Yield, Resistance and Fiber Quality Performance of Reniform Nematode resistant QTL *Ren*^{barb2} in BARBREN-713 Derived Upland Cotton Lines

by

Huawei Wang

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 12th, 2015

Keywords: cotton, reniform nematode, SSR marker, quantitative trait loci

Approved by

David Weaver, Chair, Professor of Crop, Soil and Environmental Sciences
Charles Chen, Associate Professor of Crop, Soil and Environmental Sciences
Kathy Lawrence, Professor of Entomology and Plant Pathology

Abstract

Reniform nematode (*Rotylenchulus reniformis* Linford and Oliveria) is a major pest of upland cotton (*Gossypium hirsutum L.*) in Alabama. BARBREN-713 is a germplasm with high resistance to reniform nematode (RR) released by combined effort of Texas A&M and USDA-ARS. The quantitative trait locus (QTL) named Ren^{barb2} was identified as closely linked to genes that contribute the most resistance. An interest has grown to test the real world performance of resistance genes associated with this QTL in terms of both yield and fiber quality in field studies. A population numbered 148 was developed at Auburn University by crossing BARBREN-713 with an elite breeding line AU3202. This population was tested with gel based electrophoresis and two groups were selected based on the presence or absence of Ren^{barb2}, with each group containing 20 lines. In spring of 2014, several greenhouse screening tests were conducted and nematode egg count data was obtained to determine RR resistance. Field testing was done at Tennessee Valley Research and Extension Center (TVREC) in Belle Mina, Alabama in two locations: one field infested with RR and one field not infested. Yield, nematode egg count, and fiber quality data were obtained. Data showed that the QTL Ren^{barb2} did contribute majorly to resistance to RR even in a different genetic background, and yield of those lines homozygous positive for the marker were 22% higher in a heavily RR infested field. However, a slight reduction in yield potential was observed in the nematode-free field, and no significant effect on fiber quality was observed.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	analysis of variance
bp	base pair
cM	centimorgan
CRD	completely randomized design
DAP	days after planting
DAI	days after inoculation
ELO	elongation
kg ha^{-1}	kilogram per hectare
log_c	logarithm 10 of nematode egg count
mL	milliliter
m	meter
M	molarity
MIC	micronaire
mM	micromolarity
PCR	polymerase chain reaction
QTL	quantitative trait loci
RCBD	randomized complete block design
RFW	root fresh weight
RIL	recombinant inbred line
RR	reniform nematode

SFC	short fiber content
SFW	shoot fresh weight
SSR	simple sequence repeat
STR	fiber strength
Tm	melting temperature
TVREC	Tennessee Valley Research and Extension Center
UHM	upper half mean
UI	uniformity index
μL	microliter
USDA-ARS	United States Department of Agriculture, Agricultural Research Service

Literature Review

Upland Cotton and Reniform nematode

Cotton (*Gossypium spp.*) is a major textile crop planted for its fiber. Over 26 million metric tons of cotton lint is currently produced worldwide and ranked as the world's 16th most valuable crop (FAOSTAT, 2012). There are four major species used in commercial production, and upland cotton (*Gossypium hirsutum L.*) is the most widely grown cotton species, accounting for more than 90% of world lint yield.

Originating in Mexico, upland cotton is an allotetraploid (AADD genome, 2n=4x=52) plant. The A genome was native to Africa, and was carried to the New World around 1~2 million years ago by transoceanic dispersal. Hybridized with the Mexican D genome, it gave rise to the diverse allotetraploid species including *G. hirsutum* (Wendel, 1989).

Also being the most popular cotton species in the U.S., a total of 4.4 million hectares were devoted to upland cotton production in 2014 (National Cotton Council, 2014). With such a large acreage, there are many different pests of cotton and management of these pest stresses is always a challenge. Historically, the boll weevil (*Anthonomus grandis*) was the most costly pest in the U.S., causing billions of dollars in losses as measured in both yield loss and the expense of insecticide application. With USDA's eradication program the cotton industry was finally released from the stress of the boll weevil, and started to focus on other problems (National Cotton Council, 2009).

Reniform nematode (*Rotylenchulus reniformis* Linford and Oliveria, abbreviated as RR) was first described in 1940 by Linford and Oliveria on cowpeas (*Vigna unguiculata*) in Hawaii (Linford and Oliveria, 1940), and then recognized to be a cotton pest in the 1950s. According to Linford and Oliveria (1940), RR is similar to other plant parasitic nematodes in that they have a

stylet for penetration. There are four juvenile stages and an adult stage, where adult females are the only ones that infest plants. Feeding behaviors have never been observed on males. Mainly dwelling in tropical and subtropical areas, they prefer silty soil to sandy soil, which is a very distinctive feature that sets them aside from other parasitic nematodes. Speaking of distinctive features, Linford and Oliveria (1940) observed that their molted cuticles do not disappear, but stay around their body protecting them. RR gets its name because the adult females are kidney shaped. After infestation, females embed half their bodies inside the host's root, and the posterior part starts to enlarge into reniform. Females then lay eggs on the exterior after 25 days into their lifecycle on average. Unlike root-knot nematodes (*Meloidogyne incognita*), such feeding behavior does not induce galls on the roots. Moreover, above-ground symptoms are usually only wilting and stunted growth. Such features of RR made them a very difficult pest to deal with. Cuticles provide them protection against harmful environments, including pesticide chemicals. Fast generation propagation grants them the ability to rapidly build up numbers and recover from destruction. Hardly noticeable symptoms on the host may cause misdiagnosis of the pest infestation or even let them slip under farmers' eyes entirely.

As early as 1959, Jones et al. (1959) studied the effect of RR infestation on cotton yield. Fumigant nematicide (ethylene dibromide) in an infested field at Baton Rouge, Louisiana increased the yield of two cultivars by 29.2% and 69.6% respectively. Later repeats of the same study showed a 35.3% increase on average. Moreover, Jones et al. (1959) showed that fumigation increased boll size consistently for all four years of their study (0.35g to 0.91g increase per boll, all $p<0.05$). In two (1954 and 1956) out of four years, they observed significant increase in the lint percentage (turnout ratio) after applying ethylene dibromide (1.4% and 0.3% increase, $p<0.01$ and $p<0.05$ respectively for 1954 and 1956). Such results suggested that RR can

not only decrease yield, but also affect yield components, such as boll size and turnout ratio, negatively. Lawrence et al. (1990) treated an infested field with a set of different nematicides at two different sites. Comparing the group treated by the most effective nematicide (1,3-dichloropropene + aldicarb) with the control group, yield difference was 60% and 33% at each site respectively. Since then, the pest has spread to various areas in the south, including Texas, Georgia, Alabama, and some other major cotton producing states. Currently, US Cotton Council estimates that RR is responsible for the loss of 4% of US total cotton yield (US Cotton Council, 2012), sharing the title of "major cotton pest" side by side with the Southern Root-knot nematode (*Meloidogyne incognita*). It is quite obvious that this pest threatened profits of cotton farmers and should be managed more effectively.

Management of RR

Traditionally, crop rotation and nematicides have been used to effectively manage RR infestation. According to Robinson (1997), studies have shown that RR has an exceptionally wide host range, which can make the use of crop rotation as a management tool more challenging. Cauliflower (*Brassica oleracea* var. *botrytis*), papaya (*Caprica papaya*), muskmelon (*Cucumis melo*) and many other tropical crops are all known hosts. Moreover, common weeds such as bindweed (*Convolvulus arvensis*), lamb's quarters (*Chenopodium album*), indian lovegrass *(Eragrostis pilosa*), bindii (*Tribulus terrestris*) and many more are all susceptible to RR. As a matter of fact, 314 out of the 364 plant species reviewed in Robinson's literature are all hosts. Fortunately, peanut (*Arachis hypogaea*), corn (*Zea mays*) and sorghum (*Sorghum bicolor*) are known to be non-host crops (Robinson et al., 1997; Windham and Lawrence, 1992; Birchfield, 1983). There are also resistant soybean cultivars available (Robbins et al., 1999). However, peanut requires special infrastructures for production, which adds

unnecessary cost and is not preferred. Studies then have focused on the latter three crops. A two-year rotation of upland cotton with corn reduced early season nematode population even when compared to aldicarb treated non-rotation groups (Stetina et al., 2007). Significant population reduction during early, mid-season and harvest was detected, and such reduction in RR population resulted in yield loss reduction in the most effective rotation sequence (cotton-corn-corn-cotton) (Stetina et al., 2007). Due to the short planting season of corn and RR's ability to infect many weeds, post season weed management is also extremely important in corn rotation strategies. Besides corn, contiguous soybean or peanut rotation sequences (cotton-soybean-soybean-cotton, cotton-peanut-peanut-cotton) reduced RR population by as high as 70% and increased yield by 22% when compared to a non-rotated control group (Gazaway et al., 2007). Rotation with a highly reniform nematode resistant soybean cultivar (Hyperformer HY 798) showed the same result in a different study (Davis et al., 2003).

Nematicides are also an effective management tool. As mentioned above, aldicarb and 1,3-dichloropropene (1,3-D) are two of the most popular and effective nematicides (Lawrence et al., 1990). K-pam (potassium N-methyldithiocarbamate), Vapam (sodium methdithiocarbamate) are new members of the fumigant family. Recently, seed treatment products for nematode protection are available from several vendors. AVICTA (Thiamethoxam & Abamectin) and Aeris (Thiodicarb & Imidacloprid) are just two of these. Bayer CropScience has also made available a biological seed treatment Poncho/VOTiVO. It contains a systematic insecticide clothianidin for other insect problems, and a strain of the bacteria *Bacillus firmus* which can attack plant parasitic nematodes.

However, there are several challenges. RR can exist in substantial numbers at a much deeper depths (Westphal and Smart 2003) than other soil-borne pests and may be out of reach of

nematicides. With such attributes, it usually requires a multi-year rotation with non-host crops to control RR population and keep them within in an acceptable range (Westphal and Smart 2003). Last but not least, some of the very effective nematicides (aldicarb, for example) are no longer available, further narrowing the management options for farmers. In heavily infested fields, yield loss due to RR could go as high as 66.9% (Lawrence et al. 1990). Alabama is right at the center of high RR population and large yield loss (Blasingame and Patel, 2012), thus it is highly desirable to study this pest and come up with new methods to control damage.

Genetic resistance for RR in cotton

Genetic resistance in the form of resistant cultivars seems to be a desirable solution to management of RR. Unfortunately, the discovery, study and adaptation of genetic resistance for RR is much later than that for root-knot nematode in cotton, and reports have not found appreciable level of tolerance, let alone resistance, in upland cotton cultivars or germplasm (Robinson et al., 1999; Usery et al., 2005).

Some accessions of *G. hirsutum* were reported to have moderate resistance after an extensive search in the gene pool. Yik and Birchfield (1984) reported that three wild accessions of *G. hirsutum* were resistant, while Weaver et al. (2007) found seven accessions to be moderately resistant. However, these reports were not been confirmed in later studies (Robinson and Percival 1997; Sürmelioğlu et al., 2010).

The search was then extended to other species in the Malvaceae family for a practical level of resistance. Yik and Birchfield (1984) evaluated 200 accessions in this family and reported that *G. longicalyx*, *G. arboreum*, *G. stocksii*, *G. somalense* and *G. barbadense* 'Texas 110' were resistant. Stewart and Robbins (1995) found some accessions of *G. arboreum* to be resistant also. Attempts had been made to incorporate resistance in these species into *G.*

hirsutum. Sacks and Robinson (2009) crossed G 371 (a hexaploid ADD genome *G. hirsutum/G. aridum* breeding line) with *G. arboreum* accession A_2-190 (determined to be resistant by Stewart and Robbins, 1995) to incorporate such resistance into the *G. hirsutum* genome. Interestingly, while evaluating resistance of the progeny, Sacks and Robinson found that the parent, G 371, is also highly resistant to RR, supporting only 10% RR population compared to susceptible check 'Deltapine 16'. Since no considerable resistance has been found in the *G. hirsutum* genome, they concluded that such resistance must come from *G. aridum*. Romano et al. (2009) later mapped the resistance QTL as Ren^{ari} onto chromosome 21 (D genome) and found flanking SSR markers BNL3279_132 and BNL2662_90 to be within 1cM of the QTL. Since the two SSR markers were also found in other *G. aridum* accessions, they confirmed that the resistance is from *G. aridum*.

Another source, *G. longicalyx*, is especially worthwhile because it is almost immune to RR (Yik and Birchfield, 1984). However, incorporating this source of resistance into *G. hirsutum* is not an easy task due to chromosomal incompatibility. *G. longicalyx* is very different from *G. hirsutum* not only because of ploidy (*G. hirsutum* is allotetraploid and *G. longicalyx* is diploid), but also because *G. longicalyx* is the only species with the F genome (Wendel and Cronn, 2003). A triple species hybrid referred to as HLA (*hirsutum-longicalyx-armourianum*) was developed by the combined effort of USDA-ARS, Texas A&M AgriLife Research and Cotton Incorporated. It expressed a high level of resistance and served as the bridging hybrid in a backcross program (Bell et al., 2014). In 2007, LONREN-1 and LONREN-2 germplasm lines were developed with desirable agronomic characteristics, and made available to researchers (Bell et al., 2013). The QTL for resistance was mapped to chromosome 11 (A genome) and named Ren^{lon} later (Dighe et al., 2009). SSR marker BNL3279_114 was found to be tightly linked with the QTL (within 1.4cM distance).

Although LONREN lines were released with desirable agronomic characteristics in mind, extensive field studies of these lines showed that stunting and development issues were present under severe nematode pressure (Nichols et al., 2010; Weaver et al., 2013). Greenhouse experiments showed that an increase in initial inoculum level resulted in a decrease in root mass of LONREN and LONREN-derived lines (Sikkens et al., 2011). Susceptible checks, on the other hand, showed an increase in root mass in response to nematode attack. Moreover, pathogenic fungi *Rhizoctonia solani* has been isolated from necrotic roots and reported to affect lines carrying Ren^{lon} more severely than those that do not (Bell et al., 2012). Speculations have been made that these lines may be vulnerable to fungi attacks, or that the reaction to RR attack is of a hypersensitive nature. Hypersensitivity basically means that plant cells commit suicide after the nematode attacks them, thus starving the nematodes and restricting their reproduction. However, plants also sacrifice too much root system, so the decrease in root mass is observed.

Despite the fact that LONREN lines consistently perform very well in nematode reproduction tests and support very low levels of nematode reproduction, the stunting issues have heavily limited their applicability in field situations. Because of this, other sources of resistance are being tested and used in breeding programs. Robinson et al. (2004) confirmed the resistance of *G. barbadense* accession 'Texas 110' reported by Yik and Birchfield (1984), and further identified another *G. barbadense* accession, GB-713, as being highly resistant. Three QTLs related to RR resistance in GB-713 were mapped and named Ren^{barb1}, Ren^{barb2} and Ren^{barb3} respectively (Gutiérrez et al., 2011). Ren^{barb1} and Ren^{barb2} are located on chromosome 21 at position 168.2cM and 182.7cM respectively. They are flanked by SSR markers BNL1551_162, GH132_199, and BNL4011_155 and BNL3279_105 respectively. The third QTL, Ren^{barb3}, is located on chromosome 18 at position 42.0cM, and is flanked by SSR markers BNL1721_178

and BNL569_131. Preliminary tests on GB-713-derived germplasm lines have shown very promising results. Sikkens et al. (2014) tested BAR 41, BARBREN-713 (both developed by USDA-ARS and Texas A&M AgriLife Research), and five M713 variations (developed by USDA-ARS and Mississippi Agriculture and Forestry Experiment Station) in adjacent fields, one with heavy nematode infestation and the other without. In the heavily infested field, GB-713 derived lines averaged less than 10% yield loss, while susceptible checks and LONREN derived lines averaged 65% and 70% yield loss respectively. The GB-713 source of resistance seems to be the most commercially promising.

BARBREN-713 and its derivative population ACX148

The germplasm line BARBREN-713 was released to breeders in 2007, and the official release notice was published later (Bell et al., 2014). BARBREN-713 was developed by backcrossing the hybrids resulting from the cross GB-713 × 'Acala NemX' to the recurrent parent Acala NemX. Acala NemX is known to be resistant to root-knot nematode (Weaver, 2015). According to the release notice, the BARBREN-713 germplasm line contains three QTLs of interest, two of which are from GB-713 related to RR resistance (*Ren*^{barb2} and *Ren*^{barb3}) and the third is related to root-knot nematode resistance. Although there is a third QTL (*Ren*^{barb1}) related to RR resistance in *G. barbadense* accession GB713 (Gutiérrez et al., 2010), it was not mentioned in the release note, thus we have designed our experiments assuming its absence. Taking advantage of this improved source of resistance, a breeding program at Auburn University crossed it with several advanced breeding lines and developed several populations. ACX148 is one of those populations, derived from the cross BARBREN-713 × AU3202. AU3202 is an elite breeding line with promising agricultural characteristics but known to be susceptible to RR. One hundred $F_{2:3}$ RIL lines were developed, and their $F_{2:5}$ open-pollinated

seeds were available in fall 2013. A seed increase attempt was performed during the spring of 2014, but failed due to thrips (*Frankliniella occidentalis*) infestation. Nonetheless, there were still enough seeds for several greenhouse screening tests and an entire year of field study. The plants in the greenhouse screening test and the field test were all $F_{2:5}$ plants germinated from these seeds. However, plant materials for DNA marker analysis were sampled by my previous colleague in summer 2013 in Prattville, AL, and were from $F_{2:4}$ plants.

Materials & Methods

DNA Extraction

DNA extraction was done according to the mini-prep method published by Zhang et al. (2000). In short, one newly unfolded young cotton leaf from each plant was taken and put into 1.5mL autoclaved centrifuge tubes for best extraction results. The samples were immediately stored in liquid nitrogen, and then ground with an electrical drill in 500μL DNA extraction buffer [0.1 M Tris-HCl, pH 8.0; 1.0 M NaCl; 0.02 M EDTA, pH 8.0; 2% (w/v) cTAB; 2% (w/v) polyvinlypyrrolidone-40; 1mM 1,10-phenoanthroline; 0.2% (v/v) β-mercaptoethanol]. The resulting mix was incubated in water at 65 ℃ for at least 15 minutes, and then thoroughly shake-mixed with an equal volume of chloroform:isoamyl alcohol (24:1 ratio v/v) solution. After centrifuging at 12,000 × g for 10 minutes, the water based supernatant was transferred into a fresh centrifuge tube, where 500μL of -20 ℃ isopropanol was added. The tube was then inverted several times until a precipitate appeared. One slight modification from the published procedure was that the mixture was kept at 20 ℃ overnight instead of 1 hour. On the second day, the tubes were then centrifuged again at 12,000 × g for 10 minutes, and this time all liquid phase was discarded. The resulting precipitate was then washed by 70% and 100% ethanol sequentially, and dried under an air hood for approximately 30 minutes. The DNA pellet was then dissolved in low salt Tris-EDTA solution [10 mM Tris-Hcl, pH 8.0; 1 mM EDTA] in a 65 ℃ water bath. The cleaning procedure immediately followed. 500μL of cleaning solution [0.05 M Tris-HCl, pH 8.0; 0.05 M EDTA, pH 8.0; 2% (w/v) cTAB; 2.05% (w/v) NaCl; 0.02% (w/v) 1, 10-phenanthroline] was added to each tube and the tubes were shaken on a Vortex at low setting for 2 hours. The solution was then centrifuged at 12,000x g for 5 minutes. Discarding again the liquid phase, the

precipitate was washed by 80% ethanol + 15 mM ammonium acetate solution, followed by another wash with 100% ethanol. The resulting pellet was again dried, dissolved in low salt Tris-EDTA buffer, and kept at -20 ℃ for storage.

Primer Design

Primer sequences from CottonMarker.org for BNL3279 and BNL569 were used at the beginning of the project. While BNL3279 yielded great result, BNL569 performed very inconsistently and presented quite a few difficulties. The PCR product yield was too low to be useful, so it was suggested that we design a new set of primers. The regional sequence around BNL569 was also obtained from CottonMarker.org, and all design procedures tightly followed the instruction published by Thornton and Basu (2010). The free online software Primer3 was chosen as the tool. Most parameters were kept at default. Maximum primer size was increased to 28 and minimum melting temperature decreased to 50 ℃ to accommodate the fact that there was not enough sequence upstream of the target sequence. Also, GC content was left as-is instead of increased as instructed by the paper. Finally, max self-complimentary was increased to 4 to limit the number of possible primers. The designing procedure was run twice, the first time targeting the same region as that published by CottonMarker.org, and the second time targeting a downstream region where higher quality primers could be designed. Eleven primer pairs in total were obtained and tested. Unfortunately, there was no polymorphism discovered for the downstream region within our genotypes (AU3202, BARNBREN-713 and Population 148), and thus it cannot be used as a microsatellite marker to track the Ren^{barb3} QTL. However, several higher yielding primer pairs were found for the original region, and BNL569_4 of the ten pairs was selected (see Table 1 for the complete set of primer pairs). The final forward primer was

modified with a leading sequence (GAGTTTTCCCAGTCACGAC) to complement that of the dyed tail primer.

PCR protocol

A touchdown protocol was used to ensure the best quality of product was made ready for gel electrophoresis. The protocol was adapted from that used by the Auburn University School of Fisheries, Aquaculture and Aquatic Sciences. In short, 70 × master mix [1 × Taq Polymerase Buffer; 2.5 mM $MgCl_2$; 200 μM each dNTP; 0.4 μM forward primer; 0.6 μM reverse primer; 0.02 μM 700-dyed tail primer, or 0.04 μM 800-dyed tail primer; 0.05 U/μL Taq Polymerase; add water to 8 μL] was made for 64 reactions. 2μL of DNA template (50 ng/μL) was added to make the final volume of each mix 10μL. A 96 well plate was used to hold eight PCR tube strips for each run, and each strip contained eight tubes. After adding master mix and DNA template, the plate was mixed well on a Vortex. A thermal cycler was then used to perform the PCR program. It is listed as follows: 1) 95 ℃ for 3 min 30 sec; 2) 95 ℃ for 30 sec; 3) 55 ℃ (BNL3279)/ 53 ℃ (BNL569) for 30 sec; 4) 72 ℃ for 30 sec; 5) Repeat 2-4 for 19 more times; 6) 94 ℃ for 30 sec; 7) 51 ℃ (BNL3279)/ 48 ℃ (BNL569) for 30 sec; 8) 72 ℃ for 30 sec; 9) Repeat 6-8 for 14 more times; 10) 72 ℃ for 5 min; 11) 4 ℃ storage. The post-elongation phase was not necessary and could be omitted. All reagents were handled with the lights turned off because the dyed tail primers are light sensitive. 2μL of products were then diluted by adding 6μL of formamide loading dye [0.02 M EDTA, pH 8.0; 0.0008% (w/v) Bromphenol Blue; both dissolved in formamide]. This final product was kept at 4 ℃ overnight until the gel was prepared and ready to run electrophoresis.

Electrophoresis

The next step was to run a polyacrylamide gel electrophoresis on the Licor DNA Analyzer 4300. Recipes and protocols were also from the Fishery Lab of Auburn University. The gel used was 7% acrylamide with urea [7% (w/v) Acrylamide; 7M Urea; 1x TBE buffer], and was prepared with 250µL of 10% APS solution and 25µL of TEMED. Gel apparatus was assembled the next day after PCR was done. Each run contained at most 64 samples; two of which were always the parents (BARBREN-713 and AU3202). Diluted samples were loaded with syringes, and a 50-330 bp ladder was added to both end of the gel to provide standard size locator. The gel was run at 1400 V and 40 ℃ for 1.5 to 2 hours, depending on the length of product fragment. Then, each sample was scored individually as **a** (homozygous for the BNL569_131 allele), **h** (heterozygous), or **b** (homozygous for the BNL569_135 allele, which occurred in AU3202).

Greenhouse screening tests

The greenhouse screening for nematode resistance was designed as a completely randomized experiment (CRD) and was done in spring 2014. Methods of nematode inoculation and egg extraction were similar to those of Weaver et al. (2007). In short, 40 germplasm lines from population 48 plus 5 controls were planted in 150mL cone-tainers [50% sand and 50% silty soil from The E. V. Smith Research Center, Plant Breeding Unit, 19:6:12 fertilizer at 7.4g/L of soil], with two seeds each. The five controls include the two parents (BARBREN-713 and AU3202), a susceptible control (‘SG747’), a resistant control (LONREN 21-4) and a fiber quality control (experimental genotype E103-123). There were five repetitions for each line, making a total of 225 experimental units. The seedlings were transplanted and removed as needed to fill each cone with exactly one seedling one day before inoculation. Seven days after planting (DAP), the entire set was randomized, and each cone was inoculated with 5,000 RR

eggs and vermiforms. The cone-tainers were kept in five racks, and each cone-tainer had four empty spaces around it so that each plant had approximately 25 cm^2 space for growth. Nematode extraction was done sixty days after inoculation (DAI) using the sieve and sugar centrifuge method to extract vermiforms, and Clorox method to extract eggs from root.

Nematode Extraction

Before extraction, plant height was measured and above ground portion was cut off and weighed (shoot fresh weight). Soil in the cone-tainer was then washed over a number 65 sieve and a number 225 sieve in sequence. Material collected in the 225 sieve was then washed into a container for sugar centrifuging. The fresh root was laid on a paper towel for at least fifteen minutes to dry off. After air drying, the root was weighed (fresh root weight) and kept in a container until ready for Clorox extraction. A solution of 45.4% w/v sugar water was added to the sieve extraction material and the mixture was stirred thoroughly and centrifuged at 2,000 rpm for 2 minutes. Supernatant was then poured over a number 200/500 sieve apparatus. The apparatus was rinsed thoroughly with tap water, and the remainder in the number 500 sieve was poured into a flask. The root material was submerged in 10% v/v Clorox solution and shook for exactly 4 minutes. After pouring off the Clorox solution, remaining root material was then rubbed by hand over a number 200/500 sieve apparatus and rinsed with tap water. The remainder in the 500 sieve was subject to sugar water centrifuging and the result was also kept in a flask. Contents in each flask was then poured into squared petri-dishes and eggs were counted. The counted number was then normalized by dividing the total number of eggs by the root fresh weight obtained earlier.

Field test

On May 8th 2014, seeds of the 40 lines from ACX148 and the 5 controls used in the inoculation experiments were planted at the Tennessee Valley Research and Extension Center (TVREC) in Belle Mina, AL. There were two independent experiments, one was in a RR-infested field, the other in a RR-free field. Both experiments were designed similarly as an augmented randomized complete block design (RCBD). The two treatment groups were defined by the presence or absence of the BNL3279_105 marker, and the five controls were included only for comparison. The site of planting in both fields was divided into five blocks. Each block contained all 45 lines planted as one row plots, where each plot was approximately 1.01 m $\times$ 7.62 m in dimension. Please refer to Figure 2 through 4 for actual pictures taken during the planting season for a better idea of where the two experiments were located. Plant height and vigor ratings (from 0 to 5, where 0 means very low vigor and 5 means excellent vigor) were taken at early season (June 16th) and mid-season (July 23rd). One plant from each plot in the RR infested field was also dug with as much of its root system as possible and brought back for nematode extraction at the early season rating date. Nematode extraction protocol was similar to that used to extraction greenhouse screening tests, and height, shoot fresh weight and root fresh weight were recorded. In early October, boll samples (25 bolls) were collected by hand from each plot and ginned on a table-top saw tooth gin. Seed cotton weight, seed weight and lint weight were all recorded, and the turnout ratio was calculated. One hundred grams of cotton lint from each plot was collected and sent to Cotton Incorporated (Cary, NC) for fiber quality tests. Micronaire (MIC), upper half mean length (UHM), uniformity index (UI), fiber strength (STR), elongation (ELO) and short fiber content (SFC) were determined for each sample. Later the same month, seed cotton was machine-harvested separately for each plot by the station to determine plot yield.

Statistical Analysis

All statistical analyses were done with the R software package for statistical analysis. A mixed model was built for the field test results with the help of the lme4 package by Bates et al. (2015). Experiment factors were presence vs. absence of the marker (fixed), genotypes nested within marker groups (random) and blocks (random). According to Robinson et al. (2004), variances for nematode egg count are highly positively correlated to the associated means, suggesting that the egg count data is of lognormal distribution. Thus, a logarithm transformation was performed on the nematode egg count per gram of root data for both the greenhouse screening and field test experiments. Everything else, including the blocking effect, was assumed to be normally distributed. A generalized linear model with presence/absence of the marker BNL3279_105 as the fixed factor was built for the greenhouse screening test. ANOVA test was then run based on such models. The p value was obtained for the fixed effects, and variance contribution was obtained for the random effects. Figures and graphs were plotted with the help of the ggplot2 package (Wickham, 2009).

Results and Discussion

Genotyping ACX148

One hundred $F_{2:3}$ lines from population ACX148 were sampled in summer 2013 by my colleague Ruijuan Li in Prattville, AL. The technique for sampling DNA was actually used to sample a new population derived from BARBREN-713 to learn the process in spring 2014. The SSR marker BNL3279 was also genotyped by my colleague, and the protocols were only used later to verify her results. 20 RES lines (homozygous positive for BNL3279_105) and 20 SUS lines (homozygous negative for BNL3279_105) were selected, and these 40 lines were then genotyped by the above method for the SSR marker BNL569. In summer 2014, the primer sequence from CottonMarker.org was used and the PCR yield was consistently low (Figure 1a). After redesigning the primers, primer pair BNL569_4 showed the best yield and produced clear bands on the polyacrylamide gel (Figure 1b). The primer pair BNL569_4 was only a frame shifted version of the original pair provided by CottonMarker.org (Table 1). Such huge difference in yield may suggest a 3' mispairing in the original forward or reverse primer, possibly caused by a point mutation. This mutation occurred in both parents, as well as the $F_{2:3}$ lines. However, BNL569 was used to create linkage maps (Zhao et al., 2012) and mapped the *Ren*^{barb} QTLs in BARBREN-713 (Gutiérrez et al., 2010). None of these literature citations mentioned anything about such a point mutation. Since it was not in the release documentation of BARBREN-713, the source of it is still unknown.

Unfortunately, there were no valuable polymorphisms discovered in the 40 lines for BNL569 marker. Although the BARBREN-713 sample correctly showed the BNL569_131 band, it is not the actual parent to the population but another BARBREN-713 plant sampled later. Only one out of 40 lines (148024) was scored **a**, and the rest were all **h** or **b**. The complete genotypes

for markers BNL3279 and BNL569 for all 40 lines can be seen in Table 2. It departed very far from the expected ratio of 1:2:1 for a:h:b genotypes (χ^2=83.4, df=2, p<0.0001), suggesting that the original BARBREN-713 plant used to make the cross may not be homozygous for BNL569_131. It is well known that cotton is hard to keep genetically pure because it has open-pollinated flowers. The difficulties encountered while genotyping the BNL569 marker may also contribute to the loss of purity. Since it is very hard to get an acceptable yield for BNL569 PCR products, people may not monitor every plant for the presence of BNL569_131. Thus, the distributed seeds may have contamination and impurity.

Regarding the technique used in genotyping population ACX148, the mini-prep method introduced by Zhang et al. greatly simplified the entire process of genotyping. It was very fast and efficient. A single person could extract more than forty samples each day. Each young cotton leaf yielded 500μL of 200ng/μL DNA templates on average. The templates were high in quality also (>2.0 260/280 and >1.5 260/230), in spite of the fact that no column or filter were used to purify them. Such quality was sufficient for electrophoresis analysis.

On the other hand, PCR was the part that required most attention. The BNL569 marker was very sensitive to $MgCl_2$ concentration as opposed to most other markers. Standard concentration was 1.5 mM and required no additional $MgCl_2$ solution, which was used for BNL3279 and caused no problem. However, the yield at this concentration was not good even using the new BNL569 primer pair. After increasing the concentration in 0.5mM steps, I discovered that 2.5mM was enough to produce acceptable results, and further addition will not increase yield visually.

Although genotyping the samples was a trivial task, it provided us with precious information about the actually genotype of the plants, making the experiment design for field

study much easier. The 40 lines chosen for genotyping BNL569 were also selected as test subjects in the following greenhouse screening and field tests, and the genotype of BNL3279 was used as the fixed factor to separate treatment groups.

Greenhouse and field study

Because of the double planting strategy, we were able to run the greenhouse screening test without missing data points. There were no significance differences observed for shoot fresh weight and root fresh weight between the RES lines and SUS lines. RES lines were significantly taller than SUS lines ($p<0.01$), and had less nematode eggs per gram of root ($p<0.001$). All greenhouse results are summarized in Table 3. It confirmed that the QTL *Ren*^{barb2} does contribute to significant RR resistance. Interestingly though, when plotted by lines (Figure 5), the experimental line with the lowest average egg count was from the SUS group (148023). However, this is only one screening test, and further repetitions are needed to make any valuable conclusions from such an observation.

Yield, nematode count and some phenotypic data of the field experiments are summarized in Table 4 and Table 5, for the RR infested field and RR free field respectively. Briefly put, the RES group had higher shoot fresh weight ($p<0.05$), root fresh weight ($p<0.05$) and plant height ($p<0.001$) early season in the RR infested field. The plant height difference continued until mid-season ($p<0.001$). The RES group also had lower nematode egg count per gram of root ($p<0.001$), confirming the result from the greenhouse screening test and providing evidence to support the effectiveness of the gene associated with QTL *Ren*^{barb2} in the field. More significantly, such resistance also resulted in higher yield for the RES group ($p<0.001$). The RES group yielded approximately 1074 kg ha^{-1} of lint on average in the RR-infested field, which was 22% higher than the 834 kg ha^{-1} yield from the SUS group. On the other hand, there was no

difference observed between the two groups in the RR-free field for shoot height. However, the RES group yielded only approximately 1374 kg ha^{-1} of lint, which was 10% lower than the 1553 kg ha^{-1} lint yield from the SUS group, a significant difference ($p<0.01$). The turnout ratio was negatively affected in both environments, with the RES group having a lower turnout ratio (42.2% in the RR-infested field and 41.6% in the RR-free field) than that of the SUS group (42.7% in the RR-infested field and 42.6% in the RR-free field) ($p<0.01$ in RR-infested field, $p<0.001$ in RR-free field).

On the other hand, there were no differences between the two groups with respect to fiber quality (Table 6 and Table 7 for the RR-infested and RR-free field respectively). The exception was UHM, where the SUS group was significantly higher in both fields ($p<0.001$ in both fields). E103-123 showed a higher STR and a lower SFC when the estimated means of different groups were plotted (the controls were not tested against ANOVA), confirming that it is indeed a high quality germplasm line.

Such results from both the greenhouse and field tests were consistent, proving that the QTL Ren^{barb2} is indeed contributing resistance to RR. As a result of such resistance, it provided less yield loss under RR pressure compared to those susceptible lines. However, such resistance does not come without a price. The yield drag observed in the RR free field, as well as the lower turnout ratio and UHM observed in both fields, suggested that there may be some deleterious effects bounded to the QTL region. However, a similar two-year experiment on LONREN derived material suggested that the Ren^{lon} QTLs do not have such deleterious effect on yield (Weaver et. al., 2013). Further repetitions are required to confirm such observation and uncover more such effects, but that is a topic left for the interested researchers to take over. Despite the fact that the planting locations were physically close, there were still significant variances

observed in different blocks. The consistency of *Ren*^{barb2}'s performance across different locations and different years is still unknown, which is another topic that can be taken over by the interested. The fiber quality data didn't show any surprises. Most parameters of most lines showed no significant difference from others, including the controls. It showed a lack of effort in breeding for a higher fiber quality germplasm in general. An earlier study on LONREN derived material (Weaver et. al., 2013) showed that the *Ren*^{lon} QTLs actually enhance fiber strength in upland cotton. It could be a valuable effort in the BARBREN-713 derived lines also, since the *Ren*^{lon} and *Ren*^{barb} QTLs have been mapped to homeologous chromosomes (Bell et. al., 2014; Guti èrrez et. al., 2011). One QTL from each source was identified to be linked with the SSR marker BNL3279 in some way, suggesting a very similar location on respective chromosomes. It could be an indication that they may have similar effects. Moreover, BARBREN-713 was developed with crosses to *G. barbadense* which is known to have longer and stronger fiber. As opposed to the LONREN derived germplasm lines tested in earlier studies (Weaver et al., 2013), BARBREN-713 derived lines did not show stunting in their early growing stage in RR infested fields and the average yield was higher than that of the susceptible lines, meaning that we have finally found a commercially valuable source of resistance. Combining the possibility of making better fiber and the fact that they can make more fiber, BABREN-713 derived lines, including population ACX148, are the worthwhile material for breeding nematode resistant and agriculturally favorable cultivars.

References

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67(1):1-48. doi: 10.18637/jss.v067.i01.

Bell, A. A., J. Quintana, X. Zheng, D.M. Stelly, and R.L. Nichols. 2012. A protocol for isolating and enumerating *Thielaviopsis basicola* from cotton roots: Applications to LONREN stunt. In Proceedings of the Beltwide Cotton Conferences, Orlando, FL. 3–6 Jan. 2012. National Cotton Council of America, Memphis TN. p. 329–340.

Bell, A. A., A. F. Robinson, J. Quintana, N. D. Dighe, M. A. Menz, D. M. Stelly, X. Zheng, J. E. Jones, C. Overstreet, E. Burris, R. G. Cantrell, and R. L. Nichols. 2014. Registration of LONREN-1 and LONREN-2 germplasm lines of upland cotton resistant to reniform nematode. J. of Plant Registrations 8:187–190. doi: 10.3198/jpr2013.11.0069crg.

Bell, A.A., A.F. Robinson, J. Quintana, S. E. Duke, J. L. Starr, D. M. Stelly, Z. Zheng, S. Prom, V. Saladino, O. A. Gutiérrez, S. R. Stetina, and R. L. Nichols. 2015. Registration of BARBREN-713 germplasm line of Upland cotton resistant to reniform and root-knot nematodes. J. of Plant Registrations. 9:89–93.

Birchfield, W. 1983. Wheat and grain sorghum varietal reaction to *Meloidogyne incognita* and *Rotylenchulus reniformis*. Plant Dis. 67:41–42.

Blasingame, D., and M. V. Patel. 2012. Cotton disease loss estimate committee report. p. 1242–1246. In S. Boyd, M. Huffman, B. Robertson (ed.). Proc. Beltwide Cotton Conf. San Antonio, TX.

Davis, R. F., S. R. Koenning, R. C. Kamerait, T. D. Cummings, and W. D. Shurley. 2003. *Rotylenchulus reniformis* management in cotton with crop rotation. J. Nematol. 35:58-64.

Dighe, N. D., A. F. Robinson, A. A. Bell, M. A. Menz, R. G. Cantrell, and D. M. Stelly. 2009. Linkage mapping of resistance to reniform nematode in cotton following introgression from *Gossypium longicalyx* (Hutch. & Lee). Crop Sci. 49:1151–1164.

Food and Agriculture Organization of the United Nations Statistics Division. 2014. Food and agricultural commodities production ranking, 2012. http://faostat3.fao.org/browse/rankings/commodities_by_regions/E; Verified November 5, 2015.

Gazaway, W. S., K. S. Lawrence, and J. R. Akridge. 2007. Impact of crop rotation and fumigation on cotton production in reniform infested fields. p. 1357–1360. In S. Boyd, M. Huffman, D. Richter, and B. Robertson (ed.) Proc. Beltwide Cotton Conf. New Orleans, LA.

Gutièrrez, O. A., J. N. Jenkins, J. C. McCarty, M. J. Wubben, R. W. Hayes, and F. E. Callahan. 2010. SSR markers closely associated with genes for resistance to root-knot nematode on chromosomes 11 and 14 of Upland cotton. Theor. Appl. Genet. 121:1323–1337.

Gutiérrez, O.A., A. F. Robinson, J. N. Jenkins, J. C. McCarty, M. J. Wubben, F. E. Callahan, and R. L. Nichols. 2011. Identification of QTL regions and SSR markers associated with resistance to reniform nematode in *Gossypium barbadense* L. accession GB713. Theor. Appl. Genet. 122:271–280.

Jones, J. E., L. D. Newsom, and E. L. Finley. 1959. Effect of the reniform nematode on yield, plant characters, and fiber properties of upland cotton. Agron. J. 51:353–356.

Lawrence, G. W., K. S. McLean, W. E. Batson, D. Miller, and J. C. Borbon. 1990. Response of *Rotylenchulus reniformis* to nematicide applications on cotton. Suppl. J. Nematol. 22:707–711.

Linford, M. B., and J. M Oliveira. 1940. *Rotylenechulus reniformis*, nov. gen., n. sp., a nematode parasite of roots. Proc. of the Helminthological Society of Washington. 7:35–42.

National Cotton Council. 2009. Boll weevil eradication program. http://www.cotton.org/tech/pest/bollweevil/index.cfm; Verified November 6, 2015.

National Cotton Council. 2013. U.S. Beltwide yield losses to nematode damage. https://www.cotton.org/tech/pest/nematode/losses.cfm; Verified November 2, 2015.

National Cotton Council. 2014. U.S. Cotton planted acres. http://www.cotton.org/econ/cropinfo/production/us-cotton-planted-acres.cfm; Verified November 2, 2015.

Nichols, R. L., A. Bell, D. Stelly, N. Dighe, F. Robinson, M. Menz, J. Starr, P. Agudelo, J. Jones, C. Overstreet, E. Burris, C. Cook, R. Lemon, and D. Fang. 2010. Phenotypic and genetic evaluation of LONREN germplasm. p. 798–799. In S. Boyd, M. Huffman, B. Robertson (ed.). Proc. Beltwide Cotton Conf. New Orleans, LA.

Robbins, R.T., L. Rakes, L.E. Jackson, and D.G. Dombek. 1999. Reniform nematode resistance in selected soybean cultivars. Suppl. J. Nematol. 31:667–677.

Robinson, A. F., and A. E. Percival. 1997. Resistance to *Meloidogyne incognita* race 3 and *Rotylenchulus reniformis* in wild accession of *Gossypium hirsutum* and *G. barbadense* from Mexico. J. Nematol. 29:746–755.

Robinson, A.F., C.G. Cook, and A.E. Percival. 1999. Resistance to *Rotylenchulus reniformis* and *Meloidogyne incognita* race 3 in the major cotton cultivars planted since 1950. Crop Sci. 39:850–858.

Robinson, A. F., A. C. Bridges, and A. E. Percival. 2004. New sources of resistance to the reniform (*Rotylenchulus reniformis* Linford and Oliveira) and root-knot (*Meloidogyne*

incognita (Kofoid & White) Chitwood) nematode in upland (*Gossypium hirsutum* L.) and sea island (*G. barbadense* L.) cotton. J. Cotton Sci. 8:191–197.

Romano, G. B., E. J. Sacks, S. R. Stetina, A. F. Robinson, D. D. Fang, O. A. Guti èrrez, and J. A. Scheffler. 2009. Identifcation and genomic location of a reniform nematode (*Rotylenchulus reniformis*) resistance locus (*Ren*^{ari}) introgressed from *Gossypium aridum* into upland cotton (*G. hirsutum*). Theor. Appl. Genet. 120:139–150.

Sacks, E.J., and A. F. Robinson. 2009. Introgression of resistance to reniform nematode (*Rotylenchulus reniformis*) into upland cotton (*Gossypium hirsutum*) from *Gossypium arboreum* and a *G. hirsutum*/*Gossypium aridum* bridging line. Field Crops Res. 112:1–6.

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.

Sikkens, R. B., D. B. Weaver, K. S. Lawrence, and R. L. Nichols. 2014. Comparative performance of reniform nematode resistant germplasm lines. In Proceedings of the Beltwide Cotton Conferences, New Orleans, LA. 6–8 Jan. 2014. National Cotton Council of America, Memphis TN. p. 652–659.

Stetina, S. R., L. D. Young, W. T. Pettigrew, and H. A. Bruns. 2007. Effect of corn-cotton rotations on reniform nematode populations and crop yield. Nematropica 37:237–248.

Stewart J. M., and R. T. Robbins. 1995. Evaluation of Asiatic cottons for resistance to reniform nematode. p. 165–168. In Oosterhuis DM (ed) Proceedings of the 1994 cotton research meeting and 1994 summaries of cotton research in progress, Fayetteville. Arkansas Agricultural Experiment Station Special Report 166, AR.

Sürmelioğlu, Ç., R. B. Sikkens, R. R. Sharpe, S. R. Moore, E. van Santen, K. S. Lawrence, and D. B. Weaver. 2010. Resistance to reniform nematode from *G. hirsutum* and *G. longicalyx* sources: a comparison. p. 787–788. In S. Boyd, M. Huffman, B. Robertson (ed.). Proc. Beltwide Cotton Conf. New Orleans, LA.

Thornton B., and C. Basu. 2011. Real-time PCR (qPCR) primer design using free online software. Biochem. Mol. Biol. Educ. 39(2):145–154.

Weaver, D. B., K. Lawrence, and E. van Santen. 2007. Reniform nematode resistance in upland cotton germplasm. Crop Sci. 47:19–24.

Weaver, D. B., R. B. Sikkens, K. S. Lawrence, Ç. Sürmelioğlu, E. van Santen, and R. L. Nichols. 2013. *Ren*^{lon} and its effects on agronomic and fiber quality traits in upland cotton. Crop Sci. 53:913–920.

Weaver, D. B. 2015. Cotton nematodes. p. 547 – 570. In D. Fang (ed.) Cotton. American Society of Agronomy Monograph. Madison, WI.

Wendel, J. F. 1989. New World cottons contain Old World cytoplasm. Proc. Nat. Acad. Sci. USA 86:4132–4136.

Wendel, J. F., and R. C. Cronn. 2003. Polyploidy and the evolutionary history of cotton. Adv. Agron. 78:139–186.

Westphal, A., and J. R. Smart. 2003. Depth distribution of *Rotylenchulus reniformis* under different tillage and crop sequence systems. Phytopathology 93:1182–1189.

Wickham, H. 2009. ggplot2: elegant graphics for data analysis. Springer New York.

Windham, G. L., and G. W. Lawrence. 1992. Host status of commercial maize hybrids to Rotylenchulus reniformis. J. Nematol. 24:745–748.

Usery, S. R., K. S. Lawrence, G. W. Lawrence, and C. H. Burmester. 2005. Evaluation of cotton cultivars for resistance and tolerance to Rotylenchulus reniformis. Nematropica 35:121–134.

Yik, C. P. and W. Birchfield. 1984. Resistant germplasm in Gossypium species and related plants to Rotylenchulus reniformis. J. Nematol. 16:146–153.

Zhang, J., and J. M. Stewart. 2000. Economical and rapid method for extracting cotton genomic DNA. J. Cotton Sci. 4:193–201.

Table 1. BNL569 primer pairs designed by Primer3.

Primer Pair Name	Product Region	Product Size(bp)	Forward Sequence	Tm (℃)	Reverse Sequence	Tm (℃)
BNL569	original	131	TTGAGAAGTACTACCATTAATTATCCA	55.6	GACTGATGCCAGTTGACCCT	58.5
BNL569_1	downstream	104	AGGGTCAACTGGCATCAGTC	60.1	TCCTGCAGTTGCTGATTCAT	59.4
BNL569_2	original	141	TGAGAAGTACTACCATTAATTATCCAA	56.8	GAGACTGATGCCAGTTGACC	58.2
BNL569_3	original	133	CCATTAATTATCCAAAAATAAGAAA	55.2	AGAAGAGACTGATGCCAGTTG	57.1
BNL569_4	original	131	CCATTAATTATCCAAAAATAAGAA	53.9	AAGAGACTGATGCCAGTTGA	55.9
BNL569_5	downstream	138	CAGGTGCCACCATTCTAAATC	59.4	TCCTGCAGTTGCTGATTCAT	59.4
BNL569_6	original	145	TGAGAAGTACTACCATTAATTATCCA	55.5	AGAAGAGACTGATGCCAGTTG	57.1
BNL569_7	downstream	140	GACAGGTGCCACCATTCTAA	58.5	TCCTGCAGTTGCTGATTCAT	59.4
BNL569_8	original	102	GGTCAACTGGCATCAGTCTCT	59.3	TCCTGCAGTTGCTGATTCAT	59.4
BNL569_9	downstream	116	TCTTTTTATTTCTCCAGGGTCAA	59.1	TGCAGTTGCTGATTCATTCTG	60.0
BNL569_10	downstream	117	ATCTTTTTATTTCTCCAGGGTCA	58.2	TGCAGTTGCTGATTCATTCTG	60.0
BNL569_11	original	149	ACCATTAATTATCCAAAAATAAGA	54.8	CTGCAGGTTCTCTGAAGAAG	55.3

Table 2. Genotypes of the 40 RIL lines in population ACX148 with regard to SSR marker BNL3279 and BNL569.

Lines	BNL3279 score	BNL569 score	Lines	BNL3279 score	BNL569 score
ACX148018	RES	b	ACX148060	RES	b
ACX148019	RES	b	ACX148062	RES	b
ACX148020	SUS	b	ACX148063	RES	b
ACX148022	SUS	b	ACX148064	SUS	b
ACX148023	SUS	h	ACX148065	RES	b
ACX148024	SUS	a	ACX148068	SUS	b
ACX148029	RES	b	ACX148069	SUS	b
ACX148030	RES	b	ACX148071	SUS	h
ACX148040	SUS	b	ACX148072	SUS	b
ACX148041	RES	b	ACX148076	RES	b
ACX148042	RES	b	ACX148081	RES	b
ACX148043	SUS	b	ACX148083	RES	b
ACX148049	RES	b	ACX148084	SUS	h
ACX148050	RES	b	ACX148085	SUS	h
ACX148052	SUS	b	ACX148086	SUS	b

40 lines out of 100 were genotyped and recorded. 20 of which are homozygous positive for BNL3279_105 and scored RES, while the other 20 are homozygous negative for BNL3279 and scored SUS. There are three scores for the BNL569 marker: a (homozygous positive for BNL569_131), h (heterozygous), and b (homozygous negative for BNL569_131). One out of 40 is homozygous positive (ACX148024), five others are heterozygous (ACX148056, ACX148071, ACX148084, ACX148085), and the rest are all homozygous negative.

Table 3a. Greenhouse screening results.

Phenotypic parameters	RES group			SUS group			Difference	p value of ANOVA test
		95% CI			95% CI			
	Mean	Lower	Upper	Mean	Lower	Upper		
SFW (g)	5.047	4.766	5.329	5.058	4.775	5.341	-0.010	0.959
RFW (g)	3.109	2.824	3.393	3.076	2.792	3.361	0.032	0.874
Height (cm)	32.725	31.549	33.901	30.364	29.182	31.546	2.361	<0.01*
Normalized Log_c	3.539	3.429	3.649	3.987	3.877	4.097	-0.448	<0.001*

There were no significant difference observed in SFW and RFW data. However, the RES group makes taller plants than the SUS groups, indicating that RR affects plant height more than their weight in greenhouse conditions. Nematode egg count per gram of root is, without surprises, significantly lower for the RES group.

Table 3b. Comparison of all groups in greenhouse screening test.

Treatment group	SFW (g)		RFW (g)		Height (cm)		Normalized Log_c	
	Mean	Std err.	Mean	Std err.	Mean	Std err.	Mean	Std err.
RES	5.047	0.142	3.109	0.142	32.725	0.585	3.539	0.056
SUS	5.058	0.143	3.076	0.142	30.364	0.588	3.987	0.056
BARBREN-713	6.206	0.636	4.690	0.634	32.400	2.617	3.890	0.249
AU3202	4.842	0.636	3.312	0.634	35.500	2.617	4.006	0.249
SG747	6.128	0.636	3.006	0.634	27.600	2.617	4.028	0.249
LONREN 21-4	4.926	0.636	2.232	0.634	25.400	2.617	1.950	0.249
E103-123	5.440	0.636	3.972	0.634	34.000	2.617	2.638	0.249

The controls were only included for comparison not testing. The table shows that LONREN 21-4 and E103-123 both have great resistance to RR, as they are closely related germplasm lines derived from the same LONREN breeding program. The susceptible controls (AU3202 and SG747), on the other hand, supports a much higher level of nematode reproduction.

Table 4. Yield, turnout ratio, weight, height and resistance of RES and SUS group in RR infested field.

Phenotypic parameters	RES group			SUS group			Difference	p value of ANOVA test
		95% CI			95% CI			
	Mean	Lower	Upper	Mean	Lower	Upper		
Lint Yield (kg ha^{-1})	1074.96	922.88	1227.05	834.42	682.34	986.51	240.53	<0.0001*
Turnout ratio (%)	42.2	41.9	42.6	42.7	42.4	43.1	-0.5	0.0049*
SFW (g)	32.60	26.48	38.72	29.56	23.44	35.68	3.03	0.0378*
RFW (g)	4.33	3.58	5.07	3.89	3.14	4.64	0.43	0.0341*
Early season height (cm)	20.10	18.24	21.95	18.60	16.74	20.46	1.49	<0.001*
Mid-season height (cm)	57.38	52.36	62.39	49.90	44.88	54.91	7.48	<0.0001*
Normalized Log_c	2.63	2.49	2.78	2.95	2.81	3.10	-0.32	<0.0001*

The RES group performed better than the SUS group in almost all parameters except the turnout ratio. Resistance can be well estimated by measuring plant height as early as 40 DAP, and it is a better measurement than both shoot weight and root weight. Estimated lint yield of the RES group was a little over one thousand kilograms per hectare, approximately 22% higher than that of the SUS group.

Table 5. Yield and turnout ratio of RES and SUS group in RR free field.

Phenotypic parameters	RES group			SUS group			Difference	p value of ANOVA test
		95% CI			95% CI			
	Mean	Lower	Upper	Mean	Lower	Upper		
Lint Yield (kg ha^{-1})	1394.45	1264.82	1524.07	1553.46	1423.84	1683.09	-159.01	<0.001*
Turnout ratio (%)	41.6	41.0	42.2	42.6	41.9	43.2	-0.99	<0.0001*

The RES group suffered from yield drag in non-stressed environment. Estimated yield was approximately 10% lower than that of the SUS group. The turnout ratio was lower too, consistent with the result in RR infested field.

Table 6. Fiber quality parameters of RES and SUS group in RR infested field.

Phenotypic parameters	RES group			SUS group			Difference	p value of ANOVA test
	Mean	95% CI		Mean	95% CI			
		Lower	Upper		Lower	Upper		
MIC	5.230	5.142	5.318	5.159	5.071	5.247	0.071	0.088
UHM (mm)	29.314	28.977	29.650	29.973	29.636	30.309	-0.659	<0.0001*
STR	31.113	30.714	31.511	31.234	30.835	31.632	-0.121	0.599
ELO	4.586	4.409	4.763	4.478	4.301	4.654	0.109	0.185
UI (%)	84.304	84.020	84.588	84.619	84.335	84.903	-0.315	0.032*
SFC (%)	7.386	7.256	7.517	7.335	7.205	7.465	0.051	0.461

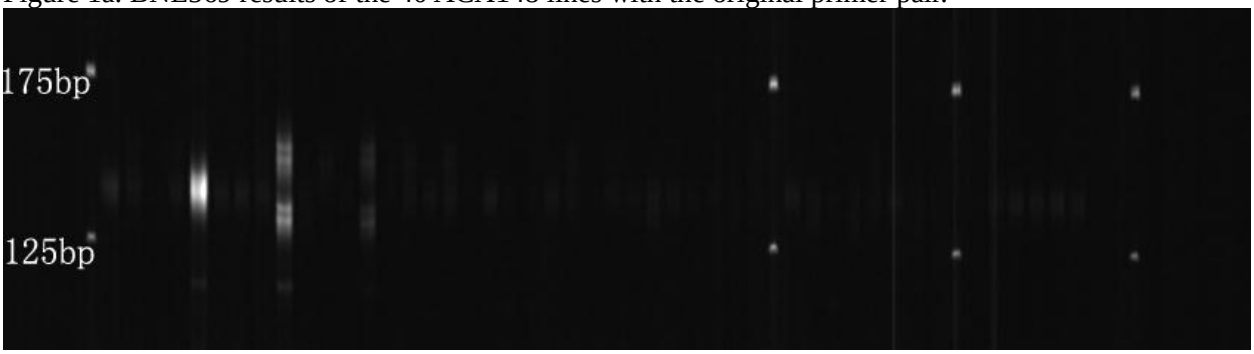
Most parameters, except UHM and UI, were not significantly different between the two treatment groups. The RES group, however, performed worse than the SUS group in the two significantly different categories, where the estimated UHM of fiber was 0.659 mm shorter, and 0.315% less uniform. Despite the statistical significance, the differences are minuscule, and are of no real world importance.

Table 7. Fiber quality parameters of RES and SUS group in RR free field.

Phenotypic parameters	RES group			SUS group			Difference	p value of ANOVA test
	Mean	95% CI		Mean	95% CI			
		Lower	Upper		Lower	Upper		
MIC	5.059	4.936	5.181	5.000	4.878	5.122	0.059	0.151
UHM (mm)	29.426	28.805	30.048	30.020	29.398	30.642	-0.594	<0.0001*
STR	31.273	30.671	31.874	31.050	30.448	31.652	0.223	0.352
ELO	4.664	4.530	4.797	4.714	4.580	4.847	-0.050	0.471
UI (%)	84.549	83.918	85.180	84.516	83.885	85.148	0.032	0.854
SFC (%)	7.291	6.945	7.638	7.365	7.018	7.712	-0.074	0.430

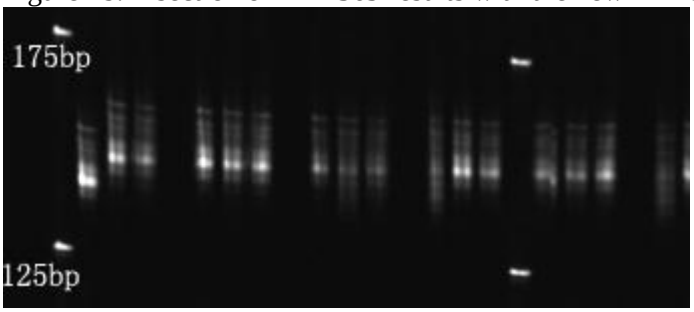
There are no really differences from the RR infested field. The estimated UHM of the RES group was 0.594 mm shorter than that of the SUS group, which is statistically significant, but not important.

Figure 1a. BNL569 results of the 40 ACX148 lines with the original primer pair.



Our product of interest BNL569_131 is located between the size standard 125bp and 175bp bands. As one can see, the bands are very faint and smeared.

Figure 1b. A section of BNL569 results with the new BNL569_4 primer pair.

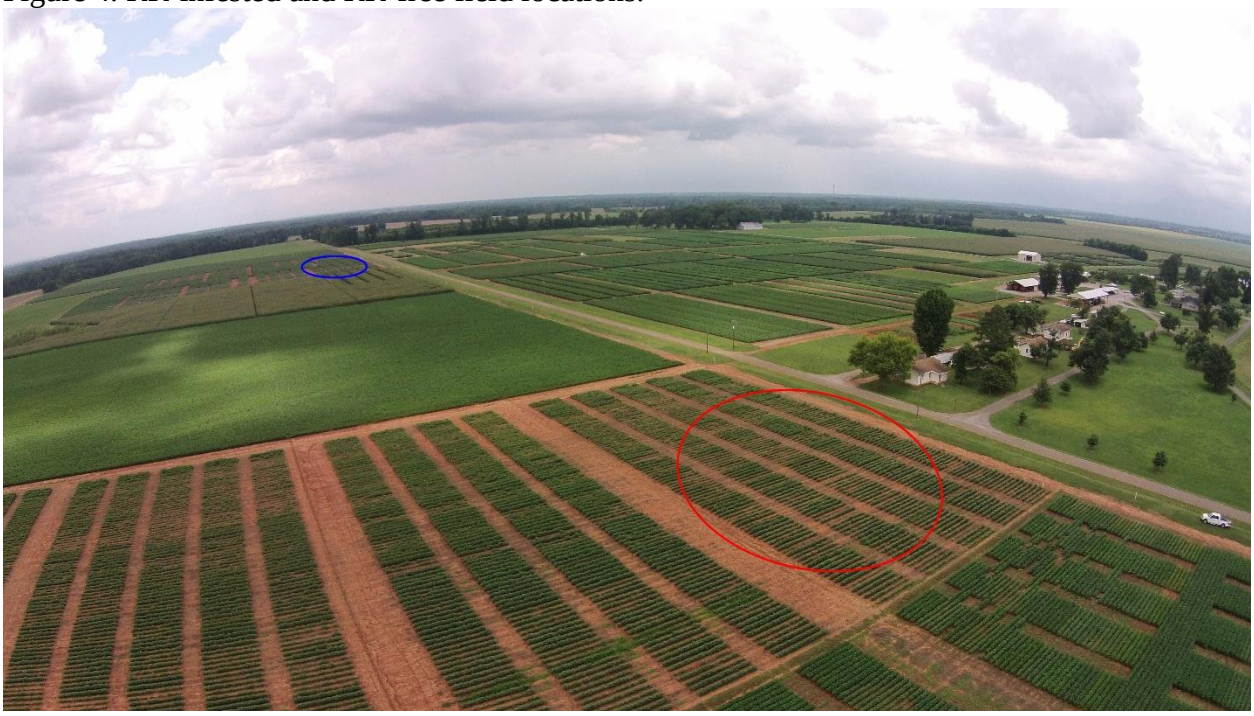


. It is quite clear that the bands are much clearer and easier to score than the previous picture.

Figure 2. RR-infested field view during early season.

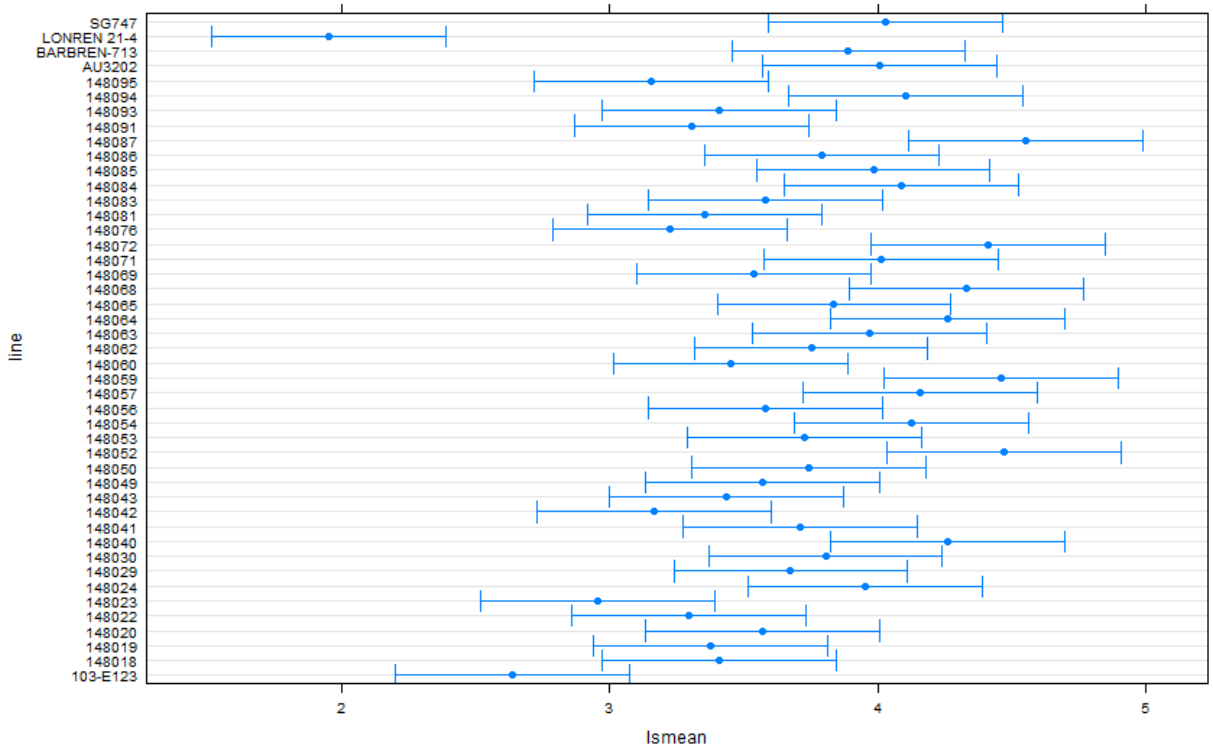
Figure 3. RR-free field view during early season.

Figure 4. RR-infested and RR-free field locations.



Near red circle: RR-infested field test location at TVREC.
Far blue circle: RR-free field test location at TVREC.

Figure 5. Estimate of average egg count of different germplasm lines in greenhouse



LONREN derived material continues to show their extremely high resistance here. LONREN 21-4 and E123-103 are outperforming everything else. 148023 had the lowest egg count among all the experimental lines. It is interesting to point out because it scored SUS for the SSR marker BNL3279_131.