EVALUATION OF THE TEMPORAL AND SPATIAL OCCURRENCE OF *FUSARIUM OXYSPORUM* F. SP. *VASINFECTUM* RACES AS INFLUENCED BY SELECTED COTTON GENOTYPES IN THE NATIONAL COTTON FUSARIUM WILT EVALUATION FIELD IN ALABAMA

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Abstract

Fusarium oxysporum f. sp. *vasinfectum* (FOV) is pathogenic to cotton not only in the United States but also in all major cotton growing regions around the world. Different biotypes known as races have been documented infecting cotton and inducing symptoms of wilting, stunting, chlorosis and necrosis of leaves, vascular discoloration, and even plant death. This pathogen also interacts with nematodes such *Meloidogyne incognita*, the root-knot nematode (RKN) to cause even more damage to cotton. Multiple races of the FOV pathogen have been found coexisting in a single field. Some races are more commonly found at certain times of the cotton season, such as race 4 in the western part of the United State commonly infecting and causing stand reductions in the early part of the season. This study was initiated to 1) Determine the in-season temporal variability of FOV races on selected Upland, Pima, and Acala cotton cultivars with known tolerance or resistance to RKN and certain FOV races. 2) Map the spatial variability of FOV races across a naturally infested field. Fungal isolates were taken from plants exhibiting symptoms of FOV infection at weekly intervals for the first six weeks and bi-weekly for the remainder of the season. Portions of the Translation elongation factor (EF-1α) were used to identify race designation for each isolate of FOV collected and this information was used to determine the temporal and spatial distribution of the pathogen.

Introduction

Fusarium wilt is caused in many crops worldwide by the soilborne fungal pathogen *Fusarium oxysporum*. This diverse group of fungal pathogens has more than 120 forma specialis according to the host crop that they infect. *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) is a cotton pathogen that was first discovered in 1892 from samples collected in Alabama and Arkansas (Atkinson 1892). This pathogen is capable of causing large amounts of damage and crop loss. It is estimated that during the 2017 cotton production season fusarium wilt caused a crop loss of 0.33% or more than 69,000 cotton bales across the United States cotton belt (Lawrence et al. 2018). However, this pathogen is not only a problem in the United States cotton industry. Since its discovery, the disease has now been found affecting cotton in every major cotton growing region around the world with the most recent being Australia where they found the disease in 1993 (Kochman 1995). Throughout the world and here in the United States, this disease is caused by multiple genotypes of the FOV pathogen which are known as races.

One way to differentiate between these races is by using a classification system originally proposed by Armstrong and Armstrong (1958). This method classified races using a host differential test which determined the ability of FOV isolates to cause disease on Yelredo soybeans, Gold Dollar tobacco, Grimm alfalfa, and a range of cotton species and cultivars (Armstrong and Armstrong 1958). The validity of this method of separating FOV into races has been questioned and now races are commonly determined by sequencing portions of several genes and comparing these to sequences of references isolates from each race. Genes commonly used for this are the translational elongation factor (EF-1α), Beta-tubulin (BT), Phosphate:H^+ symporter (PHO), and the intergenic spacer regions (IGS) (O'Donnell et al. 1998; Tooley et al. 2001; O'Donnell et al. 2000; Appel and Gordon 1996). Not all isolates match one of the original races and a different designation is used when new genotypes of FOV are discovered such as LA-108, LA-110, and MDS-12.

It has been reported that a single cotton field can contain multiple races of the pathogen (Smith 2015). However, little is known about how these diverse races of FOV interact with one another within a field. Some races such as race 4 are thought to occur only early, in the first few weeks of the season, while others are known to infect cotton throughout the season (Hutmacher et al. 2011). This study investigated the temporal and spatial distributions of FOV races within a single field previously documented as having a diverse population of the pathogen (Smith 2015).

Materials and Methods

Testing was conducted at the Plant Breeding Unit of Auburn University's E. V. Smith Research Center in Tallassee, AL in the National Cotton Fusarium Wilt Evaluation Field. This field has been used for 66 years to evaluate new cotton genotypes and breeding lines for their resistance or susceptibility to fusarium wilt. The trial field is a Kalmia loamy sand soil type consisting of 80% sand, 10% silt, and 10% clay. Past testing has revealed this field to have a diversity of FOV races and it is known to contain an established population of *Meloidogyne incognita* (root-knot nematode). This cotton test was planted on May 17, 2018 in a Latin Square design with 10 replications using a John Deere MazEmerge (John Deer; Moline, IL) planter with Almaco cone planters (Almaco; Nevada, IA). Each test plot consisted of one row that was 7.6 meters long with a 0.9-meter row spacing and a 1.8-meter alley between each replication. Each plot was planted with one of eight cotton cultivars (Table 1) that were chosen for this test based on their resistance or susceptibility traits for either FOV or root-knot nematode (RKN). Cotton cultivars Rowden and M-315 were used as a FOV susceptible and resistance checks respectively and were included in the test twice to help standardize the test.

Table 1: List of cotton cultivars and their resistance traits to either *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) or the root-knot nematode (RKN).

Cotton Cultivar	Resistance traits
Upland Cotton – *Gossypium hirsutum*	
Rowden	FOV and RKN susceptible
M-315	RKN Resistant
Stoneville 4946 GLB2	RKN tolerant
PhytoGen 480 W3FE	RKN resistant
DeltaPine 1558NR B2XF	FOV susceptible and RKN resistant
Acala Cotton - *Gossypium hirsutum*	
PhytoGen 72	Moderately susceptible to FOV
Pima Cotton – *Gossypium barbadense*	
PhytoGen 800	FOV race 4 resistant
Pima S7	FOV susceptible

Sample collection of symptomatic FOV-infected plants began two weeks after cotton planting and continued on a weekly basis for the first six weeks; thereafter samples were taken every other week until the cotton was defoliated in preparation for harvest. At each sampling date, any cotton plants from the test plots that exhibited visual symptoms of fusarium wilt (ex. wilting) were removed from the soil using a shovel and transported to the lab for fungal isolation. Fungal isolations were accomplished by splitting the stem and upper taproot of the cotton plants using a scalpel. Three small sections of the vascular tissue were removed from each plant. Each section was surface sterilized in 95% ethanol for 30 seconds and a 0.625% NaOCl solution for 1 minute and placed onto a Petri dish containing half-strength acidified potato dextrose agar (APDA). These Petri dishes were incubated at room temperature for three to five days allowing for fungal growth; pure cultures were transferred to new half strength APDA plates.

Race identification of each FOV isolate collected throughout the growing season was obtained by sequencing portions of a particular gene in the FOV genome and comparing these to sequences from specific reference isolates. DNA was first extracted from each fungal isolate by transferring a morphologically pure cultural from each isolate to a new half strength APDA plate containing a sterile cellophane sheet (Bennett et al. 2013). These fungal isolates were allowed to grow for 5-10 days and then the mycelium was harvested from each Petri dish by scraping the surface of the cellophane sheet with a sterilized scalpel. DNA was extracted from this mycelium using a Quick-DNA Fungal/Bacterial Miniprep Kit following the manufacturer's protocol. Samples were stored at -20°C until further use.

Fragments of the EF-1α gene were sequenced for identification of the isolates (Table 2). PCR amplification was carried out in 0.2 ml PCR tubes containing 25 µL of JumpStart™ REDTaq® ReadyMix™ reaction mix (Sigma-Aldrich; St. Louis, MO), 10 mM of each primer, 3 µl of DNA template, and 20 µL of nuclease-free water.

Amplification was carried out in a MultiGene thermocycler (Labnet international; Edison, NJ) as follows: 94°C for 2 minutes followed by 40 cycles of (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute) and a final extension of 72°C for 5 minutes (Kim et al. 2005). PCR products were sent to Eurofins Genomics (Louisville, KY) for purification and sequencing. Primers used for sequencing were the same as were used for amplification.

Table 2: Primers used for race identification of FOV isolates

Locus	Primer Sequence	Reference
Translational elongation factor (EF-1α)		
EF-1	ATGGGTAAGGAAGACAAGAC	(O'Donnell et al. 1998)
EF-2	GGAAGTACCAGTGATCATGTT	
Beta-tubulin (BT)		
BT 3	CGTCTAGAGGTACCCATACCGGCA	(Tooley et al. 2001)
BT 5	GCTCTAGACTGCTTTCTGGCAGACC	
Phosphate:H^+ symporter (PHO)		
PHO 1	ATCTTCTGGCGTGTTATCATG	(O'Donnell et al. 2000)
PHO 6	GATGTGGTTGTAAGCAAAGCCC	
Intergenic spacer regions (IGS)		
CNS1R	GAGACAAGCATATGACTAC	(Appel and Gordon 1996)
28F	CTGAACGCCTCTAAGTCAGAA	

DNA sequencing results were aligned using BioEdit Sequence Alignment Editor and were manually adjusted (Hall 1999). Sequence alignments were compared to previously published reference sequences downloaded from GenBank (Holmes et al. 2009). Phylogenetic analyses were conducted using (MEGA7) Molecular Evolutionary Genetics Analysis version 7 (Kumar et al. 2016). A phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Branching patterns were determined by a bootstrap method with 1000 replicates.

Results and Discussion

Throughout the season more than 300 plant samples were collected from the FOV field used in this study. A total of 126 FOV isolates were obtained during the 2018 cotton season. Upon race identification of each of these isolates, a diverse population of FOV was found (Figure 1). Seven races were identified in the field at some point during the season (Table 3). During the season, 3 isolates were found that matched race 4 and race 4-like (MDS-12) type isolates. The primers used in the DNA sequencing process so far are not specific enough to differentiate between these two races of FOV. FOV race 4 is a very problematic pathogen that so far has only been found in California and Texas in the United States, and it is not known to exist in the state of Alabama (Kim et al. 2005; Halpern et al. 2017). However, the MDS-12 isolates of FOV are of much less concern and were originally found from samples in Alabama and Mississippi (Bennett et al. 2013). The isolates found in this study are believed to be the MDS-12 isolates for a few reasons. First, all of these isolates collected were obtained from the Rowden cultivar. Rowden is an upland cotton which is known to be less susceptible to FOV race 4 than Pima cottons (Hutmacher et al. 2011). The second reason to believe that these isolates are the MDS-12 type is the times which they were collected from the field. The isolates were collected throughout the season with one being found at the last sampling date (September 10th) just before

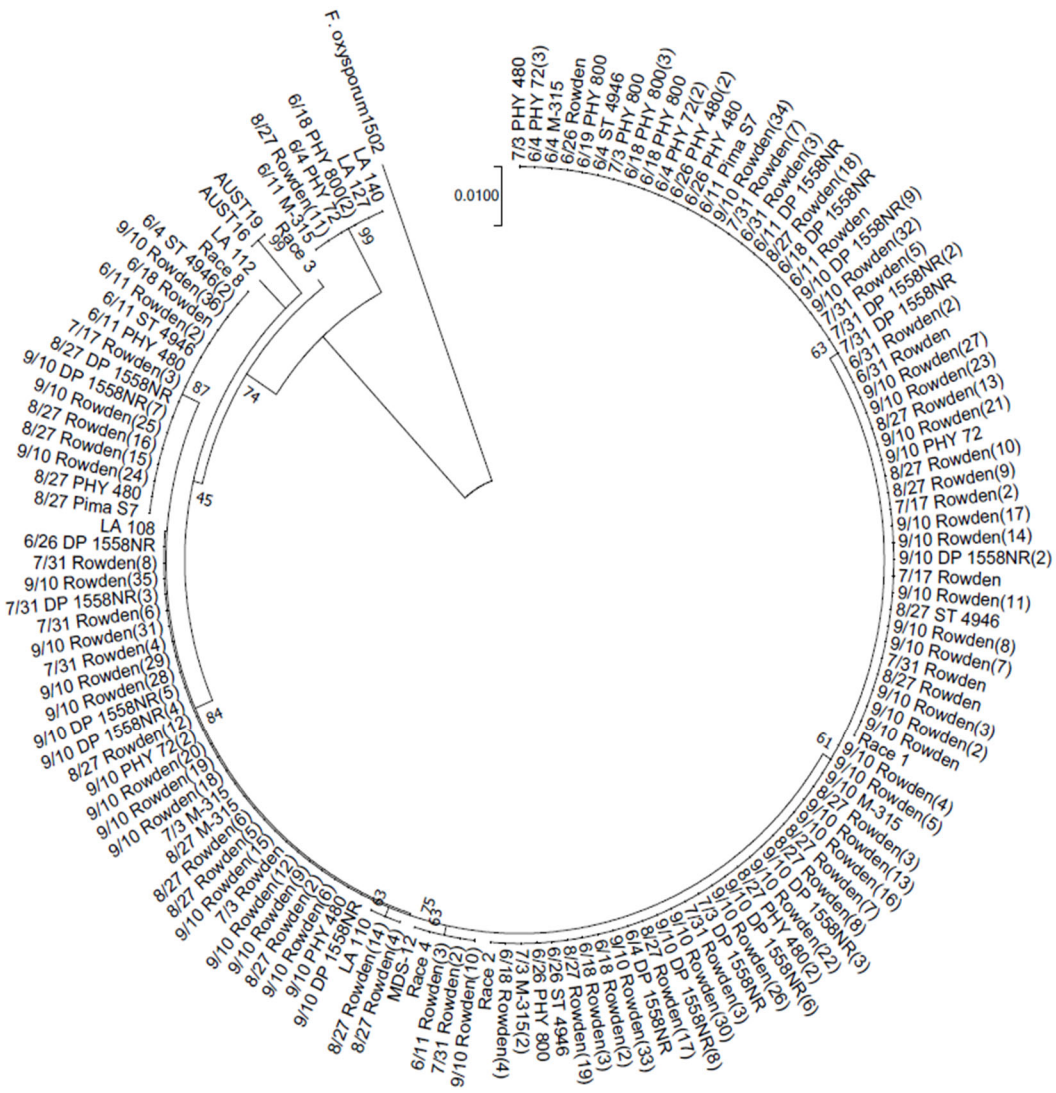

Figure 1: Phylogenetic tree of FOV isolates collected in 2018 season using sequences of the translation elongation factor 1-α. The tree was constructed in MEGA7 using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-1261.60) is shown. Bootstrap frequencies from 1,000 replication are noted next to every branch. Isolates collected during this study are labeled in the tree as the date the sample was collected followed by the cotton cultivate that the samples was collected form. For example isolate 7/3 PHY 800 was collected on July 3 from a plot containing the PHY 800 cultivar. If more than one sample was collected on the same date from the same cultivar an isolate number in parenthesis will follow the cultivar name, for example 6/4 PHY 72(3). A saprophytic *F. oxysporum* (isolated 1502) was used as an outgroup to root the tree. Reference isolates used as comparison were Race 1 isolate ATCC 16421, Race 2 isolate ATCC 1661, Race 3 isolate ATCC 16612, Race 4 isolate ATCC 16613, Race 8 isolate 031665, MDS-12 isolate AL-KL25, and Australian biotypes isolate AUST16 and Aust19.

defoliation of the cotton. This would be unusual if these were race 4 isolates which have been found predominantly in the early season and less commonly found after the first few weeks of cotton growth (Hutmacher et al. 2011). Confirmation of isolate ID, race 4/MDS-12, will be confirmed upon further sequencing using the intergenic spacer region.

Table 3: Total number of FOV isolates separated by race/genotype ID and by cotton cultivar source from the E.V. Smith Research Center, Tallassee, AL 2018 field season.

	Race 1	Race 2	Race 4 / MDS-12	Race 8	LA-108	LA-110	LA 127/140	**Total**
Upland Cotton – *Gossypium hirsutum*								
Rowden	28	17	3	8	19	2	1	**78**
M-315	1	2	0	0	1	0	1	**5**
ST 4946GLB2	2	1	0	2	0	0	0	**5**
PHY 480W3FE	3	1	0	2	1	0	0	**7**
DP 1558NR B2RF	6	5	0	2	5	0	0	**18**
Acala Cotton - *Gossypium hirsutum*								
PHY 72	1	0	0	1	0	0	0	**2**
Pima Cotton – *Gossypium barbadense*								
PHY 800	4	1	0	0	0	0	1	**6**
Pima S7	2	0	0	0	1	0	1	**4**
Total	**47**	**27**	**3**	**15**	**27**	**2**	**4**	

Cotton cultivar played a large role in the number of FOV isolates that were collected. FOV was isolated from each of the cultivar groups and cultivars included in the test (5 upland, 1 Acala, and 2 Pima) including the cultivars that were resistant to either FOV of RKN. The cultivar with the highest incidence of FOV was the susceptible check Rowden where a total of 78 FOV isolates were identified. At least one isolate of each race found in the test was recovered from the Rowden cultivar. The highest incidence was race 1 which had 28 isolates recovered from the Rowden cultivar. All three of the isolates that grouped with race 4 and MDS-2 were recovered from plots containing the Rowden cultivar. However, this high rate of infection is no surprise due to the high susceptibility of this cultivar to both FOV and RKN. Test plots containing the cultivar Deltapine 1558NR B2RF resulted in the next highest recovery of FOV with a total of 18 isolates during the season, even with the cultivar's high resistance to RKN. This demonstrates that nematode resistant alone is not enough to entirely protect a plant from fusarium wilt if it is susceptible to the FOV pathogen. The lowest amount of infection came from plots planted with the Pima S7 cultivar only 2 isolates were recovered throughout the season. This variety is susceptible to some races of FOV but has been used in tests as a resistant check for FOV race 1 (Hutmacher et al. 2013). This resistance and the fact that race 1 was the predominate FOV race in the field most likely resulted in the low isolation rate in this cultivar.

Plants samples symptomatic of FOV infection were collected throughout the entire season at each of the sampling dates included in this study. Sampling began May 31, two weeks after cotton planting and at this time, four isolates with FOV symptoms were collected. Three of these isolates grouped with race 1 in the phylogenetic analysis and one isolate grouped with race 4/MDS-12 isolates. Plant samples with FOV symptoms were acquired at every subsequent sampling date of the season. A sharp increase in the number of symptomatic plants was observed from the end of July until the cotton was defoliated. During this time 83% of the samples collected belonged to either the Race 1, 2 or LA-108 genotypes. There was an increase in the frequency of isolation in all three of these genotypes late in the season which was not observed in the other races and genotypes of FOV found during this study. This demonstrates that these three races of FOV are active through the entire season. Also, plants may become more susceptible to infection of one of these races late in the season when they are undergoing stress of flowering and boll production.

Fusarium oxysporum f. sp. *vasinfectum* isolates were found well distributed through the test field. No areas of the field were found to be free of FOV. However, some areas of the field appeared to have higher rates of infection caused by specific races of FOV, and these races were not found or only found at low levels in other parts of the field. This pattern was primarily observed for races 1 and 2. Race 1 was primarily found in the southwestern corner of the cotton field while race 2 was more predominant in the northeastern corner (Figure 2). Some overlap was observed between the two races; however, a distinct pattern was observed. It was not clear what the reason was for

the separation between these two races or if it was a result of abiotic effects such as changes in soil type or soil moisture across the field. Other races of FOV that were collected from the field were either found to be well

Figure 2: Field diagrams demonstrating the percentage of plants infected with race 1 and race 2 of *Fusarium oxysporum* f. sp. *vasinfectum* as indicated by the color of each plot. Each square in the figures represents one field plot. The top number within each plot represents the plot number (ex. 101, 102, and 103), the bottom numbers indicates the cultivar of cotton grown in that plot.

distributed (Race 8 and LA-108) across the field or were only found at low levels (Race 4/MDS-12, LA-110, LA-127/140) and no pattern of infection could be discerned.

Summary

A diversity of FOV races were found coexisting within this cotton field. In total, eight different races and genotypes of FOV were collected throughout the 2018 cotton season. The most predominate races isolated from the field were race 1, 2 and LA-108. The majority of the isolates were collected in the latter part of the season when the cotton was under stress from boll production. The majority of the samples collected in this test were obtained from the Rowden cotton cultivar. This cultivar was included in the test as a susceptible check so it is no surprise that the infection rates were so high. The cultivar Deltapine 1558NR B2RF had the second highest number of FOV isolates. This cultivar is resistant to RKN and demonstrates that a cotton cultivar cannot be fully protected from FOV using only RKN resistance. If the cotton is susceptible to the FOV pathogen and the field contains sufficient populations of that pathogen, high levels of infection can still occur. Some races (race 1 and 2) were spatially segregated within the cotton field. It is unclear as of yet what field effect(s) may be impacting these races or if similar trends will be observed in future research. So far, these isolates have been identified using portions of a single, EF-1α, gene. Future work will include sequencing of the BT PHO, and IGS regions of the DNA to confirm the race of each isolate collected (Table 2).

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