUNT

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Abstract

Thielaviopsis basicola is a slow growing fungus that is easily overwhelmed by bacteria and other fungi when whole tissues are plated on media. Developing selective media is complicated by sensitivity of the fungus to many antibiotics. A selective medium was developed, that allows ready recovery of the fungus from diluted fine tissue particles produced from surface sterilized roots by a polytron tissue generator. Dilution series of dispersed particles with drop applications to media allows determination of colony forming units (cfu) per gram of fresh root tissues. Transplanting 48-hour-old seedlings into soil cores and analyzing roots after 3 to 7 days shows the inoculum potential of soils. Measuring shoot or root weight after 10-14 days or measuring hypocotyl and first internode lengths of mature plants provides quantitative measures of stunting. Stunting of LONREN lines carrying the Ren_1^{lon} gene compared to susceptible sibs (- Ren_1^{lon}) or the cultivar Fibermax 966 was generally correlated with reniform nematode and *T. basicola* concentrations in soils. Other fungi also influenced the degree of stunting. *Fusarium oxysporum* f. sp. *vasinfectum*, especially races 3 and 4, enhanced stunting while a mixture of *F. solani* and *F. verticillioides* greatly decreased stunting. *Talaromyces flavus* and *Trichoderma virens* concentrations were also negatively correlated with stunting. Three approaches were taken to dissociate stunting from resistance to reniform nematode: 1) Five lines with Ren_1^{lon} placed in chromosome 21 instead of 11 were developed, 2) stunt resistant lines that contained the close co-dominant marker BNL3279_114 for Ren_1^{lon} were selected and evaluated for resistance to reniform nematode and presence of the repulsion marker BNL1231, and 3) the Ren_1^{lon} gene was recombined with the Ren_2^{GB713} gene which confers resistance to stunting. Each approach failed to dissociate the Ren_1^{lon} gene from stunting caused by a reniform nematode-*T. basicola* complex.

Introduction

The germplasm lines LONREN-1 and LONREN-2 were released in 2007 as new sources of resistance to reniform nematode (*Rotylenchulus reniformis*). The lines suppressed nematode reproduction by 95-98% in growth chamber or greenhouse bioassays and had excellent agronomic properties when grown in fields free of the nematode (Robinson *et al.*, 2007). Resistance appeared to be due to a single gene, Ren_1^{lon} located on chromosome 11, and was tightly linked to the co-dominant marker BNL3279_114 (Dighe *et al.*, 2009). The gene also could not be disassociated from the repulsion marker BNL1231.

When LONREN lines were planted in fields infested with reniform nematode, they often were severely stunted compared to sister lines or commercial cultivars that lacked Ren_1^{lon} (Figure 1). Similar symptoms developed when 2-day-old seedlings were transplanted into cores of the field soil placed in pasteurized sandy loam soils (Figure 2). In the latter model system stunting could be prevented by the fungicides Terrazole or Benlate indicating that fungi were involved in the stunting (Bell *et al.*, 2011).

While symptoms of LONREN stunt resembled those of seedling root rots, especially black root rot, attempts to isolate *T. basicola* were unsuccessful. We report here the development of a new selective medium and isolation protocols that readily allow detection and enumeration of *T. basicola* in infected cotton roots. These methods were used to determine the role of *Thielaviopsis* and other fungi in stunting. Mixtures of the nematode and fungi were also used as inoculum in an attempt to separate stunt susceptibility from nematode resistance.

Figure 1. Stunting of a reniform nematode-resistant LONREN line (*Ren*$_1^{lon}$ gene) compared to its susceptible sibs on each side at the Texas AgriLife Research Farm in Snook, Texas.

Figure 2. Stunting of the LONREN line MR-19 (left) compared to the Fibermax 966 cultivar (center) and the stunt resistant BARBREN 713 (right) when planted in cores of soil containing root-knot nematode plus *Thielaviopsis basicola*.

Materials and Methods

Development of Selective Media

Fungi were isolated from reniform-infested field soils using established methods (Singleton *et al.*, 1992). Sensitivity of fungi to antibiotics was determined by adding antibiotics dissolved in 10 ml of 70% ethanol or pure ethanol to molten potato dextrose agar at 42-45ºC just prior to pouring medium. Agar sections or conidia from cultures on PDA were placed on test antibiotics and colony size compared to that on PDA alone was determined after 4 and 8 days of incubation at 25ºC. Composition of the TB-CEN selective medium (Specht and Griffin, 1985) used for

comparisons is presented in Table 1, and composition of the TB-RCTT selective medium developed from the antibiotic studies is shown in Table 2.

Table 1. TB-CEN selective medium (Specht and Griffin, 1985).

Carrot juice	40-60ml
Chlortetracycline HCI	30 mg
Etridiazole	400 mg
Nystatin	250,000 units (48 mg)
Streptomycin sulfate	500 mg
Calcium carbonate	1 g
Agar	15-20 g
Deionized water	1 L

►Add all ingredients to molten water agar at 42-45ºC just prior to pouring.

Table 2. TB-RCTT selective medium (Bell et al., 2012).

Carrot juice	100 ml	
Rifampicin	50 mg	Dissolved in 10 ml ethanol.
Carbenicillin	100 mg	
Terraclor (75% a.i.)	0.7 g	Dissolved in 10 ml ethanol.
Terrazole (35% a.i.)	1.4 g	
Agar	20 g	
Deionized water	1 L	

►Add antibiotics and fungicides to molten water agar/carrot juice at 42-45ºC prior to pouring.

Isolation and Enumeration of *Thielaviopsis basicola*

Diseased seedlings from the field were collected 1 or 2 weeks after planting and tested for presence of *T. basicola.* Various concentrations of sodium hypochlorite, ethanol, and hydrogen peroxide were used to treat roots for various time intervals to determine the most effective treatment to prepare roots for plating directly on media or for comminution by a Polytron tissue generator model PTA205.

For fungal trapping experiments, acid-delinted seed, that had been gravity graded in acetone and dried, were rolled in seed germination towels wet with sterile RO purified water and incubated for 24 hours at 30ºC followed by 24 hours at 15ºC. The seedlings, usually with radicles 2-4 cm in length, were then transplanted into test soils using a wash bottle to firm the soil around the radicle. Seedlings were pulled from the soil 3-7 days after planting, washed with 3% hydrogen peroxide, and comminuted in 10-50 ml sterile water for 30 seconds at a power setting of 6 with a Kinematica Polytron-Aggregate instrument. Dispersed tissues were either diluted or applied directly to selective media to determine cfu of fungi per gram or milligram of tissue.

Disease Evaluations

A 3:1 mixture of sandy loam and fine sand soil was placed in 16 oz. polystyrene (PS) cups drilled to provide 3 drainage holes and fitted with nylon screen over the holes to retain soil. Cups were thoroughly wet with RO purified water and after 48 hours were pasteurized with aerated steam at 75ºC for 16 hours. For critical experiments, pasteurization was repeated after an additional 48 hours.

A 1 × 2½ inch core of the pasteurized soil was removed from the center of cups and the cavity was filled with 50 g of natural field soil or a sand culture of a fungus. Procedures for preparing fine sand medium are presented in Table 3 and the set up for inoculating cups is shown in Figure 3.

Fine sand medium was inoculated with fungal spore suspensions or 4 mm squares of agar cultures as appropriate and was incubated with intermittent mixing for at least 6 weeks before using as inoculum. Nematodes were prepared and inoculated into cups as described by Robinson *et al.* (2007). Usually 2-4 vermiform reniform nematodes were used per gram of soil. Root balls remaining from nematode bioassays were blended in a cement

mixer for 10-20 minutes, screened through ¼ inch hardware cloth and stored at 18ºC for future use as inoculum or to extract nematodes.

Table 3. Fine Sand Medium for Fungi.

Dry, fine sand	50 g
Dry cotton-root powder	1.5 g
Water	10 ml
Carrot juice	5 ml

1) Combine first three components in a 250 ml flask and sterilize at 15 lbs. of pressure for 20 minutes.

2) After 24 hours add carrot juice and sterilize for 15 minutes.

3) Inoculate with suspension of fungal spores or mycelial plugs and incubate for at least one month with periodic mixing before using as inoculum.

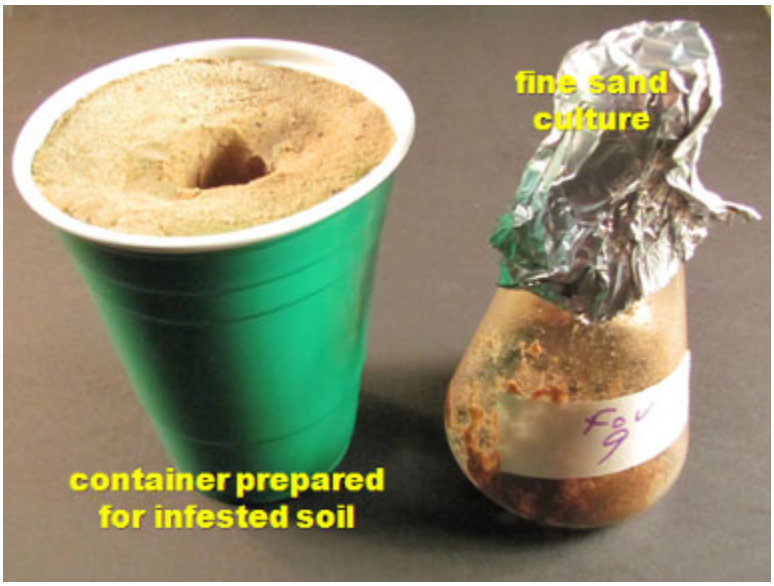

Figure 3. 16 oz. cup containing pasteurized sandy loam prepared for inoculation with sand culture.

Development of Cotton Hybrids Containing the *Ren*$_1^{lon}$, *Ren*$_2^{GB713}$, and *Ren*$_3^{GB713}$ Genes

The development of HLA and HHL triple species hybrids used to incorporate the *Ren*$_1^{lon}$ gene into each of the homologous chromosomes 11 and 21, respectively, were described by Bell and Robinson (2004). The development of LONREN lines containing *Ren*$_1^{lon}$, using marker assisted selection, are described by Robinson *et al.* (2007) and Dighe *et al.* (2009). BARBREN 713 was released as a new germplasm line in 2012 and contains the *Ren*$_2^{GB713}$ and *Ren*$_3^{GB713}$ genes which confer resistance to reniform nematode. BARBREN 713 was crossed with LONREN MR-19 to produce F_1 plants containing all three resistance genes.

Results and Discussion

Typical field stunting of a LONREN line containing the *Ren*$_1^{lon}$ gene compared to sibs lacking the gene is shown in Figure 1. Similar stunting is shown in the model system using sandy loam soils containing *Thielaviopsis basicola* plus 50 reniform nematodes per gram of core soil (Figure 2). Note that while the LONREN line containing *Ren*$_1^{lon}$ is severely stunted the BARBREN 713 line containing *Ren*$_2^{GB713}$ and *Ren*$_3^{GB713}$ genes is more resistant to stunting than the cultivar Fibermax 966. The TB-CEN selective medium for *Thielaviopsis basicola* (Table 1; Specht and Griffin, 1985) is recommended for isolating *Thielaviopsis basicola* from soils with diseased roots (Singleton *et al.*, 1992). However, when we used this medium for plants or soils from the Texas AgriLife Research farm, we did not obtain *T. basicola* from more than 200 plants and 50 soil samples. Instead we isolated primarily *Chaetomium*

funicola and *Fusarium solani*. Consequently, we isolated an array of soil-borne pathogens from this field using live carrot disks and PDA amended with 50 ppm of chlortetracycline. The sensitivity of the fungi isolated from infected roots or soil is shown in Table 4. All of the antibiotics in TB-CEN inhibited growth of *T. basicola* more than one or more other fungi. *C. funicola* which commonly degrades cellulose in soils was not inhibited by any of the antibiotics, explaining its prevalence in attempted isolations. While it colonized cotton roots it was not pathogenic.

Table 4. Relative growth rates of fungal species on PDA amended with antibiotics from the TB-CEN selective medium.

	Fungal Species							
Antibiotic	T.b.	C.f.	F.s.	P.e.	D.g.	M.p.	R.s.	A.a.
Streptomycin	2	4	4	4	Sect.	3	4	2-4
Chlortetracycline	2	4	4	4	4	4	4	4
Nystatin	1	4	2	0-1	1-2	0	2	0-1
Etridiazole	4	4	3	3	2	3	4	4

T.b. = *Thielaviopsis basicola*; C.f. = *Chaetomium funicola*; F.s. = *Fusarium solani;* P.e. = *Phoma exigua*; M.p. = *Macrophomina phaseoli*; R.s. = *Rhizoctonia solani*; A.a. = *Alternaria alternata*.

We tested a wide array of antibiotics and fungicides to develop the TB-RCTT medium presented in Table 2. The sensitivity of various soil-borne fungi to the antibiotics in this medium are presented in Table 5. This medium allowed the fastest growth rate of *T. basicola* at 22-25ºC and the highest plating efficiency of any that were tested. It did allow some bacterial growth, but this did not interfere with enumerating the fungus or obtaining selectivity of *T. basicola* growth. For bacterial-free growth the medium could be further modified by substituting 50 mg of chlortetracycline for carbenicillin. This slowed growth rate but did not change plating efficiency.

Table 5. Relative growth rates of fungal species on PDA amended with antibiotics/fungicides from the TB-RCTT selective medium.

	Fungal Species							
Antibiotic	T.b.	C.f.	F.s.	P.e.	D.g.	M.p.	R.s.	A.a.
Rifampicin	4	4	4	4	4	4	4	4
Carbenicillin	4	4	4	4	Sect.	4	4	4
Etridiazole	4	4	3	3	2	3	4	4
PCNB	4	1	3	3	1	1	1	1

T.b. = *Thielaviopsis basicola*; C.f. = *Chaetomium funicola*; F.s. = *Fusarium solani;* P.e. = *Phoma exigua*; M.p. = *Macrophomina phaseoli*; R.s. = *Rhizoctonia solani*; A.a. = *Alternaria alternata*.

Among the surface sterilization variables tested, soaking in 3% hydrogen peroxide for 1-3 minutes gave the best results. This treatment allowed recovery of *T. basicola* from a greater percentage of roots and from a greater percentage of the root surface as illustrated in Figure 4.

The Polytron homogenizer reduced cotton roots to a dispersion of fine particles as shown in Figure 5. These tissue dispersions were diluted so that a single drop would contain a specific amount of tissue, e.g., 1000, 200, and 40 µg as shown in Figure 6 for 4-day-old radicles collected from 3 soil lots after incubation at 25/20ºC and a 13-hour light/11-hour dark schedule, respectively. In this case, *T. basicola* infection potential varied 5-25 fold among lots. This data could also be converted to mean number of cfu/gram.

From our root-baiting, drop-plating studies we were able to discern several facts. All sandy loam soils sold by commercial dealers in our area were infested with *T. basicola*. Pasteurization of soils in 16 oz. cups for 16 hours at 75ºC (165ºF) will not kill *T. basicola* if soils are not thoroughly wet and allowed to set for at least 24 hours before treatment. Preferably, soil should be pasteurized a second time 48 hours after the first treatment. More than 200 samples of reniform nematodes extracted from various soils in Bauerman funnels failed to yield *T. basicola*, even when high concentrations of the fungus were present in soils. The extracts often did yield *Fusarium* and *Verticillium* species. Finally, we found that reniform nematode-infested fields had higher *T. basicola* infection potential than fields free of the nematode in the same area.

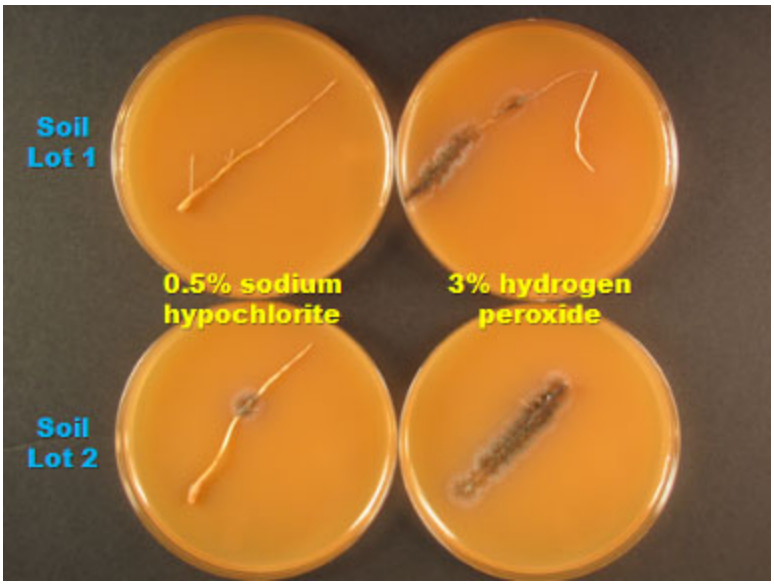

Figure 4. Recovery of *Thielaviopsis basicola* from roots grown for 5 days in two different soil lots and surface sterilized with 0.5% sodium hypochlorite or 3% hydrogen peroxide for 1 minute before placing on TB-RCTT medium.

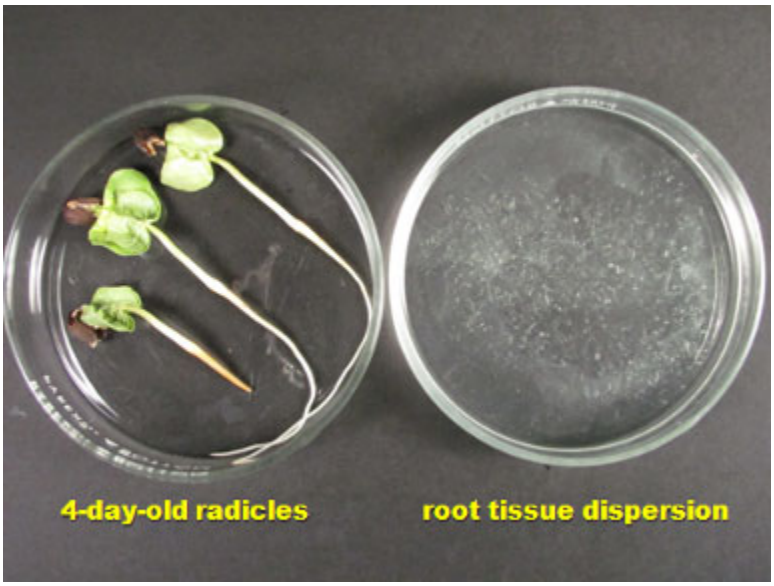

Figure 5. Root tissue dispersions formed by 30 seconds of comminution of 4-day-old radicles with a polytron homogenizer.

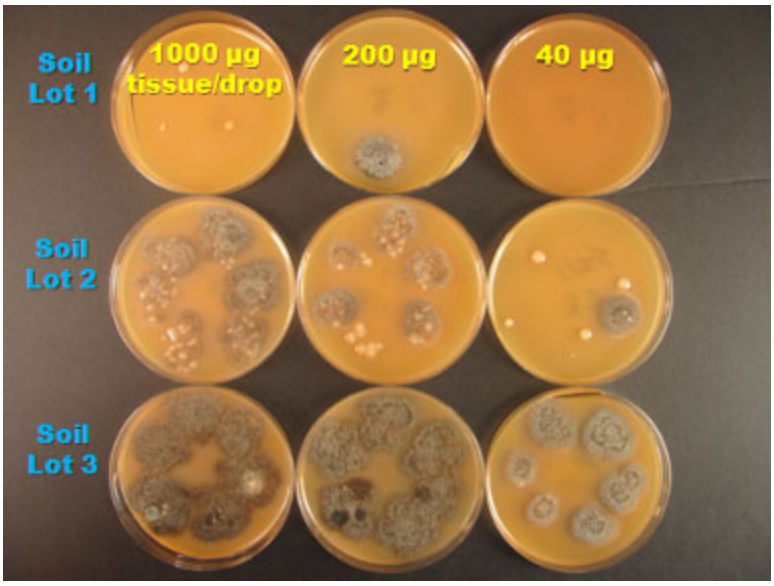

Figure 6. Drop plating of dilutions from tissue dispersions of 5-day-old radicles grown in three different soil lots.

Stunting from *T. basicola* and *Rhizoctonia solani*.
The stunting of LONREN (LON 21-4) and BARBREN (BAR 6-1-2) cotton lines compared to their parents DP 5415S and M-315 RNR, respectively, by *T. basicola* and *R. solani* alone is presented in Figure 7. In the absence of nematodes (Figure 7), LON 21-4 was not stunted any more than its parent by either fungus. However, in the presence of reniform nematode (Figure 8), LON 21-4 was stunted much more severely (significant at *P*=5%, student T test) than its parent. BAR 6-1-2 showed greater resistance than its parents to either fungus, with or without nematodes.

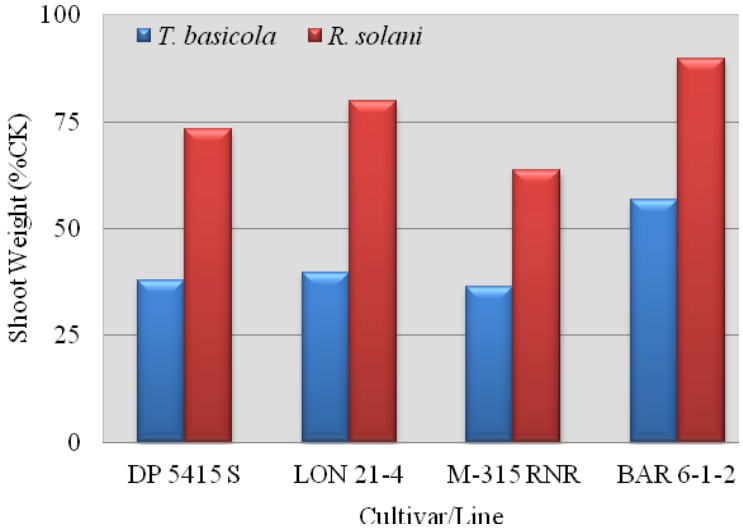

Figure 7. Resistance of LONREN 21-4 and BAR 6-1-2 to *Thielaviopsis basicola* and *Rhizoctonia solani*.

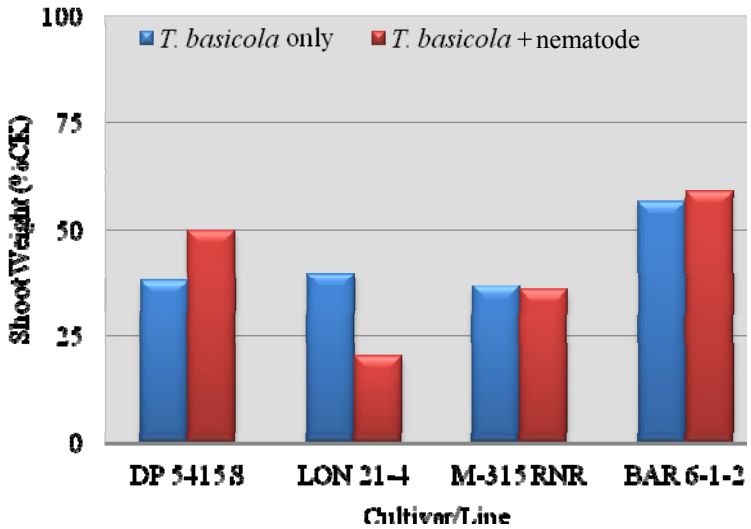

Figure 8. Effects of reniform nematode on resistance of LONREN 21-4 and BAR 6-1-2 to *Thielaviopsis basicola*.

Factors Affecting Stunting

The LONREN MR-19 line and Fibermax 966 cultivars were grown in more than 60 different soil lots from reniform infested fields or from completed nematode bioassays to determine the stunting potential (% reduction in shoot weight of MR-19 compared to FM 966) of the soils. Soils were then arrayed in descending order of percentage stunt and organized in groups of 4 or 5 to reach general conclusions about factors affecting stunting (Tables 6, 7 and 8).

Table 6. Relationships among stunting, reniform nematode concentrations, frequency of *Thielaviopsis basicola*, and bacterial concentrations.

Soil Group*	% Stunt Shoot	% Stunt Root	Nematodes per gram of soil	*T. basicola* infections (%)	Bacterial Conc. (R/S)
1	76.4	82.3	54.8	76	3.8
2	72.5	80.0	52.2	75	3.9
3	62.1	73.0	49.0	68	2.6
4	39.0	38.7	20.0	64	2.2
5	9.2	15.4	25.7	74	1.6
CK	0.7	14.1	0	0	0.9

*Arranged in descending order of % stunt caused to the LONREN line compared to its susceptible sibling. Groups 1-3 included four soils each, groups 4 and 5 included five soils each.

Table 7. Relationships among stunting, reniform nematode concentrations, shoot weight of FM 966, and cfu of *Thielaviopsis basicola* in 8-day-old plants.

Soil Group*	% Shoot Stunt	Nematodes per gram of soil	FM 966 Shoot Wt. (g)	T.b. (cfu/mg)
1	77.8	62.8	3.41	23.0
2	64.5	30.0	1.75	38.5
3	55.5	44.4	2.52	21.0
4	53.3	48.5	1.64	35.0
5	37.5	46.5	2.30	42.0
6	6.8	15.6	3.65	29.8

*Arranged in descending order of % stunt caused to the LONREN line compared to FM 966. All groups had four soil lots each.

Table 8. Relationships among stunting, reniform nematode concentrations, and frequency of fungal genera in different soil lots.

Soil Group*	% Stunt	Nematodes per gram of soil	Frequency (%) of Fungal Genera in Roots			
			Thielaviopsis	*Fusarium*	*Trichoderma*	*Talaromyces*
1	77.6	168.9	42	100	7	8
2	60.2	62.4	83	100	33	0
3	28.5	23.3	92	100	58	17
4	4.3	42.1	100	100	58	17

*Arranged in descending order of % stunt caused to the LONREN line compared to its susceptible sibling. All groups had four soil lots each.

Stunting of root development was generally greater than that of shoot development (Table 6) indicating that root deterioration was the primary cause of stunting. Soils that caused stunting always contained reniform nematode and *Thielaviopsis basicola* (Tables 6-8). Stunting severity was directly related to nematode numbers. *T. basicola* concentrations were not always related to stunt severity, possibly because high *T. basicola* concentrations also severely stunted FM 966 (Table 7) so that relative differences were smaller. Some soil lots caused only limited stunting even though they contained moderate concentrations of both reniform nematodes and *T. basicola*. In these cases we often found high concentrations of biocontrol fungi, such as *Talaromyces flavus* and *Trichoderma* species (Table 8). *Fusarium* spp. were invariably isolated from roots, but *F. oxysporum* appeared mostly with severe stunt. Therefore, stunting was assessed in soils containing relatively high numbers of different *Fusarium* groups as well as moderate levels of *T. basicola* and reniform nematode (Figure 9). A mixture of *F. oxysporum* f. sp. *vasinfectum* races 1, 2 and 8 greatly increased the severity of stunting of LONREN MR-19 in each of two different soil lots from

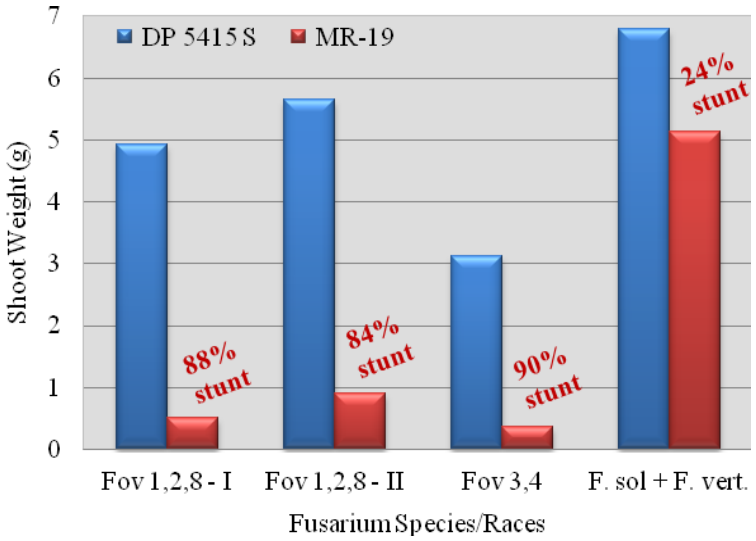

Figure 9. Effect of *Fusarium* species on stunting caused by reniform-*Thielaviopsis* complex.

two different *Fusarium* experiments. A mixture of *F. oxysporum* f. sp. *vasinfectum* races 3, 4 and a related molecular clad caused even more severe stunting, especially of the cultivar DP5415 S. None of the *Fusarium* races alone combined with reniform nematode caused appreciable stunting. Consequently, the effects of *Fusarium* appeared to be in enhancing the pathogenicity of *T. basicola*. In contrast a mixture of six *F. solani* and *F. verticillioides* isolates greatly decreased stunting, even though moderate levels of *T. basicola* were present. Clearly, third party organisms can strongly affect stunting associated with the Ren_1^{lon} gene.

Attempts to Separate Stunting from Nematode Resistance.

Three approaches were taken to try to separate susceptibility to stunting from resistance to reniform nematodes in LONREN lines. Since introgression of Ren_1^{lon} into chromosome 11 might have displaced normal resistance genes found at the same location, we also introgressed Ren_1^{lon} into homologous chromosome 21 using the HHL triple

species hybrid (Bell and Robinson, 2004). Five such lines were developed to the BC_7 generation and crossed with commercial cultivars. The mean results for LONREN 17-29 (Ren_1^{lon} in chromosome 21), its F_1 hybrid and several commercial cultivar parents are shown in Figure 10 compared with mean results for several MR Lines (Ren_1^{lon} in chromosome 11) crossed with Fibermax 966. Stunting occurred in association with Ren_1^{lon} regardless of chromosome location, and it was largely dominant in its expression.

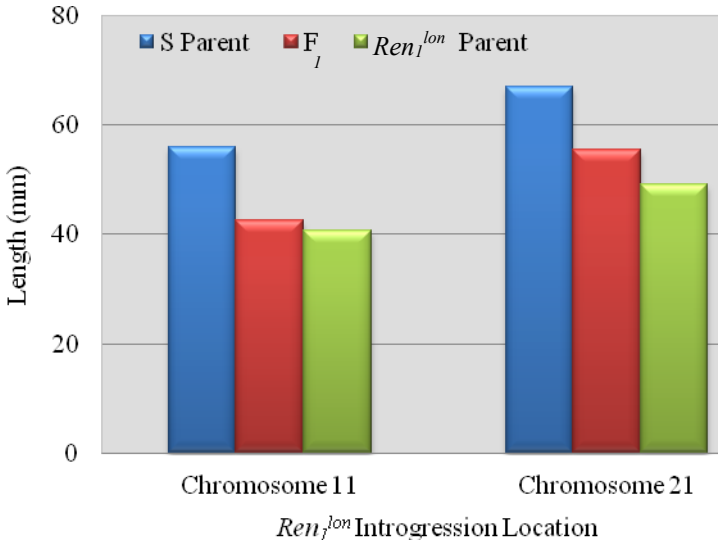

Figure 10. Mean effects of Ren_1^{lon} substituted into chromosome 11 compared to chromosome 21 on stunting (hypocotyl length) of F_1 hybrids and parents homozygous for Ren_1^{lon}.

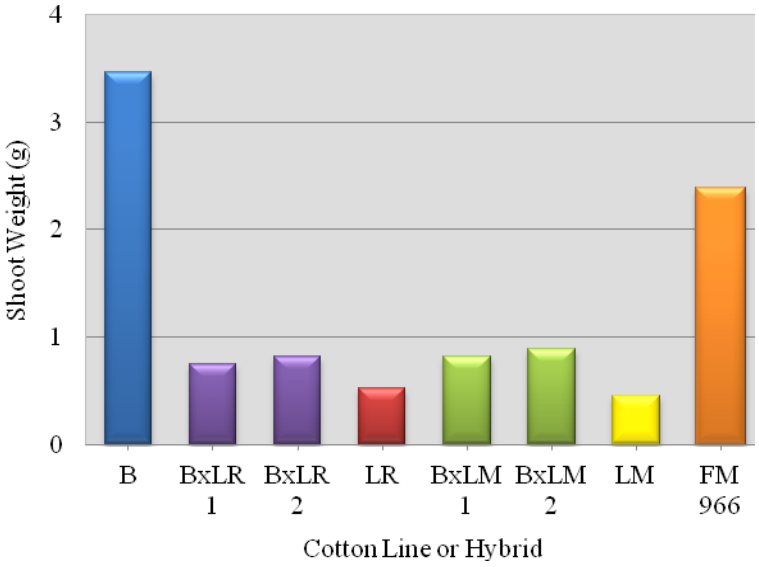

Figure 11. Shoot weights of 18-day-old hybrids of LONREN (LR, LM) x BARBREN (BAR 6-1-2; B) compared to parents and Fibermax 966.

The codominant DNA marker closest to Ren_1^{lon} is BNL3279_114. Therefore, we screened F_2 progeny for resistance to stunt and presence of the marker in hope that some of these plants might be resistant to both stunt and nematodes. To date, more than 10,000 F_2 progeny derived from more than 2,000 F_1 plants heterozygous for BNL3279_114 and resistant to reniform nematode have been screened for resistance to stunt caused by a reniform nematode-*Thielaviopsis* complex. These plants represented all 28 LONREN families. More than 3,000 stunt-resistant plants,

mostly nematode-susceptible sibs, have been screened for BNL3279_114. About 200 of the F_2 plants had the marker and selfed seed was produced from each of these to retest for stunt resistance and obtain plants homozygous for it as well. F_3 and F_4 seedlings from plants confirmed to be stunt resistant and homozygous for BNL3279_114 were screened for resistance to reniform nematode. Seven and two stunt resistant lineages homozygous for BNL3279_114 on chromosome 11 and 21, respectively, were obtained. All of these lines lost resistance to the nematode and the BNL1231 repulsion marker for Ren_1^{lon}, which has been indissociable from the gene.

The third approach to disassociate Ren_1^{lon} from stunting was to hybridize LONREN lines with BARBREN lines that have the Ren_2^{GB713} and Ren_3^{GB713} genes and are resistant to stunting. The results in Figure 11 show that stunting associated with Ren_1^{lon} is dominant even when combined with other *Ren* genes that are associated with resistance to stunting.

Summary

Development of improved selective media, use of tissue dispersion by a Polytron tissue generator to measure root invasion, and development of inoculation techniques to mimic stunting in the field have allowed critical evaluation of the role of *Thielaviopsis basicola* in severe stunting of LONREN lines in reniform nematode-infested fields. Severity of stunting caused by reniform-*Thielaviopsis* complexes depends on what other fungal species inhabit the root as well as inoculum concentrations of the nematode and *T. basicola*. *F. oxysporum* f. sp. *vasinfectum* increases the severity of stunting apparently by favoring pathogenicity of *T. basicola*. In contrast, *F. solani*, *F. verticillioides*, *T. flavus* and *Trichoderma* spp. appear to act as biological antagonists to reduce stunt severity. Attempts to genetically disassociate stunting from the Ren_1^{lon} gene are ongoing but unsuccessful to date.

The mode of action of Ren_1^{lon} in causing a marked increase in stunt is unknown. However, changes directed by Ren_1^{lon} resemble those of the NPR1gene from *Arabidopsis thaliana* which has been introgressed into cotton. The NPR1 gene causes increases in resistance of cotton to many soil-borne pathogens including reniform nematode and *T. basicola* (Parki *et al.*, 2010a; Rathore, unpublished). Yet it causes increased susceptibility to the defoliating strain of *Verticillium dahliae* (Parki *et al.*, 2010b). Our preliminary evidence indicates that Ren_1^{lon} also diversely affects resistance to many soil-borne pathogens.

Acknowledgements

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