MULTI-YEAR STUDY OF THE TEMPORAL VARIABILITY OF *FUSARIUM OXYSPORUM* F. SP. *VASINFECTUM* RACES AS INFLUENCED BY COTTON CULTIVAR IN THE NATIONAL COTTON FUSARIUM WILT EVALUATION FIELD IN ALABAMA

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Abstract

Fusarium wilt of cotton, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), can be found in cotton globally. Different races and genotypes have been documented infecting cotton and inducing symptoms of wilting, stunting, chlorosis and necrosis of leaves, vascular discoloration, and even plant death. Some races of this pathogen also interact with nematodes such as *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 causing disease at certain times of the cotton season, such as race 4 in the western part of the country, commonly infecting and causing stand reductions in the early part of the season. This study was initiated to 1) Assess the population diversity of FOV in the National Cotton Fusarium Wilt Evaluation Field located in Tallassee, AL. 2) To determine the in-season and between season temporal variability of FOV races on selected Upland, Pima, and Acala cotton cultivars with known susceptibility or resistance to RKN and certain FOV races. Plants exhibiting symptoms of FOV infection were collected from the field for fungal isolation at weekly intervals for the first six weeks and bi-weekly for the remainder of the season. From each FOV isolate collected from the field, portions of the Translation elongation factor (EF-1α), β-tubulin (Bt) and the phosphate permease (PHO) were sequenced to identify the race designation for each isolate. In total, eight different races and genotypes of FOV were collected throughout the 2018 and 2019 cotton seasons. The most predominant races isolated from the field were race 1, LA-108, race 2, and race 8, samples of these races appeared throughout the growing season.

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 2019 cotton production season fusarium wilt caused a crop loss of 0.3% or more than 59,600 cotton bales across the United States cotton belt (Lawrence et al. 2020). 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.

Armstrong and Armstrong (1958) devised a classification system that 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. 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 permease (PHO), and the intergenic spacer regions (IGS) (O'Donnell et al. 1998; Tooley et al. 2001; O'Donnell et al. 2000; O'Donnell et al. 2009). 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 mostly 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 distribution 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 more than 60 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 genotypes and it is known to contain an established population of *Meloidogyne incognita* race 3 (root-knot nematode). Three cotton tests were planted on May 17, 2018 (one test) and April 24, 2019 (two tests) in a Latin Square design with 10 replications using a John Deere MaxEmerge (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. Correlations were analyzed using Proc Corr via SAS 9.4 (SAS Institute, INC; Cary, NC) with a significance level of $P \leq 0.05$.

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
PhytoGen 480 W3FE	RKN resistant
DeltaPine 1558NR B2XF	FOV susceptible and RKN resistant
Stoneville 4946 GLB2	RKN tolerant, FOV resistant
Acala Cotton - *Gossypium hirsutum*	
PhytoGen 72	FOV resistant, RKN susceptible
Pima Cotton – *Gossypium barbadense*	
PhytoGen 800	FOV race 4 resistant, RKN susceptible
Pima S7	FOV and RKN susceptible

Sample collection of plants showing symptoms commonly associated with fusarium wilt (wilting, chlorosis or necrosis of leaves) began two weeks after cotton planting and continued on a weekly basis for the first six weeks. After this time 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 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 particular genes 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 culture 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 translational elongation factor (EF-1α), Beta-tubulin (BT), and phosphate permease (PHO) genes were sequenced for identification of each isolate collected in 2018; only data from the EF-1α gene portion sample data from 2019 is reported (Table 2). PCR amplification was carried out in 0.2 ml PCR tubes containing 12.5 µL of JumpStart™ REDTaq® ReadyMix™ reaction mix (Sigma-Aldrich; St. Louis, MO), 0.5 µl of each primer (10 mM), 1.5 µl of DNA template, and 10 µL of nuclease-free water. Amplification was carried using specific primers and thermocycler setting listed in Table 2. 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. Use of these three gene fragments is sufficient to identify most races of FOV however they are not specific enough to differentiate between FOV race 4 and MDS-12 isolates. These two types of FOV are identical at the sequenced part of the EF-1α, BT, and PHO, however, they are very different in terms of pathogenicity and impact to the cotton industry of the isolates.

Table 2: Primers used for race identification of FOV isolates

Locus	Primer Sequence	Reference	Thermocycler Settings
Translational elongation factor (EF-1α)			
EF-1	ATGGGTAAGGAAGACAAGAC	(O'Donnell et al. 1998)	94°C for 2 min followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min with a final extension of 72° for 5 min
EF-2	GGAAGTACCAGTGATCATGTT		
Beta-tubulin (BT)			
BT 3	CGTCTAGAGGTACCCATACCGGCA	(Tooley et al. 2001)	94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1.5 min with a final extension of 72° for 10 min
BT 5	GCTCTAGACTGCTTTCTGGCAGACC		
Phosphate permease (PHO)			
PHO 1	ATCTTCTGGCGTGTTATCATG	(O'Donnell et al. 2000)	97°C for 1 min followed by 35 cycles of 96°C for 30 sec, 50°C for 1 min, and 72°C for 1 min with a final extension of 72° for 10 min
PHO 6	GATGTGGTTGTAAGCAAAGCCC		
Race 4 specific primers			
R4F	GCTCCGTGTCWGAGCTTCTT	(Yang et al. 2006)	94°C for 3 min followed by 10 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec, 25 cycles 90° for 30 sec, 59° for 30 sec, and 72° for 15 sec with a final extension of 72° for 1 min
R4R	TGCTCATCGTGGAGCATAAC		
Multiplex PCR for detection of TƒO1, MULE/ TƒO1, and MITE/ TƒO1 insertions in the PHO gene			
FovP-f	GGCCGATATTGTCGGTCGTA	(Ortiz et al. 2017 and Bell et al. 2019)	94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 40 sec with a final extension of 72° for 5 min
FovM-R	CCGCCATATCCACTGAACA		
FovT-R	ATCTGTCTTTCGTCGGCAAT		
FovP-R	CTCCAGTGCAGTGCTTGGTA		
Intergenic spacer regions (IGS)			

CNS1	GAGACAAGCATATGACTAC	(O'Donnell et al. 2009)	94°C for 90 sec followed by 40 cycles of 94°C for 30 sec, 58°C for 90 sec, and 68°C for 3 min with a final extension of 68° for 5 min
NL11	CTGAACGCCTCTAAGTCAG		
iNL11	AGGCTTCGGCTTAGCGTCTTAG		Used for sequencing only
NLa	TCTAGGGTAGGCKRGTTTGTC		
CNSa	TCTCATRTACCCTCCGAGACC		
iCNS1	TTTCGCAGTGAGGTCGGCAG		

Any isolates that were identified as race 4 or MDS-12 were tested further using a three-step approach to identify the isolates. First, a multiplex PCR analysis using primers FovP-F, FovM-R, FovT-R, and FovP-R was conducted to look for gene insertions in the PHO gene of the isolates. Some race 4 isolates that have been collected in the United States have one of 3 insertions in the PHO gene which do not exist in the MDS-12 genotype. After the PCR was conducted samples were run through a 1.5% agarose gel electrophoresis to view the DNA fragment size. Isolates without a gene insertion will have a DNA fragment size of 396 bp while an isolate containing one of the gene insertions will range in size from 426-663 bp. Secondly, PCR was conducted on all isolates in question using the race 4 specific primer set R4F and R4R following the protocol outlined by Yang et al. (2006). Finally, nearly complete sequences of the IGS gene were obtained using the primers listed in Table 2 and following the protocol outlined by O'Donnell et al. (2009). These sequences were then compared to the same references isolates used for the EF-1α, BT, and PHO sequencing.

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. Phylogenetic analyses were conducted using (MEGAX) Molecular Evolutionary Genetics Analysis (Kumar et al. 2018). 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

A total of 126 FOV isolates were obtained during the 2018 cotton season and 488 isolates during the 2019 cotton season. Upon race identification of each of these isolates, a diverse population of FOV was found (Figure 1). Seven races or genotypes were identified in the field during 2018 and 8 races or genotypes were found during 2019. Figure 2 shows the breakdown of races found on each cultivar and how many isolates of that race were found. During the two seasons, Race 1 was the most common isolate found (39%). However, high amounts of the genotype LA 108 (23%), race 2 (16%) and race 8 (15%) were also found. Samples of the genotypes MDS-12, LA 110, LA 112, and LA 127-140 were also found at low levels. Samples of the genotype LA 112 were not found during 2018 test but were found at low levels during the 2019 year. During both years of testing 14 isolates were found that aligned with both the MDS-12 and race 4 references isolates when sequenced with the EF-1 α, BT, PHO primers. However, these primers are not specific enough to differentiate between these two types of isolates so further testing was conducted to determine if these isolates were of the MDS-12 or race 4 types.

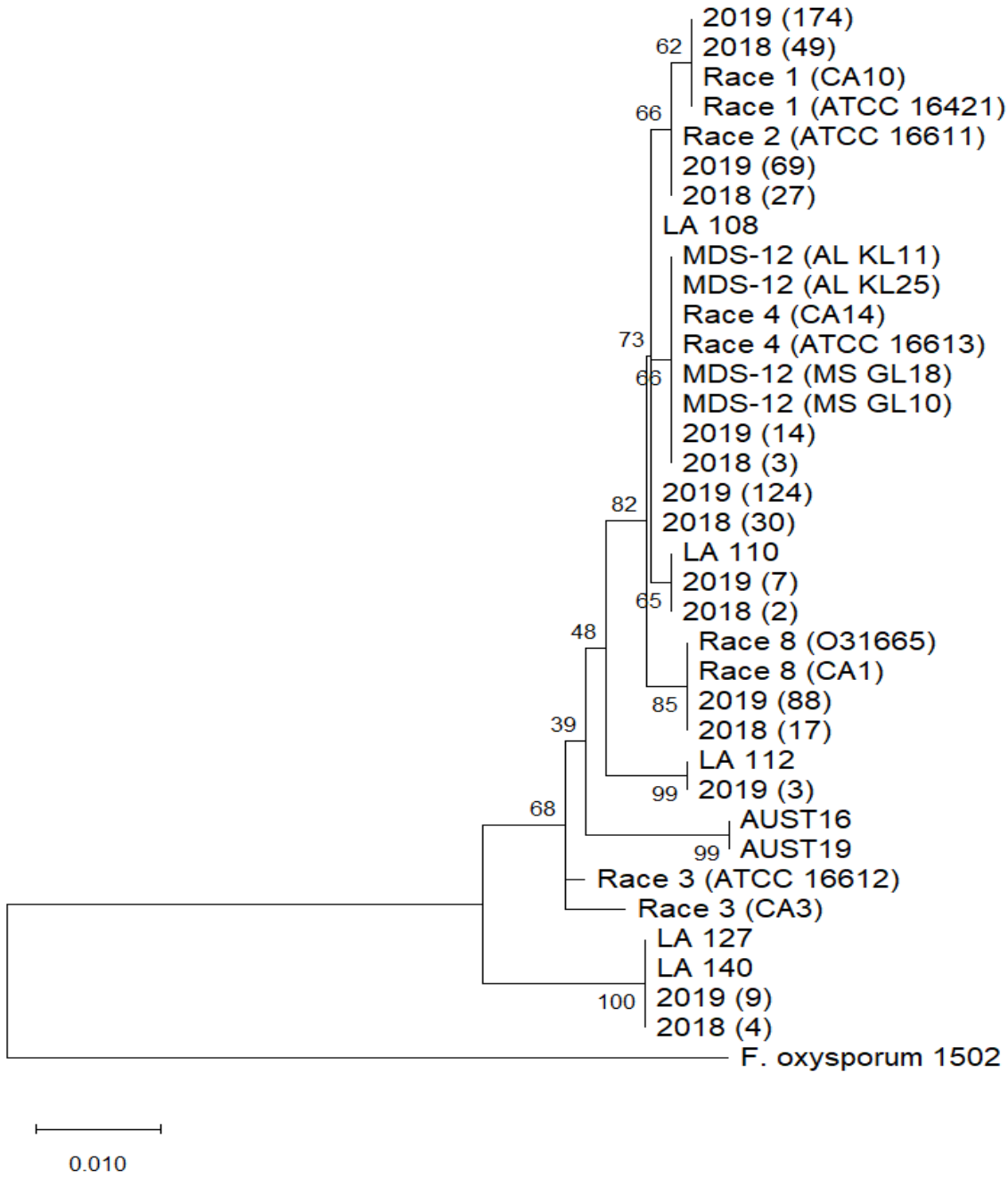

Figure 1. Condensed phylogenetic tree of FOV isolates collected during the 2018 and 2019 cotton seasons using an analysis of the Translation elongation factor. Tree was conducted in MEGAX using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-1362.35) is shown. Bootstrap frequencies form 1,000 replications are noted next to every branch. Isolates with identical sequences collected during the study are repressed by a single isolate in the tree and are labeled with the year of collection and the number of identical isolates that were found. For example, isolate 2019 (174) represents 174 identical isolates that were collected in 2019. References isolates used for comparison are labeled by the race followed by an isolate name, for example Race 1 (CA10) is a race 1 reference isolate identified as CA 10. A saprophytic *Fusarium oxysporum* (isolate 1502) was used as an outgroup to root the tree.

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). When further testing was conducted to look for TƒO insertions into the PHO gene none were found. The PCR and gel electrophoresis produced a DNA fragments size of 396 bp for all of the isolates in question (Figure 3). This led us to believe that these isolates were not of the race 4 type but did not provide definitive proof as some isolates of races 4 have been found to not contain one of these gene insertions. The next step was to run a PCR analysis using race 4 specific primes. During this testing, there was no amplification for any of the isolates in question further confirming that these isolates were of the MDS-12 genotype. One more analysis of these isolates confirmed these findings further. Upon obtaining nearly full sequences of the IGS gene from each isolates in question and conducting a phylogenetic analysis it was found that all of these isolates were contained in one of two clades containing MDS-12 references isolates and none were found in the race 4 clade (figure 4).

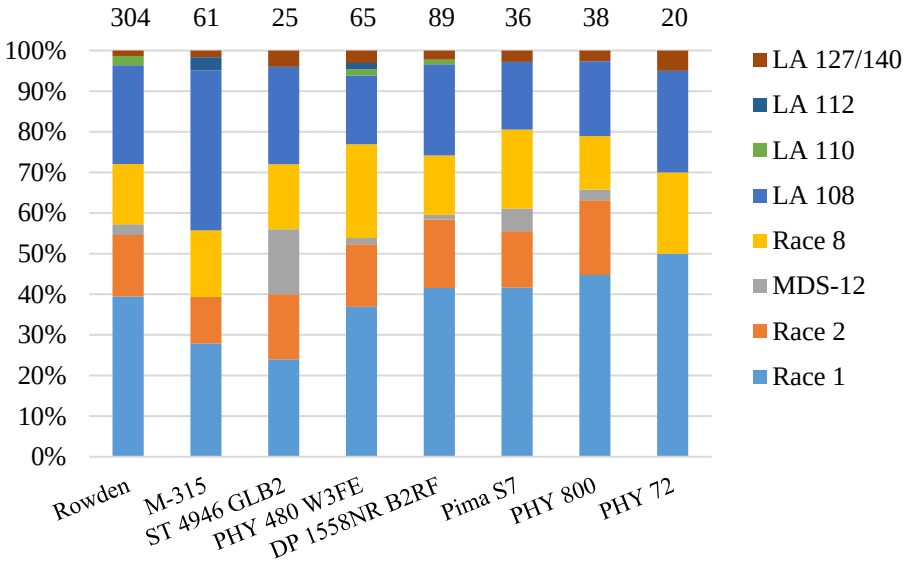

Figure 2. Shows the breakdown of the race population found on each of the cotton cultivars included in the test. The different colors in each bar indicate the percentage of isolates found on that cultivar is made up by that race. The numbers above each bar indicate the total number of isolates that were found on that cotton cultivar. For example 39.5% of the total 304 isolates found on the Rowden cultivar were race one as indicated by the light blue portion of the bar.

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 and/or RKN. The cultivar with the highest incidence of FOV was the susceptible check Rowden where 49% of the total FOV isolates were identified; this high rate of infection is no surprise due to the high susceptibility of this cultivar to both FOV and RKN. At least one isolate of each race found in the test was recovered from the Rowden cultivar with the exception of the LA 112 genotype. This genotype was only isolated from M-315 and PHY 480 W3FE cultivars. The cultivar with the second highest incidence of FOV (14%) was DP 1558NR B2XF. This cultivar is resistant to RKN and supported a low population density of the nematode; this demonstrates that nematode resistance alone is not enough to entirely protect a plant from fusarium wilt. The lowest amount of FOV infection 3% was observed on the cultivar PHY 72 which is an Acala type of cotton with resistance to FOV. During 2018 all three of the isolates identified as the genotype MDS-12 were recovered from plots containing the Rowden cultivar. In the 2019 season in which FOV infection was more severe, MDS-12 isolates were collected from all cultivars except M-315 and PHY 72.

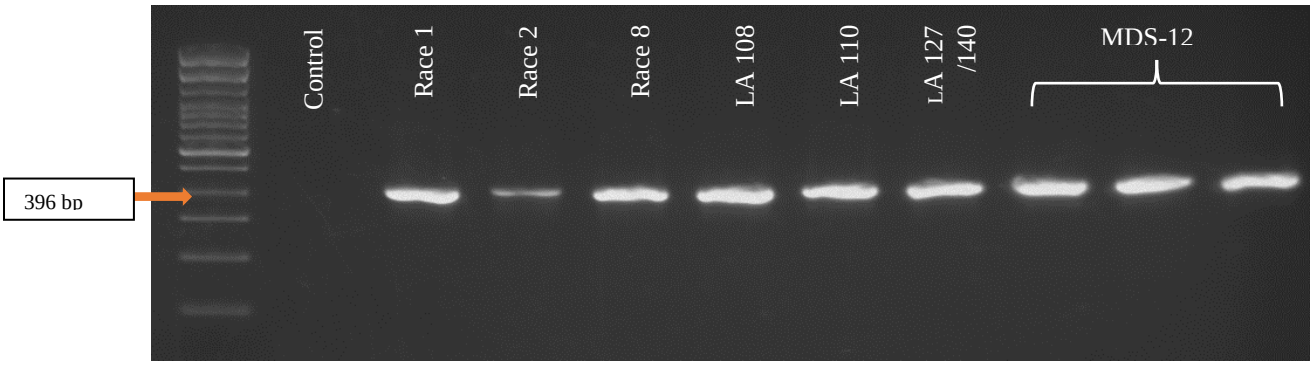

Figure 3. Detection of T*f*o1, MITE/T*f*o1, MULE/T*f*o1 insertions into the PHO gene of FOV isolates collected in 2018. Bands will appear at 396 bp when no insertion is present, 583 bp when the T*f*o1 insertion is present, 426 bp when the MULE/T*f*o1 insertion is present, and 663 bp when the MITE/T*f*o insertion is present. Isolates shown are lane 1, water control; lane 2, race 1; lane 3, race 2; lane 4, race8; lane 5, LA 108; lane 6, LA 110; lane 7, LA 127/140; lanes 8-10 MDS-12. Only 3 isolates of MDS-12 are shown, however, all isolates produced identical sized bands. All isolates produced a band at 396 bp showing the lack of a T*f*o insertion into the PHO gene.

Fusarium oxysporum f. sp. *vasinfectum* isolates were collected throughout the entirety of both growing seasons. Sampling began May 31, 2018 and May 15, 2019 two weeks after cotton planting. Plant samples with FOV infection were acquired on these original sampling dates and all subsequent sampling dates of the seasons. A sharp increase in the number of symptomatic plants was observed from the end of July until the cotton was defoliated in both years (Figure 5). At this time of the season, there was a sharp increase in the four predominant races and genotypes that were found in this study (race 1, race 2, race 8, and LA 108). However, there was not a statistical correlation between the time of the season and any of these four races, as samples of each were found throughout the season in both years of testing.

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 and 2019 cotton seasons. The most predominant races isolated from the field were race 1, LA-108, race 2, and race 8. 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. Cotton cultivars that were susceptible to RKN but resistant to FOV, such as PHY 72, held up better to the high pressure of FOV in this testing. So far, these isolates have been identified using portions of a single, EF-1α, gene. Future work will include sequencing of the BT and PHO regions of the DNA for isolates collected during 2019 to confirm the race of each isolate collected (Table 2).

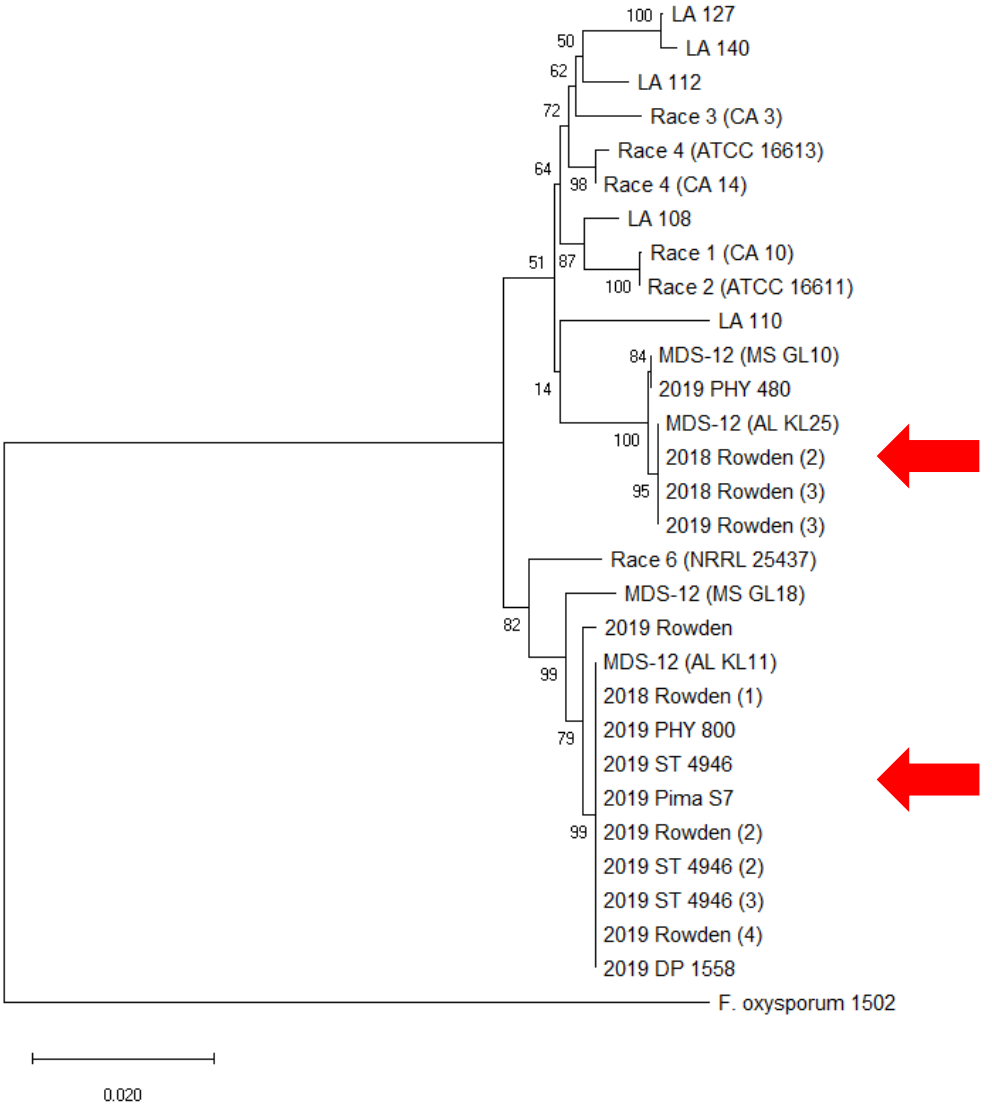

Figure 4. Phylogenetic tree of FOV isolates collected during the 2018 and 2019 cotton seasons using an combined analysis of the translation elongation factor and intergenic spacer. Tree was conducted in MEGAX using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-7043.89) is shown. Bootstrap frequencies form 1,000 replications are noted next to every branch. Isolates are labeled with the year that they were collected followed by the cultivar from which they were collected, in the case of isolates having the identical name number was added in parenthesis. For example, isolate 2018 Rowden (1) represents the first isolate collected from the Rowden cultivar in 2018. References isolates used for comparison are labeled by the race followed by an isolate name, for example Race 1 (CA10) is a race 1 reference isolate identified as CA 10. A saprophytic *Fusarium oxysporum* (isolate 1502) was used as an outgroup to root the tree.

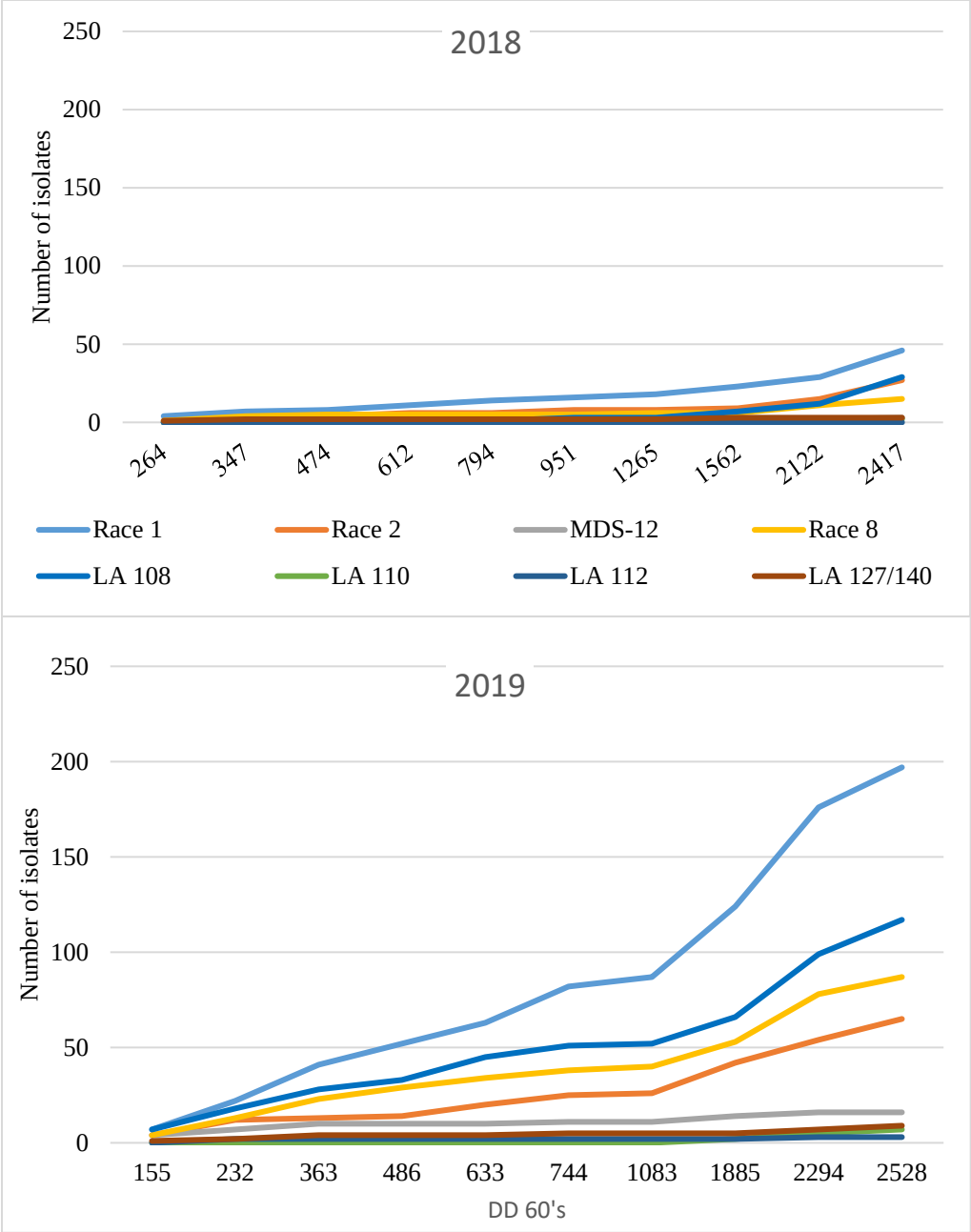

Figure 5. The 2018 and 2019 temporal distribution of *Fusarium oxysporum* f. sp. v*asinfectum* (FOV) throughout the cotton growing season. The x axis displays the growing degree day (DD 60's) accumulation and the y axis is the number of FOV samples collected.

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