Genetic Resistance to Reniform Nematode (*Rotylenchulus reniformis*) in Upland Cotton (*Gossypium hirsutum*)

by

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Abstract

Reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira, is one of the most destructive nematode pests of cotton (Gossypium spp.), and upland cotton (*G. hirsutum* L.), the most common cultivated type, is lacking in resistance. Differing levels of resistance to reniform nematode has been reported in several species of *Gossypium*. Our objectives in the first part of this research were to compare levels of resistance in two *G. hirsutum* wild accessions (TX 245 and TX 1419) with those germplasm lines with resistance derived from *G. longicalyx*, a wild cotton relative that has been reported to be immune, and to determine if resistance in *G. hirsutum* is a heritable trait. We evaluated $F_{2:3}$ lines from crosses derived from TX 245 and TX 1419 and an adapted susceptible genotype for resistance to reniform nematode in the greenhouse using LONREN-1, a highly resistant *G. hirsutum* type with resistance derived from *G. longicalyx* for comparison. There were no differences among the $F_{2:3}$ lines for vermiform reproduction. Zero heritability of resistance was determined within these populations, and LONREN-1 resistance was far superior. In the second part of this study, $F_{2:3}$ lines derived from a cross between LONREN-1 and an adapted susceptible line were tested for reniform nematode resistance. Twenty-one of 100 $F_{2:3}$ lines were found to be highly resistant and not different from the LONREN-1 parent. We concluded that the previously observed resistance in *G. hirsutum* was not repeatable, but resistance derived from *G. longicalyx* was highly heritable and repeatable.

The aim of the second part of this research was to evaluate the impact of age and condition of seedlings on reniform nematode reproduction in upland cotton by investigating the relationship between the seedling vigor and genetic resistance to reniform nematode. Seeds of two cultivars (PM 1218 and FM 966, susceptible), two wild accessions (TX 245 and TX 1419, previously showing erratic results with regard to resistance), and LONREN-2 (resistant) were exposed to adverse stress conditions (accelerated aging) to simulate reduced seedling vigor caused by time and improper storage. Genotypes were then tested for reniform nematode resistance by evaluating nematode reproduction, to determine the possible effects on seedling vigor on nematode reaction. Plant height was recorded once a week during the evaluation period. Dry and fresh weights of roots and shoot dry weights were measured at the end of the study. Results showed that germination rate and seedling vigor decreased with increasing length of exposure time to adverse conditions. Reniform nematode numbers for each genotype were not significantly different due to accelerated aging. Consequently, there was no direct relationship between the age and condition of the cotton seedlings and their response to reniform nematode.

The objective of the third part of this research was to evaluate the potential use of Simple Sequence Repeats (SSR) to monitor upland cotton populations for resistance to reniform nematode. This was performed by molecular screening of 38 out of 100 $F_{2:3}$ lines of the cross LONREN-1 × FM 966 in addition to a control group that included both of the parents, LONREN-1 and FM 966 as resistant and susceptible controls, respectively. The SSR markers amplified most of the 38 DNA samples showing that

phenotype-based resistance determined in our previous study was reliable except some unexpected results which is remained for further evaluation. Results also show that marker-assisted screening can be easily applied to analyze upland cotton populations for reniform nematode resistance and to select good candidates for further breeding studies.

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CHAPTER 1

LITERATURE REVIEW

Cotton, *Gossypium spp*., is a significant fiber crop which is harvested in almost every tropical country as well as in many parts of the subtropical world (Mondal *et al*., 2008) and has 45 diploid and 5 allotetraploid species (Fryxell, 1992). Only 4 of 50 species of *Gossypium* (*G. arboreum*, *G. barbadense*, *G. herbaceum*, and *G. hirsutum*) have been domesticated due to their useful seed lint (Smith and Cothren, 1999). *G. arboretum* and *G. herbaceum* are diploid (2n = 26) whereas *G. barbadense* and *G. hirsutum* are tetraploid (4n = 52) species (Pundir, 1972).

Species in the genus *Gossypium* have varying characteristics. Some have short-brown or long-white fibers, others are small trees or perennials, and are unique among fiber crops (Smith and Cothren, 1999). This uniqueness of cotton makes its value more important all over the world in both industrial and agricultural retail. Although cotton is not native to the United States, its importance as a fiber crop is high. Seventeen of the southern U.S. states grow cotton commercially (Smith and Cothren, 1999) with the U.S. being one of the ten largest producers of cotton (China, the United States, India, Pakistan, Brazil, Turkey, Greece, Australia, Syria, and Mali, ranked from top to bottom respectively) in the world (Workman, 2007). Respectively, Texas, Georgia, North

Carolina, Arkansas, and Mississippi were the five highest most cotton producing states in the U.S. in 2010 (USDA-NASS, 2011).

Cotton continues to be an important agricultural commodity, with Alabama ranking 10th among U.S. states with production of 490,000 bales in 2010. This represents an increase of 145,000 bales compared with 2009 production, and harvested acreage was increased nearly 38,445 ha (USDA-NASS, 2010). Cotton is the fourth most important row crop in the U.S. and the most important industrial crop, with a farm-value of more than $5.6 billion in 2007. Nowadays, cotton remains the most important export of the southern United States, and a majority of the world's annual cotton crop is short-staple American cotton (Yafa, 2004). Cotton's diversity of use in different areas such as textile, food and feed, and chemical industries makes it a miracle crop and cotton will continue to be a major agricultural commodity in the upcoming years.

Cotton has many biotic pests. Thrips (*Frankliniella occidentalis*), aphids *(Aphis gossypii*), boll weevils (*Anthonomous grandis*), spider mites (*Tetranychus* spp.), various weed and nematode species are some of the major ones. These pests cause different damage symptoms such as chlorotic and necrotic areas, deformation of plant tissue, defoliation, crinkling and malformation of leaves, discolored lint, gall formation, etc. (Smith and Cothren, 1999). Insect and mite pests reduced cotton yield about 3.8% and the total loss and management cost was $543 million in 2008 (Williams, 2008). Pest management in cotton has been done in several ways. Biological control is one management option and can be done using parasites, predators, or pathogens of the pest.

Assasin bugs (*Zelus renardii*), big-eyed bugs (*Geocoris punctipes* and *Geocoris uliginosus*), damsel bug (*Nabis* sp.), flower bug (*Orius tristicolor*), and green lacewings (*Chrysoperla carnea*) are some beneficial predators living with cotton (UOA, 2006).

Chemical control of cotton pests is another way of management. People started to use insecticides for control of arthropod pests after the 1920s and has been quite effective. Carbamates, organophosphates, and pyrethroids are types of pesticides used on cotton worldwide. Cotton producers use these chemicals due to their efficacy and economical advantages but their disadvantages are also important. Chemical insecticides pose high risks to nontarget species including humans (Smith and Cothren, 1999). They may also interfere with the normal metabolism of the plant, leading to yield losses, and target species often find ways to develop resistance against insecticides and use of those chemicals may become a problem rather than a solution.

Cultural controls such as tillage, delayed planting, or application of plant growth regulators also can help manage cotton pests. Fall or spring tillage helps prevent regrowth of cotton stalks or weeds whereas plant growth regulators are used for earlier maturity of the cotton crop (Smith and Cothren, 1999). Additionally, use of cover crops and application of organic amendments are other cultural practices for managing nematode pests (Koenning *et al.,* 2004).

Besides the several pests affecting cotton, nematodes are of major concern. Nematodes are obligatory parasitic, microscopic invertebrates that feed with or on

bacteria, fungi, plants, or other nematodes and protozoa (Veech, 1984). All nematodes have similar life cycles starting with an egg and developing into an adult in four molts. Each developmental stage is called a juvenile, and can be either male or female. They may be in vermiform, fusiform, reniform, lemon, globose, or pyriform shapes. Plant parasitic nematodes have stylets that allow them to feed in or on plant roots. These endo- or ecto-parasites damage crops directly by feeding, by transmitting viruses, and serving as an entry site for bacterial and fungal infections. Infected plants are stunted and/or discolored and also weak under environmental stress. Their root systems are reduced and galls may exist in roots (Coyne and Talwana, 2000). Nematode damaged areas also provide an entry point for other plant pathogens to attack the root and further weaken the whole plant, thus causing a reduction of roots' water and nutrient absorbing capacity (Lucas, 1998).

The southern root-knot nematode, *Meloidogyne incognita*, and the reniform nematode, *Rotylenchulus reniformis* (Figure 1.01) are the most damaging nematode pests of cotton in the U.S. (Robinson and Cook, 2000; Robinson, 2007). Root-knot nematodes are pests of a huge variety of plants including many agronomic crops and weeds mostly of warm climates and cause 5% yield loss of crops in the world (Agrios, 2005). The six common hosts of nematodes from genus *Meloidogyne* are cotton (*Gossypium* spp.), peanut (*Arachis hypogaea*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), pepper (*Capsicum* spp.), and watermelon (*Citrullus lanatus*) (Koenning *et al*., 2004). Galls on secondary roots are the symptoms with adult female root-knot nematodes living endoparasitically (Smith and Cothren, 1999). Reniform nematode was first found

by Linford and Oliveira by examining pineapple (*Ananas comosus L.*) in Hawaii in 1940. Like root-knot nematodes, reniform nematodes are also pests of a huge variety of plants mostly in tropical climates and are able to reproduce on almost 314 plant species. However, there are also plants such as rice (*Oriza sativa* L.), nutmeg (*Myristica fragans* Houtt.), and onion (*Allium cepa* L.) that are resistant or tolerant to this nematode (Robinson *et al*., 1997). *Gossypium longicalyx, G. herbaceum, G. arboreum* are wild cotton accessions that are highly resistant to reniform nematode (Yik and Birchfield, 1984). Also, fifty commercial hybrids of maize (*Zea mays*) were found resistant to this nematode by Windham and Lawrence in 1992 (Windham and Lawrence, 1992) . Symptoms on susceptible host plants are not usually apparent but stunting (Figure 1.02) and discoloration of lower leaves are some visual indicators (Smith and Cothren, 1999). Delayed fruiting is also a symptom of reniform nematode infection in cotton (Jones *et al*., 1959). Although reniform nematodes do not produce galls on roots of host plants, Robinson and Cook (2001) observed galling on some kenaf plant roots that were infested with this nematode. In the same study, Robinson and Cook tested sunn hemp (*Crotalaria juncea L.*) and kenaf (*Hibiscus cannabinus L.*) for resistance and identified sunn hemp as resistant to both *M. incognita* and *R. reniformis*. Also, reniform nematode populations were several magnitudes bigger than root-knot nematode populations in infested cotton fields according to Robinson and Cook (2001). According to another study conducted by Robinson and Percival in 1997, 'Deltapine 16', an upland cotton genotype, and all Pima and California Acala cultivars (Robinson *et al*., 1999) are susceptible hosts of reniform nematode whereas Auburn 623 RNR, another upland cotton genotype selected for resistance to root-knot nematode, is also not resistant to the same nematode.

Upland cotton, *Gossypium hirsutum*, is a predominantly self-pollinated crop, but also up to about 30% cross-pollination might occur depending on vectors. It is allotetraploid with 26 pairs of chromosomes and two genomes with 13 chromosomes each (2n=52) (Acquaah, 2007). The reniform nematode was first reported in upland cotton in Georgia (Smith, 1940) and Louisiana (Smith and Taylor, 1941) and has been a rising problem of cotton in the U.S especially in Alabama, Louisiana, and Mississippi (Robinson, 2007). There has been no correlation found between reniform nematode presence and soil and water quality in the environment (Robinson *et al*., 1990). On the other hand, Robbins *et al*. (2003) found a correlation between soil nematode populations and plant height. They observed that there was no height difference among reniform tolerant cotton plants while there was an increase in the nematode population as the heights were decreasing among susceptible cotton plants. No disease symptoms have been determined with reniform-affected cotton plants (Weaver *et al*., 2007). Thus, the sedentary semi-endoparasitic parasite affects cotton through reductions in yield, boll size, and lint percentage (USDA, 2007; Cook *et al*., 1997; Jones *et al*., 1959).

Upland cotton has no resistance to reniform nematode. In 2005, Lawrence *et al*. tested 52 cotton cultivars and none of the cultivars were found to be either resistant or tolerant to reniform nematode. However, six accessions of upland cotton, TX-25, 748, 1586, 1828, 1860, and 2469 were classified as moderately resistant to reniform nematode in 2004 (Robinson *et al*., 2004). Moreover, only one plant, upland cotton accession TX-502, out of 46 that were tested for reniform nematode resistance was detected as resistant

but this plant had the typical characteristics of *G. barbadense* with flower, boll, and leaf morphology (Robinson, 2007).

Options for management of reniform nematode, such as crop rotation and nematicide application, have been suggested. For example [from Smith and Cothren, 1999: p.602]: "Agricultural consultants in Mississippi and Alabama recommend nematicide application if population densities of vermiform stages of *R. reniformis* exceed 2 nematodes cm^{-3} soil in the spring or 10 nematodes cm^{-3} soil in winter" (Blasingame and Patel, 1987). Unfortunately, these options may not be good from an economic standpoint and so are not preferred by producers. However, there are other less costly management options such as reducing water and nutrient stress for decreasing yield loss caused by reniform nematode. Crop rotation with resistant peanut *(Arachis hypogaea)* has been found effective against reniform nematode in the southern U. S. (Smith and Cothren, 1999). However, rotation with tobacco and vegetable crops is not suggested (Koenning *et al*., 2004). Consequently, genetic resistance would be a very attractive management option for both breeders and producers. Since the nematode causes a $400 million loss annually in cotton production (an estimate based on a price of $0.75 per pound of cotton, the price of which is now over $2.00 per pound), a reniform nematode resistant upland cotton germplasm would be wonderful for the cotton industry (USDA, 2007).

Adapted upland cotton cultivars lack genetic resistance and so only nematicides and crop rotation have been used as management options. One example of an adapted

cotton cultivar that may show tolerance to reniform nematode is Paymaster 1218 (PM 1218) (Sciumbato, 2005). The use of tolerance is not preferred by producers, due to no effect on reducing nematode numbers on cotton plants. A few studies have evaluated the USDA germplasm collection for resistance, finding accessions TX 110, TX 1347, and TX 1348 to be resistant. Further study was done within upland cotton, *Gossypium hirsutum*, and Weaver *et al*. (2007) evaluated 2066 accessions and identified seven of them (TX 245, TX 378, TX 500, TX 1419, TX 1472, TX 1565, and TX 1765) as behaving better than the control, PM 1218, which is one of the best adapted lines in terms of resistance. Robinson and Percival (1997) tested forty six accessions of upland cotton for reniform nematode resistance and TX-1347 and TX-1348 were the only resistant accessions among all. They also tested two *G. barbadense* accessions and observed one of them, TX-110, as resistant to reniform nematode. This accession was also tested as resistant to the same nematode earlier by Yik and Birchfield in 1984.

Recently, molecular approaches have been applied in the development of reniform nematode resistance in upland cotton, particularly in the area of searching for and incorporating resistance from resistant related species. *G. hirsutum,* being an allotetraploid makes introgression from related species difficult since other cotton species are mostly diploids (Koenning *et al*., 2004). Recent studies have mostly focused on the use of simple sequence repeats (SSR) that are between 1 and 6 base pairs as molecular markers for reniform nematode resistance. These are codominant PCR based markers, and highly polymorphic and abundant supporting their use for genetic linkage mapping. Their small locus size is also one of their advantages (Liu, 2007). Saha *et al*. (2004)

studied SSR markers and developed PCR based EST (Expressed Sequence Tag) markers of cotton plant. They detected 52% polymorphism between *G. hirsutum* and *G. barbadense*, and 49% polymorphism between these two cotton species were detected earlier by Reddy *et al*. in 2001. These two studies on polymorphism between *G. hirsutum* and *G. barbadense* show that the possibility of introgression between these two species is highly encouraging.

Weaver *et al*. (2007) also stated that there are probably different genes responsible for resistance due to the big diversity among geographic distributions of a few moderately reniform nematode resistant upland cotton accessions which they tested. In 2007, Robinson *et al.* created two 52-chromosome trispecies hybrids HLA [(*G. hirsutum* × *G. longicalyx*) chromosome - doubled × *G.armourianum*] and HHL [(*G. hirsutum* × *G. herbaceum*) chromosome - doubled × *G. longicalyx*] and these germplasms (LONREN-1 and LONREN-2) were later released from the USDA and the Texas Agricultural Experiment Station (Starr *et al*., 2007). To create marker-assisted genotyping of reniform resistant genes within cotton genotypes, Dighe *et al*. (2009) examined these trispecies hybrids and screened fifty two BNL-SSR primer pairs which may be linked to the reniform resistance gene on chromosomes 11 and 21 and indicated that marker BNL836 was found in ten of the twelve resistant cotton plants whereas the marker was not seen in twelve susceptible cotton plants. Also, they observed the primer BNL1066 was present in all resistant cotton plants while it was absent in all susceptible cotton plants. The results were the same for the primer BNL3279 and it was also seen only in resistant plants. Dighe *et al*. also reported that homozygous and heterozygous

reniform nematode resistant cotton plants have seeds with green fuzz whereas reniform nematode susceptible cotton plants produce seeds with white fuzz. This study might be a good guide for introgression of resistance to reniform nematode from resistant species into *G. hirsutum.*

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Figure 1.01 Compound microscope view of a juvenile female reniform nematode

(Source: http://www.mgel.msstate.edu/nematode.htm)

Figure 1.02. Stunted cotton plants suffering from reniform nematode

(Source:http://www.lsuagcenter.com/en/crops_livestock/crops/Cotton/Diseases/Cotton+Nematodes.htm)

CHAPTER 2

RESISTANCE TO RENIFORM NEMATODE FROM *G. HIRSUTUM* AND *G. LONGICALYX* SOURCES: A COMPARISON

Abstract

Reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira, is one of the most destructive nematode pests of cotton (*Gossypium* spp.), and upland cotton (*G. hirsutum* L.) the most common cultivated type is lacking in resistance. Differing levels of resistance to reniform nematode has been reported in several species of *Gossypium*. Our objectives were to compare levels of resistance in two *G. hirsutum* wild accessions (TX 245 and TX 1419) with those of *G. longicalyx*, a wild cotton relative that has been reported to be immune, and to determine if resistance in *G. hirsutum* is a heritable trait. We evaluated $F_{2:3}$ lines from crosses derived from TX 245 and TX 1419 and an adapted susceptible genotype for resistance to reniform nematode in the greenhouse using LONREN-1, a highly resistant *G. hirsutum* type with resistance derived from *G. longicalyx* for comparison. There were no differences among the $F_{2:3}$ lines for vermiform reproduction. Zero heritability of resistance was determined within these populations, and LONREN-1 resistance was far superior. In the second part of this study, $F_{2:3}$ lines derived from a cross between LONREN-1 and an adapted susceptible line were tested for reniform nematode resistance. Twenty-one of 100 $F_{2:3}$ lines were found to be highly resistant and not different from the LONREN-1 parent. We concluded that the previously

observed resistance in *G. hirsutum* was not repeatable, but resistance derived from *G. longicalyx* was highly heritable and repeatable.

Introduction

Cotton, *Gossypium* spp., is a significant fiber crop because of its importance as a natural textile fiber all over the world. According to the U.S. Department of Agriculture, total U.S. cotton production reached 102.5 million 218 kg bales in 2009 and the U.S. is the third largest cotton producer in the world (Cotton Inc., 2010). Upland cotton, *Gossypium hirsutum* L., is the primarily type of cotton grown in the U.S. and accounts for 97 percent of all U.S. cotton (USDA, 2009).

The reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira, is a plant parasitic microscopic roundworm. It has a wide host range mostly in the tropical and subtropical parts of the world. It has been reported as a pest from more than 38 countries and the U.S. is one of the most affected countries (Riggs *et al*., 1993). This sedentary semi-endoparasitic parasite, reniform nematode, affects cotton through reductions in yield, boll size, and lint percentage (Cook *et al*., 1997; Jones *et al*., 1959).

Reniform nematode was first reported in upland cotton in Georgia (Smith, 1940) and Louisiana (Smith and Taylor, 1941) and has been increasing as a problem of cotton in the U.S especially in Alabama, Louisiana, and Mississippi (Robinson, 2007). Yield losses due to reniform nematode in upland cotton are typically between 10% and 25% but

up to 50% loss may occur under water stress (Robinson, 1999). Unobvious symptoms of infected cotton plants make the evaluation for resistance difficult (Weaver *et al*., 2007).

Gossypium longicalyx, G. herbaceum, and *G. arboreum* are wild relatives of cotton that are highly resistant to reniform nematode (Yik and Birchfield, 1984). However, no resistance to reniform nematode has been found in upland cotton (Cook and Robinson, 2005; Starr *et al*., 2007; Lawrence *et al*., 2005). On the other hand, Robinson *et al*. (2004) reported six accessions of upland cotton, TX-25, 748, 1586, 1828, 1860, and 2469 as moderately resistant to reniform nematode. Only nematicide application and crop rotation with non-hosts of reniform nematode such as corn *(Zea mays)* and peanut *(Arachis hypogaea)* have been found to be useful in reducing yield losses (Lawrence *et al*., 2008; Davis and Webster, 2005).

In the previous study by our research group (Weaver *et al*., 2007), seven upland cotton accessions (TX245, TX378, TX500, TX1419, TX1472, TX1565, and TX1765) were found to be moderately resistant to reniform nematode after greenhouse evaluations of 1973 cotton accessions from the USDA primitive *G. hirsutum* collection . Two of these seven accessions, TX 245 and TX 1419, supported significantly lower nematode numbers in comparison to the control 'PM 1218', an adapted susceptible cultivar. In the present study, TX 245 and TX 1419 were crossed to PM 1218 in an attempt to introgress resistance into adapted germplasm. LONREN is a germplasm release from the USDA and the Texas Agricultural Experiment Station (Starr et al., 2007) that incorporates resistance to reniform nematode from *G. longicalyx* into *G. hirsutum* via a tri-species

hybrid (Robinson *et al*., 2007). LONREN was released by USDA as two reniform-resistant BC_7 lines, LONREN-1 and LONREN-2, in 2007. Other research has focused on the reniform resistance and agronomic traits of these two germplasm lines and found them acceptable under both greenhouse and field conditions (Starr *et al*., 2007).

This study focuses on comparison of resistance to reniform nematode from *G. hirsutum* accessions TX 245 and TX 1419 and *G. longicalyx* (LONREN-1) sources. Objectives of our study were to evaluate resistance to reniform nematode from *G. hirsutum* and *G. longicalyx* sources and to determine the inheritance of resistance to reniform nematode within $F_{2:3}$ progenies of resistant parent LONREN-1 (*G. longicalyx* source), moderately resistant TX 245 and TX 1419 (*G. hirsutum* source), adapted PM 1218, and susceptible FM 966.

Materials and Methods

Reniform nematode screening was conducted in two greenhouse experiments. The first experiment was conducted on 60 $F_{2:3}$ progenies of TX 245 × PM 1218 and 60 $F_{2:3}$ progenies of TX 1419 × PM 1218 crosses. LONREN-1 and parents TX 245, TX 1419, and PM 1218 were included as the control group. In the second experiment, 100 $F_{2:3}$ lines of the cross LONREN-1 × FM 966 were evaluated and Fibermax 966 (FM 966, susceptible), Paymaster 1218 (PM 1218, susceptible), LONREN-1, LONREN-1m, and LONREN-2 (highly resistant) were the control group. LONREN-1 and LONREN-1m are genetically identical; only the source of seed was different. LONREN-1 came directly from the developers in Texas; LONREN-1m came from a winter nursery in Mexico. In

the first experiment, seed came from F_2 plants which were grown at the Plant Breeding Unit, E.V. Smith Research Center, Shorter, Alabama in May 2008 and harvested in November 2008. In the second experiment, seed was produced at the same location and was both planted and harvested in 2007.

Entries in both of the experiments were evaluated in sets (10 reps/set). Progenies of TX lines were evaluated in 6 sets (23 entries, including controls, in each set) whereas LONREN progenies were evaluated in 5 sets (25 entries, including controls, in each set). In the first experiment, TX 245 × PM 1218 progenies were included in sets 1, 3, and 5 while TX 1419 × PM 1218 progenies were included in sets 2, 4, and 6. Seeds were planted in the greenhouse at the Alabama Agricultural Experiment Station Plant Science Research Center on the Auburn University campus, Auburn, AL. All plants were grown in 150- mL cone-tainers (Ray Leach Cone-tainers, Stuewe & Sons, Inc; 2.5-cm diameter by 20-cm depth) filled up with loamy sand soil (a mixture of 72.5% sand, 25% silt, 2.5% clay; 1% organic matter; pH 6.4). The soil was sterilized by autoclaving twice at 121˚ C and 103.4 kPa for two hours.

One week after planting, seedlings were inoculated with reniform nematodes which were grown on ST 5599 BG/RR cotton at the Plant Science Research Center. The inoculum was standardized as 1000 vermiform life stages in 1mL of water per each seedling. Sixty days after inoculation (approximately 2 life cycles), nematodes were extracted from the whole root system via the Baermann funnel technique. Each plant root was carefully removed from the cone-tainers and put on a 470 ml plastic funnel after

being enclosed in a Kimwipe and a pliable screen. Funnels were filled with tap water and left for 2 to 3 days before draining 100-mL of water from each funnel into individual beakers. After draining some water, roots were gently washed and fresh weight of roots was recorded. Nematodes in the drained water in beakers were poured through a 500-µm pore sieve and standardized and quantified under a Nikon TS100 inverted microscope at 40x magnification to determine nematode reproduction.

Reniform nematode number per gram of plant root was calculated and data were analyzed using a comparison of LSmeans (Fisher's protected Least Significant Differences; $P \leq 0.05$) in Proc Glimmix (SAS institute, Inc., Cary, NC).

Results and Discussion

Experiment 1:

Reniform nematode reproduction on $F_{2:3}$ progenies in sets 1, 3, and 5 (TX245 × PM1218) ranged from 1559 to 2657 vermiform counts per gram of plant root and in all cases was significantly higher than LONREN-1 (Figure 2.01). In the previous study (Weaver *et. al*., 2007), TX 245 had low nematode reproduction in comparison to PM 1218, the adapted susceptible genotype. However, across all three sets in this study, nematode counts of parental line TX 245 were not different from PM 1218 and significantly higher than the resistant control LONREN-1, which supported very low numbers of reniform nematode in all six sets of the first experiment. Vermiform populations in sets 2, 4 and 6 were similar to those of progenies in sets 1, 3, and 5 (Figure 2.02). For the parent line TX 1419, average vermiform numbers ranged from 263 to

4087. Reproduction of reniform nematode was also high on adapted control PM 1218 in sets 2, 4, and 6. Although the average vermiform counts per gram of plant root for both progenies and parent lines TX 1419 and PM 1218 decreased in set 6 as compared to other sets, it was still higher than the resistant control LONREN-1. The highly resistant control LONREN-1 had consistently the lowest nematode counts in the first experiment whereas PM1218, the adapted susceptible control, always had significantly more vermiform count per gram of root than LONREN-1. Neither the progenies nor the parents (PM 1218, TX 1419, and TX 245) showed resistance to reniform nematode in comparison to the highly resistant LONREN-1. Furthermore, there were no significant differences among progenies for either population, resulting in zero genetic variation for nematode resistance, and thus zero heritability. Results from the previous study by Weaver *et al*. (2007) regarding moderate resistance of two *G. hirsutum* accessions, TX 245 and TX 1419, could not be repeated in the current study. This has been observed in previous studies in evaluating for reniform resistance within *G. hirsutum*. Yik and Birchfield (1984) reported three wild *G. hirsutum* accessions as resistant, but later work by Robinson and Percival (1997) reported the same accessions as not different from the susceptible 'Deltapine 16'. Robinson *et al*. (2004) also identified six *G. hirsutum* accessions as having a moderate level of resistance to reniform nematode. However, five of these lines were later found to be not largely different from the adapted susceptible 'PM 1218' in their reaction to *R. reniformis* by Weaver *et al*. (2007). Thus heritability of resistance to reniform nematode in both TX245 and TX1419 was zero in these populations. Age of cotton seed, environmental conditions under which they were produced, vigor of cotton seedlings, and possible heterogeneity among plants within a

particular accession might be the factors that resulted in the lack of repeatability of the results obtained in the research by Weaver *et al*. (2007).

***Experiment 2*:**

In each of the 5 sets evaluated in experiment 2, the LONREN control group (LONREN-1, LONREN-1m, and LONREN-2) had the lowest reniform nematode reproduction whereas the susceptible parent FM 966 had the highest vermiform reproduction (Figure 2.03). Average vermiform counts per gram of plant root supported by the progenies was always between the two parents in all five sets. Distribution of reniform nematode count among the 100 LONREN-1 × FM 966 progenies is shown in Figure 2.04. Of these 100 $F_{2:3}$ progenies, 21 progenies had low nematode numbers similar to, and were not significantly different from resistant parent LONREN-1, and were considered to be descended from homozygous resistant F_2 plants. This is consistent with the reported inheritance of the resistance gene in LONREN (REN^{lon}) (Robinson *et al*., 2007) as a simple dominant gene ($\chi^2 = 0.85$, P = 0.25 – 0.50). Reniform nematodes were able to feed freely on the adapted control, PM 1218, in all sets. Overall, nematode numbers were consistent for all entries and sets.

It is worthwhile to conduct further evaluations, both field and greenhouse, of the 21 resistant lines among the 100 $F_{2:3}$ progenies of LONREN-1 and FM 966 cross in order to determine if any of those lines are good candidates for further breeding purposes. Also it should be possible to compare the agronomic and fiber traits of these 21 lines with sister susceptible lines to more precisely determine the effect of REN^{lon} on these traits.

In conclusion, resistance previously reported in TX 245 and TX 1419 was not repeatable; *G. hirsutum* is still lacking in resistance. However, *G. longicalyx* source LONREN-1 is much superior to *G. hirsutum* in resistance to reniform nematode. Genetic resistance to reniform nematode in *G. hirsutum* still remains to be discovered.

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Figure 2.01. Reniform nematode count per gram of root for TX245 x PM1218 Lines (sets1, 3 and 5). Vertical bars for TX 245 × PM 1218 cross represent the average of 20 entries with 10 replications in every set.

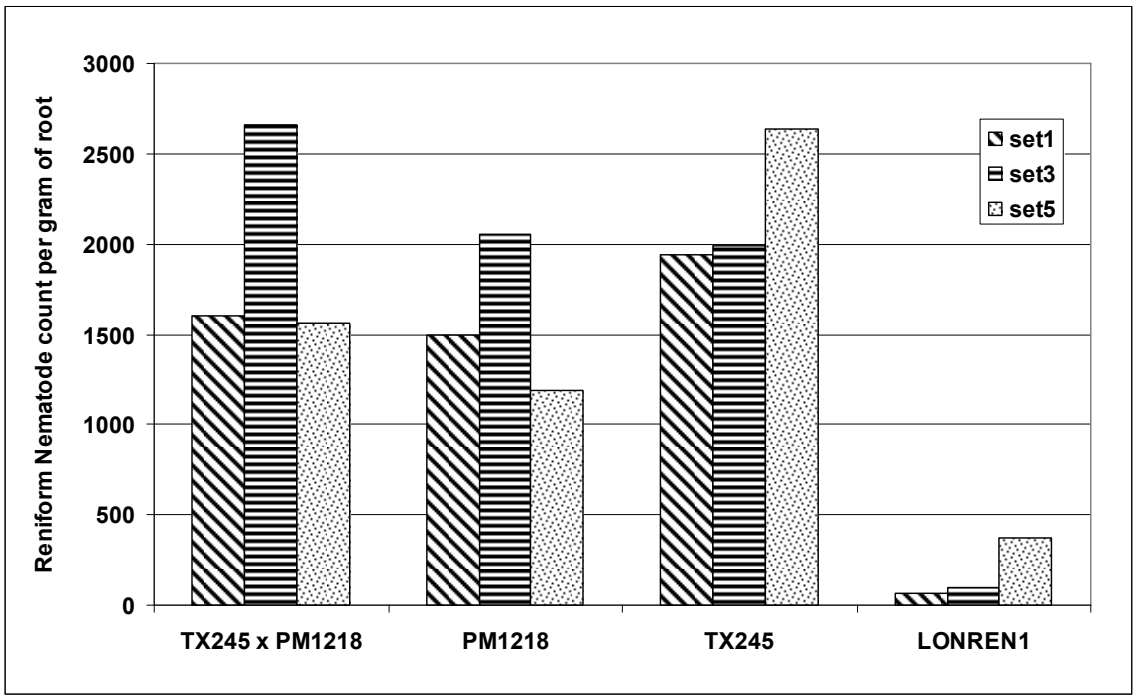


Figure 2.02. Reniform nematode count per gram of root for TX1419 x PM1218 Lines (sets 2, 4 and 6). Vertical bars for TX 1419 × PM 1218 cross represent the average of 20 entries with 10 replications in every set.

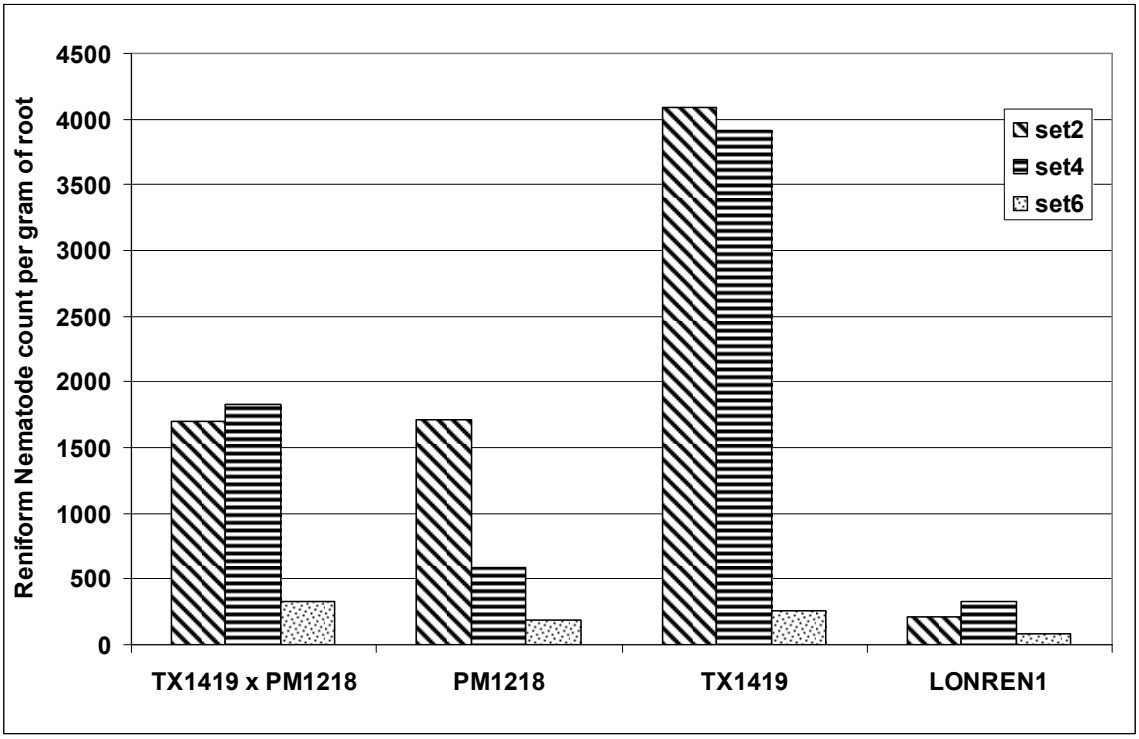


Figure 2.03. Reniform nematode count per gram of root for sets 1 to 5 of the second experiment. The dotted first bar in each set represents the average of 20 entries with 10 replications. Every set includes different 20 entries of F2:3 lines of LONREN-1 × FM 966 cross adding up to 100 different entries among all five sets.

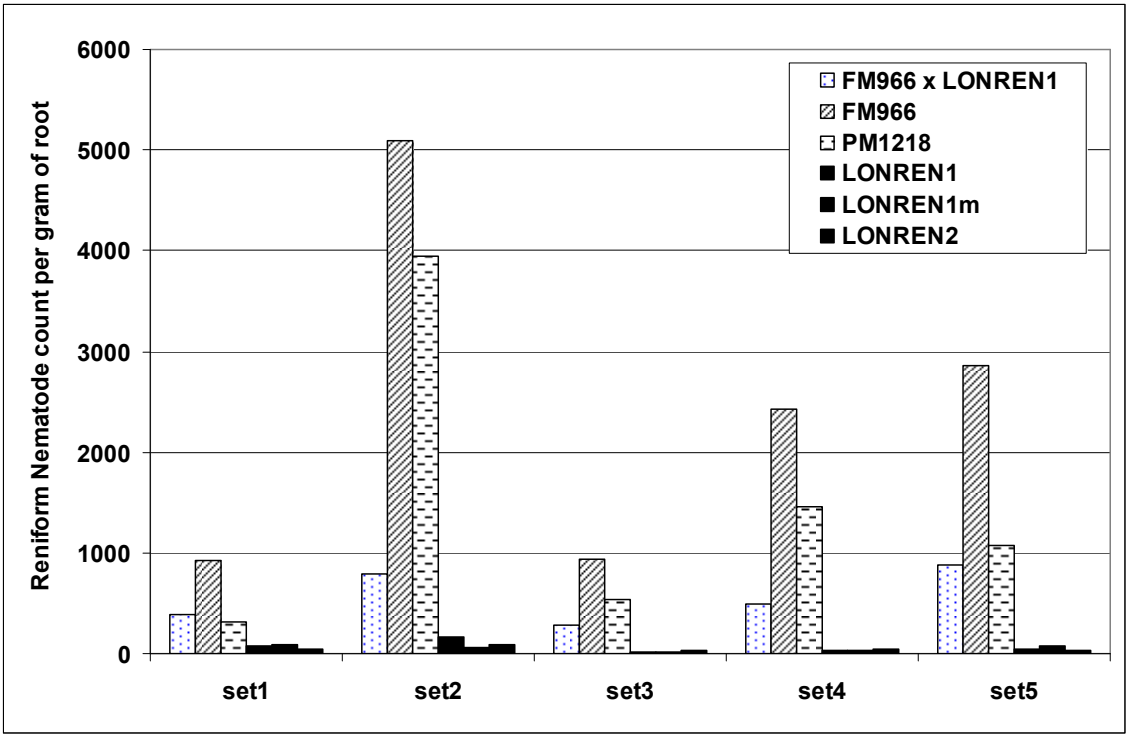
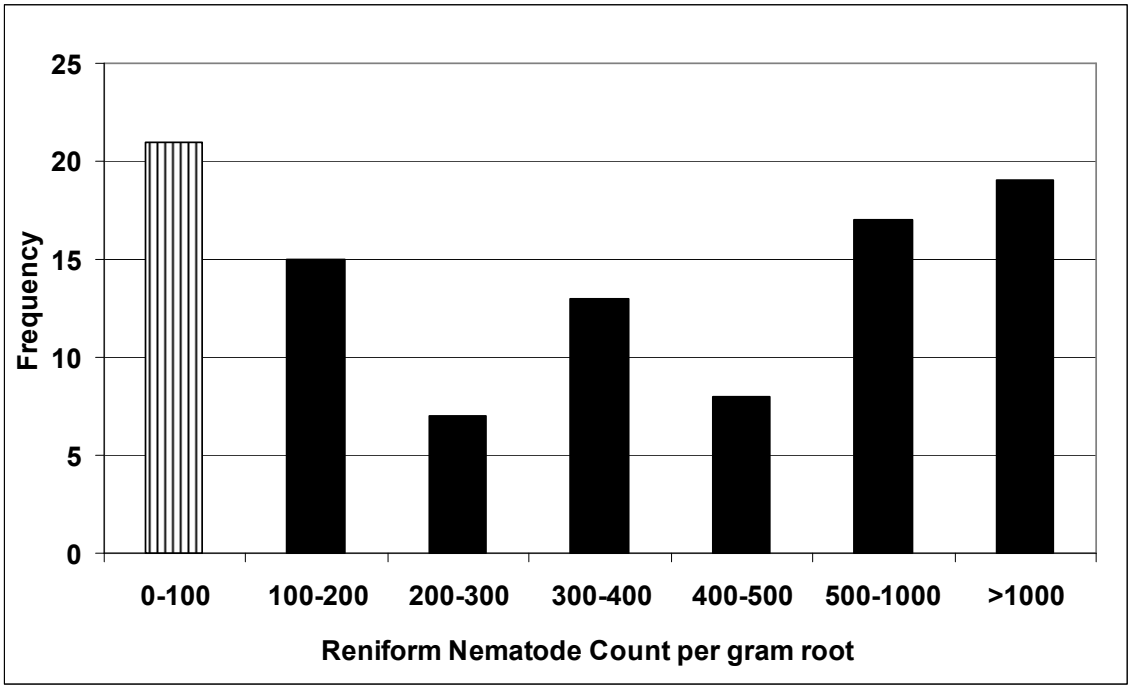


Figure 2.04. Distribution of reniform nematode count per gram of root among 100 LONREN-1 x FM966 progenies. Vertically lined bar indicates lines with the highest level of resistance.



CHAPTER 3

SEEDLING VIGOR EFFECTS ON EVALUATION OF GENETIC RESISTANCE TO RENIFORM NEMATODE IN *GOSSYPIUM HIRSUTUM*

Abstract

American upland cotton (*Gossypium hirsutum* L.) is the leading cotton species and dominates cotton production worldwide. Reniform nematode (*Rotylenchulus reniformis*) has been a serious problem of upland cotton causing approximately $100 million loss every year in the U.S. cotton belt. Currently no *G. hirsutum* genotypes have been identified that show a high level of resistance to reniform nematode. Zero heritability of resistance to *Rotylenchulus reniformis* in *Gossypium hirsutum* has been shown in our previous study. The aim of this study was to evaluate the impact of age and condition of seedlings on reniform nematode reproduction in upland cotton by investigating the relationship between the seedling vigor and genetic resistance to reniform nematode. Seeds of two cultivars (PM 1218 and FM 966, susceptible), two wild accessions (TX 245 and TX 1419, previously showing erratic results with regard to resistance), and LONREN-2 (resistant) were exposed to adverse stress conditions (accelerated aging) to simulate reduced seedling vigor caused by time and delayed harvest. Genotypes were then tested for reniform nematode resistance by evaluating nematode reproduction, to determine the possible effects on seedling vigor on nematode reaction. Plant height was recorded once a week during the evaluation period. Dry and

fresh weights of roots and shoot dry weights were measured at the end of the study. Results showed that germination rate and seedling vigor decreased with increasing length of exposure time to adverse conditions. Reniform nematode numbers for each genotype were not significantly different due to accelerated aging. Consequently, there was no direct relationship between the age and condition of the cotton seedlings and their response to reniform nematode.

Introduction

Cotton, *Gossypium* spp., is a significant fiber crop because of its importance as a natural textile fiber all over the world. According to the U.S. Department of Agriculture, total U.S. cotton production reached 102.5 million 218 kg bales in 2009 and the U.S. is the third largest cotton producer in the world (Cotton Inc., 2010). Upland cotton, *Gossypium hirsutum* L., is the primarily type of cotton grown in the U.S. and accounts for 97 percent of all U.S. cotton (USDA, 2009).

The reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira, is a plant parasitic microscopic roundworm. It has a wide host range mostly in the tropical and subtropical parts of the world. It has been reported as a pest from more than 38 countries and the U.S. is one of the most affected countries (Riggs *et al*., 1993). This sedentary semi-endoparasitic parasite, reniform nematode, affects cotton through reductions in yield, boll size, and lint percentage (Cook *et al*., 1997; Jones *et al*., 1959).

Reniform nematode was first reported in upland cotton in Georgia (Smith, 1940) and Louisiana (Smith and Taylor, 1941) and has been increasing as a problem of cotton in the U.S especially in Alabama, Louisiana, and Mississippi (Robinson, 2007). Yield losses due to reniform nematode in upland cotton are typically between 10% and 25% but up to 50% loss may occur under water stress (Robinson, 1999). Unobvious symptoms of infected cotton plants make the evaluation for resistance difficult (Weaver *et al*., 2007).

Gossypium longicalyx, G. herbaceum, and *G. arboreum* are wild relatives of cotton that are highly resistant to reniform nematode (Yik and Birchfield, 1984). However, no resistance to reniform nematode has been found in upland cotton (Cook and Robinson, 2005; Starr *et al*., 2007; Lawrence *et al*., 2005). On the other hand, Robinson *et al*. (2004) reported six accessions of upland cotton, TX25, 748, 1586, 1828, 1860, and 2469 as moderately resistant to reniform nematode. Only nematicide application and crop rotation with non-hosts of reniform nematode such as corn *(Zea mays)* and peanut *(Arachis hypogaea)* have been found to be useful in reducing yield losses (Lawrence *et al*., 2008; Davis and Webster, 2005).

In the previous study by our research group (Weaver *et al*., 2007), seven upland cotton accessions (TX 245, TX 378, TX 500, TX 1419, TX 1472, TX 1565, and TX 1765) were found to be moderately resistant to reniform nematode after repeated greenhouse evaluations of 1,973 cotton accessions from the USDA primitive *G. hirsutum* collection . Two of these seven accessions, TX 245 and TX 1419, supported significantly lower nematode numbers than the control ‘PM 1218’, an adapted susceptible cultivar.

However, resistance in these two accessions, TX 245 and TX 1419, was studied again and expression of resistance could not be repeated (Sürmelioğlu *et al*., 2010). There might be different reasons of lack of repeatability of resistance to reniform nematode in these genotypes. One possible explanation could be the effect of seedling vigor on nematode response. Cotton seeds lose their vigor over time in storage. Less vigorous seedlings may have lower resistance to pests such as reniform nematode or may support lower population levels due to lower seedling growth. Most methods of evaluating cotton for reniform nematode resistance are based on nematode reproduction (Robinson *et al*., 2006; Weaver *et al*., 2007).

One cause of seed deterioration in cotton is storage for long periods or under conditions of high temperature and humidity. (Helmer, 1965). Delouche and Baskin (1973) conducted research on accelerated aging techniques by exposing seed samples to high temperatures (40 to 45°C) and near 100% relative humidity for 2 to 3 or more days and then evaluating the exposed seeds in a standard germination test. They thus established a protocol for accelerated aging of cotton seed by exposure to high temperature and humidity for short periods.

In the current study, seed condition and seedling vigor as a possible factor in evaluation of cotton for genetic resistance to reniform nematode was investigated in 5 genotypes: two wild accessions, TX 245 and TX 1419 (previously showing erratic results with regard to resistance); two cultivars, Paymaster 1218 (PM 1218, susceptible) and Fibermax 966 (FM 966, susceptible); and one germplasm line, LONREN-2

(Robinson *et al.*, 2007), with resistance derived from the highly resistant species, *G. longicalyx*. The objective of this experiment was to understand the effect of seedling vigor on expression of resistance to reniform nematode resistance in *Gossypium hirsutum* as evaluated based on nematode reproduction.

Materials and Methods

Accelerated aging treatments

Seeds of all genotypes in the experiment were produced in a common field environment in 2008 at the Plant Breeding Unit in Tallassee, AL to eliminate any confounding due to seed source. Immediately after harvest and acid delinting, all seed were then exposed to accelerated aging using protocols similar to those described by Delouche and Baskin (1973). Three hundred fifty seeds from each genotype were put into boxes which were designed according to the description by Wilburn (1983) and were kept in an aging chamber at the Plant Science Research Center, Auburn, AL. Stress conditions were set up as described by Delouche and Baskin (1973) which consisted of a constant temperature of 40°C and 100% relative humidity for six different exposure times (0, 24, 48, 72, 96, 120 hours). Seed samples were air dried for about 24 hours following their removal from the aging chamber.

Standard germination test

Standard germination tests were conducted on 100 seed from each entry and for each age-accelerated exposure time. Seeds of each genotype were aligned on separate wetted germination papers and labeled with the entry name. Germination papers then

were rolled up and put into germinators for 7 days at a temperature of 30°C. Following the first set, a second standard germination test was conducted with an additional 48 seeds from each entry under the same conditions. To evaluate the % germination rate, germinated seeds were counted. Radicle lengths of all germinated seeds were measured. Seedling vigor was determined based on average radicle length and germination percentage at the end of 7 days.

Cool germination test

Two sets of seed samples of genotypes TX 245, TX 1419, FM 966, PM 1218, and LONREN-2 were tested for cool germination. The first set included 100 seeds of each age-acceleration treatment and 48 seeds were used in the second set. Seeds were aligned on wetted germination papers as described previously. Cool germinator conditions were the same for both of the tests where seed samples were kept at 18°C for 7 days. Data were collected by evaluating the % germination rates and radicle length of each genotype at the end of 7 days.

Nematode resistance evaluation

Genotypes were tested for reniform nematode resistance in the greenhouse after the standard germination tests were conducted. Four identical sets of all five genotypes were tested in the spring and fall of 2009. In each set eight seeds from each genotype and each accelerated aging treatment were planted in the greenhouse at the Plant Science Research Center in Auburn, AL. All plants were grown in 150-mL individual cone-tainers (Ray Leach Cone-tainers, Stuewe & Sons, Inc; 2.5-cm diameter by 20-cm depth)

filled with loamy sand soil (a mixture of 72.5% sand, 25% silt, 2.5% clay; pH 6.4). The soil was sterilized by autoclaving twice at 135 °C and 27 kPa for 2 h on 2 consecutive days. All cone-tainers were labeled with accession name and exposure time and were randomized. One week after planting, each seedling was inoculated with 1000 reniform nematodes (vermiform and eggs) in one mL of water. Inoculated plants were left in the greenhouse for 60 days (approximately 2 nematode life cycles). Meanwhile, growth observation was done by measuring the plant height every week. Sixty days after inoculation, final leaf and node counts were recorded before nematode extraction. Nematodes were extracted from the whole root system via the Baermann funnel technique. Each plant root was carefully removed from the cone-tainers and put on a 470 ml plastic funnel after being enclosed in a Kimwipe and a pliable screen. Funnels were filled with tap water and left for 2 to 3 days before draining 100-mL of water from each funnel into individual beakers. After draining the water, roots were gently washed and fresh and dry weights of roots were recorded. Aboveground parts were also dried and weighed. Nematodes in the drained water in beakers were poured through a 500-µm pore sieve and standardized and quantified under a Nikon TS100 inverted microscope at 40x magnification to determine nematode reproduction. Final leaf and node count, weekly plant height measurement, shoot and root dry weight measurements were only done for sets 3 and 4.

Data analysis

Generalized linear mixed models procedures as implemented in SAS® vs. 9.2 PROC GLIMMIX (SAS Institute Inc., Cary, NC) were used to analyze the data. A

lognormal distribution function was used for reniform response data, whereas the normal distribution function sufficed for plant responses. Experimental repeat and block within experimental repeat were random effects, whereas line, age treatment and their interaction were fixed effects. The Dunnett-Hsu test was used to compare differences between the reniform reproduction of LONREN-2 and other lines (TX 245, TX 1419, FM 966, and PM 1218).

Results and Discussion

Germination tests

Germination rates of cotton seed decreased significantly as the length of exposure time in the accelerated aging treatments increased from 0 to 120 hours (Table 3.01). Within each exposure duration, germination was better in seeds in the standard germination tests (30°C) than in cool germination tests (18°C). Germination was always highest in PM 1218 seeds and lowest in the exotic TX accessions at the 120-hour exposure period. PM 1218 had more than 20% reduction in germination over 120 hours during the standard germination test whereas TX 1419 showed a reduction as large as 42%. In the cool germination test (Table 3.02), germination rate of accelerated-aged PM 1218 seeds was highest and was reduced by 52% over the aging period. TX 245 showed the largest decrease in germination rate during the cool test with 59% decrease after 120 hours of exposure time to adverse conditions. Based on these data, we concluded that the accelerated aging test was effective in reducing seed germination as measured by standard and cool germination tests.

Seedling vigor observations

Genotypes did not differ for average radicle length in the standard germination test for the control (0 days of accelerated aging) (Table 3.03). Over the 120 hours of standard germination test, PM 1218 showed the least reduction (1.2%/ day) whereas FM 966 had the highest decrease in average radicle length (6.2 %/ day). In the cool germination test, average radicle lengths ranged between 20 and 23 mm for the control (0 days of exposure) (Table 3.04). Average radicle lengths decreased between 8.9 % (TX 245) and 13.9 % (FM 966) per each day over the 120-hour exposure period during the cool germination tests. As measured by radicle length in both the standard and cool germination tests, the accelerated aging treatments significantly decreased seedling vigor after 7 days.

Nematode resistance evaluation

Reniform nematode numbers per gram of fresh root weight were not significantly different among genotypes except for LONREN-2 which is highly resistant to reniform nematode (Figure 3.01). For the genotypes TX 245, TX 1419, PM 1218, and FM 966, average vermiform numbers ranged from 579 (TX 1419 at 48 hrs) to 1324 (FM 966 at 120 hrs and TX 245 at 96 hrs). However, the resistant control LONREN-2 supported very low numbers of reniform nematode in all four sets. Among all genotypes included in the study, there was no significant correlation between nematode reproduction on cotton seedlings and the reduction in seedling vigor caused by adverse aging conditions.

Growth observations (plant heights, final leaf and node counts), root fresh and dry weight and shoot dry weight are shown in Tables 3.05 and 3.06. There was no significant difference among the plant heights of all genotypes during the run of the greenhouse screening. Final node and leaf counts were also approximately similar among all five genotypes. No significant difference due to accelerated aging was found among weight measurements of dry root and shoots as well. There appears to be a relationship between the cotton seedlings exposed to adverse aging conditions and fresh root weights of the genotypes. Longer exposure to accelerated aging times resulted in lower fresh root weights, and may be related to reduced seedling vigor caused by the accelerated aging.

In conclusion, expression of genetic resistance to reniform nematode as measured by nematode reproduction is not significantly influenced by the age and condition of cotton seeds. However, the Accelerated Aging Techniques can be applied to simulate reduction in seedling vigor of upland cotton.

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Table 3.01. Standard germination rates (% weighted averages) after 7 days for accelerated aging times up to 120 hrs.

	Genotypes					
Exposure time		FM 966	PM 1218	TX 245	TX 1419	LONREN-2
	0 hr	91.1	91.1	86.9	79.5	93.3
	24 hrs	91.7	92.8	76.2	77.8	91.3
	48 hrs	83.4	93.3	84.0	73.2	90.9
	72 hrs	83.1	91.6	73.2	66.9	87.6
	96 hrs	81.0	88.1	75.3	56.7	78.1
	120 hrs	72.5	86.1	65.3	46.6	77.4

Table 3.02. Cool germination rates (% weighted averages) after 7 days for accelerated aging times up to 120 hrs.

	Genotypes					
Exposure time		FM 966	PM 1218	TX 245	TX 1419	LONREN-2
	0 hr	81.1	93.2	35.1	63.8	77.2
	24 hrs	87.9	91.2	51.7	58.0	78.3
	48 hrs	73.5	92.4	36.5	53.8	82.8
	72 hrs	70.5	83.1	24.0	59.1	39.7
	96 hrs	55.0	65.1	24.8	40.9	38.4
	120 hrs	38.9	72.6	14.4	28.0	39.7

Table 3.03. Radicle length in standard germination test following accelerated aging treatments of 0 and 120 hr.

Genotype	Control (0 hr aging) length (mm)	120 hr aging length (mm)	Reduction in length	
			(mm/day)	(% / day)
FM 966	62.8	40.2	4.5	6.2
PM 1218	55.0	48.4	1.3	1.2
TX 245	57.5	41.4	3.2	6.0
TX 1419	64.9	58.7	1.3	4.4
LONREN-2	51.0	41.2	1.9	5.9

Table 3.04. Radicle length in cool germination test following accelerated aging treatments of 0 and 120 hr.

Genotype	Control (0 hr aging) length (mm)	120 hour aging length (mm)	Reduction in length	
			(mm/day)	(% / day)
FM 966	19.8	5.9	2.8	13.9
PM 1218	20.3	8.5	2.4	12.1
TX 245	21.8	11.2	2.1	8.9
TX 1419	19.2	8.3	2.2	13.1
LONREN-2	19.7	9.8	2.0	11.3

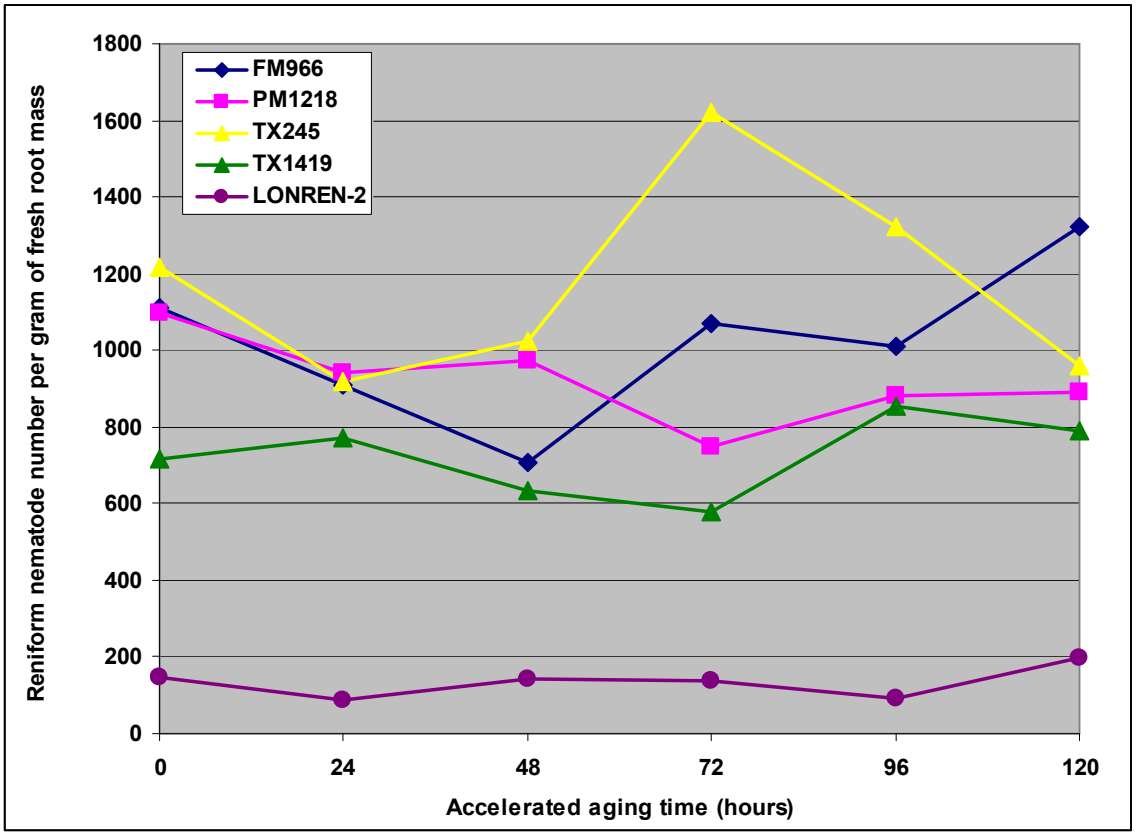
Table 3.05. Results of the third set of the nematode resistance evaluation test.

entry	AAT time	height (cm)	nodes	leaves	average shoot dry weight (g)	average root fresh weight (g)	average root dry weight (g)
FM966	0 hrs	16.7	4.0	4.0	0.59	3.60	0.57
	24 hrs	17.6	4.0	4.0	0.65	5.27	0.54
	48 hrs	19.2	4.3	3.4	0.82	5.42	0.52
	72 hrs	19.3	4.4	3.6	0.80	4.48	0.50
	96 hrs	19.0	4.0	4.0	0.85	4.82	0.52
	120 hrs	17.9	4.0	4.4	0.81	4.66	0.53
PM1218	0 hrs	19.2	4.8	3.1	0.67	5.56	0.60
	24 hrs	20.1	5.2	3.0	0.72	6.19	0.81
	48 hrs	20.5	4.6	3.3	0.93	5.65	0.72
	72 hrs	20.2	4.9	3.5	0.76	7.03	0.66
	96 hrs	20.5	4.8	3.1	0.82	6.73	0.76
	120 hrs	20.8	4.9	2.9	0.79	6.59	0.66
TX245	0 hrs	20.9	6.2	3.2	0.42	7.31	0.57
	24 hrs	20.7	6.4	3.2	0.41	7.41	0.71
	48 hrs	22.9	6.2	3.8	0.55	6.57	0.78
	72 hrs	18.8	7.1	2.6	0.33	6.38	0.66
	96 hrs	20.1	6.7	2.9	0.43	6.56	0.71
	120 hrs	21.4	7.4	2.9	0.43	7.06	0.74
TX1419	0 hrs	23.5	4.6	4.0	0.96	6.49	0.70
	24 hrs	22.0	4.3	3.9	0.91	6.00	0.81
	48 hrs	23.6	5.5	3.6	0.88	7.29	0.78
	72 hrs	22.5	5.1	3.3	0.90	7.21	0.79
	96 hrs	23.0	4.8	3.8	1.02	5.48	0.67
	120 hrs	19.7	4.7	4.0	0.81	6.02	0.56
LONREN-2	0 hrs	15.8	3.1	5.2	1.02	3.28	0.30
	24 hrs	18.4	3.1	4.9	1.13	4.17	0.43
	48 hrs	17.7	3.0	5.0	1.20	3.85	0.42
	72 hrs	18.8	3.6	4.8	1.16	3.75	0.36
	96 hrs	19.0	2.9	5.1	1.17	3.04	0.46
	120 hrs	19.3	2.7	6.0	1.14	2.59	0.43

Table 3.06. Results of the fourth set of the nematode resistance evaluation test.

entry	AAT time	height (cm)	nodes	leaves	average shoot dry weight (g)	average root fresh weight (g)	average root dry weight (g)
FM966	0 hrs	15.3	4.2	2.6	0.51	2.19	0.29
	24 hrs	14.5	4.3	2.9	0.49	2.01	0.28
	48 hrs	14.3	4.9	2.7	0.44	2.07	0.35
	72 hrs	15.1	4.5	3.1	0.46	2.00	0.27
	96 hrs	14.2	4.3	3.2	0.45	1.68	0.25
	120 hrs	15.0	3.5	3.5	0.45	1.74	0.23
PM1218	0 hrs	14.7	4.4	2.6	0.48	2.21	0.37
	24 hrs	15.7	5.2	2.2	0.50	2.19	0.40
	48 hrs	15.5	4.4	2.8	0.50	2.31	0.42
	72 hrs	15.9	4.3	2.7	0.60	2.38	0.41
	96 hrs	16.4	4.2	2.8	0.66	2.24	0.37
	120 hrs	16.2	4.6	2.8	0.60	2.40	0.39
TX245	0 hrs	14.9	6.0	2.1	0.29	2.61	0.53
	24 hrs	16.0	7.3	2.4	0.23	2.55	0.48
	48 hrs	15.9	7.2	1.6	0.21	2.52	0.48
	72 hrs	14.7	6.4	2.8	0.21	2.51	0.44
	96 hrs	16.2	7.2	2.3	0.31	2.84	0.52
	120 hrs	16.4	7.1	2.4	0.25	2.32	0.42
TX1419	0 hrs	19.3	5.0	3.0	0.63	2.57	0.49
	24 hrs	16.9	4.3	3.6	0.54	2.62	0.50
	48 hrs	16.7	4.3	3.4	0.58	2.71	0.48
	72 hrs	18.7	5.1	2.8	0.58	2.71	0.58
	96 hrs	18.2	4.5	3.5	0.53	2.41	0.45
	120 hrs	18.8	5.1	3.4	0.57	2.89	0.53
LONREN-2	0 hrs	14.9	2.7	4.9	1.98	2.18	0.37
	24 hrs	14.9	3.6	4.0	0.79	2.11	0.37
	48 hrs	14.7	2.8	4.5	0.76	2.25	0.41
	72 hrs	13.8	3.2	4.0	0.78	1.96	0.37
	96 hrs	13.3	2.7	4.3	0.65	2.19	0.36
	120 hrs	12.4	3.1	4.1	0.71	2.22	0.43

Figure 3.01. The effect of accelerated aging time on reniform nematode numbers per gram of fresh root mass of five different genotypes. Each point represents the average of four identical sets.



CHAPTER 4

MARKER ASSISTED SCREENING OF UPLAND COTTON FOR RENIFORM NEMATODE RESISTANCE

Abstract

Cotton, *Gossypium* spp., is the fourth most important row crop in the U.S. and the most important industrial crop, with a farm-value of more than $5.6 billion in 2007. Reniform nematode, *Rotylenchulus reniformis*, is the number one nematode pest of cotton in the U.S. Adapted upland cotton cultivars lack genetic resistance. The objective of this study was to evaluate the potential use of Simple Sequence Repeats (SSR) to monitor upland cotton populations for resistance to reniform nematode. This was performed by molecular screening of 38 out of 100 $F_{2:3}$ lines of the cross LONREN-1 × FM 966 from our previous study in addition to a control group that included both of the parents, LONREN-1 and FM 966 as resistant and susceptible controls, respectively. The SSR markers amplified most of the 38 DNA samples showing that phenotype-based resistance determined in our previous study was reliable except some unexpected results which will require further evaluation. Results also show that marker-assisted screening can be easily applied to analyze upland cotton populations for reniform nematode resistance and to select good candidates for further breeding studies.

Introduction

Cotton, *Gossypium* spp., is a significant fiber crop because of its importance as a natural textile fiber all over the world. According to the U.S. Department of Agriculture, total U.S. cotton production reached 102.5 million 218 kg bales in 2009 and the U.S. is the third largest cotton producer in the world (Cotton Inc., 2010). Upland cotton, *Gossypium hirsutum* L., is the primary type of cotton grown in the U.S. and accounts for 97 percent of all U.S. cotton (USDA, 2009).

The reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira, is a plant parasitic microscopic roundworm. It has a wide host range mostly in the tropical and subtropical parts of the world. It has been reported as a pest from more than 38 countries and the U.S. is one of the most affected countries (Riggs *et al*., 1993). This sedentary semi-endoparasitic parasite, reniform nematode, affects cotton through reductions in yield, boll size, and lint percentage (Cook *et al*., 1997; Jones *et al*., 1959).

Reniform nematode was first reported in upland cotton in Georgia (Smith, 1940) and Louisiana (Smith and Taylor, 1941) and has been increasing as a problem of cotton in the U.S especially in Alabama, Louisiana, and Mississippi (Robinson, 2007). Yield losses due to reniform nematode in upland cotton are typically between 10% and 25% but up to 50% loss may occur under water stress (Robinson, 1999). Unobvious symptoms of infected cotton plants make the evaluation for resistance difficult (Weaver *et al*., 2007).

Gossypium longicalyx, G. herbaceum, and *G. arboreum* are wild diploid relatives of cotton that are highly resistant to reniform nematode (Yik and Birchfield, 1984). However, no resistance to reniform nematode has been found in upland cotton (Cook and Robinson, 2005; Starr *et al*., 2007; Lawrence *et al*., 2005). On the other hand, Robinson *et al*. (2004) reported six accessions of upland cotton, TX-25, 748, 1586, 1828, 1860, and 2469 as moderately resistant to reniform nematode. Only nematicide application and crop rotation with non-hosts of reniform nematode such as corn *(Zea mays)* and peanut *(Arachis hypogaea)* have been found to be useful in reducing yield losses (Lawrence *et al*., 2008; Davis and Webster, 2005). Unfortunately, these options may not be good from an economic standpoint and so are not preferred by producers. Thus, genetic resistance would be a very attractive management option for both breeders and producers. Recently, molecular approaches have been applied in the development of reniform nematode resistance in upland cotton, particularly in the area of searching for and incorporating resistance from resistant related species. *G. hirsutum,* being an allotetraploid makes introgression from related species difficult since other cotton species are mostly diploids (Koenning *et al*., 2004). Recent studies have mostly focused on the use of simple sequence repeats (SSR) that are between 1 and 6 base pairs in length as molecular markers for reniform nematode resistance. These are codominant PCR based markers, and highly polymorphic and abundant supporting their use for genetic linkage mapping. Their small locus size is also one of their advantages (Liu, 2007).

In the previous study by our research group (Sürmelioğlu *et al*., 2010), 100 $F_{2:3}$ lines of the cross LONREN-1 × FM 966 were evaluated in terms of reniform nematode

resistance and Fibermax 966 (FM 966, susceptible), Paymaster 1218 (PM 1218, susceptible), LONREN-1, LONREN-1m, and LONREN-2 (highly resistant) were the control group. LONREN is a germplasm release from the USDA and the Texas Agricultural Experiment Station (Starr et al., 2007) that incorporates resistance to reniform nematode from *G. longicalyx* into *G. hirsutum* via a tri-species hybrid (Robinson *et al*., 2007). LONREN was released by USDA as two reniform-resistant BC_7 lines, LONREN-1 and LONREN-2, in 2007. Other research has focused on the reniform resistance and agronomic traits of these two germplasm lines and found them acceptable under both greenhouse and field conditions (Starr *et al*., 2007).

Most methods of evaluating cotton for reniform nematode resistance are based on nematode reproduction (Robinson *et al*., 2006; Weaver *et al*., 2007). Unfortunately, this method of repeated sampling in either greenhouses or fields is not good from an economic standpoint and also is a time-consuming, labor-intensive process. However, marker-assisted DNA analysis is less time-consuming and allows the researcher to see the results sooner.

The study described herein focuses on evaluation of 20 promising lines from our previous work (Sürmelioğlu *et al*., 2010) which were phenotype-based observed as highly resistant to reniform nematode similar to the highly resistant parent LONREN-1, and were considered to be descended from homozygous resistant F_2 plants. This is consistent with the reported inheritance of the resistance gene in LONREN (REN^{lon}) (Robinson *et al*., 2007) as a simple dominant gene ($\chi^2 = 0.85$, P = 0.25 – 0.50, for 20 lines

out of a population size of 100). Objectives of our study were to differentiate resistant from susceptible $F_{2:3}$ progenies of LONREN-1 × FM 966 cross and to compare the molecular genetic analysis of susceptible and resistant progenies which were derived from one resistant (LONREN-1) and a susceptible (FM 966) parent.

Materials and Methods

Plant materials

Evaluation of 20 promising lines from our previous work (Sürmelioğlu *et al*., 2010) was done with the $F_{2:3}$ progenies of the cross LONREN-1 × FM 966. Seeds came from 100 F_2 plants which were grown at the Plant Breeding Unit, E.V. Smith Research Center, Shorter, Alabama in May 2008 and harvested in November 2008. In the greenhouse at the Auburn University Plant Science Research Center (PSRC), all entries which were previously reported as either homogeneous resistant (20 entries) or homogeneous susceptible (18 entries) were planted individually in plastic pots in fall of 2009 (Table 4.01). The parental genotypes, LONREN-1 and FM 966, and two other genotypes, PM 1218 and LONREN-2 were also planted in the same manner. Two cotyledons from each healthy plant were removed and placed in a freezer (-20°C) until required for DNA extraction.

Extraction buffer and cleaning solution preparation

Plant DNA extraction buffer was hand-prepared according to the recipe described by Zhang and Stewart (2000). The buffer consisted of 0.1 M Tris-HCl, 1.0 M NaCl, 0.02 M EDTA (Ethylene Diamine Tetraacetic Acid), 2% (w/v) cTAB (cetyl Trimethyl

Ammonium Bromide), 2% (w/v) polyvinlypyrrolidone-40, 1 mM 1,10-phenanthroline, and 0.2% (v/v) β-mercaptoethanol. Another solution which was referred to as cleaning solution by Zhang and Stewart (2000) was also hand-prepared in advance with the following ingredients: 0.05 M Tris-HCl, 0.05 M EDTA, 2% (w/v) cTAB, 2.05% (w/v) NaCl, and 0.02% (w/v) 1, 10-phenanthroline. The pH of both of the solutions was adjusted to 8.0 to prevent crystallization of the contents.

DNA extraction

Plant DNA was extracted according to the Mini-prep method described by Zhang and Stewart (2000) with slight modifications. Briefly, one handpicked cotyledon of each plant was chopped with a razor blade and placed in sterile 1.5 mL microcentrifuge tubes. Meanwhile, a hot water bath was preheated and the samples were kept on ice during the process. Each tube was filled with 0.5 mL of extraction buffer and the plant tissues were macerated using an electric drill and a stainless-steel micropestle (Scienceware® Microcentrifuge Tube Sample Pestle) vigorously for about 5 minutes. The razor blade and the micropestle were rinsed with 100% ethanol between each sample. After tissue maceration, the tubes were incubated in the hot water bath at 65°C for about 1 hour. Following the hot water bath, 0.5 mL of chloroform:isoamyl alcohol (24:1, v/v) was added to the samples and the tubes were centrifuged at the highest speed for 10 minutes. The yellowish supernatant was transferred carefully into a new sterile 1.5 mL microcentrifuge tube and the rest was discarded. One half mL of isopropanol was added and the tubes were vortexed briefly and kept in freezer (-20°C) for about 1 hour for DNA precipitation. Then, the centrifugation was repeated and the supernatant was discarded

carefully. The pelletized samples in tubes were washed with 0.5 mL of ethanol (70% and 100%, respectively) by spinning at full speed. After washing, ethanol was discarded and the pellets (extracted DNA) in the tubes were air dried with the help of a blower fan for about 20 minutes. The tubes were filled with 0.3 mL of TE buffer (Tris-EDTA, pH 8.0) for DNA suspension. Extra cleaning of DNA samples was done as follows: 0.5 mL of cleaning solution was added to DNA samples in TE buffer and the tubes were shaken for 2 hours on a Vortex-Genie® 2 Vortex (MO BIO Laboratories, Inc. Carlsbad, CA). Then, the mixture in tubes was centrifuged for 5 minutes and the supernatant was immediately discarded. Washing twice with 0.5 mL of 80% ethanol and a solution of 100% ethanol (0.5 mL) and 15 mM ammonium acetate was performed respectively. Following the discard of the liquids, the pure DNA pellet was air dried as earlier described. After ethanol completely evaporated, 0.3 mL of TE buffer was added into each tube for DNA resuspension. The DNA samples were kept in a freezer (-20°C) overnight before the estimation of DNA yield.

DNA yield

The DNA concentration and quality were estimated by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). For pure nucleic acid, the 260/280 ratio was 1.8 whereas the 260/230 ratio was between 1.8 and 2.2. DNA from each sample was diluted to 10 ng/µL with TE buffer (pH 8.0) and stored at -20°C until needed for amplification.

SSR markers

3 SSRs (BNL836, BNL1066, and BNL3279; mapped to the A-genome of *G. hirsutum* (Guo *et al*., 2006)) which were developed by Brookhaven National Laboratory (BNL, Upton, New York, USA) were used to detect polymorphisms among the $F_{2:3}$ progenies of the cross LONREN-1 × FM 966. The SSR primer sequences are described in Cotton Marker Database (http://www.cottonmarker.org/). Forward and reverse primer sequences of the 3 SSR markers used in this study are shown in Table 4.02. Each forward primer was labeled with infrared fluorescent dye (IRD 700 or 800 with the sequence: 5'-GAGTTTTCCCAGTCACGAC-3') at the 5'-end. Fifty nanomole scale SSR markers were ordered from Eurofins MWG operon.

Polymerase chain reaction (PCR) analysis

DNA from the phenotype-based 18 susceptible and 20 resistant progenies (Sürmelioğlu *et al*., 2010) and four other genotypes (PM 1218, FM 966, LONREN-1, and LONREN-2) were PCR amplified in a total of 10 µL volume consisting of 2 µL of template DNA (20 ng), 4.5 µL of nanopure water, 1 µL of 10X PCR buffer, 0.4 µL of MgCl (50 mM), 0.8 µL of dNTPs (2.5 mM each), 0.4 µL of IRD-labeled forward primer (10 µM=10 pmol/ µL), 0.6 µL of reverse primer, 0.2 µL of labeled tail primer (700 or 800), and 0.1 µL of Platinum® Taq DNA Polymerase (5 U/µl, Invitrogen, Carlsbad, CA). A touchdown PCR program was used for PCR amplification (PTC-200, Bio- Rad Laboratories, Inc., Italy) under the following conditions: 95ºC for 4 minutes (denaturation), 57ºC for 30 seconds (annealing), 72ºC for 30 seconds (extension), 20 cycles of 95ºC for 30 seconds, 94ºC for 30 seconds, 53ºC for 30 seconds, 72ºC for 15 minutes (final extension), and 15 cycles of 94ºC for 30 seconds. After PCR, PCR

products were then centrifuged at 2000 × g / 1 min. and 5 µL of loading dye (Formamide Loading Dye 95%) was added to each 10 µL reaction. After that, the products were denatured at 95ºC for 5 minutes and centrifuged again. The final PCR products were kept in a walk-in freezer for no more than 3 days until required for electrophoresis.

Electrophoresis

PCR products were run on 10% (v/v) acrylamide gels on LI-COR 4300 DNA Analyzer (LI-COR Inc.). Although it is more expensive and more time consuming, electrophoresis was done with acrylamide gel instead of agarose gel due to small fragment sizes (25bp to 700bp) of SSR markers. For preparation of microsatellite gels, 10% APS (Ammonium persulfate), 10% Acetic acid, Bind silane solution (3-(trimethoxysilyl) propyl methacrylate), TEMED (Tetramethylethylenediamine), 10X TBE buffer (pH 8.3), and 10% acrylamide were needed and prepared in advance, and stored in a walk-in refrigerator. Before gel assembly, glass plates were washed with distilled water and dried with paper towel and Kimwipe making sure they are really clean. For gel assembly, 40 µL of acetic acid and equal volume of Bind silane solution were mixed in a microcentrifuge tube by vortexing well. This mixture was applied with a cotton-tipped swap to the area of the cleaned glass plate where the comb will be inserted. Twenty-five mL of 10% acrylamide were gently mixed with 250 µL of APS and 25 µL of TEMED in a 250 mL beaker without creating bubbles. A rectangular well comb was gently inserted in the notch and the gel mixture was poured evenly between the plates which were hooked to each other with two rails. Tapping periodically onto the front plate while pouring the gel is required to prevent creating bubbles. A casting plate was inserted

between the rails and knobs of the rails were tightened. The gel was left overnight for air dry before installing on the LI-COR machine. The outside of the glass plates which were holding the microsatellite gel was washed with distilled water and dried. Then, the gel was hanged into the machine and upper and lower buffer tanks were also installed. The tanks were filled up evenly with 1 liter of TBE buffer (1X, diluted with nanopure water) and the comb was removed. Gel was pre-run for approximately 30 minutes before loading the PCR products into the wells. 0.8 µL of each denatured PCR product was loaded into the gel by injecting samples into the wells with an 8-channel Hamilton syringe. In addition to the samples, 0.2 µL of 50-350 bp concentrated sizing standard (IRDye® 700 or IRDye® 800, LI-COR Inc.) was also loaded into the wells for fragment size estimation. Following the loading, the electrophoresis was run for 2.30 hours and sequence products were analyzed according to the segregation pattern at the end of the run.

Results and Discussions

DNA yield

The DNA with the highest quantity belonged to the resistant parent LONREN-1 and yielded 286.2 ng/µL (Figure 4.01) after extraction with the Mini-prep method. Some samples yielded very low due to the difficulty of DNA re-suspension in TE buffer. Samples that yielded less than 10 ng/µL were discarded. Minimization of this problem may be possible through careful application of the extraction step where the samples were left for ethanol evaporation before re-suspension. Although PVP (polyvinlypyrrolidone-40) was included in the extraction solution (Horne *et al*., 2004),

there was difficulty with getting DNA samples with applicable quality since cotton DNA is rich with polyphenolics.

Electrophoresis

The PCR products which were amplified by the three SSR markers, BNL836, BNL1066, and BNL3279, included 20 resistant and 18 susceptible $F_{2:3}$ plants of LONREN-1 × FM 966 cross with assorted control groups (LONREN-1, LONREN-2, FM 966, and PM 1218). Screening of BNL1066 showed that a 156-bp band was present only in the resistant plants including the resistant control LONREN-1 (Figure 4.02). This has been observed in previous studies in evaluating for reniform resistance within *G. longicalyx* (Dighe *et al*., 2009). Regarding LONREN-2, there was no 156-bp band, which was expected due to the release report of these two germplasm lines by USDA (2007). According to the release report, resistance of LONREN-2 can only be detected with the SSR primer BNL3279. Among the 20 phenotype-based most resistant plants, two plants (A122 and A202) were missing and did not show up with a banding. This may be due to a pipette error or there was no amplification with these plants based on a PCR error. Seventeen of the 20 resistant plants showed the 156-bp banding including 5 of them (B133, A110, B216, B204, and B215) with another 131-bp band. These five lines were considered as heterogeneous reniform nematode resistant whereas the rest were considered to be homozygous resistant. The 156-bp banding obviously differentiated resistant from susceptible plants. However, one of the phenotype-based resistant plants (B106) showed up with only a 131-bp band which is also seen with the susceptible plants. This 131-bp band was also present in the susceptible control parent FM 966, revealing

that this allelic size was special to the reniform nematode susceptible group. Further molecular and phenotypic screenings of this line, B106, may result in better understanding of the extraordinary banding or this plant may be evaluated in the field or greenhouse to compare the agronomic and fiber traits of B106 with other molecular-based resistant plants. It is possible that there could have been a recombination event between the locus for reniform nematode resistance (*Ren*) and the SSR marker locus in a previous generation resulting the lack of the marker BNL1066 in this line. This unexpected banding was also present among the 18 susceptible plants where 4 of them (A213, B105, B132, and B213) showed similar banding as the resistant group. Assumption of the yield of BNL1066 banding sizes was made as 156-bp and 131-bp according to a recent study (Dighe *et al*., 2009).

Upon screening of the SSR marker BNL3279, similar banding (Figure 4.03) resulted among the 19 phenotype-based reniform nematode resistant $F_{2:3}$ plants of LONREN-1 × FM 966 cross as previously shown by Dighe *et al*. (2009). Based on the molecular sizing standard, the banding of PCR products were estimated around 145-bp. No banding was observed in one of the plants (B122) due to same type of error as previously described. On the other hand, the banding throughout the susceptible plants was not clearly identified due to too dark bands of PCR products.

The electrophoresis of the DNA samples amplified by the SSR marker BNL836 yielded 3 bands that were estimated as 242, 252, and 261 base pairs, respectively, in 19 out of 20 phenotype-based resistant plants (Figure 4.04). The 252-bp band apparently

differentiated resistant from susceptible plants. Two LONREN-1 plants (diluted and undiluted) also had the 252-bp band whereas this band was not present in the susceptible control FM 966. However, the second resistant control, LONREN-2, did not show the same banding. This was expected due to the release report of this germplasm by USDA (2007). Unpredictably, the 252-bp band was seen in 4 out of 18 susceptible plants (A213, B134, B207, and B227). It is worthwhile to evaluate these four plants with further greenhouse or field screenings.

In conclusion, using molecular screening methods such as marker-assisted DNA analysis has several advantages over repeated sampling in either greenhouses or fields including the time-friendly process and skill level requisite. The results of this current study show that SSR markers BNL836, BNL1066, and BNL3279 can be successfully used to analyze upland cotton populations for reniform nematode resistance and to select good candidates for further breeding studies.

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Table 4.01. Entry names with reniform counts of 20 phenotype-based resistant and 18 phenotype-based susceptible $F_{2:3}$ lines of LONREN-1 × FM 966 cross from greenhouse screening.

Resistant		Susceptible	
Entry name	Rr/g*	Entry name	Rr/g*
A122	26	A123	1125
A110	53	A101	2330
B101	47	A109	1084
B133	90	A209	1394
A202	43	A213	1375
B106	80	B128	1791
B219	75	B211	1329
A107	49	B227	2636
B103	34	B108	1797
B129	21	B212	1146
B216	76	B105	1909
B226	23	B114	1392
B231	52	B132	1000
B104	39	B134	2886
B136	41	B135	1021
B202	30	B207	1076
B204	39	B213	1382
B122	71	B230	2245
B215	56		
B217	53		

* Reniform nematode numbers / plant root gram. Average of 5 identical sets.

Table 4.02. SSR marker names with the forward and reverse primer sequences.

Marker Name	Forward Primer	Reverse Primer
BNL836	ATCTTGTTGATTTTCTGACTACAGG	CAGACATTCCCCTTCCTTGA
BNL1066	ACATTTCCACCCAAGTCCAA	ACTCTATGCCGCCTCTCGTA
BNL3279	CATGTCCAATGGATGTGTCA	GGGCCACTTAAAGGCATTCT

Figure 4.01. DNA high yield obtained after extraction with Mini-prep method.

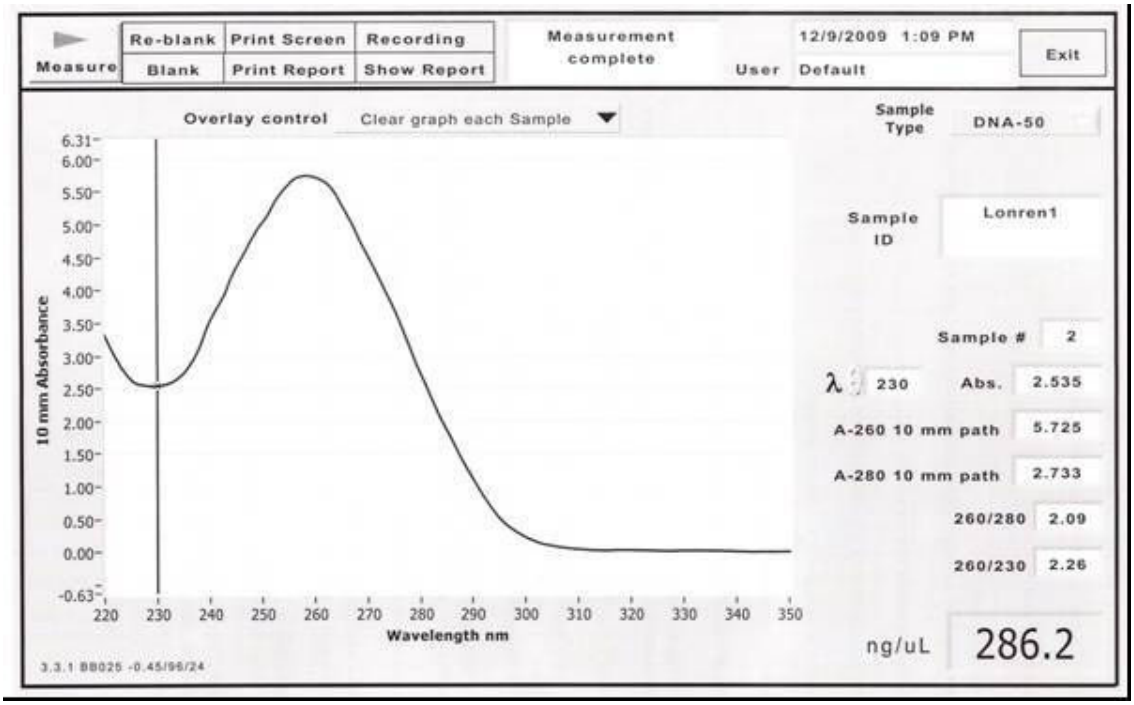

Figure 4.02. BNL1066 primer pair differentiated resistant from susceptible $F_{2:3}$ plants with a 156-bp band being present only in the resistant plants. R, resistant; S, susceptible; M, marker size standard. “R” or “S” abbreviations for the progenies were labeled according to the banding.

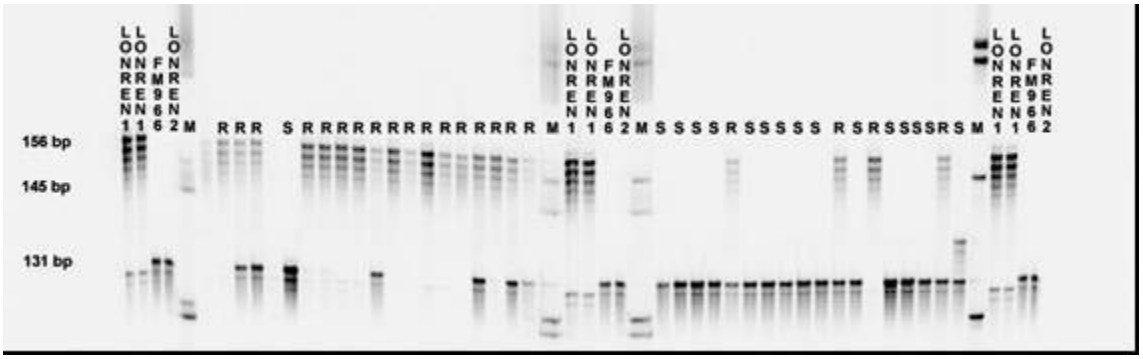

Figure 4.03. Electrophoresis image of DNA samples amplified with BNL3279 primer pair. R, resistant; mR, missing resistant; S, susceptible; M, marker size standard.

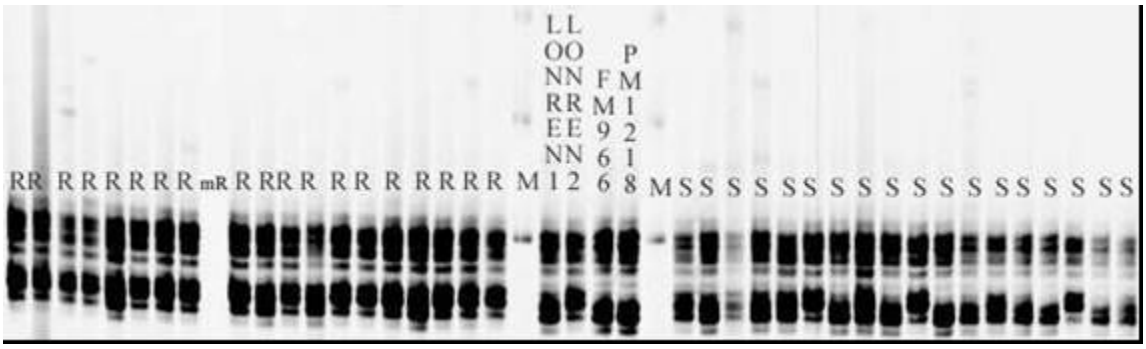

Figure 4.04. BNL836 primer pair differentiated resistant from susceptible $F_{2:3}$ plants with a 252-bp band being present only in the resistant plants. R, resistant; mR, missing resistant; S, susceptible; M, marker size standard.