SINGLE PLANT SELECTION AS A SCREENING METHOD FOR RESISTANCE TO *RHIZOCTONIA SOLANI* AND *PYTHIUM ULTIMUM* IN COTTON

A Thesis

by

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ABSTRACT

Upland cotton, *Gossypium hirsutum* L., is grown extensively in the southern United States with an annual farmgate value of $6 billion and an annual national economic impact of over $120 billion. Damage due to biotic pests, including what is known as the cotton seedling disease complex (CSDC), contribute to these losses. Two particular CSDC pathogens, *Rhizoctonia solani* and *Pythium ultimum,* are the most significant soilborne pathogens of cotton in the United States. A program for *R. solani* and *P. ultimum* resistant cotton germplasm was established at Texas A&M University AgriLife Research. Five germplasm families selected for elevated levels of condensed tannins were evaluated for resistance to *R. solani* and *P. ultimum*. Two generations of single plant selections resulted in three generations, C_0 (original families or Cycle 0) C_1, selected from the C_0 family, and C_2, selected from the C_1 generation. C_1 and C_2 were putative resistant families after one or two generation(s) of selection, respectively. Individual plants from the three generations within five families were challenged with either or both *R. solani* or *P. ultimum* to evaluate the progress of single plant selection for resistance. A susceptible cultivar for *R. solani*- and *P. ultimum*-resistance respectively, were included. Different *R. solani* and *P. ultimum* families from each generation of selection were evaluated at three inoculation levels with four replications per family. Differences in level of resistance between each generation were evaluated by comparing disease level in a randomized complete block. Cross-resistance was evaluated, i.e., C_2 families originally screened under *R. solani* pressure were inoculated

and screened for P. *ultimum resistance* and vice versa. Individual plant selection (IPS) in an artificial environment may be a useful and important tool in developing seedling disease-resistant cotton germplasm. Furthermore, it can be concluded that the family evaluated is of importance to determine the amount of progress made in terms of disease resistance with IPS. Individual plant selection when challenged with appropriate levels *R. solani* and *P. ultimum* appears to be an effective tool for selection of germplasm resistant to these seedling disease causing pathogens.

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NOMENCLATURE

DIR	Disease index rating
TDIR	Arc sin transformed disease index rating
HT	High-tannin
TAM	Texas A&M University
IPS	Individual plant selection
C_0	Cycle zero (parental material)
C_1	Cycle one
C_2	Cycle two
MS	Mean squares
DF	Degrees of freedom

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1. INTRODUCTION

Upland cotton, *Gossypium hirsutum* L., is grown extensively in the southern United States with an annual farmgate value of about $6 billion and an annual national economic impact of over $120 billion. In the southern United States, cotton is produced in Alabama, Florida, Georgia, Arkansas, Louisiana, Mississippi, Texas, Oklahoma, Kansas, New Mexico, Arizona, California, North Carolina, and South Carolina. Although this region is highly productive in terms of cotton production, significant annual yield losses are attributed to pest infestations, including what is known as cotton seedling disease complex (CSDC). Cotton seedling disease occurs when an individual pathogen, or a complex of individual pathogens, infect the seed or the early seedling stages of plant development (Minton, 1983). Such pathogens include *Fusarium spp., Pythium spp., Rhizoctonia solani,* and *Thielaviopsis basicola.* Two particular CSDC pathogens, *Rhizoctonia solani* and *Pythium ultimum* are the most significant soilborne pathogens of cotton in the United States (Disfani and Zangi, 2006). Production losses amount to over 170 million dollars and account for approximately 3% yield loss annually (NCC, 2013). General symptoms of the diseases include poor plant stands, both pre- and post-emergence damping off, seed rot, seedling root rot, delayed growth, hypocotyl lesions and stunting that delays maturity (Stanton et al., 1994). The development of cotton cultivars with resistance to *R. solani* and *P. ultimum* would reduce these losses as well as decrease costly control methods, such as direct pesticide application and seed treatments.

Rhizoctonia solani is the asexual stage of a basidiomycete fungus, *Thanatephorus cucumeris* in the order Cantharellales. *R. solani* reproduces asexually as vegetative mycelium (thread-like hyphae) and sclerotia (bundled mycelium) with no asexual spore production (no conidia) and rare sexual spore production. Sclerotia serve as both the primary inoculum and as the overwintering (or survival) stage (Cubeta and Vilgalys, 1997). The sclerotia survive in the soil for indefinite periods of time, even when fields are fallow. There are several anastomosis (AG), or hyphal fusion, groups of *Rhizoctonia* that are based on serological properties, ecological soil behavior, host range and morphological characteristics. Of the five identified AG groups, AG-4 is the group that contains the *Rhizoctonia* genotypes that affects cotton (Anderson, 1982). *R. solani* can infect cotton from seedling stage until the plant is approximately 15 cm tall. However, *R. solani* most commonly infects the seedling and serves as the leading cause of postemergence damping off in cotton seedlings worldwide (Nelson, 1987). Specific symptoms caused by *R. solani* include "girdling" hypocotyl lesions near the soil level and weak, cankerous abrasions commonly referred to as soreshin. Seedlings will often damp-off after infection, but the surviving seedlings can be stunted with delayed flowering and boll maturity (Brown and McCarter, 1975). *R. solani* is less temperature dependent than *P. ultimum*, but tends to be more common under slightly warm conditions, from 18 C to 32 C, on sandy soils and in moderate to well-drained moisture conditions (Davis, 1997).

Pythium ultimum is an oomycete, commonly referred to a water mold, in the order Pythiales. *P. ultimum* reproduces asexually through sack-like structures with spherical sporangia that produce hyphae and asexual spores called zoospores that persist in water and plant tissues (Allen et al., 2004). Oospores are the primary inoculum whereas both oospores and sporangia serve as overwintering structures (Johnson and Arroyo, 1983 and Nelson, 1987). Oospores and sporangia survive in the soil, particularly well in high-moisture, water-logged conditions. Oospores are disseminated by water flow and can infest multiple areas if not an entire production field. *P. ultimum* primarily causes preemergence damping off and can infect the cotton seed embryo in less than 24 hours. *P. ultimum* can also infect plant parts below ground, such as the roots and lower stem, during the seedling stage (Nelson, 1987). Symptoms of *P. ultimum* include stunted roots due to root cortical tissue rot at all plant stages, seedling desiccation, stunting, chlorosis and hypocotyl lesions if plant survives seedling stage. *P. ultimum* is most common in cool, 15 C - 20 C, moist environments (Davis, 1997 and Omar et al., 2007). The severity of *P. ultimum* increases with greater soil moisture, with maximum disease families at field saturation point. Increased moisture strengthens the resilience of the *P. ultimum* by enhancing growth and dispersal of zoospores via water transport in the soil. Water-logged conditions weaken the cotton plant, if the infected plant germinated and survived infection, by lowering the temperature of and decreasing the oxygen availability in the soil (Agrios, 1997). Cotton seeds and seedlings are more at risk to *P. ultimum* and *R. solani* in areas where deeper planting depths are required to achieve

moisture requirements because soil temperatures are cooler and the depth extends seedling emergence, increasing exposure of hypocotyl tissue to the pathogen.

Both pathogens cause a variety of symptoms, including pre and post-emergence damping-off, discolored and deteriorated roots and lesions on the hypocotyl (Davis, 1997). A seed infected by either *R. solani* or *P. ultimum* may deteriorate before germination, resulting in reduced plant stands and ultimately lower yield. The diseased seed usually is pliable and oozes seed contents under pressure. If the seedling is in the stage of initial infection, the cotton plant has a chance of survival but with a weakened stem and root system (Stockwell, 1983). Plants that emerge but are infected by *R. solani* and *P. ultimum* usually are stunted with crooked hypocotyls formed from the soreshin lesion, which causes irregular growth of the stem. The cotyledons remain near soil level for an extended time due to the malformed growth habits of the plant, and the cotyledons essentially are buried in the soil as the plant grows sideways due to a crooked stem (Minton, 1983).

Currently, the only means for CSDC control in cotton are fungicides applied to the seed and or seed furrow, crop rotations and selection of planting date to avoid conditions most favorable for disease development (Franke, 1999). Nearly all commercial cotton planting seed is treated with one or more fungicides to protect the seed from both preemergence and postemergence disease. Protectant and systemic fungicides may be used in conjunction to ensure greater seed and seedling protection. The use, and sometimes overuse, of these fungicides are not compliant with society's effort to limit chemical application and environmental protection (Minton, 1983).

Fungicides that control *R. solani* and *P. ultimum* add to the pesticide load in the environment, an avoidable environmental contaminant.

Crop breeding programs have been successful in achieving various levels of resistance to some CSDC pathogens for other crops species, such as corn (Franke, 1999). However, limited success has been achieved in development of acceptable genetic resistance to *R. solani* and *P. ultimum* in cotton through breeding. Achieving acceptable levels of disease resistance in cotton has proven to be an onerous and difficult breeding task due to the ever-advancing strains of fungi and bacteria (Wilkins, 2000). In addition, Poswal et al. (1986) reported that resistance to both *R. solani* and *P. ultimum* in cotton is controlled by a complex of minor genes, which has added to the breeding difficulty in cotton. Furthermore, the heritability for disease resistance in cotton has been reported as low, but detectible. Some breeding programs, such as the multi-adversity-resistant (MAR) Program at Texas A&M AgriLife Research use methods such as selection for rapid germination and seedling emergence to avoid elongated exposure to soilborne and seedborne pathogens. The MAR Program achieves avoidance, and thus host plant tolerance, to such pathogens by selecting cultivars with low temperature tolerance so that these MAR cultivars can be planted earlier in the growing season (McCarty and Percy, 2001). These cultivars can be planted earlier in the year, when the temperature is below optimum temperatures for *R. solani* and *P. ultimum*, decreasing chances of active pathogen infectation. Bush et al. tested 37 MAR lines for resistance to CSDC and discovered significant differences among lines at moderate inoculum levels (1978). Although some significant improvements have been documented among individual

MAR lines, no single MAR line has proven to be highly resistant at a commercially acceptable level (Bush et al., 1978).

Other studies have been conducted on the resistance level of current cultivars to *R. solani* and *P. ultimum*. In 1968, Hefner reported that only one of the ten cultivars of that year exhibited a sucessful seed stand due to high levels of resistance against *R. solani* (Hefner, 1968). More recently, Wang and Davis (1997) evaluated 12 upland cotton cultivars for resistance to both pathogens as well as *Thielaviopsis basicola*. Of those, three cultivars (DeltaPine 6100, ChemBred 7 and Royale) demonstrated initial resistance to *R. solani* in terms of seedling survival, but had significant post-emergence damping-off losses. In addition, four other cultivars (DeltaPine 6166, Prema, DeltaPine 6100, and Maxxa) demonstrated a high level of resistance to *P. ultimum* both pre- and post-emergence (Wang and Davis, 1997 and Garber et al,. 1991). The level of resistance must be further evaluated in terms of location, different pathogen strains and a commercially acceptable level of resistance.

Studies have attempted to determine the factor or factors attributing to disease resistance expressed in adult cotton plants related to the resistance, or lack of resistance, in cotton seedlings. This relationship may help cotton breeding programs develop more efficient screening techniques that better identify genotypes for disease resistance. One theory relies on the effectiveness of plant defense compounds as a mechanism for resistance. Allelochemicals, such as tannins and phenolic acids, and compounds, such as gossypol, have been reported as plant defense compounds that effect pest resistance in cotton. The accumulation of these chemicals and biochemical compounds in plant

tissues have antibiotic and toxic effects on pests such as insects and pathogens (Cheng et al., 2007). In 2006, Ray Kennett (2006) at Texas A&M University concluded that tannin, a polyphenol often associated with plant defense compounds, production along with other defense compounds, may not be produced until late developmental stages within the plant and thus are not effective in limiting seedling disease. These compounds may not be produced until a disease infection or insect attack occurs on the plant (Franke, 1999). Furthermore, Hunter et al. (1978) concluded that hypocotyl accumulation of terpenoid, a plant metabolite produced in flowers and vegetative tissues, in cotton seedlings contributes to resistance to *R. solani* after infection.

Further efforts in screening and developing cotton cultivars with resistance to *R. solani* and *P. ultimatum* would enhance plant stand, reduce losses, and decrease on-farm inputs (Wilkins, 2000). However, resistant cultivars, although cheaper and more environmentally friendly than alternative pathogen control, must be highly consistent and reliable due to the effective options, such as treated seed, currently available for control. An effective screening method for disease resistance is necessary to develop resistant cultivars.

2. RESEARCH OBJECTIVES

The objectives of this study were to 1) evaluate and make individual plant selections from six high-tannin experimental families (Smith et al., 1990a and 1990b; Schuster et al., 1990) and an obsolete cultivar, Luxor (Thaxton and El-Zik, 2003; PI 9900394) for resistance to *R. solani* and *P. ultimum,* 2) produce germplasm with increased resistance to *R. solani* and *P. ultimum*, and 3) determine if single plant selection is an effective method to make quantifiable progress in achieving improved disease resistance to *R. solani* and *P. ultimum* in cotton.

3. MATERIALS AND METHODS

3.1 Project History

One approach to CSDC resistance is to elevate certain plant defense compounds in current high-yielding cotton cultivars. Previous evaluation of specific high-tannin (HT) families for resistance to *R. solani* and *P. ultimum*, conducted at Texas A&M AgriLife Research under the supervision of Ray Kennett, suggested that improved resistance to these pathogens through appropriate selection was possible (Kennett, 2009). Kennett evaluated the effect of plant defense polyphenols in cotton, called tannins, on resistance. These compounds are considered to be an essential plant defense mechanism against disease and insects (Levin, 1973). Kennett evaluated 37 germplasm lines developed by Smith and associates (1990a and 1990b; Schuster et al., 1990) with elevated tannin levels. These HT families were developed by Texas A&M AgriLife Research in 1989 through pedigree breeding methods with selections based on tannin concentration. The program included HT accessions collected from Mexico, Belize and India. The objective of the original breeding project was to utilize tannin plant compounds as a feeding deterrent to the cotton bollworm (*Helicoverpa zea*) and other common cotton pests. Although the tannin level or the plant compound itself did not result in bollworm resistance, the families may be a source of genes for resistance to other cotton pests such as those that cause cotton seedling disease.

A portion of Kennett's evaluation entailed evaluating different HT families after a single cycle of selection for resistance to *R. solani*. Plants were inoculated with a high concentration of inoculum to ensure that the seedling was exposed to the pathogen

(Kennett, 2006). The gain from one cycle of selection was evaluated and determined to be significant as shown (Table 1). Resistance scores were standardized to the resistant control at 100% of the plants surviving. After one cycle of IPS, the selected families averaged 169% of the standardized check, Tamcot SP 21, while the unselected families averaged only 58%, suggesting selectable variability within the HT families. Significant improvement in resistance to *R. solani* was observed in four of the 37 tested HT families. Further testing was desirable to determine if additional progress was possible and to further quantify the improvements in resistance. This study investigated Kennet's results using six of the 37 HT families in Table 3 and Table 4 (TAM $86I^3$-16, TAM $86J^3$-1, TAM $86I^3$-11, TAM86 E-8, TAM 87N-6 and $TAMI^3$-24) to determine the effectiveness of single plant selection over multiple generations in selected families for resistance to *R. solani* or *P. ultimum* respectively.

Table 1. Mean values for resistance to *R. solani* and *P. ultimum*, respectively, after one cycle of single plant selection following soil inoculation. Two replications were conducted under greenhouse conditions at College Station, TX in December 2004 (Kennett, 2009).

HT Family	Tannin content g kg^{-1} fresh weight	Percentage survival unselected†	Percentage survival selected†
‡ TAM $86I^3$-16	1.17	17	133
‡ TAM $86J^3$-1	0.82	67	100
‡ TAM $86I^3$-11	0.64	0	242
§ TAM 86 I^3-26	0.83	150	189
§ TAM 87N-6	0.80	22	226
§ TAM 86 E8	0.71	28	176
¶ Sphinx (resistant check)	1.26	100	n/a
¶ WD18 (susceptible check)	0.38	0	n/a

† Resistance scores have been standardized with the resistant check set to 100%
‡ Families screened for *R. solani* resistance in 2012 and 2013.
§ Families screened for *P. ultimum* resistance in 2012 and 2013.
¶ Commercial check.

Table 2. Texas A&M AgriLife Research germplasm families challenged with *R. solani* or *P. ultimum* for resistance to Cotton Seedling Disease Complex.

Family	Pedigree	Pathogen
TAM 86I^3-11	'Roger's 10N' / Texas Race Collection #1041 PI540287	*R. solani*
TAM 86I^3-16	'Lankart 571' / Texas Race Collection #1142 PI 540289	*R. solani*
TAM 86J^3-1	'Stoneville 213' / 'Rogers Glandless' PI 540294	*R. solani*
Luxor	CABUCAHUGS-1-88 / CABUCAG8US-1-88 PI 9900394	*R. solani*
TAM 86E-8	Roger's 10N / Texas Race Collection #789 PI 540306	*P. ultimum*
TAM 87N-6	Stoneville 213/ Texas Race Collection #1055/2/'Empire Glandless' PI 540300	*P. ultimum*
TAM 86I^3-26	Lankart 571 / Texas Race Collection #1124 PI 540292	*P. ultimum*

Table 3. Percent seedling survival of 32 HT† germplasm families, Tamcot SP21, and TAM 96WD-18 following inoculation with *R. solani* under greenhouse conditions at College Station, TX in 2004 and 2005 (Kennett, 2009).

Family	Percent Survival‡
[§] Tamcot SP21	65 a
TAM 87 N 4	59 ab
[#] TAM 86 III 11	57 abc
TAM 87 M 48	57 abc
TAM 86 E 3	55 abcd
TAM 86 DD 17	52 abcde
TAM 87 N 7	51 abcdef
[¶] TAM 87 N 6	50 abcdefg
TAM 86 DD 12	50 abcdefg
TAM 87 N 5	50 abcdefgh
TAM 86 DD16	50 abcdefgh
Tamcot 96WD18	50 abcdefgh
TAM 86 CC 7	49 abcdefgh
TAM 86 III 22	48 bcdefgh
TAM 86 CC 18	47 bcdefgh
TAM 86 CC 13	47 bcdefgh
[¶] TAM 86 E 8	46 bcdefghi
TAM 86 DD 11	45 bcdefghi
[#] TAM 86 J 1	44 bcdefghij
TAM 87 M 41	44 bcdefghij
TAM 86 III 15	44 bcdefghij
TAM 86 E 9	43 bcdefghij
TAM 86 III 31	41 cdefghij
TAM 86 CC 17	41 cdefghij
TAM 86 CC 11	40 defghij
TAM 86 III 7	39 efghij
[¶] TAM 86 III 26	38 efghij
[#] TAM 86 III 16	38 efghij
TAM 86 E 7	38 efghij
TAM 86 CC 12	34 ghij
TAM 86 III 24	33 hij
TAM 86 E 4	30 ij
TAM 86 E 19	28 j
TAM 86 III 8	28 j

† HT, high tannin.

‡ Means followed by the same letter are not different according to Waller LSD at K=100.

[§] Commercial check.

[¶] Families screened for *P. ultimum* resistance in 2012 and 2013.

[#] Families screened for *R. solani* resistance in 2012 and 2013.

Table 4. Percentage seedling survival of 32 HT† germplasm families, Tamcot Sphinx, Stoneville213, and Suregrow 747 after infection by *P. aphanidermatum*. Four reps were performed in an incubator at 30º C in March – June 2006 (Kennett, 2009).

Family	Percent Survival‡
¶ TAM 86 III 26	80 a
¶ TAM 87 N 6	80 a
TAM 87 N 7	76 ab
TAM 87 N 5	74 abc
¶ TAM 86 E 8	74 abc
§ Tamcot SP21	72 abcd
# TAM 86 III 11	72 abcd
TAM 86 III 8	72 abcd
TAM 87 M 48	70 abcde
TAM 86 E 9	70 abcde
TAM 86 DD 12	69 abcde
TAM 86 E 7	69 abcdef
TAM 86 E 19	68 abcdefg
TAM 87 M 41	68 abcdefg
TAM 86 III 15	67 abcdefg
TAM 86 CC 7	67 bcdefgh
TAM 86 E 20	67 bcdefgh
# TAM 86 III 16	67 bcdefgh
# TAM 86 J 1	65 bcdefghi
TAM 86 III 24	64 bcdefghi
TAM 86 DD 16	63 cdefghij
TAM 86 E 3	60 defghij
TAM 86 DD 11	60 defghij
TAM 86 III 22	60 defghij
TAM 86 III 7	59 efghij
TAM 87 N 4	56 fghij
TAM 86 E 6	56 ghij
TAM 86 E 14	54 hijkl
TAM 86 CC 13	53 ijkl
TAM 86 CC 12	50 jklm
TAM 86 E 4	46 klm
TAM 86 DD 18	44 klm
Stoneville 213	42 lm
TAM 86 CC 11	38 m
TAM 86 CC 17	22 n
Sure Grow 747	14 no
TAM 86 II 31	13 no
TAM 86 DD 17	7 o

†HT, high tannin.
‡ Means followed by the same letter are not different according to Waller LSD at K=100.
§ Commercial check.
¶ Families screened for *P. ultimum* resistance in 2012 and 2013.
# Families screened for *R. solani* resistance in 2012 and 2013.

3.2 General Procedure

The breeding program for resistance to *R. solani* and *P. ultimum* at Texas A&M AgriLife Research utilized six high-tannin (HT) families previously shown to have potential for resistance through single plant selection and an obsolete, resistant cultivar, Luxor (Thaxton and El-Zik, 2003). Three HT families plus Luxor were challenged with *R. solani* and three HT families plus Luxor were challenged with *P. ultimum.* The pedigree of these six HT families and Luxor and the pathogen for which they were screened in Table 2.

3.3 *R. solani* and *P. ultimum* Screening and Selection

R. solani inoculum was prepared on a potato dextrose agar (PDA) in a 100 mm x 15 mm petri dish for 10 days at room temperature (20 C to 25 C). The culture was transferred to 25 g of autoclaved wheat bran mixed with 25 ml of tap water. After incubating the culture at room temperature for five days, the wheat bran inoculum was refrigerated at 10 C and stored up to one year. Previously conducted preliminary assays determined the LD_{90} concentration to be 0.125 g wheat bran inoculum per kg of soil. LD_{90} for this experiment was defined as the least amount of inoculum that results in 10% survival of the susceptible controls14 days after inoculation. At planting, the inoculum bran was thoroughly mixed with 1kg soil, a sandy loam mixture, and then mixed thoroughly with ca 500 cm^3 peat and 100 ml of water to ensure uniform inoculum distribution (Djonovic et al., 2007). A standard planting tray (0.37 x 0.52 m), separated into six lanes (56 mm wide x 57 mm deep) with plastic dividers, containing 1 kg of inoculated soil per lane, was utilized for seedling evaluation. Twenty seeds of each of

the C_0 families were planted in separate lanes (two trays used for a total of 10 lanes planted in a randomized manner), for a total of 200 seeds per family. The C_0 families included:the HT familesTAM 86J^3-1, TAM 86I^3-11, TAM 86I^3 -16, the resistant check Luxor, and the susceptible check SU 2004, a *G. hirsutum* of unknown origin (pers. comm., J. L.Starr). An uninoculated control was included to confirm that symptoms did not occur in the absence of the pathogen. After planting, each lane was wetted to field capacity and covered with plastic wrap to prevent moisture loss. Trays were placed in a growth chamber at 20 C +/- 1 and watered daily. At seven days after planting (DAP), the plastic wrap was removed and emergence and the number of symptomless plants were recorded. At 10 DAP, susceptible plants, as indicated by failure to emerge (pre-emergence damping off), non-surviving seedlings (post-emergence damping off), and plants showing visible symptoms of infection (such as lesions, poor root systems, crooked hypocotyl growth etc.), were discarded. The surviving, symptomless C_1 plants were transplanted into 19 liter C-1100 pots filled with Sunshine Professional Growing Mix™. After transplanting, some C_1 plants developed *R. solani* symptoms such as irregular hypocotyl growth, soreshin lesions and necrotic cotyledons and were discarded. Bolls from symptomless, resistant C_1 plants were harvested, ginned and seeds were delinted with concentrated sulfuric acid, providing C_1 generation seed. The C_1 experimental families were pruned and allowed to regrow in the greenhouse to produce additional seed. The previously explained screening process was repeated to produce putatively *R. solani* resistant cycle two (C_2) seed.

P. ultimum was cultured on the V8™ media prepared from 200 ml of V8™ juice and centrifuged with 2.5 g $CaCO_3$ for 30 min at 13,200 x g. The pellet was discarded and distilled water was added to the supernatant to bring the volume to one liter. One ml of cholesterol stock (15 mg ml^{-1} dissolved cholesterol in 95% EtOH) was added, stirred for 5 min then autoclaved for 30 min. Twelve ml aliquots of the V8™ media was added to each 100-mm x 15-mm petri dish, and then each dish was inoculated with a 10-mm plug of *P. ultimum* from a 10-day old corn meal agar. The agar was supplemented with 10 μg ml^{-1} of rifampicin stock solution of 50 mg ml^{-1} in methanol to eliminate potential bacterial contamination. The petri dish culture was incubated at room temperature in a dark laboratory cabinet for 10 days as a still culture. The *P. ultimum* mycelial mat was removed from the petri dish and macerated in 50 ml of tap water using a Waring blender for approximately 30 sec (Howell, 2002). Previously conducted preliminary assays determined the appropriate inoculum concentration to be three mycelial mats/kg of soil to achieve a LD_{90}. The inoculum water mixture was added to 1kg soil and mixed thoroughly to ensure uniform inoculum distribution. Standard planting trays (0.37 m x 0.52 m) divided into six lanes (56 mm wide, 57 mm deep) as discussed in the *R. solani* screening description were used for seedling evaluation. Each lane was filled with the 1,000 g soil/mycelial mat mixture and 20 seeds were planted per lane. Ten replications of 20 seeds each the HT familiesTAM 86E-8, TAM 87N-6 and TAM $86I^3$-26), the susceptible check SU-2004 and the resistant check Luxor were tested in the C_0 generation. Each lane was wetted to field capacity, the tray covered with plastic wrap and placed in a 20 C +/- 1 growth chamber. At seven days after planting (DAP),

the plastic was removed and the number of symptomless *P. ultimum* C_1 plants and the overall plant stand was documented for cotton line. At 10 DAP the surviving and symptomless *P. ultimum* C_1 plants were transplanted into 19 liter C-1100 pots of Sunshine Professional Growing Mix. After transplanting, some *P. ultimum* C_1 plants showed *P. ultimum* symptoms such as watery lesions, stunted growth and chlorosis of the cotyledons. Any plants that exhibited such symptoms were discarded and were not included for the next generation of selection. The symptomless *P. ultimum* C_1 plants were grown to maturity in the greenhouse for approximately 5 months. Bolls from symptomless, resistant *P. ultimum* C_1 plants were harvested, ginned and seed acid delinted. The previous screening process was repeated with *P. ultimum* C_2 seed.

C_1 seed of the *R. solani* and *P. ultimum* families were planted into inoculated soil as described above. Seedlings were evaluated for *R. solani* and *P. ultimum* resistance, respectively, and those showing no symptoms (C_2) were transplanted to the greenhouse and grown to maturity to produce C_2 seed for evaluation and designated as C_2 families. Cycle one produced a number of plants within each family that became the foundation plant for a new, resistant strain of upland cotton, designated as C_2 families. For example, each of the TAM HT experimental families and Luxor produced two to four C_1 families, which then produced multiple plants (C_2) showing no symptoms when challenged with *R. solani* and *P. ultimum*. A single C_2 plant from each of the two to four C_1 families was advanced and thus the number of families evaluated for genetic gain for resistance to *R. solani* was 20 and 12 for *P. ultimum* (Table 5). The C_1 and C_2 plants in the greenhouse were pruned and allowed to regrow to produce additional C_1 and C_2 seeds.

Table 5. Percentage of plants selected for *R. solani* and *P. ultimum* resistance, respectively, over two cycles of individual plant selection in growth-chamber experiments from six cotton families and one cultivar, Luxor.

		Selection Cycle		
	Family	Cycle 0 (C_0)	Cycle 1 (C_1)	Cycle 2 (C_2)
R. solani	TAM 86J^3-1	1.0%	1.0%	4.0%
	TAM 86I^3-11	1.0%	1.0%	4.0%
	TAM 86I^3-16	1.0%	1.5%	4.5%
	Luxor	2.0%	2.0%	4.5%
P. ultimum	TAM 86E-8	0.5%	0.5%	2.0%
	TAM 87N-6	1.5%	3.0%	2.0%
	TAM 86 I^3-26	0.0%	0.0%	0.0%

3.4 Quantify Genetic Gain for Resistance

The C_0, C_1, and C_2 generations from the HT families and Luxor, along with the in-house susceptible family, SU-2004, were challenged with *R. solani* or *P. ultimum*, in a bulk seedling test to determine improvement in resistance. Each experiment was replicated four times and challenged with three inoculation levels (0, original selection level, and 2x original selection level). The experimental design was a factorial of a randomized complete block design. Families were randomized by lane within each planting flat having a total of 6 lanes as described above. Ten seeds from each family were planted per lane, and thus each planting flat represented one replication (6 lanes per flat therefore 6 families per flat). Ten C_0, C_1, and C_2 seeds per replication were tested based on the protocols previously discussed. The susceptible and resistant checks were grown in the outside border row of the flat. At 10 days after planting, the surviving seedlings in each flat were inspected for disease damage that occurred both above and below the soil surface.

Plants tested for *R. solani* resistance were given a susceptibility rating based on a scale derived from Stanton et al. (1993) and Wang and Davis (1997). The scale ranges from 0 to 6 where 0 = no symptoms, 1 = one, small lesion, 2= multiple and/or large, surface lesions, 3 = deep, dark lesion(s) with normal root and tissue growth, 4 = deep, dark lesion(s) with loss of all cortical tissue and few or no healthy roots and 5 = dead and 6 = pre-emergence damping off. Plants tested for *P. ultimum* resistance were given a susceptibility rating based on a scale similar to that used for *R. solani* ratings. The scale ranges from 0 to 6 where 0 = no symptoms (healthy plant), 1 = small lesion, normal

roots, 2= small lesion, stunted roots, 3 = multiple and/or large lesion, 4 = multiple, large lesions with diminished roots, 5 = seedling emerged but did not survive and 6 = pre-emergence damping off. The experiment dealing with each pathogen was conducted twice (run) to evaluate potential confounding factors due to the environment.

Resistance was evaluated based on the improved emergence rate that is the final plant stand, as well as a calculated disease index rating. Disease index rating (DIR) was calculated for each family using the following formula (Powell et al., 1971):

Disease Index Rating:

$$\frac{(\text{no.plants in class 1 x 1})+(\text{no plants in class 2 x 2})\ldots+(\text{no.plants in class 6 x 6})}{\text{Total no.plants in treatment x 6}}$$

Cross-resistance was evaluated for the different test families and two pathogens. Two C_2 *R. solani* families (TAM 86I[3] -16 C_2 and Luxor 3 C_2) and two C_2 *P. ultimum* families (TAM 86E-8 C_2 and TAM 87N-6 C_2,) were evaluated for cross-resistance, i.e., *R. solani* C_2 families were inoculated and screened for *P. ultimum* resistance and *P. ultimum* C_2 families were inoculated and screened *R. solani* resistance. Both screening procedures followed the above protocols for *R. solani* and *P. ultimum,* i.e., a factorial design of a RCB with three pathogen treatments (no inoculum, selection rate, and 2x selection rate). The same seedling screening method and rating scale were followed as outlined above.

3.5 Data Analysis

Bartlett's Test for Homogeneity of Variances was applied to the data over both runs for percentage survival and disease index rating to ensure that the variances were proportionate. The test indicated that percentage survival data were homogeneous. The DIR data variances were found not to be homogeneous, as is expected with ordinal disease rating data, so the data were transformed with several different transformation methods for reevaluation. Arc sine transformation provided the closest to normal distribution and was utilized in the analysis of variance. It should be noted, however, that none of the transformations, including arc sine, resulted in completely homogeneous variances. DIR was analyzed using the transformed DIR (TDIR) data, but the non-transformed mean values were reported in all tables and discussions.

Analyses of variances were conducted using the SAS® statistical software, version 9.3 of the SAS program for Windows. PROC GLM, the general linear model, was used to analyze all data. All main effects were considered fixed effects, with the exception of run. Run is the top main effect and is the only one-way interaction with rep and is therefore confounded. Means were separated by Waller LSD at k =100, which represents the 5% probability level. Significant interactions were noted and evaluated in this discussion.

Correlation between DIR and percentage survival was evaluated. PROC CORR, the correlation procedure, was used to analyze all data. A correlation model, using DIR as the y-variable and percentage survival as the x-variable, was provided for interpretation and discussion.

4. RESULTS AND DISCUSSION

4.1 *R. solani* and *P. ultimum* Improved Resistance

The ANOVA for *R. solani* resistance among the five families evaluated over three cycles of selection and three inoculation levels indicated significant variation among all main effects except the two repeats of the experiment (Table 6). Families did not respond the same to cycles of selection or the three levels of inoculation tested. The average performance of all families combined was not consistent over the three cycles of selection as indicated by significant interactions. Thus the ANOVA indicates that main effects cannot be separated and are considered apart from a discussion of interactions of those effects. Since three-way interactions are difficult to present and discuss, means for percent survival and DIR are presented in Tables 7 and 8 in a two-way interaction format with cycles within families (hereafter referred to as Family x Cycle lines) separated within inoculum rate. This presentation of the data allows for the determination of individual Family x Cycle lines, i.e., plants derived by each cycle of selection to be compared for their potential as *R. solani* or *P. ultimum* resistant germplasm lines. Separating original families within each cycle or families within inoculation level or inoculation levels impact on each cycle of selection, i.e., two-way interactions are not as informative from a breeding perspective. Although percent survival means within each Family x Cycle could be separated statistically, there is no biological reason for this variation in response to *R. solani* since no disease pathogen was applied (Table 7).

Survival in the absence of the pathogen ranged from 55 to 85% across the 21 Family x Cycle lines, including the SU-2004 control. When these 21 Family x Cycle

lines were challenged with the 1x selection rate, i.e., the selection level of *R. solani* pathogen, survival clearly improved with selection within each Family x Cycle with only TAM $86I^3$-16-3-C_2 compared with its Family C_0 and in Luxor C_1 compared with Luxor C_0. Within the 1x rate of inoculum, all C_0 Family x Cycle lines were not different than the unselected SU-2004 control but Luxor C_0 had significantly better survival than the C_0 Family x Cycle lines from the three HT families. This suggests that the MAR program as established by Bird (Bush et al., 1978) was successful in selecting for a low level of *R. solani* resistance.

When the Family x Cycle lines were challenged with a 2x inoculation rate, i.e., twice the amount of pathogen that was used to challenge the populations during the selection process, percent survival was either zero or not significantly different than zero for all Family x Cycle lines and the SU-2004 control except for TAM $86J^3$-1-2-C_1 and Luxor -3-C_1 and C_2 and Luxor-5-C_1 (Table 7). These data again support the seedling disease resistance in the MAR Luxor (Bush et al., 1978) cultivar but also suggest some variation in the HT material, especially TAM $85J^3$-1 germplasm line.

Data in Table 7 also confirm that inoculum rate had a significant impact on percent survival. While the lower case letters indicate mean separation within columns, the upper case letters indicate significant differences among values within rows of data. In almost all cases, the percentage survival of the Family x Cycle lines was significantly lower with 1x rate of inoculum except in the case of Luxor and TAM$86J^3$-1-2-C_1 where survival was not significantly decreased under pathogen pressure. Again, in almost all cases, the survival of the Family x Cycle lines was decreased under the 2x inoculation

rate, except in the case of TAM86J^3-1-4-C_1, TAM86I^3-16-3-C_2, and Lucor-5-C_1 where survival was comparable under both 1x and 2x inoculum pressures.

Disease Index Rating, DIR, in the absence of the pathogen was zero across the 21 Family x Cycle lines, including the SU-2004 and Luxor controls (Table 8). When these 21 Family x Cycle lines were challenged with the 1x selection level of *R. solani* pathogen, DIR improved, i.e., decreased, with selection within each set of Family x Cycle lines, except for TAM 86J^3-1-4-C_1 when compared to TAM 86J^3-1- 4-C_2. Within the 1x rate of inoculum, all C_0 Family x Cycle lines were not statistically different than the susceptible SU-2004 control, but were significantly less resistant than resistant Luxor control. DIR values for Luxor-3-C_1, Luxor-5-C_1, Luxor-3-C_2, and Luxor-5-C_2 were significantly lower than for all other Family x Cycle lines, suggesting that the MAR program releases contain additional variation for resistance to *R. solani*. When the Family x Cycle lines werechallenged with a 2x inoculation rate, DIR was either 100% diseased or not significantly different than 100% for all Family x Cycle lines.

Data in Table 8 also confirm that inoculum rate had a significant impact on DIR. As indicated above for Table 7, the lower case letters indicate mean separation within columns, while the upper case letters indicate significant differences among values within rows of data. In all cases, the DIR of the Family x Cycle lines was significantly more severe when inoculum at the 1x rate as compared to the control. Under 2x inoculum pressure, as compared to the 1x rate of inoculum, almost all Family x Cycle lines demonstrated greater disease symptoms except for TAM 86J^3-1-C_0, TAM 86I^3-11-

C_0, TAM $86I^3$-16-C_0 and SU-2004 in which DIR for the 1x rate was near 100% and not statistically different than the SU-2004 DIR.

Overall, Pearson's correlation of DIR and percentage survival was -0.90. Decreasing DIR values, i.e., symptoms were less severe, were associated with higher percentage survival as one would expect from the means in Tables 7 and 8. In summary, these data suggest that while no single HT family contained complete resistance to *R. solani*, there is selectable variation for resistance within these HT families, as well as within the cultivar Luxor. However, C_1 and C_2 of the HT families did not out perform C_1 and C_2 of Luxor, which suggests that these HT families may not be the most useful sources of resistance to *R. solani*.

Table 6. ANOVA for sources of variation on percentage survival and disease index rating (DIR) after infection by *R. solani*. Four reps were performed in a growth chamber at 30C repeated in two separate experiments in 2013.

R. solani		Percentage Survival	DIR
Source	df	Mean Square	Mean Square
run	1	0.078	0.0003
error a	6	0.017	0.0010
family	4	0.125**	0.0006*
run*family	4	0.017	0.0002
error b	24	0.016	0.0002
cycle	2	0.199**	0.0078**
run*cycle	2	0.069	0.0005
family*cycle	6	0.053*	0.0007
run*family*cycle	6	0.017	0.0002
error c	47	0.020	0.0004
inoc	2	14.143**	0.1201**
run*inoc	2	0.050	0.0007
family*inoc	8	0.127**	0.0112**
cycle*inoc	4	0.303**	0.0034**
run*family*inoc	8	0.018	0.0002
run*cycle*inoc	4	0.006	0.0001
family*cycle*inoc	12	0.043*	0.0008**
run*family*cycle*inoc	12	0.014	0.0002
error d	349	0.020	0.0002

*, ** Significant at $p<0.05$ and $p<0.01$, respectively.

Table 7. Percentage survival of C_0, C_1 and C_2 families under 0, 1x and 2x selection level of *R. solani* inoculum. Four replications were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

R. solani Percentage Survival over experiments

Family	No inoculum		1x selection rate†		2x selection rate‡	
TAM 86J^3-1-C_0	73 bc	A	10 f	B	0 d	C
TAM 86J^3-1-2-C_1	55 g	A	45 bcd	A	22 ab	B
TAM 86J^3-1-4-C_1	71 bcd	A	24 de	B	14 abcd	B
TAM 86J^3-1-2-C_2	66 cde	A	37 bcde	B	15 abcd	C
TAM 86J^3-1-4-C_2	73 bcd	A	27 cde	B	7 bcd	C
TAM 86I^3-11-C_0	79 ab	A	9 f	B	3 cd	C
TAM 86I^3-11-1-C_1	63 defg	A	24 de	B	0 d	C
TAM 86I^3-11-2-C_1	61 efg	A	28 cde	B	8 bcd	C
TAM 86I^3-11-1-C_2	64 cde	A	43 bcde	B	8 bcd	C
TAM 86I^3-11-2-C_2	64 cde	A	39 bcde	B	15 abcd	C
TAM 86I^3-16-C_0	85 a	A	9 f	B	0 d	C
TAM 86I^3-16-3-C_1	58 fg	A	34 bcde	B	0 d	C
TAM 86I^3-16-6-C_1	59 fg	A	30 cde	B	0 d	C
TAM 86I^3-16-3-C_2	61 efg	A	12 ef	B	12 abcd	B
TAM 86I^3-16-6-C_2	68 cdef	A	29 cde	B	7 bcd	C
Luxor-C_0	61 efg	A	24 de	B	0 d	C
Luxor-3-C_1	76 bc	A	63 ab	A	18 abc	B
Luxor-5-C_1	64 cde	A	27 cde	B	23 ab	B
Luxor-3-C_2	74 bc	A	86 a	A	27 a	B
Luxor-5-C_2	66 cdef	A	56 abc	A	15 abcd	B
SU-2004 C_0	80 ab	A	8 f	B	0 d	B

Values within columns followed by the same lower case letter and values within rows followed by the same upper case letter are not different at $p < 0.05$.

† 1x rate = 0.125g wheat bran inoculum/1,000 g soil.
‡ 2x rate = 0.250g wheat bran inoculum/1,000 g soil.

Table 8. *R. solani* disease index rating on selected families in each cycle under growth chamber conditions. Four replications were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

R. solani Disease Index Rating (DIR)[§] over experiments						
Family	No inoculum		1x selection rate[†]		2x selection rate[‡]	
TAM 86J^3-1-C$_0$	0 a	A	96 a	B	100 a	B
TAM 86J^3-1-2-C$_1$	0 a	A	80 bc	B	96 a	C
TAM 86J^3-1-4-C$_1$	0 a	A	89 ab	B	97 a	C
TAM 86J^3-1-2-C$_2$	0 a	A	62 d	B	91 a	C
TAM 86J^3-1-4-C$_2$	0 a	A	87 ab	B	97 a	C
TAM 86I^3-11-C$_0$	0 a	A	95 a	B	99 a	B
TAM 86I^3-11-1-C$_1$	0 a	A	88 ab	B	100 a	C
TAM 86I^3-11-2-C$_1$	0 a	A	88 ab	B	97 a	C
TAM 86I^3-11-1-C$_2$	0 a	A	74 c	B	97 a	C
TAM 86I^3-11-2-C$_2$	0 a	A	81 bc	B	94 a	C
TAM 86I^3-16-C$_0$	0 a	A	97 a	B	100 a	B
TAM 86I^3-16-3-C$_1$	0 a	A	93 ab	B	100 a	C
TAM 86I^3-16-6-C$_1$	0 a	A	85 b	B	100 a	C
TAM 86I^3-16-3-C$_2$	0 a	A	80 bc	B	95 a	C
TAM 86I^3-16-6-C$_2$	0 a	A	72 c	B	99 a	C
Luxor-C$_0$	0 a	A	78 bc	B	100 a	C
Luxor-3-C$_1$	0 a	A	58 de	B	94 a	C
Luxor-5-C$_1$	0 a	A	67 cd	B	93 a	C
Luxor-3-C$_2$	0 a	A	31 f	B	93 a	C
Luxor-5-C$_2$	0 a	A	50 de	B	95 a	C
SU-2004 C$_0$	0 a	A	97 a	B	100 a	B

Values within columns followed by the same lower case letter and values within rows followed by the same upper case letter are not different at $p < 0.05$.

[†] 1x rate = 0.125g wheat bran inoculum/1,000 g soil.
[‡] 2x rate = 0.250g wheat bran inoculum/1,000 g soil.
[§] Note that raw DIR averages were reported, arcsin transformed values were analyzed.

The ANOVA for *P. ultimum* resistance among the four families evaluated over three cycles of selection and three inoculation levels indicated significant variation among all main effects except the two repeats of the experiment (Table 9). Only two of the three HT families, TAM 87N-6 and TAM 86E-8, selected on the work of Kennett (2009) were advanced. In the original selection experiments, the third HT family, TAM $86I^3$-26, did not produce any symptomless plants and, therefore, was not advanced to the C_1 generation of selection. Luxor was not advanced due to the *P. ultimum* resistant behavior of Luxor parental material in the original screening relative to the HT families. Unlike under *R. solani* pressure, Luxor demonstrated resistant behavior to *P. ultimum* as compared to the test families. Under *R. solani* pressure, Luxor performed comparatively to the test cultivars and thus was advanced as a test line, not a control.

Averaged over cycles and levels of inoculation, families responded similarly, $p<0.05$, while cycles of selection were significantly different when averaged over families and inoculation levels, as were inoculation levels (Table 9). However, families did not respond the same to the cycles of selection nor to inoculation levels relative to percent survival. Cycles also did not produce similar responses to inoculation levels. However, the three-way interaction of family*cycle*inoculation was significant for percent survival and DIR as it was in the *R. solani* portion of this study and thus the discussion relative to selecting for resistance to *P. ultimum* will follow the same format, i.e., means for percent survival and DIR under *P. ultimum* selection are presented in a two-way interaction format with cycles within families separated within inoculum rate.

Survival in the absence of the pathogen ranged from 38 to 88% across the 14 Family x Cycle lines, including the SU-2004 C_0 and Luxor C_0 as controls (Table 10). When the 12 Family x Cycle lines plus the two controls were challenged with the 1x selection rate, i.e., the selection level of *P. ultimum* pathogen, percentage survival of TAM 87N-6 and TAM 86E-8 improved significantly from the C_0 generation over each selection cycle. Percentage survival of two of the other three Family x Cycle lines of family 87N-6, TAM 87N-6-3 and TAM 87N-6-6, improved significantly compared with the C_0 generation with one cycle of selection but the second cycle of single plant selection did not result in a greater survival over the single cycle within the 1x rate of inoculum, all C_0 Family x Cycle lines were significantly more resistant than the susceptible SU-2004 control, but less resistant than resistant Luxor control.

When the Family x Cycle lines were challenged with a 2x inoculation rate, i.e., twice the amount of pathogen that was used to challenge the populations during the selection process, percent survival was significantly greater than zero for all Family x Cycle lines except for the susceptible SU-2004 control. Improvement in percentage survival at the 2x inoculation rate followed the same pattern as with the 1x rate in that only Family x Cycle line TAM87N-6-2-C_1 exhibited significantly greater survival than the respective C_0 generation but all Family x Cycle lines showed greater survival than the C_0 population. When compared only with the C_1 cycle, only TAM86E-8-1-C_2 showed a significant improvement in survival. These data suggest some variation in the HT material, both in TAM 87N-6 and TAM 86E-8 germplasm lines.

Data in Table 10 also confirm that inoculum rate had a significant impact on percentage survival. As was demonstrated in Table 7 for *R. solani* screening, the lower case letters indicate mean separation within a column, the upper case letters indicate significant differences among values within rows of data. The percentsge survival of the Family x Cycle lines was significantly lower with 1x rate of inoculum in all cases except for TAM 87N-6-2-C_1, TAM 87N-6-6-C_1, TAM 86E-8-C_0, and Luxor, in which percentage survival was not significantly lowered under 1x inoculum pressure compared to no inoculum present. However, these exceptions did not hold when Family x Cycle lines were challenged with the 2x inoculation rate. Under 2x selection rate pathogen pressure, the survival of lines significantly decreased compared to the 1x rate for all Family x Cycle lines and Luxor, but not for SU-2004, which was essentially zero survival under both inoculation rates.

DIR in the absence of the pathogen was zero across the 14 Family x Cycle lines and the SU-2004 and Luxor controls (Table 11). When these 12 Family x Cycle lines plus the two controls were challenged with the 1x selection level of *P. ultimum* pathogen, DIR significantly improved with each cycle of selection within each family. Within the 1x rate of inoculum, all C_0 Family x Cycle lines were not statistically different than the susceptible SU-2004 control, but were less resistant than resistant Luxor control. Within the 1x inoculation rate, DIR produced a clearer representation of the effect of individual plant selection as a tool for improving resistance to *P. ultimum*.

When the Family x Cycle lines were challenged with a 2x inoculation rate, DIR was significantly reduced for all Family x Cycle lines except for TAM 87N-6-1-C_1 and

TAM 87N6-2-C_1 compared with their respective C_0 cycle (Table 11). A second cycle of single plant selection against the 1x rate significantly reduced DIR compared with the C_1 cycle. These data confirm that progress can be made in resistance to *P. ultimum* through the use of single plant selection. Data in Table 11 also confirm that inoculum rate had a significant impact on DIR. The DIR of the Family x Cycle lines was significantly higher with the presence of inoculum in all cases. Under the 2x selection rate, DIR was more severe in all cases except for TAM 87N-6-C_0, TAM 87N-6-1-C_2, TAM 86E-8-C_0 and SU-2004, in which DIR did not increase significantly and as expected with 2x inoculum pressure compared to 1x inoculum. In the case of TAM 87N-6-C_0, TAM 86E-8-C_0 and SU-2004, DIR was at 100 or not statistically different than 100 under both the 1x and 2x inoculum rates. TAM 87N-6-1-C_2, however, remained between 75 and 79 under each inoculation rates, respectively. DIR was reduced to approximately 50 in C_2 of the TAM 87N-6 family as well as in C_2 of the TAM 86E-8 family under 1x selection pressure.

Pearson's correlation for improvement in *P. ultimum* resistance of all lines related to decreased DIR and increased percentage survival was -0.845. That is, as DIR rating decreased, i.e., symptoms were less severe, percent survival values increased. Overall, these data suggest that while no single HT family contained complete resistance to *P. ultimum*, there is selectable variation for resistance within these HT families. Because two of the five advanced HT families outperformed the resistant check Luxor, these families may be useful sources of germplasm resistance to *P. ultimum.* This superior performance was only indicated by percent survival, and not reflected by a significant improvement in DIR.

Table 9. ANOVA for sources of variation on percentage survival and disease index rating (DIR) after infection by *P. ultimum*. Four replications were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

P. ultimum		Percentage Survival	DIR
SOV	df	Mean Square	Mean Square
run	1	0.036	0.001
error a	6	0.056	0.014
family	1	0.083	0.004
run*family	1	0.015	0.004
error b	6	0.035	0.003
cycle	2	1.009**	0.560**
run*cycle	2	0.002	0.001
family*cycle	2	0.371**	0.020**
run*family*cycle	2	0.0007	0.002
error c	24	0.023	0.002
inoc	2	2.100**	0.899**
run*inoc	2	0.004	0.0002
family*inoc	2	0.219*	0.002
cycle*inoc	4	0.417**	0.176**
run*family* inoc	2	0.043	0.009
run*cycle* inoc	4	0.004	0.001
family*cycle*inoc	4	0.154*	0.018*
run*family*cycle*inoc	4	0.011	0.002
error d	213	0.032	0.006

*, ** Significant at $p<0.05$ and $p<0.01$, respectively.

Table 10. Percentage survival of C_0, C_1 and C_2 families selected for *P. ultimum* resistance under 0, 1x and 2x selection levels of *P. ultimum* inoculum. Four replications were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

P. ultimum Percentage Survival over experiments						
Family	No inoculum		1x selection rate†		2x selection rate‡	
TAM 87N-6-C_0	85 a	A	31 fg	B	9 d	C
TAM 87N-6-1-C_1	73 bc	A	48 def	B	17 cd	C
TAM 87N-6-2-C_1	61 d	A	69 c	A	35 ab	B
TAM 87N-6-3-C_1	79 ab	A	61 cd	B	19 cd	C
TAM 87N-6-6-C_1	61 d	A	59 cde	A	20 bcd	B
TAM 87N-6-1-C_2	79 ab	A	41 efg	B	25 abc	C
TAM 87N-6-2-C_2	74 bc	A	89 ab	B	37 a	C
TAM 87N-6-3-C_2	56 d	A	73 bc	B	27 abc	C
TAM 87N-6-6-C_2	39 e	A	61 cd	B	29 abc	C
TAM 86E-8-C_0	38 e	A	26 g	A	7 d	B
TAM 86E-8-1-C_1	65 cd	A	44 def	B	21 bcd	C
TAM 86E-8-1-C_2	58 d	A	97 a	B	38 a	C
SU-2004 C_0	88 a	A	9 a	B	0 a	B
Luxor C_0	74 b	A	70 b	A	21 bcd	B

Values within columns followed by the same lower case letter and values within rows followed by the same upper case letter are not different at $p < 0.05$.

† 1x rate of pathogen = 3 mycelial mats/1,000 g soil.
‡ 2x rate = 6 mycelial mats/1,000 g soil.

Table 11. *P. ultimum* disease index rating (DIR) on selected families in each cycle under growth chamber conditions. Four replications were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

P. ultimum Disease Index Rating (DIR)† over experiments						
Family	No inoculum		1x selection rate[a]		2x selection rate[b]	
TAM 87N-6-C_0	0 a	A	93 a	B	98 ab	B
TAM 87N-6-1-C_1	0 a	A	84 b	B	95 abc	C
TAM 87N-6-2-C_1	0 a	A	74 c	B	88 cde	C
TAM 87N-6-3-C_1	0 a	A	75 c	B	93 abcd	C
TAM 87N-6-6-C_1	0 a	A	73 c	B	90 bcd	C
TAM 87N-6-1-C_2	0 a	A	75 c	B	79 ef	B
TAM 87N-6-2-C_2	0 a	A	54 de	B	76 g	C
TAM 87N-6-3-C_2	0 a	A	59 d	B	85 def	C
TAM 87N-6-6-C_2	0 a	A	59 d	B	78 fg	C
TAM 86E-8-C_0	0 a	A	96 a	B	99 a	B
TAM 86E-8-1-C_1	0 a	A	84 b	B	95 abc	C
TAM 86E-8-1-C_2	0 a	A	50 e	B	82 efg	C
SU-2004 C_0	0 a	A	99 a	B	100 a	B
Luxor C_0	0 a	A	36 f	B	69 h	C

Values within columns followed by the same lower case letter and values within rows followed by the same upper case letter are not different at $p < 0.05$.

† 1x rate of pathogen = 3 mycelial mats/1,000 g soil.
‡ 2x rate = 6 mycelial mats/1,000 g soil.
§ Note that raw DIR averages were reported, arcsin transformed values were analyzed.

4.2 Cross-resistance between *R. solani* and *P. ultimum*

Cross-resistance of four randomly selected families, TAM 87N-6, TAM 86E-8, Luxor and TAM86I^3-16, was tested. Luxor and TAM 86I^3-16, families originally tested and selected for *R. solani* resistance, were inoculated and evaluated for resistance at the zero and 1x selection rate of *P. ultimum* (Table 12).

Percentage survival of Luxor and TAM 86I^3-16 families was not significantly different when challenge with *P. ultimum*, although the two families responded differently to the two levels of inoculum (Table 12). This significant interaction between family and inoculum level caused a direction of response interaction with percentage survival increasing from 71% when germinated under no inoculum pressure to 80% survival when 1x rate of inoculum was added to the germination medium, while the opposite occurred for TAM 86I^3-16 family, survival decreasing from 71% to 60%. While these numbers may have statistical significance, they don't appear to biologically important. Percentage survival improved from an average of 55% at C_0 averaged over the two families to 71% at C_1 and 85% at C_2, suggesting that single plant selection for *R. solani* did result in a measurable improvement in resistance to *P. ultimum*. The cycle x inoculum level interaction was the result of a low percentage survival under the 1x rate for C_0 and an almost immune response (99% survival) under the 1x rate of *P. ultimum* for C_2 Family x Cycle lines.

The three way interaction of inoculum x family x cycle for DIR statistically required the separation Family x Cycle lines within inoculum levels, although family and cycle sources of variation for DIR were significant (Table 12). The inoculum levels would obviously result in a significant difference between levels because by definition, DIR for the no inoculum level must be zero and thus any impact of the pathogen would result in a biological difference. While mean separation in Table 14 indicates a significant difference among the Family x Cycle lines, the general trend in DIR within the 1x selection inoculum rate was a decrease with each cycle of selection within both Luxor and TAM 86I^3-16.

Table 12. ANOVA for sources of variation on percentage survival and disease index rating (DIR) after cross infection by *P. ultimum* on lines originally screened for *R. solani* resistance. Four reps were performed in a growth chamber at 30 °C repeated in two separate experiments in 2013.

		Percentage Survival	DIR
SOV	df	Mean Square	Mean Square
run	1	0.039	0.038
error a	6	0.016	0.012
family	1	0.043	0.157*
family*run	1	0.001	0.018
error b	6	0.010	0.030
cycle	2	0.571**	0.999**
cycle*run	2	0.007	0.018
cycle*family	2	0.015	0.018
cycle*run*family	2	0.001	0.037
error d	24	0.003	0.013
inoc	1	19.85**	0.103*
inoc*run	1	0.038*	0.007
inoc*family	1	0.043*	0.466**
inoc*cycle	2	0.571**	1.030**
inoc*run*family	1	0.0001	0.005
inoc*run*cycle	2	0.007	0.001
inoc*family*cycle	2	0.015	0.097*
inoc*run*family*cycle	2	0.001	0.002
error d	36	0.004	

*, ** Significant at $p<0.05$ and $p<0.01$, respectively.

Table 13. Cross-resistance percentage survival of Luxor and TAM 86I[3]-16 following two cycles of single plant selection for resistance to *R. solani* were challenged with *P. ultimum* under growth chamber conditions.

Cycle of selection	No inoculum	1x selection rate[†]
C_0	73 a A[‡]	38 c B
C_1	68 a A	75 b B
C_2	72 a A	99 a B

[†] 1x rate = 3 mycelial mats/1,000 g soil

[‡] Values within columns followed by the same lower case letter, and values within rows followed by the same upper case letter, are not different at $p < 0.05$.

Table 14. Cross-resistance DIR[§] on families originally bred for *R. solani* resistance, in each cycle of selection when challenged with *P. ultimum* inoculum. Four replications were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

Family	No inoculum	1x selection rate[†]
Luxor-C_0	0	87 de
Luxor-3-C_1	0	63 g
Luxor-3-C_2	0	47 h
TAM 86I^3-16-C_0	0	95 abc
TAM 86I^3-16-6-C_1	0	74 fg
TAM 86I^3-16-6-C_2	0	45 h

[†] 1x selection rate = 3 mycelial mats/1,000 g soil.
[§] Note that raw DIR averages were reported, arcsin transformed values were analyzed.

TAM 87N-6 and TAM 86E-8 families originally tested and screened for *P. ultimum* resistance, were inoculated and evaluated for resistance at the zero and 1x selection rate of *R. solani* inoculum. Percentage survival of TAM 87N-6 and TAM 86E-8 families were not significantly different when challenge with *R. solani*, and the two families responded similarly to the two levels of inoculum as indicated by a nonsignificant interaction between family and inoculum (Table 15). Selection cycle had a significant impact on survival and the two families responded similarly to the two cycles as indicated by non-significance for inoc*family. A significant inoc*cycle interaction suggested that the average performance of each family within each cycle was not consistent within inoculum levels. The inoc*cycle interaction was caused by extremely low survival in the C_0 lines (Table 16). Percentage survival improved from an average of 10% at C_0 averaged over the two families to 36% at C_1 then decreased to 28% at C_2, when challenged with a 1x rate of *R. solani*, indicating that single plant selection for *P. ultimum* did result in a measurable improvement in resistance to *R. solani*. As was true for Luxor and TAM $86I^3$-16, the three way interaction of inoculum x family x cycle for DIR statistically required the separation Family x Cycle lines within inoculum levels although family and cycle sources of variation for DIR were significant (Table 15). The inoculum levels result in a significant difference between levels because DIR for the no inoculum level is zero and thus any impact of the pathogen would result in a biological difference. While mean separation in Table 17 indicates a significant difference among the Family x Cycle lines, the general trend in DIR within the 1x rate was a slight decrease in DIR with each cycle of selection within both TAM 87N-6 and TAM 86E-8.

Table 15. ANOVA for sources of variation on percentage survival and disease index rating (DIR) after cross infection by *R. solani* on lines originally screened for *P. ultimum* resistance. Four reps were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

		Percentage Survival	DIR
SOV	df	Mean Square	Mean Square
run	1	0.076	0.069
error a	6	0.015	0.016
family	1	0.004	0.116*
family*run	1	0.001	0.001
error b	6	0.019	0.010
cycle	2	0.166**	0.099*
cycle*run	2	0.026	0.032
cycle*family	2	0.003	0.133
cycle*run*family	2	0.025	0.022
error c	24	0.015	0.011
inoc	1	14.64*	4.766**
inoc*run	1	0.076	0.049
inoc*family	1	0.004	0.359
inoc*cycle	2	0.333**	0.039
inoc*run*family	1	0.001	0.001
inoc*run*cycle	2	0.053	0.003
inoc*family*cycle	2	0.007	0.129*
inoc*run*family*cycle	2	0.050	0.002
error d	36	0.013	0.018

*, ** Significant at $p<0.05$ and $p<0.01$, respectively.

Table 16. Cross-resistance percentage survival of TAM 87N-6 and TAM 86E-8 following two cycles of single plant selection for resistance to *P. ultimum* were challenged with *R. solani* under growth chamber conditions.

Cycle of selection	No inoculum	1x selection rate[†]
C_0	62 a A[‡]	10 c B
C_1	72 b A	36 a B
C_2	58 a A	28 b B

[†] 1x rate= 0.125 g wheat bran inoculum/1,000 g soil

[‡] Values within columns followed by the same lower case letter, and values within rows followed by the same upper case letter, are not different at $p < 0.05$.

Table 17. Cross-resistance DIR[§] on families originally bred for *P. ultimum* resistance, in each cycle of selection when challenged with *R. solani* inoculum. Four replications were performed in a growth chamber at 30º C repeated in two separate experiments in 2013.

Cross-resistance: DIR[§] over experiments		
Family	No inoculum	1x selection rate[†]
TAM 87N-6-C_0	0	95 ab
TAM 87N-6-3-C_1	0	89 cd
TAM 87N-6-3-C_2	0	79 ef
TAM 86E-8-C_0	0	98 a
TAM 86E-8-1-C_1	0	93 bcd
TAM 86E-8-1-C_2	0	85 de

[†] 1x rate= 0.125 g wheat bran inoculum/1,000 g soil.
[§] Note that raw DIR averages were reported, arcsin transformed values were analyzed.

Pearson's correlation for improvement in cross-resistance of all lines tested was -0.89. That is, as DIR decreased (symptoms were less severe), percentage survival increased. Overall, these data suggest that while response to cross inoculation varied among families, selection for resistance to one CSDC pathogen affected resistance for another CSDC pathogen within these HT and Luxor families.

5. CONCLUSIONS

The objective of this study was to analyze the effectiveness of IPS as a screening tool to select for resistance to *R. solani* and *P. ultimum* within a number of HT germplasm lines and an obsolete cultivar, Luxor. The data reported herein suggested:

- Individual plant selection is an effective tool to quickly screen cotton seedlings for resistance to *R. solani* and *P. ultimum;*
- Families responded differently to cycles of selection, as indicated by the family x cycle interaction, and indicated that the effectiveness of IPS is specific to the line being evaluated;
- Disease Index Rating is negatively correlated with percentage survival over cycles of selection; that is, resistance is increased within families with succeeding cycles of selection so that disease severity decreased while percentage survival increased within the families tested;
- Cross-resistance for resistance to both *R. solani* and *P. ultimum* in the same family is possible in certain families; further evaluation is needed to determine the mechanism for resistance to each pathogen.

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