EVALUATING COTTON GENETIC RESISTANCE TO RENIFORM NEMATODE ACROSS ENVIRONMENTS USING REMOTE MEASUREMENTS

A Thesis

by

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ABSTRACT

Reniform nematode, *Rotylenchulus reniformis*, is a highly detrimental pest in cotton and necessitates improved understanding of both in-season detection and modern management options. Newly released genetically resistant varieties have proven to be a more consistent management option than traditional nematicides to maintain yields while simultaneously suppressing reniform nematode (REN) populations. However, knowledge is lacking to inform producers when to prioritize nematode resistance over industry-leading susceptible varieties relative to nematode pressure and production environment. Trials were conducted at College Station, Wall, and Lubbock, TX with objectives to evaluate variety performance across a wide range of environments and to delineate between abiotic and biotic plant stress like nitrogen and water availability, using in-season remote measurements. In variety evaluations, two REN varieties were compared to four reniform-susceptible industry-leading varieties in the respective regions. In remote measurement trials, PHY 443 W3FE (resistant) was compared to PHY 480 W3FE (susceptible) under different irrigation regimes and fertilizer N rates to induce abiotic stress similar to reniform nematode stress. Across all site-years, resistant varieties increased lint yields by 51% compared to susceptible varieties ($p < .0001$), and suppressed nematode densities by 53% ($p < .0001$). Plant heights were affected by variety in both irrigation regimes at College Station and Wall rainfed trials, where resistant varieties were consistently taller than susceptible varieties and had larger canopy percentages. Green-Red Vegetation Index (GRVI) values indicated that in dryland systems the resistant variety was greener but in irrigated systems nitrogen influenced GRVI and the susceptible variety was greener. Reniform nematode resistant varieties resulted in greater yield gains in irrigated systems with large reniform nematode populations but at all site years genetic resistance improved lint yields and suppressed nematode populations. GRVI was not a consistent indicator of reniform nematode stress but did correlate consistently with fertilizer N rate in irrigated trials. Within irrigation regimes, plant height and canopy percentage were consistently correlated with reniform nematode stress and are likely the most useful remote measurements to estimate in-field nematode distribution and severity.

DEDICATION

Dedicated to my Papa and Grandaddy for their unwavering love and support.

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Contributors

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NOMENCLATURE

REN	Reniform nematode resistant
SUS	Reniform nematode susceptible
GRVI	Green red vegetative index
UAV	Unmanned aircraft vehicle
RACE	Replicated agronomic cotton evaluation
SAS	Statistical analysis software
RGB	Red green blue
VIs	Vegetative indices
QGIS	Quantum geographic information system
Cli-MATE	Midwest Regional Climate Center's application tools environment
NO_3-N	Nitrate nitrogen
N	Soil applied nitrogen
m	meter
cm	centimeter
mm	millimeter
cc	cubic centimeter
nm	nanometer

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

CHAPTER I

INTRODUCTION OF AGRONOMIC EFFECTS ON COTTON DUE TO RENIFORM NEMATODE

Introduction

The reniform nematode, *Rotylenchulus reniformis*, is a plant parasitic nematode attracting major attention from cotton (*Gossypium hirsutum*) growers in Texas. First discovered on Hawaiian cowpea roots in 1940 by Linford and Oliveira, the parasitic roundworms were found in cotton fields in the continental U.S. over the next 50 years where they have become an established threat to the southern cotton belt. While infestations of reniform nematodes don't affect fiber quality, they can drastically reduce cotton yield by decreasing boll size and number of bolls (Jones et al., 1959). Lawrence (2022) stated, "The reniform nematode is an economic tragedy reducing the grower's profitability by half." Robinson (2007) indicates that reniform nematodes have replaced Meliodogyne incognita (Root-knot nematode) as the costliest nematode in cotton. As a cotton plant endures environmental stress, it becomes more susceptible to other pathogens (Dasgupta et al., 1993). This makes reniform nematodes detrimental to growers already struggling with negatively contributing abiotic factors such as the persistent drought conditions of Texas. An estimated 469,000 bales were lost to all diseases in Texas in 2018, with a loss of 49,000 bales attributed to reniform nematodes (K. Lawrence et al., 2019). The overall objectives of this work were to characterize genetically tolerant cotton varieties according to different levels of nematode pressure, nitrogen, and irrigation applications.

Literature Review

Reniform nematode parasitism on cotton

The initial discovery of reniform nematodes on tropical plants in Hawaii led reniform nematodes to be classified as a subtropical pest, which reduced awareness of its pest potential in cotton (Koenning et al., 2004). Unlike the preferences of other parasitic nematodes, reniform nematodes can thrive in fine textured soils (Koenning et al., 1996). Although reniform nematodes persist in the roots of cotton, reduced fiber yields from above ground stunting is often the first noticeable symptom. In a study published in 1959, cotton plants under reniform nematode stress in a greenhouse had a 12.6% reduction in biomass, 21.3% reduction of total boll weight, and a 20.7% reduction in number of bolls (Jones et al., 1959). Alongside yield reduction, mechanical damage from nematode feeding sites leaves the plant vulnerable to fungal and bacterial infection. The presence of reniform nematodes can increase the susceptibility of seedlings to fungal borne pathogens (Dasgupta M, 1993). This is demonstrated in the relationship between Fusarium wilt and the root-knot nematode, where the injury to the roots from feeding sites allows the fungal pathogen to establish itself within the cotton root.

Reniform nematode management options

Traditionally, nematode populations have been managed using nematicides that can be costly, dangerous to apply, and produce inconsistent results (Grabau et al., 2021). Rotational cropping with non-host crops like corn or grain sorghum is effective at suppressing populations but is not often implemented due to greater near-term economic opportunity with continuous cotton. Although rotation with non-host crops can help negate subsequent cotton yield loss, the persistence of nematode populations below the plow layer limits the effectiveness of single year rotations and necessitates more long-term solutions (Koenning et al., 2000). Stetina et. al (2007) demonstrate that after one year of a non-host, nematode populations recover rapidly within a single growing season of a susceptible crop. Two years of a non-host crop, in this case corn, was necessary to suppress nematode populations below damaging levels after returning to cotton.

Nematicide options for reniform nematode are limited initially by the wide range of soil textures that they inhabit. Some commonly used nematicides are specifically effective in coarse textured soils (e.g. fumigants like telone). The most successful nematicides for reniform nematodes are applied in-furrow at planting (aldicarb and fluopyram) or applied post-emergence to the cotton (oxamyl). Efficacy of nematicides is variable, and often highly dependent on environmental conditions, including precipitation near the time of application (Grabau et al., 2021). All of these products bear additional costs and potential hazards for growers. Field research comparing various combinations of these products across Texas concluded that nematicides did not consistently improve cotton yields, and the added input cost generally accounted for a net economic loss (Dudak et al., 2020). Under ideal soil conditions, reniform nematode eggs can enter an anhydrobiotic state where they can persist in the soil for up to two years (Koenning et al., 2004). A study in 2005 indicated that populations of reniform nematode could be found at depths up to 91 cm (Robinson et al., 2005). Foliar and soil applied nematicides are ineffective on nematode populations below the plow layer, reducing the impact of nematicides as a long-term solution.

Stand establishment with strong seedling vigor is essential for cotton growing with nematode pressure. Wanjura et al. (1969), established that early emergence of seedlings has a strong positive correlation with cotton yield. High vigor seedlings are also less affected by early-season pathogen pressure, “which lessens the potential for early-season crop loss” which can be associated with reniform nematode damage (Chastain et al., 2020). Female reniform nematodes mature fully after just four weeks, at which point reproduction can occur (Lawrence, 2021). Varieties with higher vigor are more likely to overcome initial reniform nematode pressure before plant maturity. Studies as early as 1989 demonstrate that while breeding for root-knot nematode tolerance to red clover, screening for early vigor decreased root gall and egg mass scores at harvest (Quesenberry et al., 1989). Although nematicides do not consistently improve yields, the addition of nematicides prior to or at planting could suppress initial populations of nematodes allowing varieties with lower vigor to establish healthy root systems before feeding sites are established. A recent report states that a standalone option for nematode control is not sustainable and combinations of practices are necessary for management, the most important factor being the selected cultivar that all other management options will be based around (Lawrence, 2021).

Cotton varieties with genetic tolerance to reniform nematodes have recently become commercially available. Early attempts to breed cotton varieties with resistance to reniform nematode resulted in stunted plants with depressed yields, but continued efforts resulted in highly resistant cultivars without the previous yield suppression (Khanal et al., 2018). A recent study reported that although varieties with reniform nematode resistance can improve yields in infested fields, the same varieties planted along susceptible varieties in a non-infested yield were not as profitable (Plumblee & Mueller, 2021). Varieties with genetic tolerance were first commercially available in 2021 and could outgrow the early season nematode pressure without the help of additional nematicides. These resistant varieties can better tolerate the nematode parasitism which in turn allows the cultivar to maintain productive yields in infested fields. When a reniform nematode embeds itself into the roots of resistant cotton, the cells surrounding the nematodes head will lignify or collapse, referred to as programmed cell death. This reduces the rate of nematode feeding and allows the plant to overcome the parasitism (Mota et al., 2013). Reproduction still occurs, but at a much slower rate than would be possible on susceptible cotton.

CHAPTER II

EFFECTS OF COTTON GENETIC RESISTANCE TO RENIFORM NEMATODE ACROSS ENVIRONMENTS

Introduction

Rotylenchulus reniformis, reniform nematode, is an established pest in the southern U.S. cotton belt with drastic impact on upland cotton production. This microscopic roundworm establishes feeding sites in the roots of young cotton plants diverting nutrients essential for growth and early vigor. This creates chlorotic seedlings with stunting occurring in waves across a cotton field that can be easily mistaken for nutrient or water deficiency but can have a significant impact on yield (Lawrence, 2021). Nematode feeding sites on cotton make the plant more susceptible to disease and other environmental factors (e.g., drought, nutrient stress, extreme temperatures, etc.) (Dasgupta et al., 1993). Under nematode stress, cotton bolls are smaller and more likely to abort with additional stress which in turn reduces yields and growers profit margins (Jones et al., 1959). Unlike most nematodes, soil texture has little effect on reniform nematodes as they can survive in fine textured soils (Koenning et al., 1996). The soil borne pathogen is invisible to the naked eye, making it extremely easy to transfer between fields on tillage equipment, harvesters, and even soil from the tires of trucks. This contributes to the rapid spread of reniform nematodes over the cotton belt and its increasing priority in southern cotton production.

Once reniform nematodes have been introduced into a field, they can start reproducing in the presence of a host in as little as two weeks (Lawrence, 2021). This rapid reproduction makes it challenging to stay ahead of the infection and growers without the proper management tools can find themselves struggling to maintain productive cotton yields while nematode populations continue to grow. Strong seedling vigor is needed for cotton seedlings established in an infected field, nematicides can be used to give seedlings the early season advantage needed to overcome the infection. In the past, nematicides have been applied as fumigants, seed treatments, and as foliar treatments to suppress population sizes during the growing season. Nematicides can produce inconsistent results (Dudak et al., 2020) (Grabau et al., 2021) and are non-selective within the soil profile which can have a negative impact on the soil microbial system (Dasgupta et al., 1993). Crop rotation is another common tool used to suppress nematode populations, adding a non-host crop into growing rotation for more than one year can suppress nematode populations and improve production for the next cotton season (Stetina et al., 2007). This method is not easily accepted as cotton typically has a higher financial return and rotational cropping requires additional equipment.

Cotton varieties with genetic resistance have become commercially available to producer's combatting nematodes. In recent years, genetic resistance has been a more consistent management tool for reniform nematodes compared to nematicides (Dudak et al., 2020). Using a resistant variety in place of nematicides can reduce traffic through the field for secondary applications of nematicides and requires little involvement after the initial seed purchase. When genetic resistance to reniform nematodes was first introduced, producers were concerned that the development of a new trait in early commercial varieties could result in yield reduction. This yield reduction has not been a concern in modern commercially available varieties with genetic resistance to reniform nematodes. Resistant varieties can shut down cells surrounding nematode feeding sites, slowing nematode reproduction, and maintaining boll production potential. Applications of nematicides can be influenced by soil texture, application timing, and placement, whereas planting resistant seed requires little to no additional planning. Using genetic resistance as a management tool may be necessary to manage reniform nematodes, but more research is needed to best utilize resistant varieties. Previous research evaluating the value of genetic resistance as a management tool (Dudak et al. 2020), emphasized differences in resistant varieties performance under different management systems. Research is still needed to characterize resistant variety effects under different environments. The objectives of this research were to assess the efficacy of genetic resistance as a management tool under different soil types, levels of nematode pressure, and growing environments.

Methods

Field research trials to address these objectives were coordinated at three sites in Texas (Wall, College Station, and Lubbock, TX) over the 2021 and 2022 growing seasons. Sites were chosen due to their long history of commercial cotton production and the presence of reniform nematodes. The site located in Wall, Texas was on an Angelo Clay loam (Fine-silty, mixed, superactive, thermic Aridic Calciustolls) (USDA-NRCS, 2022) under continuous cotton with conventional tillage and furrow irrigation. The field site at College Station (Texas A&M University farm) was on a Belk Clay (Fine, mixed, active, thermic Entic Halpuderts) (USDA-NRCS, 2022). The soil at the Lubbock site was an Acuff loam (Fine-loamy, mixed superactive, thermic Aridic Paleustolls) (USDA-NRCS, 2022) and Olton clay (Fine, mixed, superactive, thermic Aridic Paleustols). Both the College Station and Lubbock locations are established reniform nematode research sites with AgriLife Research and Extension in continuous cotton with conventional tillage and furrow irrigation. Variety trials were coordinated at all three sites, and genetic resistance × fertilizer N rate trials were conducted at Wall and College Station.

Weather data was gathered from Cli-MATE weather stations that best represented each site (Cli-MATE Daily Observed Station Selector, 2022). Climate varied between the three locations, with lower precipitation accumulation at both West Texas sites. Average annual precipitation at College Station, Wall, and Lubbock is 1050 mm year^{-1}, 530 mm year^{-1}, and 460 mm year^{-1}, respectively, based on a 30-year annual rainfall average. Prior to planting, “pre-season” composite nematode samples were taken during moist soil conditions to establish a baseline for reniform nematode populations. In 2021 pre-season samples were examined by range under the assumption that reniform nematodes were consistent throughout the field site. In 2022 all nematode samples were examined on a per-plot basis to increase the resolution of population densities within the field.

In all three locations, reniform nematode resistant cotton varieties DP 2143NR B3XF and PHY 443 W3FE were used. To compare, four different reniform nematode susceptible varieties of similar maturity were selected for each location according to previous RACE trial results and local grower recommendations. For both Lubbock and Wall, the varieties DP 1948 B3XF, PHY 480 W3FE, FM 2398 GLTP, and NG 4098

B3XF were selected. Varieties selected for College Station included DP 1646 B2XF, PHY 400 W3FE, ST 5707 B3XF, and NG 4936 B3XF. One variety was only available with the nematicide Copeo (fluopyram). To maintain uniformity across treatments in 2021, Copeo was added (0.06 oz lb seed^{-1}) secondarily to untreated varieties. In 2022, varieties previously treated with Copeo were washed for 30 seconds in pure acetone under a vent hood and then left to dry. Test plots were planted, with washed and unwashed treatments, and stands were compared to ensure that establishment was not affected by removal of seed treatment. Plots were 12.19 m long and 4 rows wide, cotton was planted on 1.02 m rows at 111,150 seeds hectare^{-1} in all locations.

Nematode extractions were conducted using a modified Baermann funnel, the pie pan method (Thistlethwayte, 1970). 200cc of soil was added to a double layer of tissue on top of wire mesh in an aluminum pie pan. 250cc of water was then added to the pie pan and the sample was left to steep for two days to allow time for live nematodes to migrate from the soil into the water. After 48 hours, the nematode solution was removed from the pie pan. The solution was then moved to a beaker where it rested for 45 minutes to allow the nematodes to settle in the bottom of the solution. After resting, the samples were then reduced to 100cc of solution. Next, a 5cc homogenous subsample was removed and added to a grid marked petri dish. One half of the petri dish were counted and extrapolated to nematodes 200cc soil^{-1}.

Trials were arranged as a randomized complete block design with four replications. Within locations, trials were duplicated to include both a rainfed and irrigated location. Results were analyzed in SAS using linear mixed models where variety or genetic resistance, and nitrogen rate (in trials with N treatments) were fixed effects. Results were analyzed within location and irrigation regime. Year and block nested within year were treated as random. Power transformations were applied to lint yield and nematode densities according to the Box-Cox method to meet requirements for normality and variance (Box and Cox, 1964). Means were separated using Tukey's HSD (α = 0.1) and back-transformed for presentation.

Table 1. Monthly average precipitation in 2021 and 2022 and departures from the 30-year (1994-2023) averages at College Station, Wall, and Lubbock, TX.

	College Station		Wall		Lubbock	
	2021	2022	2021	2022	2021	2022
	mm					
January	69 (54) *	107 (92)	30 (9)	3 (-18)	25 (-45)	5 (-66)
February	47 (32)	58 (43)	10 (-18)	9 (-19)	6 (-55)	3 (-58)
March	44 (15)	90 (61)	13 (-25)	13 (-25)	59 (-27)	1 (-85)
April	84 (51)	70 (37)	45 (8)	3 (-34)	5 (-61)	0 (-66)
May	183 (112)	62 (-8)	36 (-41)	38 (-39)	149 (34)	91 (-24)
June	94 (35)	5 (-54)	124 (67)	19 (-38)	63 (-23)	21 (-65)
July	102 (52)	0 (-50)	93 (65)	0 (-28)	76 (20)	3 (-53)
August	56 (7)	115 (66)	135 (70)	62 (-3)	92 (7)	151 (66)
September	70 (8)	65 (3)	10 (-52)	45 (-17)	15 (-75)	21 (-69)
October	107 (65)	47 (5)	67 (4)	59 (-4)	16 (-108)	61 (-63)
November	62 (42)	131 (110)	26 (-6)	71 (38)	10 (-75)	15 (-70)
December	58 (41)	141 (124)	1 (-19)	26 (7)	6 (-87)	10 (-82)

*****Parenthetical values show departures from 30-year averages at College Station, Wall, and Lubbock, TX.

Table 2. Monthly average air temperature in 2021 and 2022 and departures from the 30-year (1994-2023) averages at College Station, Wall, and Lubbock, TX.

	College Station		Wall		Lubbock	
	2021	2022	2021	2022	2021	2022
	°C					
January	11 (6) *****	10 (5)	8 (0)	8 (0)	5 (-6)	4 (-1)
February	9 (2)	10 (3)	6 (-4)	7 (-3)	3 (-10)	4 (-3)
March	17 (5)	16 (4)	15 (0)	15 (0)	12 (-5)	11 (-1)
April	20 (4)	23 (7)	18 (-1)	22 (3)	15 (-6)	18 (2)
May	24 (3)	27 (6)	23 (-1)	28 (4)	20 (-5)	24 (3)
June	28 (2)	31 (5)	28 (0)	30 (2)	26 (-2)	27 (1)
July	29 (1)	33 (5)	28 (-1)	32 (3)	26 (-4)	30 (3)
August	30 (3)	31 (4)	28 (-1)	30 (1)	26 (-4)	27 (0)
September	27 (4)	27 (4)	26 (1)	26 (1)	24 (-3)	24 (2)
October	24 (7)	22 (5)	21 (2)	20 (1)	19 (-3)	16 (-1)
November	16 (6)	16 (6)	14 (1)	12 (-1)	11 (-5)	8 (-2)
December	19 (13)	13 (7)	16 (7)	10 (1)	11 (-1)	7 (2)

*****Parenthetical values show departures from 30-year averages at College Station, Wall, and Lubbock, TX.

Results

In 2021, College Station experienced a wetter growing season, averaging 46.5 mm more rain per month than the 30-year average between May and October. Temperatures in College Station were higher than normal in both years, with monthly averages ranging from 1-7 °C hotter during the growing season of 2021, and 4-6 °C hotter in 2022. In 2022 at College Station, May-July was drier than normal before rains started in August delaying harvest times. In Wall during 2021, June-August experienced an average of 67 mm more rain per month than the 30-year average. Conditions were extremely dry at Wall in 2022, monthly rainfall during the growing season was an average 22 mm less per month than the 30-year normal. Wall monthly temperatures in 2021 varied from the 30-year mean by only 1-2 °C during the growing season. In 2022 the growing April, May, June, and July were 3, 4, 2, and 3°C above normal, respectively.

In 2021, Lubbock received greater rainfall than normal during the summer and much less than normal in September through the remainder of the year. Similar to Wall, Lubbock was extremely dry in 2022 until August when precipitation was 66 mm more than the 30-year average, rainfall remained low for the rest of the year. While temperatures at Lubbock were 2-5°C below average in 2021, temperatures in 2022 were 1-3°C warmer than average throughout the cotton growing season.

Across all site years, apart from Lubbock dryland, variety affected cotton lint yield (Table 4). In College Station rainfed trials, PHY 443 W3FE (resistant) yielded more than NG 4936 B3XF and ST 5707 B2XF but was similar to other susceptible varieties ($p = 0.0007$). In the same environment, DP 2143NR B3XF (resistant) yielded more compared to ST 5707 B2XF. ST 5707 B2XF did not yield more than any other variety in both rainfed and irrigated conditions ($p = 0.0007$). In a rainfed environment at College Station, resistant varieties increased cotton lint yields by 29% more than susceptible varieties ($p <.0001$). Rainfed yields had more separation between means than irrigated trials. In College Station irrigated trials, PHY 443 W3FE and PHY 400 W3FE produced more cotton compared to ST 5707 B2XF ($p = 0.03$). DP 2143NR B3XF was not different from any other variety in irrigated conditions. College Station irrigated yields were increased by 22% with genetic resistance to reniform nematodes ($p <.0001$). In Wall, PHY 443 W3FE yielded more than all susceptible varieties under both rainfed and irrigated regimes. DP 2143NR B3XF yielded greater than PHY 480 W3FE in rainfed trials and both PHY 480 W3FE and FM 2398 GLTP in irrigated trials (rainfed; $p = 0.0002$, irrigated; $p <.0001$). In irrigated trials at Wall, DP 1948 B3XF yielded more than both FM 2398 GLTP and PHY 480 W3FE (*p* <.0001). In Wall, genetic resistance increased yields by 83% in rainfed trials and 113% in irrigated trials (*p* <.0001). Lubbock yields were only impacted by variety under irrigated conditions (Table 4), where PHY 443 W3FE yielded more than most susceptible varieties apart from PHY 480 W3FE (*p* = 0.005). DP 2143NR B3XF yielded greater than FM 2398 GLTP (*p* = 0.005) but was similar to PHY 443 W3FE and PHY 480 W3FE. Lubbock rainfed yields were increased by 36% with genetic resistance and 41% in irrigated conditions (rainfed; *p* = 0.05, irrigated; *p* = 0.002).

Nematode populations were affected by variety at College Station and in Wall rainfed trials (Table 3). In College Station DP 2143NR B3XF had less nematodes than DP 1646 B2XF and PHY 400 W3FE in rainfed conditions and PHY 400 W3FE in irrigated conditions. PHY 443 W3FE had similar nematode populations to all varieties in rainfed and irrigated conditions (rainfed; *p* = 0.05, irrigated; *p* = 0.07). Genetic resistance suppressed nematode populations by 67% in rainfed conditions and 53% in irrigated (rainfed; *p* = 0.02, irrigated; *p* = 0.03). In rainfed trials at Wall, DP 2143NR B3XF nematode populations were lower compared to PHY 480 W3FE but was similar to all other varieties (*p* = 0.06). PHY 443 W3FE nematode populations were not different from any other variety. At Wall in a rainfed environment, genetic resistance resulted in lower nematode populations by 38% (*p* = 0.07).

Final plant heights were influenced by variety in both irrigation regimes at College Station and in rainfed trials at Wall. At College Station in a rainfed environment, PHY 443 W3FE produced taller plants compared to most varieties except for ST 5707 B2XF where heights were similar (*p* <.0001). DP 2143NR B3XF was not taller than susceptible varieties in rainfed College Station trials (*p* <.0001). Under an irrigated regime in College Station, ST 5707 B2XF was taller than other susceptible varieties but was not different from resistant varieties (*p* = 0.0002). In Wall in a rainfed environment, PHY 443 W3FE was taller FM 2398 GLTP, NG 4098 B3XF, and PHY 480 W3FE. DP 2143NR B3XF was only taller compared to NG 4098 B3XF and PHY 480 W3FE (*p* = 0.0005).

Table 3. Cotton variety effects on lint yield, nematode densities, and final plant height within irrigation regime for College Station, TX.

	Lint yield		Nematode density		Final plant height	
Variety	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
	—— kg ha^{-1}——		—200 cc soil^{-1}—		—— cm ——	
PHY 443	815 a*****	1305 a	414 ab	529 ab	97.8 a	91.4 ab
DP 2143	686 ab	1168 ab	118 b	209 b	89 bc	91.5 ab
DP 1646	680 ab	1217 ab	856 a	439 ab	85.4 bc	83.8 bc
NG 4936	592 bc	1090 ab	390 ab	732 ab	81.3 c	76.9 c
PHY 400	748 ab	1257 a	1275 a	1096 a	83.8 c	82.1 bc
ST 5707	490 c	1029 b	575 ab	740 ab	93.3 ab	95.5 a

*****Within columns, means with the same letter are not different (α = 0.1).

Table 4. Cotton variety effects on lint yield, nematode densities, and final plant height within irrigation regime for Wall, TX.

	Lint yield		Nematode density		Final plant height	
Variety	Rainfed	Irrigated	Rainfed	Variety	Rainfed	Irrigated
	—— kg ha^{-1}——		—200 cc soil^{-1}—		—— cm ——	
PHY 443	602 a*****	961 a	3134 ab	4206 a	50.7 a	68.6 a
DP 2143	473 ab	786 ab	1399 b	3376 a	48.7 ab	69 a
DP 1948	368 bc	590 bc	3570 ab	5133 a	45.4 abc	65.1 a
FM 2398	296 bc	363 d	3305 ab	3798 a	39.6 bcd	61.8 a
NG 4098	332 bc	518 bcd	4934 a	9179 a	39.1 cd	65.9 a
PHY 480	255 c	384 cd	2236 ab	3816 a	35.7 d	63.1 a

*****Within columns, means with the same letter are not different (α = 0.1).

Table 5. Cotton variety effects on lint yield, nematode densities, and final plant height within irrigation regime for Lubbock, TX.

	Lint yield		Nematode density		Final plant height	
Variety	Rainfed	Irrigated	Rainfed	Variety	Rainfed	Irrigated
	—— kg ha^{-1}——		—200 cc $soil^{-1}$—		—— cm ——	
PHY 443	691 a*****	955 a	383 a	339 a	57.8 a	66.7 a
DP 2143	478 a	832 ab	139 a	355 a	58.4 a	53.3 a
DP 1948	458 a	639 bc	398 a	339 a	61.3 a	64.5 a
FM 2398	407 a	509 c	239 a	601 a	48.3 a	58.1 a
NG 4098	468 a	573 bc	580 a	504 a	57.8 a	53.3 a
PHY 480	405 a	722 abc	251 a	429 a	64.1 a	61.6 a

*****Within columns, means with the same letter are not different (α = 0.1).

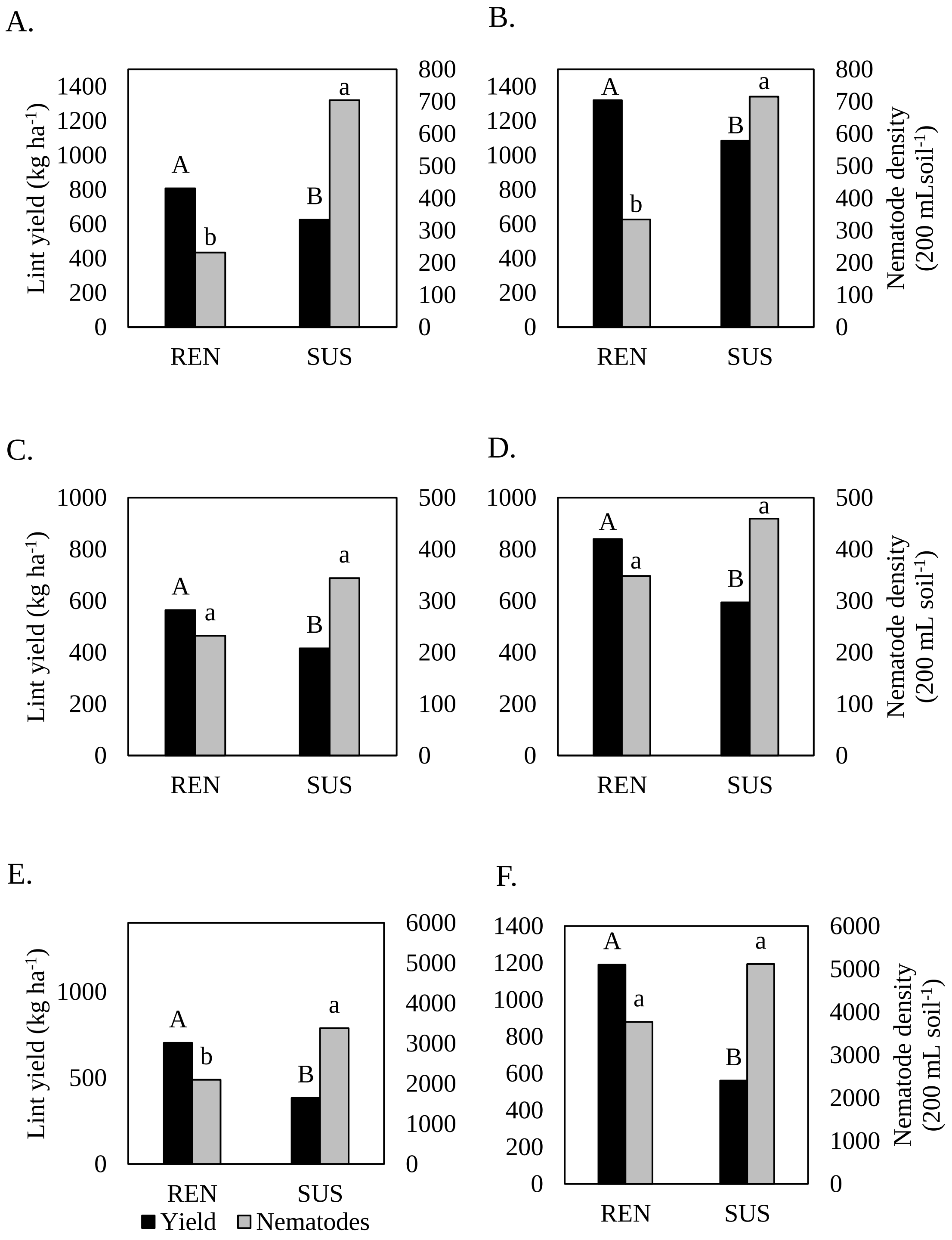

Figure 1. Cotton variety effect on lint yield and nematode densities within location and irrigation regime. (A. College Station rainfed, B. College Station irrigated, C. Lubbock rainfed, D. Lubbock irrigated, E. Wall rainfed, F. Wall irrigated.)

Discussion

The findings of this work emphasize variation between resistant variety performance among different environments and nematode densities. In lower input systems, and at sites with higher populations of reniform nematode, resistant varieties were more beneficial than when they were planted in higher input systems with lower nematode densities. Across all three sites Wall, maintained the highest nematode densities. It has been reported that reniform nematodes prefer sandier soil types, others have reported that soil texture has no effect on the distribution of reniform nematodes (Tu et al., 2003). Koenning et al. (2006), confirmed that reniform nematodes have the ability to survive and reproduce in fine textured soils. Of the three testing sites, Lubbock had the lowest populations of reniform nematode, lowest temperatures, highest latitude, and resulted in the least benefit from planting resistant varieties. Although in combined analyses, resistant varieties still increased cotton lint yields by 36% and 41% in rainfed and irrigated systems respectively (rainfed; $p = 0.05$, irrigated; $p = 0.002$). In Wall, where nematode populations and temperatures were highest there was a much greater yield benefit to planting resistant varieties. With the addition of genetic resistance, yields were increased by 83% and 113% when compared to susceptible varieties in the same environment ($p <.0001$). At both Lubbock and Wall, the yield benefit due to genetic resistance was greater in irrigated trials than rainfed. This supports that resistant cotton in lower input farming systems might be more influenced by the combination of nematode and water stress. Nematodes mobility is limited by excess soil moisture. Ahmed et al. (2014) suggested that longer breaks between irrigation timings would reduce soil moisture and increase nematode reproduction. In this work, nematode populations were higher in irrigated trials compared with rainfed; however, this may be attributed to relatively drier growing seasons resulting in shorter plants with smaller root mass and subsequently less nematode reproduction.

Nematode populations were significantly influenced by resistant varieties. Nyaku et al. (2016) stated that the hypersensitive response of resistant lines reduces reproduction rates of reniform nematodes. College Station and Wall dryland trials resulted in a reduction of nematode densities with genetic resistance. Lubbock populations were not affected by resistance, possibly due to lower densities and less rainfall events driving nematodes lower in the soil profile. Although nematode populations were lower at College Station than Wall, resistant varieties were still able to suppress populations. College Station is in a warmer and more humid environment than Lubbock which is better suited for nematode reproduction. Plants at College Station were considerably larger than plants at Lubbock, larger root masses are able to support larger populations of reniform nematodes which may explain why there were differences in College Station but not Lubbock.

Resistant varieties produce taller, larger plants with lower nematode populations below ground. Susceptible varieties were not able to overcome nematode stress and were stunted with lower yields. Plant heights and biomass measurements were less dependent on resistance as days after planting increased, indicating that reniform nematode stress has a greater effect on younger cotton plants. This is supported by previous work resulting in greater differences in plant heights between susceptible and resistant varieties at early season timings than late season (Dudak et al., 2020).

CHAPTER III

REMOTE SENSING IN COTTON TO INFORM RENIFORM NEMATODE MANAGEMENT

Introduction

Potential applications of remote sensing

In a cotton production site infested with reniform nematodes, early detection is key to prevent the spread throughout a field. Identifying a nematode infection starts when growers notice areas of reduced vigor and gradual yield declines. Current methods for diagnosis include taking a soil sample from the root zone of infected regions to be sent to the closest agricultural lab for analysis. With the help of Unmanned Aircraft Vehicles, or UAVs, growers could potentially spot the infection sooner.

UAVs can be bought at a wide price range and online tutorials allow them to be used after just a short learning curve. With practice, UAVs can be used to create a digital snapshot of a location at field scale that can be referenced long after the crop has been harvested to monitor distribution. Most UAVs on the market are equipped with a simple RGB camera, red (620-750nm) - green (495-570nm) - blue (450-495nm), that relies on visible light reflectance. RGB imagery can be manipulated to estimate remote measured canopy height, canopy area, and canopy volume as a function of the two. Many measurements can be analyzed using RGB imagery but with modifications UAVs can also be equipped with spectral or thermal sensors to increase the value of a flight.

Vegetative Indices, or VIs, are used to identify variation within a field that can be monitored over time to inform management decisions (Hatfield et al., 2019). VI's can be accurately used to estimate crop yield (Ji et al., 2021), identify drought stress (Ballester et al., 2019), and N status (Ballester et al., 2017), in cotton and small grain crops. These predictions are often correlated with a single response variable, but more research is needed to delineate between different stress factors within fields. Aerial imagery is being used to create maps at field scale, and using VIs, identify symptoms associated with nematode stress to identify potential yield loss. This is estimated using one or multiple VIs to quantify the radiation reflected off the leaf surface. Using a spectral vegetative index such as GRVI, green red vegetative index, to identify regions of stress would allow the grower to make nematode management decisions while waiting on lab results to confirm the infection. Although varieties with reniform nematode resistance have been proven to improve yields in infested fields, they may not have the same profitability when planted in non-infested fields (Plumblee & Mueller, 2021). With more research, variable application of resistant varieties, seed treatments, or nematicides could be informed by UAV imagery. The objectives of this work were to improve remote detection of reniform nematode presence and severity under different environments.

Methods

Field research trials to address this objective were conducted at two locations in Texas (Wall and College Station) over the 2021 and 2022 growing seasons. Sites were chosen due to their long history of commercial cotton production and the presence of reniform nematodes. The Wall, Texas location was on an Angelo Clay loam, (Fine-silty, mixed, superactive, thermic Aridic Calciustolls) (USDA-NRCS, 2022), and is managed for continuous cotton production with conventional tillage practices and furrow irrigation. The field site at College Station (Texas A&M University farm) was on a Belk Clay (Fine, mixed, active, thermic Entic Halpuderts)(USDA-NRCS, 2022). The College Station location is in a reniform nematode research location used by Texas A&M AgriLife Research and Extension, it is in a continuous cotton program with furrow irrigation. Varieties chosen to compare the performance of genetic resistance were PHY 443 W3FE (resistant) and PHY 480 W3FE (susceptible), selected for their similar maturity. In 2021 a composite soil sample was collected at depths of 0-15 cm and 15-30 cm at both locations to determine fertilizer recommendations and rates. In 2022 soil nitrogen samples were taken at depths of 0-15 cm, 15-30 cm, and 30-60 cm to measure residual NO_3-N content and better inform fertilizer N treatments. All NO_3-N testing was done through the Texas A&M soil and forage testing lab. Nitrogen treatments were selected based on fertilizer recommendations from pre-season nitrogen samples. Fertilizer treatments were applied at rates of 0, 22, 45, and 67 kg N ha^{-1} as a side dress application at pinhead square. Irrigation zones (rainfed vs. irrigated) were separated with a 4-row planted border between the two trials to provide a buffer zone for any irrigation runoff or lateral movement through the soil.

Aerial imagery was obtained using a DJI Phantom 4 Pro quadcopter (SZ DJI Technology Co., Ltd, Shenzhen, China) equipped with a single band red edge Sentera sensor fixed nadir, and a RGB camera on a gimble. Flight plans were configured using Pix4D Capture on an iPhone X with 80% front overlap and 70% side overlap and the

RGB camera fixed at 90 degrees. In 2021, flights were flown at a height of 54.86 m, and to increase the resolution in 2022, flights were lowered to 30.48 m. All imagery was stitched by Pix4D Mapper using the Multispectral 3D map option with additional outputs selected to isolate red, green, and blue pixels. Values including canopy volume, area, and plant height, were calculated using QGIS. Using the raster calculator, an excess green equation, (2 * (green / (red + green + blue)) - (red / (red + green + blue)) - (blue / (red + green + blue))), is used to isolate vegetation pixels. A “threshold layer” is then created to classify pixels as either plant or soil pixels. This is used to mask soil pixels from any further calculations. A grid is created in QGIS to identify treatment plots within the map to extract remote canopy measurements, i.e. canopy percentage, height, and GRVI.

Trials were managed, measured, and analyzed as described in the methods of Chapter 3. Locations were combined. When interactions occurred between N-rate and cotton variety (as was the case for final height) results were sliced by nitrogen treatment for interpretation.

Results

Cotton lint yields were influenced by variety in both irrigation regimes. In both environments, PHY 443 W3FE increased lint yields compared to PHY 480 W3FE by 34% and 39% in rainfed and irrigated trials respectively (p <.0001).

Nematode densities were affected by variety under both irrigation regimes. PHY 443 W3FE had lower nematode densities than PHY 480 W3FE by 48% and 50% in rainfed and irrigated trials, respectively (rainfed; $p = 0.0001$, irrigated p <.0001).

GRVI, green-red vegetative index, was influenced by resistance in both irrigation regimes. In rainfed systems, PHY 443 W3FE produced greener plants compared to PHY 480 W3FE ($p = 0.0002$). Under irrigated conditions, PHY 480 W3FE was greener than PHY 443 W3FE ($p = 0.006$). Fertilizer nitrogen also influenced GRVI in irrigated trials. With 67 kg N ha^{-1}, plants were greener than plants receiving 22 kg N ha^{-1} ($p = 0.02$).

Drone estimated canopy percentage was influenced by variety, but not by fertilizer N rate in both rainfed and irrigated trials. In both environments, PHY 443 W3FE had more canopy coverage than PHY 480 W3FE ($p <.0001$).

Plant height was indicative of genetic resistance mid-season (rainfed; $p = 0.005$, irrigated $p <.0001$), late-season (rainfed; $p <.0001$, irrigated $p <.0001$), and estimated from drone imagery (rainfed; $p = 0.002$, irrigated $p = 0.0005$). In all cases, PHY 443 W3FE plants were taller than PHY 480 W3FE. Nitrogen treatment had no influence on plant heights but there was an interaction between variety and nitrogen rate in rainfed treatments. In rainfed trials, susceptible varieties receiving 0 and 27 kg N ha^{-1} were shorter than other treatments ($p = 0.004$) (Figure 2). Differences in fiber quality were observed between varieties and are described in Appendix A.

Table 6. Cotton variety effect on plant heights across location and year.

	Drone Estimated Height		Mid-season Height		Final Heights	
Variety	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
	final height (cm)					
PHY 443 W3FE	10.4 a*	13.1 a	41.3 a	45.2 a	76.1 a	90.6 a
PHY 480 W3FE	8.7 b	10.5 b	35.3 b	34.6 b	67.3 b	77.1 b

*Within columns, means with the same letter are not different (α = 0.1).

Table 7. Influence of variety on GRVI and canopy percentage analyzed across location and year.

	GRVI		Canopy Percentage	
Variety	Rainfed	Irrigated	Rainfed	Irrigated
PHY 443 W3FE	0.91 a*	0.93 b	0.41 a	0.5 a
PHY 480 W3FE	0.9 b	0.92 a	0.3 b	0.34 b

*Within columns, means with the same letter are not different (α = 0.1).

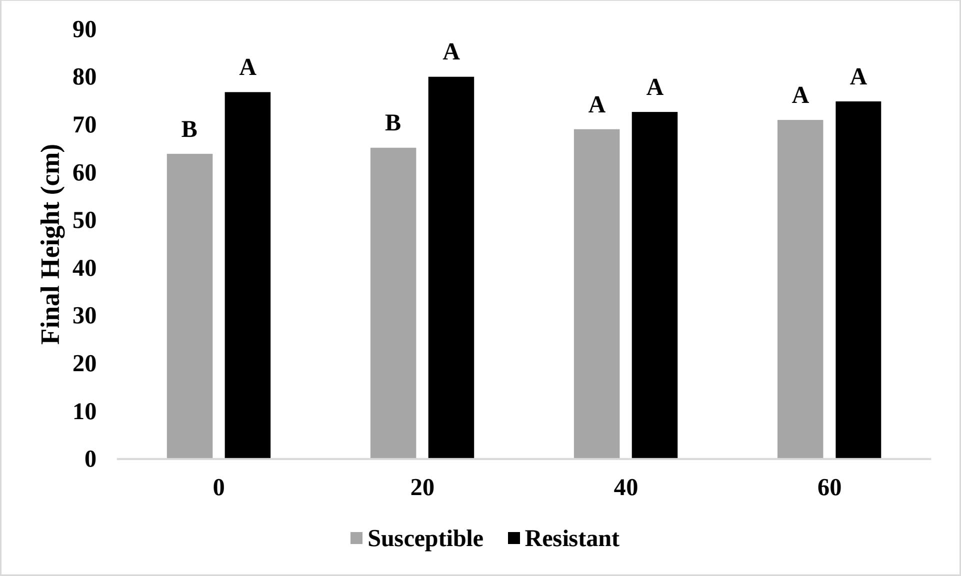

Figure 2. Effects of genetic resistance and fertilizer N rate on final plant heights in rainfed trials.

Discussion

Of the two site locations, College Station being a clay and Wall a clay loam, Wall had substantially higher nematode populations. Although reniform nematodes tend to favor loamy soil types, Tu et al. (2003), stated that soil texture had no effect on reniform nematode activity. This is also supported by Koenning et. al (2006) that confirmed reniform nematodes are able to thrive in fine textured soils. Though both sites are under a cotton-intensive crop rotation, Wall has longer periods between rainfall events which can be beneficial to nematode reproduction. Ahmed et al. (2014), speculated that higher rates of nematode reproduction occur when periods of lower soil moisture occur due to nematodes lower mobility in higher water contents.

In both irrigation regimes, the variety with genetic resistance to reniform nematodes (PHY 443 W3FE), suppressed nematode populations by 48% and 50% in rainfed and irrigated trials respectively (rainfed; $p = 0.0001$, irrigated $p < .0001$). Nyaku et al. (2016) explained that the hypersensitive response of resistant lines reduces reproduction rate of reniform nematodes. Where initial populations of reniform nematodes may have been similar prior to planting, susceptible varieties do not have the hypersensitive response to nematode feeding that could protect them and reproduction is still occurring in the root system. Susceptible treatments that have nematode stress compounded with water stress resulted in the least benefit from genetic resistance. These findings emphasize the value of managing reniform nematodes in a ‘high-input’ system with adequate nitrogen fertilization and supplemental irrigation.

As nitrogen treatments were increased, plant heights between susceptible and resistant varieties were not different. Possibly, when compounding stresses are reduced, in this case N stress, cotton plants were able to overcome the damaging effects of nematode feeding. This is supported by a study done in crookneck squash in 1980 which emphasized that applying urea at rates of over 0.4 g/kg soil reduced the negative effects of root-knot nematode damage (Rodriguez-Kabana & King, 1980).

GRVI was influenced by applied nitrogen rates in both irrigation regimes. In a rainfed environment PHY 443 W3FE produced greener plants than PHY 480 W3FE. This did not occur in irrigated trials, where PHY 480 W3FE was greener than PHY 443 W3FE. Irrigated varieties were taller with more biomass, which may have needed more nitrogen than was applied, which could explain only seeing a nitrogen effect in irrigated plots. If rainfed plots had more than sufficient nitrogen, we would not expect as apparent of differences between treatments. Resistant plants receiving supplemental irrigation possibly needed more nitrogen than was applied and were chlorotic in response. Although GRVI was correlated with nitrogen rate within an irrigated regime, it was not a consistent indicator of nematode stress. Within irrigation regimes, plant height and canopy percentage were the most consistent indicators of nematode densities and yield predictors. These measurements were extracted using RGB technology and open source software accessible to most drone operators.

CHAPTER IV

CONCLUSIONS

Genetic resistance to reniform nematodes is a consistent management option to negate reniform nematode damage to cotton. The addition of genetic resistance increased plant heights, canopy biomass, and yield while reducing nematode populations. As management systems increase input, the benefit of genetic resistance is also increased. While dryland fields with lower nematode densities still resulted in a benefit from resistant varieties, in irrigated systems with high populations of nematodes, the increase in yield was larger. Reniform nematode resistant varieties are successful in many environments due to their ability to resist nematode feeding sites. Our results didn't support any reduction of yield due to genetic resistance to reniform nematodes.

Using RGB based remote measurements, canopy percentage and plant height were a consistent indicator of reniform nematode stress. GRVI taken in an irrigated environment, indicated that resistant varieties are greener than susceptible varieties. In a rainfed system nitrogen rate did not influence GRVI but an interaction between nitrogen rate and genetic resistance indicated that susceptible varieties receiving less than 45 kg N ha^{-1} were shorter than other treatments. Susceptible varieties in a reniform nematode infected field are not able to thrive under nematode stress and as a result are shorter, smaller plants with reduced yields and supported larger nematode densities. GRVI was not a consistent indicator of reniform nematode stress. Plant height and canopy percentage within irrigation regime could be correlated to nematode stress and lower yields associated with that stress. These differences in varieties were detected using limited remote technologies with mid-season measurements.

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APPENDIX A

EFFECT OF RENIFORM NEMATODE RESISTANCE ON COTTON FIBER QUALITY

Results

Cotton management and Agronomy

Micronaire was influenced by variety in all trials and irrigation regimes. In College Station rainfed trials, DP 2143NR B3XF had higher mic than PHY 443 W3FE and PHY 400 W3FE, PHY 443 W3FE was not different from susceptible varieties (p =0.006) (Table 8). DP 1646 B2XF had greater micronaire than PHY 400 W3FE in a rainfed environment (p =0.006). In an irrigated environment at College Station DP 2143NR B3XF produced cotton lint with a higher micronaire than PHY 443 W3FE, NG 4936 B3XF, and PHY 400 W3FE but was similar to DP 1646 B2XF and ST 5707 B2XF (p =0.002). At Wall rainfed trials, DP 2143NR B3XF had higher micronaire than most varieties except for FM 2398 GLTP which was similar ($p < 0.0001$) (Table 9). In a Wall irrigated system, DP 2143NR B3XF had larger micronaire compared to all other varieties ($p < 0.0001$). At Lubbock under a rainfed regime, DP 2143NR B3XF and FM 2398 GLTP had larger micronaire than PHY 480 W3FE, PHY 443 W3FE was similar to all varieties (p =0.01) (Table 10). In Lubbock in an irrigated environment, PHY 443 W3FE had a higher micronaire than DP 1948 B3XF but was similar to other susceptible varieties (p =0.04).

Cotton lint strength was influenced by variety in Wall under both irrigation regimes and Lubbock irrigated. In Lubbock with supplemental irrigation, DP 2143NR B3XF produced stronger fibers compared to DP 1948 B3XF and PHY 480 W3FE (*p* <.0001) (Table 10). PHY 443 W3FE strength was not different from other varieties in this environment (*p* <.0001). In Wall in a rainfed environment, DP 1948 B3XF and NG 4098 B3XF were stronger than PHY 443 W3FE, FM 2398 GLTP, and PHY 480 W3FE. DP 2143NR B3XF was stronger than FM 2398 GLTP and PHY 480 W3FE in the same conditions (*p* <.0001) (Table 9). In Wall irrigated trials, DP 2143NR B3XF, DP 1948 B3XF, and FM 2398 GLTP were stronger than PHY 443 W3FE and NG 4098 B3XF.

Cotton lint length was affected by variety in Wall, College Station rainfed varieties, and Lubbock irrigated varieties. In College Station in a rainfed regime, DP 1646 B2XF produced longer fibers than most varieties, except for PHY 400 W3FE which was similar in length (*p* =0.0008) (Table 8). In Lubbock in irrigated trials, DP 2143NR B3XF and DP 1948 B3XF were longer than PHY 443 W3FE but similar to other varieties (*p* =0.05) (Table 10). At Wall in a rainfed environment, DP 1948 B3XF and NG 4098 B3XF produced longer lint than all other varieties (*p* <.0001) (Table 9). Under irrigated conditions in a similar trend, DP 1948 B3XF and NG 4098 B3XF produced the longest fiber length (*p* <.0001). In the same environment, DP 2143NR B3XF produced longer lint than PHY 480 W3FE but was similar to PHY 443 W3FE and FM 2398 GLTP (*p* <.0001).

Table 8. Cotton variety effect on cotton lint fiber strength, length and micronaire within irrigation regime at College Station, TX.

	Strength		Length		Micronaire	
Variety	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
	——g tex^{-1} ——		—— in ——		——µg in^{-1} ——	
PHY 443	26.8 a*	29 a	1.07 bc	1.09 a	4.21 bc	4.6 b
DP 2143	27.5 a	29.6 a	1.08 bc	1.09 a	4.74 a	5.12 a
DP 1646	27.2 a	28.3 a	1.12 a	1.12 a	4.58 ab	4.91 ab
NG 4936	27.8 a	27.5 a	1.05 c	1.11 a	4.32 abc	4.53 b
PHY 400	27.5 a	29.7 a	1.1 ab	1.11 a	4.1 c	4.66 b
ST 5707	28.2 a	29.6 a	1.07 bc	1.11 a	4.35 abc	4.86 ab

*****Within columns, means with the same letter are not different (α = 0.1).

Table 9. Cotton variety effect on cotton lint fiber strength, length and micronaire within irrigation regime at Wall, TX.

	Strength		Length		Micronaire	
Variety	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
	——g tex^{-1} ——		—— in ——		——µg in^{-1} ——	
PHY 443	29.4 a*	31 ab	1.06 b	1.09 bc	4.46 bc	4.34 b
DP 2143	30 a	31.7 a	1.06 b	1.12 b	4.98 a	4.91 a
DP 1948	29.6 a	29.2 b	1.11 a	1.16 a	4.47 bc	4.17 b
FM 2398	29.7 a	30.5 ab	1.06 b	1.09 bc	4.67 ab	4.27 b
NG 4098	29.6 a	31 ab	1.13 a	1.17 a	4.24 c	4.09 b
PHY 480	28 a	29.5 b	1.05 b	1.08 c	4.16 c	4.22 b

*****Within columns, means with the same letter are not different (α = 0.1).

Table 10. Cotton variety effect on cotton lint fiber strength, length and micronaire within irrigation regime at Lubbock, TX.

	Strength		Length		Micronaire	
Variety	Rainfed	Irrigated	Rainfed	Variety	Rainfed	Irrigated
	——g tex^{-1} ——		—— in ——		——µg in^{-1} ——	
PHY 443	28.3 bc*	29.3 b	1.08 a	1.11 b	4.64 ab	4.89 a
DP 2143	29.6 ab	31.1 a	1.12 a	1.16 a	4.94 a	4.6 ab
DP 1646	29.9 a	30.9 a	1.12 a	1.16 a	4.59 ab	4.32 b
NG 4936	27.8 c	29.4 b	1.1 a	1.13 ab	4.77 a	4.74 ab
PHY 400	30.8 a	31.7 a	1.12 a	1.15 ab	4.67 ab	4.43 ab
ST 5707	27.1 c	28.7 b	1.07 a	1.13 ab	4.33 b	4.51 ab

*****Within columns, means with the same letter are not different (α = 0.1).

Results

Reniform nematode management in cotton using remote technology

Micronaire was influenced by variety in Wall but not at College Station. In Wall, under both irrigation regimes, PHY 443 W3FE (resistant) produced cotton lint with a higher mic than PHY 480 W3FE (susceptible) ($p < .0001$). Fiber length was only affected by variety in College Station. In both irrigation environments, PHY 480 W3FE produced fibers that were longer than PHY 443 W3FE (rainfed; $p = 0.01$, irrigated $p = 0.04$). Fiber strength was influenced by variety in College Station rainfed trials and all trials at Wall. In College Station in a rainfed environment, PHY 443 W3FE produced stronger fibers compared to PHY 480 W3FE ($p = 0.002$). At Wall under both irrigation regimes, PHY 443 W3FE had stronger fiber than PHY 480 W3FE (rainfed; $p = 0.001$, irrigated $p = 0.004$). Lint uniformity was impacted by variety at Wall in irrigated conditions, PHY 480 W3FE had more uniformity than PHY 443 W3FE ($p = 0.05$).