**Evaluation of the genetic diversity, temporal distribution, and management of *Fusarium oxysporum* f. sp. *vasinfectum* in the National Cotton Fusarium Wilt Evaluation Field**

by

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Abstract

*Fusarium oxysporum* f. sp. *vasinfectum* (FOV), the causal agent of cotton Fusarium wilt, has been causing losses in the cotton industry around the world for more than 125 years. Large genetic diversity exists within this pathogen which is also known to form a disease complex with at least three nematode genera to cause increased disease and crop damage. The overall objective of this research was to 1) identify the genetic diversity and temporal distribution of FOV that exist within a single Alabama cotton field and 2) evaluate a potential chemical management option for its effect on the entire FOV-nematode disease complex.

In chapter 1, an in-depth review is provided of current literature with insight into the diversity of FOV races/genotypes that affect cotton farms in the United States and explains methods that are used to characterize them. This chapter also provides a synopsis of the current understanding of the disease complex formed between FOV and plant-parasitic nematodes such as *Meloidogyne incognita*.

Chapter 2 is a survey of FOV isolates collected from the National Cotton Fusarium Wilt Evaluation Field to document the population diversity and temporal distribution of FOV races and genotypes that can exist within a single Alabama cotton field. This cotton field was found to contain eight different races and genotypes of FOV at varying levels. Seven of these races/genotypes were found during both years of testing and the genotype LA-112 was found only during 2019 testing. The most predominant races/genotypes found, which account for more than 93% of the total collected samples, were race 1, 2, 8, and LA-108. Other genotypes found (LA-110, LA-112, LA-127/140, and MDS-12) were only found at very low levels. For many of the races/genotypes, including all of the predominant races/genotypes, a correlation was observed between the frequency of isolation and accumulation of growing degree days. This trend indicates

that for the races/genotypes found, disease incidence increased in the latter part of the cotton growing season.

In Chapter 3, evaluations were made of the use of Reklemel™ (a new chemical nematicide) for its ability to lower *M. incognita* population density, its effects on FOV, and its usefulness in management the FOV-nematode disease complex. The objectives of this study were 1) evaluate the impact of Reklemel™ on the growth of FOV isolates *in vitro* and 2) assess cotton growth, yield, and disease incidence with the application of Reklemel™ under greenhouse and field conditions. *In vitro* testing revealed that FOV isolates were affected by Reklemel™ at varying rates among the races/genotypes that were tested. $EC_{50}$ (effective concentration to reduce 50% of mycelial growth) values ranged from a low of 14.6 mg/L with race 2 to a high of 187.6 mg/L with the genotype LA-108. In greenhouse testing, Reklemel™ significantly reduced *M. incognita* population density but had no significant effect on Fusarium wilt incidence. However, in the field, Reklemel™ reduced both *M. incognita* population density and Fusarium wilt incidence. This reduction in FOV incidence was not observed with the treatment of Velum Total™ which had statistically similar reductions in *M. incognita* egg population density.

Lastly, the Appendix is a study to determine the yield loss of cotton by the nematode *Rotylenchulus reniformis* on seven commonly available cotton cultivars and estimate the benefit associated with the application of a chemical nematicide. Field trials were established during 2017 and 2018 in two adjacent fields; one was infested with *R. reniformis* and one where *R. reniformis* was not detected. In both fields, seven cotton cultivars were planted with and without Velum Total™ (1.02 L/ha). *Rotylenchulus reniformis* was estimated to cause a yield loss of 50% between the two years of the study. Application of the nematicide Velum Total™ significantly reduced the

population density of *R. reniformis* in both years of the study but only increased yields during the 2017 cotton season.

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List of Abbreviations

FOV.....*Fusarium oxysporum formae speciales vasinfectum*

f. sp.....*formae speciales*

DAP..... Days after planting

PDA..... Potato dextrose agar

SNA.....Spezieller Nährstoffarmer agar

µm .....micrometer

Eggs/g of root.....Eggs per gram of root

$EC_{50}$..... Median effective concentration to reduce 50% of growth

PCR.....Polymerase chain reaction

RKN ..... Root-knot nematode

EF-1α .....Translational elongation factor 1 alpha

BT .....Beta-tubulin

PHO.....Phosphate permease

IGS .....Intergenic spacer

PPM..... Parts per million

## Chapter 1: Review of Literature

### Statement of Purpose

*Fusarium oxysporum* is a devastating pathogen of more than 45 plant families with *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) causing damage to cotton in every major production region around the world (Davis et al. 2006; Edel-Hermann and Lecomte 2019). A large number of races/genotypes of the FOV pathogen exist, many of which form a synergistic interaction with nematodes to create a highly damaging disease complex. This review is intended to summarize the current understanding of the cotton Fusarium wilt and the disease complex it forms with *Meloidogyne incognita.*

### *Fusarium oxysporum* f. sp. *vasinfectum*

The concept of the genus *Fusarium* was first described in 1809, with the primary character of the genus being canoe or banana-shaped conidia (Link 1809). Species concepts within the genus of *Fusarium* have been heavily debated and changed many times since 1809. The species concept of *Fusarium oxysporum*, first used by Snyder and Hansen (1940), is still used today. *Fusarium oxysporum* is a species complex of fungi that contains many non-pathogens, human/animal pathogens, and plant pathogens. On thousands of plant species, the pathogen commonly causes vascular wilts. The term *formae speciales* is used in *F. oxysporum* and other fungi to differentiate the pathogenic forms of a species that cannot be distinguished morphologically (Leslie and Summerell 2006b). *Fusarium oxysporum* can be broken down into more than 140 *formae speciales* according to the host that they infect, which include plants from more than 45 different families (Edel-Hermann and Lecomte 2019). *Formae speciales* of *F. oxysporum* have repetitively narrow host ranges and usually only infect a few plant species.

*Fusarium oxysporum* is the most widely dispersed and economically significant of all the *Fusarium* species and due to this has been one of the most commonly studied species (Leslie and Summerell 2006c). Common morphological characteristics of *F. oxysporum* are the production of microconidia, production of chlamydospores, and the shape of macroconidia. The microconidia are very abundant and oval or kidney in shape and usually single-celled; they are produced both from false-heads in the aerial mycelium and on short monophialides (Leslie and Summerell 2006c). Chlamydospores are usually formed individually or in pairs and are produced in aerial, submerged, or surface hyphae (Leslie and Summerell 2006c). Macroconidia are produced most commonly in sporodochia but can sometimes be produced from monophialides on the surface hyphae. The macroconidia are short to medium length and may be straight to slightly curved. The apical cell of the macroconidia is tapered and can form a slight hook with the basal cell being foot-shaped (Leslie and Summerell 2006c). When isolates of *F. oxysporum* are grown on potato dextrose agar, a pale to dark violet pigment is often produced in the agar. However, some isolates may not produce any pigmentation (Leslie and Summerell 2006c). *Formae speciales* of *Fusarium oxysporum* and races or vegetative compatibility groups (VCG's) within each *formae speciale* all share similar morphological characteristics.

*Fusarium oxysporum* Schlechtend. f. sp. *vasinfectum* (Atk.) Snyd. & Hans (FOV) was first identified as the causal agent of cotton Fusarium wilt in Alabama and Arkansas in 1892 (Atkinson 1892). Since the description of the disease, FOV has been reported in all significant cotton-producing regions around the world. The most recent report of the disease is from Australia, where it was first detected in 1993 (Kochman 1995). Along with the wide geographical distribution, FOV has also been shown to occur in all four domesticated cotton species *Gossypium hirsutum, G. barbadense, G. arboretum,* and *G. herbaceum* (Armstrong and Armstrong 1960). Fusarium wilt

disease losses have been estimated over the last 55 years to cause an average annual yield loss of 62,391 bales valued at 29.9 million dollars at the cotton lint value of one dollar per pound (https://www.cotton.org/tech/pest/index.cfm).

Disease Cycle and Symptoms

The infection process of FOV begins from chlamydospores, which are dormant in the soil, and require stimulation from root exudates to germinate (Hillocks 1992). After germination, the chlamydospores either infect a root or form conidia and new chlamydospores if a suitable host is not found (Chawla et al. 2012). The germination tube of the chlamydospore grows through the soil until it makes contact with the root. The germination tubes have not been observed to penetrate the host root directly. However, they first colonize the root surface through the formation of net-like mycelium (Rodríguez-Gálvez and Mendgen 1995). From this net-like mycelium, microconidia are formed on the root surface. These microconidia germinate, and hyphae develop along the epidermal cells (Hall et al. 2013). Penetration occurs most often in the root hair zones where the tissue is colonized both intercellularly and intracellularly (Hall et al. 2013). Shortly after colonization of the epidermal cells, the fungus spreads to the xylem vessels of the vascular tissue where it commonly enters through the pits (Hall et al. 2013). Once the fungus reaches the vascular tissue, the pathogen is spread through the plant by way of mycelium growth and production of conidia (Nelson 1981). The conidia do not have free passage through the vascular system but encounter barriers (vessel end walls) every few millimeters which restrict their spread through the plant (Beckman et al. 1976). The conidia must germinate and grow through to the next vessel for the spread of the infection to proceed (Hillocks 1992). Symptoms of this disease can appear at any stage of cotton growth depending on environmental factors, plant susceptibility, and the amount of inoculum (Colyer 2001). However, in most cases the plant is most susceptible to FOV infection

from the time of flowering on, which may be the reason for symptoms more commonly appearing from this growth stage on (Beckman et al. 1976). It is unclear if this trend for later season infection is due to environmental conditions, reproductive stress on the plant, or varying disease susceptibility throughout the plant's life cycle. The exception to this is with FOV race 4 which is predominately found in the early part of the cotton season (Hutmacher et al. 2011).

The symptoms caused by FOV infection were first referred to as Frenching and later, after identification of the causal agent, changed to Fusarium wilt. Infection during the seedling stage appears as wilted cotyledons that turn chlorotic and then necrotic over time; this is often lethal to the cotton plant (Colyer 2001). Older plants show symptoms of wilting (Figure 1A), stunting, chlorosis followed by necrosis of the leaves (Figure 1C) and can lead to plant death (Davis et al. 2006). Along with these symptoms, plants infected with FOV will have a brown to black discoloration in the vascular tissue that is visible when the stem is cut (Figure 1B). This vascular discoloration occurs as a result of the oxidation and polymerization of phenolic compounds (Dimond 1970). The time of infection within the cotton life cycle is a significant factor in determining the extent of yield loss to the disease. Early infection of seedling plants usually results in the death of the plant before it produces bolls (Colyer 2001). However, plants that are not infected until later in the season can survive and produce cotton bolls (Colyer 2001).

Fusarium wilt infected plants that survive and produce cotton bolls can serve as a source of inoculum for the next season through seeds that are infected with the fungus. Elliott (1923) was the first to report FOV being transmitted by infected seeds. This report concluded that the fungus could survive in the seed from season to season when it was found that seeds harvested and delinted in September could still contain viable isolates of the fungus up to eight months later (Elliott 1923). This study was not the first to look into the ability of FOV to survive inside seeds; it was

hypothesized for many years based on the dissemination of the disease into fields where cotton was not previously grown. Many studies both before and after found negative results when testing seed transmission, which raised a dispute over Elliot's results (Fulton 1907; Gilbert 1921; Neal 1928). Seed transmission of FOV was not confirmed until the work of Taubenhaus and Ezekiel (1932), who found that seeds taken from infected cotton plants produced cotton infected with FOV even though the soil it was planted in was free of inoculum. Rates of infection were very low in this study (3.3%). However, this study confirmed that FOV could survive inside the seed coat, even through the process of acid delinting and surface sterilization (Taubenhaus and Ezekiel 1932).

In addition to seed infection, FOV can be moved through a field or to new previously uninfected areas by way of plant material, soil movement, or water movement (Davis et al. 2006). Dissemination by means of infected plant material can occur from the movement of cotton to gins or the disposal of gin trash, and this could contribute to the local distribution of the pathogen (Jeffers et al. 1984). Infected soil may be moved through cultivation, equipment, vehicles, or field workers (Davis et al. 2006). Finally, water movement as a result of some types of irrigation (furrow irrigation) or natural flooding could also play a role in the dispersal of the pathogen (Grinstein et al. 1983). While these dissemination methods would play critical roles in the local spread of the disease, seed infection most likely is the key component of the movement of the disease over long distances.

Race Classification

The first separation of FOV into different races was the work of Armstrong and Armstrong (1958). This separation came about when they found that a *Fusarium oxysporum* causing wilt of cowpeas in South Carolina was a new race of FOV, a cotton pathogen, and not a new cowpea-wilt *Fusarium* (Armstrong and Armstrong 1958). This new race was given the designation of race 2 to

separate it from the preexisting and widely distributed FOV, which was given the designation of race 1. Separation of the two races was made using a host differential test, which used a range of cotton species and cultivars as well as non-cotton hosts. In this test race 2 was separated from race 1 by its ability to cause wilt on 'Yelredo' soybean and 'Gold Dollar' tobacco as well as a range of cotton species and cultivars (Armstrong and Armstrong 1958). Using similar processes, with the addition of more cotton hosts and several alternative crops including alfalfa, okra, and lupine, six more races of FOV were identified between 1958 and 1978 to bring the total to eight; race 1-8 (Table 1) (Armstrong and Armstrong 1958, 1960, and 1978: Ibrahim 1966; Chen et al. 1958).

Later research using host differential tests and DNA sequencing was not able to distinguish between races 3 and 5 and suggested that the races were the same and indicated that races 4 and 7 were at least very similar (Nirenberg et al. 1994; Skovgaard et al. 2001). Because of the combination of these races, there are currently six races that commonly cause Fusarium wilt of cotton, and they are identified as race 1, 2, 3, 4, 6, and 8 (Edel-Hermann and Lecomte 2019). The term race used in describing FOV populations does not imply that there is a relationship between the pathogen and resistance genes within the cotton, due to the use of non-cotton host in the original testing (Davis et al. 2006). Due to this and notable inconsistencies in the testing methods, the validity of the term race to describe differing populations of FOV has been challenged by Davis et al. (1996). Because of these issues with the original classification schemes new methods have been developed for classifying the diverse population of FOV isolates. For example, Assigbetse et al. (1994) developed a system that uses a host differential test where only cotton hosts were used and combined these results with data from random amplification of polymorphic DNA. In this study, isolates of FOV were grouped into race A, race 3, and race 4 with race A containing the previously described race 1, 2, and 6 (Assigbetse et al. 1994; Fernandez et al. 1994). Because of the debate

over the use of the term race, new populations found since this time have not been given race designation.

The naming scheme from the original FOV race separation is still commonly used to identify populations of FOV. However, the process of identifying isolates of FOV now frequently employs DNA-based techniques that compare gene sequences to that of reference isolates from each race. Partial and complete sequences of many genes have been used to separates races of *F. oxysporum.* Portions of the translation elongation factor (EF-1α) were first used in a study of *F. oxysporum* to investigate the evolutionary origins of *F. oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen, a pathogen of banana (O'Donnell et al. 1998). Since this time the EF-1α region of DNA has become commonly used, often in combination with the Phosphate permease (PHO) and/or β-tubulin (BT) genes, for race identification and phylogenetic analysis of FOV (Skovgaard et al. 2001; Kim et al. 2005; Holmes et al. 2009; Liu et al. 2011; Cianchetta et al. 2015; Bell et al. 2017). Many other methods have also been used to separate races of FOV, including amplified fragment length polymorphorphism, restriction digestion analysis of the intergenic spacer region of nuclear rDNA, aesculin hydrolysis tests, and many more (Davis et al. 1696; Abd-Elsalam et al. 2004; Abo et al. 2005; Kim et al. 2005). Through the sequencing of these genes and other techniques, many new genotypes of FOV have been identified in the United States (Holmes et al. 2009). These genotypes have not been defined as new races of the pathogen, and many have been given "LA" names such as LA-108 or LA-110. While these genotypes are unique in the sequencing of all of the genes commonly used for identification of FOV isolates, they have not been found to form new VCG (an alternative method for classifying FOV isolates) but instead, fall within VCG's of race 1 (Bell et al. 2017). This finding suggests that these genotypes are variants of races 1and not entirely new races of FOV.

In Australia, where the FOV pathogen was most recently discovered in 1993, unique isolates have been identified that were named the Australian biotypes (Davis et al. 1996). The pathogenicity of these isolates on a range of *Gossypium* species and cultivars was similar to that of FOV race 6 (Davis et al. 1996). However, these isolates were found to be unique from all previously reported FOV races/genotypes in aesculin hydrolysis tests, the production of volatile compounds, and VCG analysis where the isolates were placed into newly formed groups (Davis et al. 1996; Liu et al. 2011).

While sequencing of the EF-1α, PHO, and BT are very commonly used for studies of FOV, it is not perfect, and they are not capable of identifying all race and genotypes of FOV. Partial sequences of these genes were used to identify a total of 5 isolates from Alabama and Mississippi as FOV race 4 (Scott et al. 2011; Bennett et al. 2013). These findings had significant implications due to the high virulence of FOV race 4 and the thought that it was isolated to California within the United States at the time. Upon further studies that included sequencing of the intergenic spacer region (IGS) of genomic rDNA, all of these isolates were classified as a new genotype of FOV called MDS-12 (race 4-like) (Bennett et al. 2013). These two FOV's, race 4 and MDS-12, are identical at EF-1α, PHO, and BT genes but very different in terms of pathogenicity and threat to the US cotton industry. Race 4 is a highly virulent FOV that in the U. S. has only been found in California, Texas, and most recently New Mexico (Kim et al. 2005; Halpern et al. 2017; Zhu et al. 2019). However, MDS-12 has been found in much of the southeastern U. S. but has a low virulence to cotton (Bennett et al. 2013) and has even been described as non-pathogenic (Bell et al. 2017).

Due to its potential as a very problematic pathogen, specific methods were created for the detection of FOV race 4. Race 4-specific primers, R4f and R4r, have been designed to aid in its detection based on a unique amplified fragment length polymorphisms (AFLP) marker in FOV

race 4 isolates (Yang et al. 2006). Based on these specific primers, a kit has been created that can be used to identify race 4 in the field (Crutcher et al. 2016). Both the race 4-specific primers and the test kits are able to identify FOV race 4 but also give positive results for race 7 and race 3 isolates (Crutcher et al. 2016). According to Skovgaard et al. (2001), race 7 isolates were not able to be separated from race 4 based on DNA sequences and thus, these positive results are not surprising. Recent studies have found that California isolates FOV race 4 have a unique T*fo* 1 transposon insertion in the PHO gene that has proved useful in identifying this race (Ortiz et al. 2017). Using three specific primers (FovP-F, FovP-R, and FovT-R), Ortiz et al. (2017) were able to distinguish the California race 4 from all other genotypes of FOV, as well as separate it from FOV race 4 isolates originating from India. Further studies have found a total of four genotypes within FOV race 4, three of which have different variations of a T*fo* 1 transposon insertions in the PHO gene and one that does not have an insertion (Bell et al. 2019). *Fusarium oxysporum* f. sp. *vasinfectum* isolates from around the world have been identified as N (no insertion), T (T*fo1* transposon insertion), MT (MULE insertion with the inserted T*fo1* transposon), or MiT (MITE insertion in the inserted T*fo1* transposon). All of these genotypes can be differentiated with a single multiplex PCR reaction using primers FovP-F, FovP-R, and FovT-R, and FOV M-R (Bell et al. 2019).

Many of these races and genotypes of FOV have been found in the state of Alabama. Previous studies by Scott (2012) and Smith (2015) found race 1, 2, and 8, as well as genotypes LA-127/140, LA-108, and MDS-12 (race 4-like) in an FOV infested field in central Alabama. A more extensive survey of the entire country found these and additional races/genotypes in the state. This survey found race 3, along with genotype LA-112 (Cianchetta et al. 2015). In the study conducted by Scott (2012), isolates of MDS-12 were identified as FOV race 4. Further

investigation into these isolates found them not to be race 4 but instead identified them as MDS-12 (Bennett et al. 2013). FOV race 4 has not been reported in Alabama to date.

Vegetative Compatibility

Another way of classifying the variations among isolates of FOV is by defining the vegetative compatibility of the isolates. The first studies using vegetative compatibility were conducted to research the fungus *Neurospora crassa* (Garnjobst, 1953). Puhalla (1985) used vegetative compatibility in *F. oxysporum* to define 16 VCG's across 21 isolates of *Fusarium oxysporum* and found a relationship between VCG and *formae speciales*. The process used for determining vegetative compatibility is based on the ability of hyphae from two isolates of FOV to anastomose and fuse to form heterokaryons when paired with one-another (Leslie and Summerell 2006a). Isolates that are capable of fusing to form heterokaryons are vegetatively compatible and are grouped into the same VCG while those that are not able to fuse are vegetatively incompatible and are placed into different VCG's. To conduct a VCG analysis and to force the formation of heterokaryons to occur, complementary auxotrophic mutants are required (Leslie and Summerell 2006a). Nitrate ($NO_3$) non-utilizing mutants or *nit* mutants are commonly used for this purpose in *Fusarium* studies. *Nit* mutants can be recovered without mutagen treatment by growing the isolates on a medium which contains $KClO_3$ (Leslie and Summerell 2006a). Through this process, many classes of *nit* mutants most of which are usable for conducting a VCG analysis are produced.

Once *nit* mutants have been obtained, the VCG analysis can be conducted. As previously mentioned, the VCG analysis looks for heterokaryon formation between the *nit* mutants of different strains. The formation of heterokaryons can be determined by the presence of thick growth where the two colonies touch (Puhalla 1985). The ability of two mycelial strands to form

heterokaryons and therefore be of the same VCG is dependent on vegetative incompatibility (*vic*) loci. Compatibility requires that all alleles in the *vic* loci be the same, meaning that the two fungi are very similar genetically (Leslie 1993). Puhalla (1985) suggested using a numbering system to name the VCGs that would identify the *formae speciales* and the subgroup within the *formae speciales*. For example, VCG 0111, the first three numbers (011) would identify the fungus as FOV and the last number (1), or in some cases the last two numbers, would identify a subgroup within FOV (Puhalla 1985). This numbering system is still used in *F. oxysporum* VCG analysis.

The VCG analysis provides useful information for classifying and defining populations of FOV. However, this type of study does not provide information about how different VCGs are related or their pathogenicity on cotton. Currently, 21 VCG's have been identified among races and defined biotypes of FOV (Bell et al. 2017). Race 1 isolates belong to the VCG 0111 and 0119 (Bell et al. 2017; Fernandez et al. 1994). Race 2 isolates belong to the VCG's of 0112, 0115, 0117, or 01110 (Fernandez et al. 1994). All Race 3 isolates belong to a single VCG, 0113 (Fernandez et al. 1994). VCG 0114 contains all know isolates of race 4 (Fernandez et al. 1994). Race 6 isolates are in the VCG 0116 or 0118 (Fernandez et al. 1994). VCG 01111 and 01112 consist of the isolates of the Australian biotype (Davis et al. 1996; Liu et al. 2011). Many "LA" genotypes of FOV have also been identified in the southeastern United States, and all of these genotypes are in VCG's associated with FOV race 1 (Bell et al. 2017; 2019). Despite all these attempts to characterize FOV isolates, none of these methods/techniques link the characterization to pathogenicity genes. Future work is necessary to better understand the disease mechanism and the genes which comprise it so that a FOV classification system can be developed which also confers FOV virulence.

Management of FOV

Disease management of FOV is challenging through traditional management techniques. Even with the limited host range of FOV, crop rotation is not an effective management strategy. This is due to the ability of FOV to survive for long periods when cotton is not present. Smith et al. (2001) demonstrated that FOV was able to survive at high levels in soil that did not contain cotton for ten years. Two major ways that FOV can survive for this long is by the production of chlamydospores and the ability to live without causing disease symptoms on many non-cotton plant species. Chlamydospores are thick-walled asexual spores capable of living on or in small bits of decaying organic matter that require moisture and root exudates to trigger germination (Smith et al. 2001). One study in 1975 found FOV populations in California were not only able to survive but also increase their soil inoculum density when infected fields were rotated to cereal crops (Smith and Snyder 1974). This increase was a result of the FOV reproducing on the cereal roots and weed species that were present, even though no disease symptoms were observed on these plants (Smith and Snyder 1974). While this is not the typical result of crop rotation, it stands to show the shortcomings of crop rotation in FOV management due to the ability FOV has to persist in a field without a cotton host.

Another common option for disease management is the application of chemicals. While some chemicals treatments have been demonstrated to reduce FOV damage, these options are limited and expensive (Davis et al. 2006). Some seed treatment fungicides have shown good results preventing the spread of FOV inside the seed coat (Allen and Kochman 2001). While this is helpful in controlling the long-distance spread of the disease, it has no effect on disease management once the FOV pathogen has entered and become established in a field. Due to the disease complex that is formed between FOV and nematodes, such as *Meloidogyne incognita* (Kofoid and White) Chitwood, some management strategies that target the nematode portion of the disease complex

have been successfully used. The use of chemical nematicides can be used to lower the *M. incognita* population density and in turn, reduce the incidence and severity of Fusarium wilt (Colyer 1997; Jorgenson 1978; Jorgenson et al. 1978). These chemicals have not been found to impact FOV directly but can reduce the damage of the disease impact by mitigating the nematode portion of the disease complex.

Due to the shortfalls of other management methods, resistance cultivars have become the most successful and commonly used way to manage FOV. This management practice depends on moderate to high levels of resistance to not only FOV but also *M. incognita*. Early cultivars that were developed with resistance to FOV were low yielding and had poor agronomic properties compared with susceptible cultivars (Kappelman 1980). However, in the mid to late 1970s, significant improvements were made to yield potential and fiber qualities of wilt resistant cultivars (Kappelman 1980). Many similar problems with yield were associated with *M. incognita* resistance in early lines, but now a few cultivars with moderate resistance and higher yield are available to growers (Davis et al. 2006). If FOV race 4, which does not rely on *M. incognita* to cause high levels of disease, continues to spread through the country, the use of nematode resistant cultivars to manage FOV may become less effective. Therefore, cotton cultivars with high levels of resistance to FOV, especially race 4, are becoming more important in the cotton industry. FOV race 4 is most virulent to *Gossypium barbadense* cotton, but high levels of damage can also occur on *Gossypium hirsutum* (Kim et al. 2005). Shortly after the original reporting of FOV race 4 in California, highly resistant *G. barbadense* lines were identified (Ulloa et al. 2006). Resistance in *G. hirsutum* has been much more difficult to identify. FOV race 4 resistance has only recently been identified in upland cotton lines (Ulloa et al. 2020; Zhang et al. 2020). The production of commercial cotton lines, both *G. barbadense* and *G. hirsutum*, with high levels of resistance to

FOV race 4 will become increasingly important if this race of the pathogen continues to spread throughout the country.

### *Meloidogyne incognita*

Due to the significant impact that *Meloidogyne incognita* plays in the infection of FOV, it is important to understand the nematode as well as how it interacts with the fungus. The genus *Meloidogyne* encompasses more than 90 species of root-knot nematodes (Hunt and Handoo 2010), and it is estimated to account for 5% of the global crop loss (Haydock et al. 2006). The common name for the genus refers to the characteristic galling or knotting symptoms they cause on plant roots. Miles Berkeley made the first observation of these symptoms in 1855 when he reported the galling on cucumber roots in England (Hunt and Handoo 2010). Initially, Cornu placed these nematodes into the genus of *Anguillula*, and it was not until 1887 that Göldi proposed the genus of *Meloidogyne* with the description of *M. exigua* (Hunt and Handoo 2010). In 1949, the genus of *Meloidogyne* was revised by Chitwood, adding four species: *M. exigua, M. incognita, M. javanica, and M. arenaria* (Hunt and Handoo 2010).

*Meloidogyne incognita* is a detrimental pathogen to hundreds of crops around the world. It is commonly found in the southern part of the United States, where the climate is optimal for the growth and reproduction of the nematode. Vrain et al. (1978) showed that a temperature of at least 10°C is needed for the development of the nematode. Figure 2 is a map of the southeast United States, counties marked in red indicated where *M. incognita* has been detected (Kathy Lawrence, Auburn University, personal communication; Hajihassani et al. 2018; Faske et al. 2018). The nematode is present in every US state where cotton is grown and while it can cause a greater problem when interacting with FOV, the nematode itself is one of the most damaging pathogens of cotton. This nematode alone has been estimated to be the leading cause of yield loss to the

United States cotton industry during the over the last ten years (https://www.cotton.org/tech/pest/index.cfm).

Life Cycle and Symptoms

The life cycle of *M. incognita* begins as an egg embedded in a gelatinous matrix that is usually found on the surface of infested roots. The eggs are laid at the single-cell stage but begin cell division within hours (Guiran and Ritter 1997). This cell division leads to the development of the first stage juvenile, which then molts into a J2 while it is still in the egg (Moens et al. 2010). Drawing energy from food reserves in its intestine, the J2 becomes more active in the egg and repeatedly thrusts its stylet through one end of the eggshell to create an opening (Guiran and Ritter 1997). The J2 hatches from the egg and initiates its search for a host. Once a host root has been located the J2 injects root cells with cell-wall-degrading enzymes produced in the oesophageal glands using its stylet. After infection, the nematode migrates intercellularly and intracellularly through the root to the root tip where a feeding site is established (Abad et al. 2003). Once at the feeding site, the nematode induces the differentiation of parenchymatic root cells into giant cells (Abad et al. 2010). The giant cells can enlarge to over 400 times the size of a normal root cell and can contain more than 100 nuclei (Abad et al. 2010) as a result of repeated nuclear division without the occurrence cytokinesis (Jones and Payne 1978). These giant cells work as a nutrient sink to feed and sustain the nematode throughout its life cycle (Caillaud et al. 2008). Once giant cells have been established, the nematode begins to swell and then undergoes three more molts, which take the nematode thought the third and fourth juvenile stages and into the adult form (Moens et al. 2010). Male *M. incognita* nematodes are very rare and are not known to be needed for reproduction which is accomplished by parthenogenesis (Abad et al. 2010). The female nematodes are pear-shaped and sedentary inside the root except for their head region, which has the ability to move

between the giant cells to feed (Eisenback and Hunt 2010). Once the adult females reach maturity, they begin to lay eggs which are deposited on the root surface in a protective gelatinous matrix (Abad et al. 2010).

The most common diagnostic symptom associated with *Meloidogyne* spp. infection is the formation of the galls or knots on plant roots. The process of hypertrophy, along with increased cell multiplication known as hyperplasia, cause theses characteristic galling symptoms (Taylor and Sasser 1978). Small galls are approximately one or two millimeters in size and typically contain one female nematode; however, larger galls can contain several females and be one centimeter or larger in diameter (Taylor and Sasser 1978). The galls can vary in size depending on host susceptibility and level of infection. Aboveground symptoms on infected plants can include stunting, necrosis, chlorosis, reduced yields, and overall reduced quality of the crop (Colyer 2001).

FOV Disease complex with *M. incognita*

The first report of an interaction between FOV and *M. incognita* came from Atkinson in 1892. Atkinson noted that higher severity of the disease occurred when *M. incognita* was also present in the field (Atkinson, 1892). Under field conditions, this interaction results in increased incidence and severity of Fusarium wilt symptoms when *M. incognita* is present in the field, in both wilt-susceptible and resistant cultivars (Manzanilla-Lopez and Starr 2009). The disease incidence and crop damage that result from this disease complex were demonstrated by DeVay et al. (1997) to be dependent on the population levels of both the nematode and fungal pathogens.

The exact mechanisms of the interaction between FOV and *M. incognita* are not fully understood. There have been many theories throughout the years. One hypothesis is that the nematode acts as a vector for the fungal spores. However, the nematodes stylet is too narrow to ingest either the micro- or macroconidia of FOV, and there are no reports of the nematodes

transporting the spores on their cuticle (Mai and Abawi 1987). Another school of thought is that the wounds created by the nematode feeding provide an opening for the FOV to enter the plant. Yet, research has demonstrated that infection by *M. incognita* increased FOV severity, even when the FOV is applied by way of stem puncture and not in contact with wounds created by the nematode (Katsantonis et al. 2003). Also, *M. incognita* has been reported to create very little damage to the root surface during penetration (Endo and Wergin 1973). This demonstrates that the interactions must be more complex than the nematodes simply opening an entry point for the fungus. It is now believed that the interaction is related to the biochemical and physiological changes that are brought on by the nematode infection (Starr 1998). It has also been noted that *M. incognita* induced giant cells, which are established for nematode feeding, are more susceptible to infections of FOV and other fungal pathogens (Meléndez and Powell 1967; Fattah and Webster 1983). While the mechanism of this interaction may not be fully understood, the effects of disease severity and crop damage have been well documented (Atkinson 1892; Roberts et al. 1985; Starr et al. 1989).

Other nematodes have also been reported to increase the incidence of FOV infection. *Rotylenchulus reniformis* (Linford and Oliveira) has been observed to increase wilt incidence in susceptible cultivars but not in FOV resistant cultivars (Jones et al., 1959 Khadr et al. 1972). *Belonolaimus longicaudatus* (Rau) has also been shown to interact with FOV and cause an increase in wilt incidence that can match or exceed that of *M. incognita* (Yang et al. 1976). *Belonolaimus longicaudatus* has been associated with many fields incurring losses from Fusarium wilt in the state of Georgia in the last few years (Silva et al. 2019). It is speculated that this nematode plays a more significant role in the Fusarium wilt-nematode disease complex than previously thought. However, *B. longicaudatus* has a limited geographical distribution as it generally requires a soil

type that is at least 80% sand and less than 10% clay (Overstreet and McGawley 2001). Due to this limited geographic distribution, the interaction between FOV and *B. longicaudatus* is not as widely studied as FOV and *M. incognita*.

Management of *M. incognita*

Management options for *M. incognita* include cultural practices, planting resistant cultivars, application of chemical nematicides, or biological controls (Thomas and Kirkpatrick 2001). Cultural practices include the use of sanitation, crop rotation, cover crops, soil amendments, and solarization. Sanitation is a crucial step to minimize the spread of *M. incognita* and includes the removal of infected plant material as well as cleaning of farm equipment to remove any soil partials that may contain the nematode after working in infested fields (Nyczepir and Thomas 2010). Cover crops and soil amendments are used to lessen the impact of *M. incognita* by improving overall soil health, such as increasing soil organic matter or lowering soil erosion (Nyczepir and Thomas 2010). Crop rotation is an effective method used to manage nematode population density, but its usefulness for managing *M. incognita* is limited by the polyphagous nature of the genus (Lamberti 1979). Planting resistant cultivars can be used to manage *M. incognita* population density in crops such as cotton, but historically these resistant cultivars were associated with lower yield potential (Davis et al. 2006).

Chemical nematicides are commonly divided into fumigant and non-fumigant groups. Fumigant nematicides are liquids that are injected into the soil where they volatilize to a gaseous phase. Many fumigants have not only nematicidal properties but also are proficient fungal, weed, and insect pesticides (Nyczepir and Thomas 2010). Many of these fumigant nematicides, such as Methyl Bromide, have been removed from the market in the US due to environmental and human health concerns (Nyczepir and Thomas 2010). Non-fumigant nematicides are formulated as either

granular materials which are applied to the seed furrow, or liquid materials which can be applied to the soil, as a seed treatment, or to the foliage of a crop. Unlike fumigants, these forms of nematicides, when applied as soil or seed treatments, must be dispersed through soil moisture to be effective (Nyczepir and Thomas 2010). Reduction of the nematode population density through any of these methods has also been demonstrated to reduce the incidence and severity of Fusarium wilt, lowering the overall impact of the disease complex (Colyer 1997; Jorgenson 1978; Jorgenson et al. 1978).

## Research Objectives

The overall objectives of this research study were to 1) assess the population diversity of FOV races/genotypes infecting cotton cultivars which were selected for either FOV or *M. incognita* resistance or susceptibility and to 2) to determine the in-season temporal variability of FOV races collected from these cotton cultivars, and 3) evaluate the use of a new nematicide for its ability to lower *M. incognita* population density and reduce the disease impact from the FOV-*M. incognita* disease complex.

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Table 1: Host differential test used to identify races of *Fusarium oxysporum* f. sp. *vasinfectum* found throughout the world (Davis et al. 2006).

| | Race | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Host** | 1 (US) | 2 (US) | 3 (Egypt) | 4 (India) | 5 (Sudan) | 6 (Brazil) | 7 (China) | 8 (China) |
| *Gossypium arboretum* cv. Ronzi (Tree cotton) | R[z] | R | S | S | S | R | S | I |
| *G. barbadense* cv. Ashmouni (Sea Island cotton) | S | S | R | R | S | S | ? | ? |
| *G. barbadense* cv. Sakel (Sea Island cotton) | S | S | S | R | S | S | S | I |
| *G. hirsutum* cv. Acala (Upland cotton) | S | S | R | R | R | S | S | I |
| *Glycine max* cv. Yelredo (Soybean) | R | S | R | R | ? | R | I | I |
| *Nicotiana tabacum* cv. Gold Dollar (Tobacco) | R | S | R | R | ? | R | R | I |
| *Medicago sativa* cv. Grimm (Alfalfa) | S | S | R | R | ? | R | I | S |

[z] R = Resistant (no wilt symptoms), S = Susceptible (wilt symptoms), I = Intermediate (some plants susceptible some not), ? = Not tested

Figure 1: Symptoms of Fusarium wilt on upland cotton *Gossypium hirsutum*. A) Wilting of cotton plant symptomatic of Fusarium wilt infection. B) Discoloration of the vascular tissue of a cotton stem symptomatic of Fusarium wilt. C) Leaf necrosis and wilting caused by Fusarium wilt.

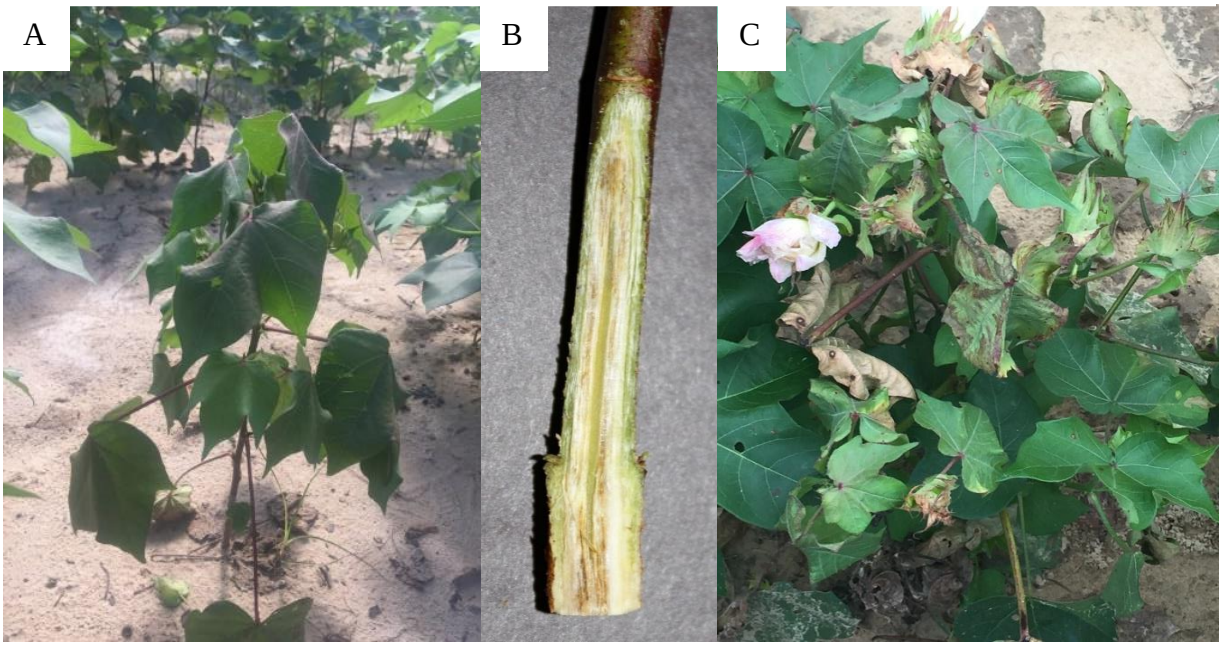
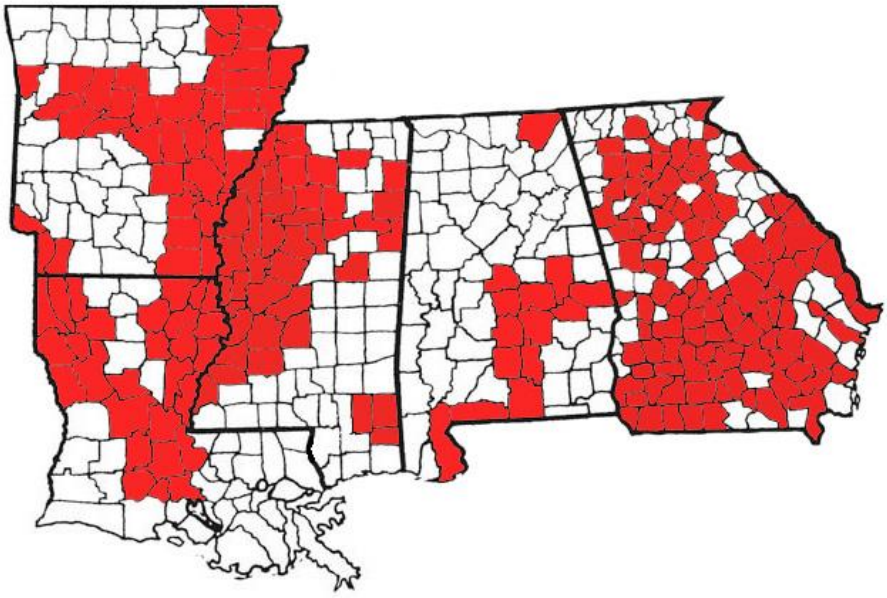


Figure 2: *Meloidogyne incognita* distribution map by county in Alabama, Arkansas, Georgia, Louisiana, and Mississippi. Counties in red are where *M. incognita* has been identified in agronomic crops. (Kathy Lawrence, Auburn University, personal communication; Hajihassani et al. 2018; Faske et al. 2018).



## Chapter 2: Diversity and temporal distribution of *Fusarium oxysporum* f. sp. *vasinfectum* races and genotypes as influenced by *Gossypium* cultivar

**Abstract:** Fusarium wilt of cotton, caused by *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), is a global disease of cotton. Multiple different races and genotypes of FOV have been documented infecting cotton and inducing symptoms of wilting, stunting, vascular discoloration, and plant death. This study was initiated in the National Cotton Fusarium Wilt Evaluation Field to 1) assess the population diversity of FOV races/genotypes infecting cotton cultivars which were selected for either FOV or *M. incognita* resistance or susceptibility and to 2) to determine the in-season temporal variability of these FOV races/genotypes. Symptomatic plants were collected from the field in each trial from two weeks after planting until crop defoliation. The race/genotype of each FOV isolate collected was identified by sequencing portions of the translation elongation factor, β-tubulin, and the phosphate permease genes. In total, eight races/genotypes of FOV were collected throughout the three site years. FOV race 1 was the most predominant in all tests (AUDPC=101.1); statistically higher numbers of isolates from LA-108 (AUDPC=59.9), race 8 (AUDPC=47.5), and race 2 (AUDPC=38.6) were also found compared to other races and genotypes collected. The genotypes MDS-12, LA-110, and LA-127/140 were found in all tests but at a low incidence, and LA-112 was only found in small numbers (three isolates) in one 2019 test. A positive correlation was observed between the accumulation of growing degree days and FOV race 1, race 2, race 8, LA-108, and LA-110.

## Introduction

Fusarium wilt is caused by the seed and soilborne fungal pathogen *Fusarium oxysporum* that produces disease in many crops worldwide. This group of fungal pathogens has more than 140 *forma speciales* capable of causing disease in plants belonging to more than 45 families (Edel-Hermann and Lecomte 2019). *Fusarium oxysporum* f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hansen (FOV) is a cotton pathogen that was first reported in 1892 from samples collected in Alabama and Arkansas (Atkinson 1892). In Atkinson's description of the disease he noted that Fusarium wilt, which was known as Frenching at the time, had increased disease incidence and severity when it occurred with *Meloidogyne incognita* (Kofoid & White) Chitwood. The disease complex formed between these two pathogens can cause severe foliar wilt symptoms and crop yield losses. The National Cotton Council of America monitors yearly cotton disease losses across the United States, and Fusarium wilt has been reported in all cotton producing states since 1965. Fusarium wilt disease losses have been estimated over the last 55 years to cause an average annual yield loss of 62,391 bales valued at 29.9 million dollars at the cotton lint value of one dollar per pound (https://www.cotton.org/tech/pest/index.cfm). However, Fusarium wilt is not only a problem in the United States. The disease affects cotton in every major cotton-growing region around the world. The most recent occurrence was in Australia, where the disease was first detected in 1993 (Kochman 1995). Fusarium wilt of cotton is caused by diverse races, vegetative compatibility groups (VCGs), and several genotypes of the FOV pathogen.

Armstrong and Armstrong (1958) devised a system that classified isolates of FOV into races using a host differential test. This test was based on the ability of FOV isolates to cause disease on a range of cotton species and cultivars and other crops. Through this type of testing Races 1, 2, 3, 4, 6, and 8 were characterized. These races vary in their virulence and impact on the

United States cotton crop. Based on the Nation Cotton Councils disease loss estimates the greatest FOV yield losses occurred between 1999-2004 when average losses amounted to 54.3 million dollars per year (https://www.cotton.org/tech/pest/index.cfm). During this time, California reported severe economic losses due to Fusarium wilt in Pima cotton even in the absence of *M. incognita*. Studies into these losses discovered FOV race 4, a highly virulent race of the disease not previously found in the United States, to be the cause of these large losses when it was identified in the San Joaquin Valley of California in 2001 (Kim at al. 2005).

The validity of using the previously described host differential method for separating FOV into races has been questioned, due to inconsistencies with the methods used in some instances, and the use of non-cotton hosts (Davis et al. 1996). Subsequently, other methods have been developed and used to characterize the diverse populations of FOV that exist throughout the world. Two commonly used methods for differentiation include classifying race/genotypes are with genetic markers and categorizing isolates using a vegetative compatibility group (VCG) system, which contains more than 21 different VCG's (Fernandez et al. 1994; Davis et al. 1996; Bell et al. 2017). In this paper, we used published, partial gene sequences to classify isolates in described races or other genotypes. Genes that were used for this classification code for the translational elongation factor (EF-1α), βeta-tubulin (BT), phosphate permease (PHO), and the intergenic spacer region (IGS) (O'Donnell et al. 1998; 2000; 2009; Tooley et al. 2001).

Using these gene sequences, new genotypes of FOV have been identified in the southeastern United States (Holmes et al. 2009). Isolates, originally collected from Georgia, were given the designations (LA-108, LA-110, LA-112, LA-127/140) when compiled and submitted to a research effort to characterize the FOV's found in the U.S. (Holmes et al. 2009). These so-called LA isolates were determined to be within the VCG containing FOV race 1, as described by Bell et

al. (2017). Another genotype, MDS-12, shares the identical base sequence of the gene for the EF-1α as does the highly pathogenic, San Joaquin Valley isolate of FOV race 4 but is less virulent and does not pose as great a threat to the cotton industry (Bennett et al. 2013; Cianchetta et al. 2015). Two unique isolates of FOV have also been found infecting cotton in Australia. These isolates could not be separated from FOV race 6 in a host differential test (Davis et al. 1996). However, they do not match any existing races or genotypes in an aesculin hydrolysis test, and they possess some unique physiological characteristics (Davis et al. 1996). These isolates are referred to as the Australian biotypes.

As mentioned, a disease complex is known to exist between FOV and some nematodes such as *M. incognita*. This interaction is hypothesized to result from the relationship between the two pathogens that modify the host plant tissue (Mai and Abawi, 1987). Similar disease complexes of Fusarium wilt and *Meloidogyne* species have been documented and studied in many other crops such as tomato (*Lycopersicon esculentum*), banana (*Musa sapientia*), and tobacco (*Solanum nicotiana*) (Mai and Abawi, 1987). The interactions of the nematode and the *Fusaria* are poorly understood and are a focus of ongoing research. This *Fusarium*-nematode disease complex is believed necessary for several of the known races/genotypes of FOV to cause significant damage, but the association is not required for all FOV.

FOV races/genotypes or VCG's can be further divided into two pathotypes known as vascular-competent and root-root pathotypes (Liu et al. 2011; Bell et al. 2017). These two pathotypes differ in their ability to move within the vascular system as well as their reliance on nematodes. The vascular-competent pathotype includes races 1, 2, 8, MDS-12, and all LA genotypes. These isolates are readily able to colonize the plant's vascular system and rely on the presence of nematodes such as *M. incognita* to cause high levels of crop damage (Liu et al. 2011;

Bell et al. 2017). The root-rot pathotype includes races 3, 4, and the Australian biotypes. This pathotype does not easily colonize the vascular system but is capable of causing significant amounts of root damage even in the absence of nematodes (Liu et al. 2011; Bell et al. 2017). Pathotype designation plays a crucial role in how Fusarium wilt is managed. The vascular-competent races/genotypes can be managed through a reduction in nematode levels, but the root-rot pathotype is managed mostly by plant resistance (Bell et al. 2017).

Smith (2015) reported that a single cotton field could contain multiple races/genotypes of the pathogen. However, little is known about how these diverse races of FOV interact with one another within a field. Some races, such as FOV race 4, are thought to infect 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 was initiated at the National Cotton Fusarium Wilt Evaluation Field, in Tallassee, Alabama to 1) assess the population diversity of FOV races/genotypes infecting cotton cultivars selected for either FOV or *M. incognita* resistance or susceptibility and to 2) determine the in-season temporal variability of FOV races/genotypes that might be isolated from these selected cotton cultivars. We hypothesized that the field would contain a diverse community of FOV races/genotypes capable of infecting all cotton cultivars included in the test throughout the cotton production season. We also anticipated a difference in the races/genotypes affecting the cotton with the *M. incognita* resistance genes compared to the cultivars without nematode resistance.

## 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 (latitude 32° 29'20.68" N longitude 85° 52'59.04" W). This field has been used to evaluate new cotton

genotypes and breeding lines for their resistance or susceptibility to the Fusarium wilt-*M. incognita* disease complex for more than 60 years (Kathryn Glass, personal communication). The trial field is a Kalmia loamy sand soil type consisting of 80% sand, 10% silt, and 10% clay with 1% organic matter and a pH of 5.3. Past testing has revealed this field has a diversity of FOV races/genotypes (Smith 2015), and it is known to contain an established population of *Meloidogyne incognita* race 3 (Groover et al. 2020). Three cotton tests were planted on May 17, 2018, (one trial) and April 24, 2019 (two trials). All trials were planted in a Latin Square design with ten replications using a John Deere MaxEmerge (John Deere; Moline, IL) planter with Almaco cone planters (Almaco; Nevada, IA) at a seeding rate of 13.1 seeds/meter of row. Of the two tests planted in 2019, one was planted into the same area used in the 2018 testing, and one was planted in a part of the field that was previously planted in soybeans. 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) chosen for this test based on their resistance or susceptibility traits for either FOV or *M. incognita*. The cotton species *Gossypium hirsutum* and *G. barbadense* were included due to documented differences in the sensitivity of these species to some races/genotypes of FOV (Kim et al. 2005).

Upland cotton, *G. hirsutum*, cultivars ‘Rowden’ and ‘M-315’ were used as susceptible and resistance checks, respectively, and were included in the test twice to help standardize the disease incidence across the field tests. The Rowden (USDA-ARS; Washington, DC) cultivar is susceptible to both FOV and *M. incognita* infection (Doan and Davis 2014). M-315 is a cultivar resistant to *M. incognita* infection (Shepherd et al. 1996). Phytogen 480 W3FE (Corteva Agriscience; Wilmington, DE) and DeltaPine 1558NR B2RF (Bayer Crop Science; Leverkusen, Germany) are both resistant to *M. incognita* with two genes for resistance (Faske et al. 2018).

Stoneville 4946 GLB2 (BASF corporation; Ludwigshafen, Germany) is a moderately resistant cultivar with one gene for resistance to *M. incognita* (Faske et al. 2018). One Acala cultivar, 'Phytogen 72', is resistant to FOV, except for FOV race 4, and susceptible to *M. incognita* (Hutmacher et al. 2013). Two Pima, *G. barbadense,* cultivars were also included in the test. PhytoGen 800 is resistant to FOV race 4 but susceptible to other races/genotypes of FOV, and susceptible to *M. incognita* infection (Mahill and Pellow 2005). Pima S-7 (USDA-ARS; Washington, DC) is susceptible to both FOV and *M. incognita* (Turcotte et al. 1992).

FOV Sample Collection, Isolation, and Identification

Sample collection of plants showing foliar symptoms (Figure 1) commonly associated with Fusarium wilt (wilting and chlorosis or necrosis of leaves) began two weeks after cotton planting. Sampling continued weekly for the first six weeks. After this time, samples were taken every other week until the cotton was defoliated before harvest. Sampling in 2018 ranged from June 4 to September 10, and 2019 sampling ranged from May 8 until September 3. In all analyses, sampling dates are given as growing degree day (DD60) values, as an alternative of date, to account for both the time of the season and cotton growth (DeVay et al. 1997). At each sampling date, any cotton plants from the test plots that exhibited foliar 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 lower 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. Cultures morphologically identified as *Fusarium* were then transferred to new half-strength APDA

plates.

Race identification of each FOV isolate collected throughout the growing season was obtained by sequencing of FOV gene fragments and comparing these to sequences from specific reference isolates. DNA was first extracted from fungal isolates by transferring cultures 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 (Zymo Research; Irvine, CA) following the manufacturer's protocol. Samples of fungal DNA were stored at -20°C until further use.

Fragments of the translational elongation factor (EF-1α), βeta-tubulin (BT), and phosphate permease (PHO) genes were sequenced for identification of each isolate collected during testing. PCR amplifications were conducted 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. Amplifications used specific primers and thermocycler settings 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. The use of these three gene fragments is sufficient to identify most races and genotypes of FOV; however, they are not adequate to differentiate between FOV race 4 and MDS-12 (race 4-like) isolates (Bennett et al. 2013; Scott 2012).

All isolates that were identified as race 4 and 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 (Table 2) was conducted to look for insertions in the PHO gene of the

isolates. Some isolates of FOV race 4 have been found to have a T*fo*1 transposon (T*fo*1), a mutator-like transposon element (MULE), or a miniature inverted-repeat transposable element (MITE) insertion in the PHO gene. After PCR was conducted, samples were run through a 1.5% agarose gel electrophoresis to view the DNA fragment size. Isolates without a gene insertion produce a DNA fragment of 396 base pairs (bp), while an isolate containing one of the gene insertions will range from 426-663 bp (Bell et al. 2019; Ortiz et al. 2017). Secondly, PCR was conducted on all isolates in question using the FOV race 4 specific primer set R4F and R4R following the protocol outlined by Yang et al. (2006). Finally, nearly full-length 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 reference 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 (https://www.ncbi.nlm.nih.gov/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.

Nematode Sampling and Extraction

Nematode samples were collected at the end of each cotton growing season just before the defoliation of the cotton crop (September 24, 2018 and September 14, 2019). Samples were collected using shovels to remove the roots of two arbitrarily selected plants from each one-row trial plot. *Meloidogyne incognita* eggs were extracted from the cotton roots by a modified version

of the methodology of Hussey and Barker (1973). The cotton roots were placed in a 0.625% NaOCl solution and shaken for four minutes at 1 g force using a Barnstead Lab-Line Max Q 5000E class shaker (Conquer Scientific; San Diego, CA). *Meloidogyne incognita* eggs were washed from the roots with tap water and collected on a 25-µm pore sieve. Eggs were then separated from the soil by modified sucrose centrifugation-flotation at 240 g for 1 minute (Jenkins 1964). After extraction, nematode eggs were enumerated under a Nikon TSX 100 inverted microscope (Nikon; Tokyo, Japan) at 40x magnification. Eggs per gram of cotton root (Eggs/g of root) were calculated by taking the ratio of total eggs extracted per the fresh root weight.

Statistical analysis

Statistical analysis was conducted in R (R Core Team 2018) using RStudio (RStudio Team 2015). Fusarium wilt incidence was collected from cotton trials throughout the three site years of the test. These data are presented as a disease index (DI) value based on a scale of 0-4 where each whole number corresponds to a 25 percent disease incidence. The DI was calculated using the equation $\mathrm{DI} = \frac{\mathrm{F}}{\mathrm{T}} * 4$, where F is equal to the total number of plants from which FOV was isolated and T is equal to the total plants in the plot obtained from 14 DAP stand counts (Schandry 2017). For each FOV race/genotype, and each cultivar included in the test, the area under the disease progress curve (AUDPC) was calculated based on the calculated DI and DD60's. AUDPC's were calculated using the Agricolae package (Mendiburu 2020) in R, and graphs were created using the ggplot2 package (Wickham, 2016). Analysis of AUDPC was conducted to compare both races of FOV and cotton cultivar by a linear mixed effect model, including test as a random effect in both analyses. Nematode eggs/g of root data were analyzed by ANOVA and means were separated using Tukey test at the $P \leq 0.05$ level. Eggs/g of root data were log-transformed to satisfy the ANOVA assumption of normally distributed residuals.

## Results

FOV Races and Genotypes

All plants sampled demonstrated typical symptoms of FOV including wilting, chlorosis and necrosis of the leaves, and discoloration of the vascular tissue in the stem of the plants (Figure 1). From the plants sampled during the three site years, FOV isolates were successfully obtained from 661 plants. Of these isolates, 126 were obtained during the 2018 test, and 535 were collected during 2019. From the 661 total isolates seven races/genotypes were identified during the 2018 test, and eight were collected during the 2019 test (Figure 2). Figure 3A displays the average AUDPC of each race/genotype in 2018 compared to the 2019 cotton season. Increased disease incidence was observed for each race/genotype during 2019 as compared to 2018. In this analysis, the curves are an average of all replications of the tests conducted in the respective year. These data represent ten replications in 2018 and twenty replications in 2019.

Figure 3B illustrates the statistical analysis of the AUDPC for each of the eight races found during these two years. Individual AUDPC values for each replication are marked with symbols that correspond to each test. The average AUDPC values and the 95% confidence intervals for each race/genotype, based on a linear mixed-effect model, are indicated with shaded boxes. FOV race 1 was the most damaging race with an average AUDPC of 101.1 and the most common field isolate in all tests making up 37.7% of the total FOV isolates (Figure 3B). However, high levels of damage were sustained from genotype LA-108, race 8, and race 2 with average AUDPC values of 59.8, 47.5, and 38.5, respectively. The genotypes LA-110 and LA-127/140 were found at low levels, 10 and 13 isolates respectively, during both years of testing. Three isolates of the genotype LA-112 were found in one of the 2019 tests (the location not used in 2018). This genotype was not found in 2018. In all three site years, a total of 16 isolates were collected that shared a 100%

identity with FOV race 4 and MDS-12 when sequenced at the EF-1α, BT, and PHO gene regions. Sequencing at these three gene regions using the primers listed in Table 2 does not allow enough differentiation to distinguish between the highly virulent FOV race 4 and the less virulent MDS-12 isolates.

Three further steps were taken to identify these isolates as either FOV race 4 or MDS-12. In a multiplex PCR analysis to look for T*fo* transposon insertions in the PHO gene, all of the isolates produced a DNA fragment size of 396 bp (Figure 4) regardless of the year of collection or cotton variety from which they were isolated. This 396 bp fragment is equivalent to the DNA fragment size produced by MDS-12 and by some FOV race 4 isolates. Second, in the PCR analysis using FOV race 4 specific primers, there was no amplification for any of the isolates in question. The final step that was taken to confirm the identity of these isolates as MDS-12 was the sequencing of the IGS gene. Sequences of these isolates shared 100% identity with previously identified MDS-12 isolates. A phylogenetic analysis of the combined EF-1α and IGS genes grouped the isolates into two separate clades with previously described MDS-12 isolates AL KL 11, AL KL 25, MS GL 10, and MS GL 18 (Figure 5). FOV Race 4 isolates included in the analysis were contained in a separate clade (Figure 5), demonstrating that the 16 isolates in question did not match FOV race 4.

Effect of Cotton Cultivar and *M. incognita*

Cotton cultivar played a significant role in the prevalence of FOV infection throughout the study. Figure 6A displays the average AUDPC values for each cotton cultivar included in the 2018 and 2019 cotton seasons to demonstrate the variation of disease impact between the two years. In this analysis, the curves are an average of all test plots containing each cultivar in each year. During both years of testing Rowden, the susceptible check, sustained increased incidence of Fusarium

wilt throughout the entirety of the cotton season. Other cultivars included in the test were affected by FOV at varying levels. All cultivars included in the test had a higher AUDPC value during the 2019 cotton season, except for PHY 800 (Figure 6A). This cultivar had a higher AUDPC rating for the 2018 season where generally less FOV infection was observed.

Figure 6B illustrates the statistical analysis of the AUDPC for each of the eight cotton cultivars included in the two years of testing. In this figure, individual AUDPC values for each replication are marked with symbols that correspond to each test plot. The average AUDPC value and 95% confidence intervals for each cotton cultivar based on a linear mixed-effect model are indicated with shaded boxes. Statistically similar AUDPC ratings were observed for Rowden (554.0), DeltaPine 1558NR B2RF (306.6), PhytoGen 480 W3FE (286.8), PhytoGen 800 (298.3), and Pima S-7 (287.1) cultivars. PhytoGen 800 and Pima S-7, two *G. barbadense* cultivars, supported the highest *M. incognita* root population density statistically similar but numerically larger that the populations supported by the Rowden cultivar (Table 3). PhytoGen 800 is known to be highly resistant to FOV race 4; however, it was susceptible to all of the races and genotypes of FOV found in this study. Pima S-7 is a cultivar known to be susceptible to all FOV races. DeltaPine 1558NR B2RF and PhytoGen 480 W3FE both have genes for resistance to *M. incognita* and sustained a significantly lower root population density of *M. incognita* compared to the Rowden susceptible check (Table 3). High levels of FOV infection were observed on these cultivars similar to that of the Rowden cultivar. However, M-315 and Stoneville 4946 GLB2 had low AUDPC values, 160 and 107 respectively. The AUDPC values for these cultivars were significantly reduced compared to the Rowden susceptible check and similar to that of the PhytoGen 72 cultivar (AUDPC 113), which is resistant to FOV race 1. M-315 is *M. incognita* resistant and sustained a lower nematode root population density compared to the Rowden

susceptible check. Stoneville 4946 GLB2 supported a similar *M. incognita* root population density to Rowden control. However, this cultivar sustained less FOV damage than did than Rowen, as measured by AUDPC.

Temporal Distribution of FOV

Infection from certain races and genotypes of FOV was affected by DD60 accumulation (Figure 7). Race 1 FOV was found in every sampling date throughout the season, and the number of samples collected was correlated with the accumulation of DD60's. FOV race 1 samples increased by 0.58 FOV samples/collection date (±0.33; ±95% C.I.) for every 100 DD60's accumulated (P-value=0.0015, $R^2$=0.31). Genotype LA-108 samples increased by 0.44 FOV samples/collection date (±0.25; ±95% C.I.) for every 100 DD60's accumulated (P-value=0.0013, $R^2$=0.31). Race 2 and 8 had similar rates of development to each other with a 0.26 FOV samples/collection date increase (±0.13; ±95% C.I.) for every 100 DD60's accumulated (P-value=0.00047, $R^2$=0.36), and a 0.21 FOV samples/collection date increase (±0.19; ±95% C.I.) in race 8 FOV samples for every 100 DD60's accumulated (P-value=0.033, $R^2$=0.15). Ten LA-110 samples were collected, and a significant correlation was observed between these samples and the accumulation of DD60's. In LA-110 a 0.06 FOV samples/collection date increase (±0.031; ±95% C.I.) was observed for every 100 DD60's accumulated (P-value=0.00024, $R^2$=0.39). Other genotypes of FOV (LA-112, LA-127/140, and MDS-12) collected during this study were not found to have a significant correlation with DD60 accumulation. However, relatively low numbers (3, 13, and 16 isolates respectively) of these genotypes were found in this research.

## Discussion

The National Cotton Fusarium Wilt Evaluation Field was found to have a more diverse population of FOV races/genotypes than were found in previous studies by Smith (2015) and Scott

(2012). In total, eight races/genotypes of FOV were found during our study. Smith (2015) did not detect the genotypes MDS-12 and LA-112 in this field. However, Scott (2012) did find the genotype MDS-12 but not LA-112. In our study, LA-112 was isolated at a very low frequency. Lack of detection of this genotype by Scott (2012) and Smith (2015) may have been due to the genotype being below detectable levels at the time of the studies. These results suggest that other fields may have more diverse FOV races/genotypes than previously recognized, and such fields can only be characterized through an extensive sampling of Fusarium wilt symptomatic plants.

Another important result of this study is what was not found. FOV race 4 was not detected in the National Cotton Fusarium Wilt Evaluation Field, despite the many germplasm lines introduced and tested at this site for at least the last 60 years. To date, FOV race 4 has not been found in the state of Alabama and has only been reported in California (Kim et al. 2005), Texas (Halpern et al. 2017), and New Mexico (Zhu et al. 2019c). In this testing some isolates were found to be identical to FOV race 4 and MDS-12 in the EF-1α, BT, and PHO gene sequences and sequencing IGS gene determined the identity of these isolates as MDS-12.

Race 4 is a highly virulent race of the FOV pathogen, which is not dependent on nematodes to cause high levels of damage. The most effective management strategy for this race of FOV is the use of tolerant cotton cultivars. To this point, there are Pima cotton cultivars with high levels of tolerance to FOV race 4 but so far, no Acala or Upland cultivars have been identified that have high levels of tolerance. In contrast, MDS-12 isolates collected in Alabama and Mississippi are of less of a concern because they were only moderately virulent to the cotton cultivars that have been tested (Bennett et al. 2013). Each of the 16 isolates, which shared the base sequence identity of the three diagnostic genes with FOV race 4, were confirmed to be MDS-12. This genotype has even been referred to as non-pathogenic (Bell et al. 2017). While the isolates collected in this study

exhibited low virulence, they were capable of infecting cotton and causing disease on both Upland cotton (*G. hirsutum*) and Pima cotton (*G. barbadense*) under field conditions in the presence of *M. incognita*.

The FOV genotype of LA-112 was only found during 2019, where the test was planted in a part of the field not included in the 2018 test. In the test where this genotype was found, it was only collected at low incidence (three total isolates) in what was otherwise a severe year for Fusarium wilt infection. A low rate of infection and sparse distribution within the testing area suggests a recent introduction to the field. This isolate was not found during a survey of this field conducted during 2010 and 2011 (Scott 2012) or 2013 and 2014 (Smith 2015). However, the genotype LA-112 was found on this farm (Kathy Lawrence, Auburn University, personal communication) in samples submitted to a nationwide survey (Cianchetta 2015). LA-112 may have been introduced into the area through contaminated seed produced in fields containing this genotype of FOV (Taubenhaus and Ezekiel 1932). Greater distribution of this genotype can also be expected with continuous cotton production as it is spread within the field by cultivation or movement with water (Grinstein et al. 1983).

Seedborne dissemination likely played a role in the diversity of FOV types that can be found in this test field. This field has been monocultured in cotton for more than 60 years as the testing site of new cotton cultivars and breeding lines for resistance to Fusarium wilt (Kathryn Glass, personal communication). Each year a large number of new cotton cultivars and breeding lines from breeders around the world were screened in this field and the seeds could have brought new and different FOV races/genotypes as seedborne inoculum. Due to this continuous introduction of germplasm from diverse locations, we expect that this field evidences a more diverse FOV community than a typical farmers field, where one or two cotton cultivars may be

planted each year. However, in this study, no Alabama farmer's fields were surveyed for FOV diversity to confirm this. Georgia farmer's fields have been reported to sometimes be infested with multiple races/genotypes of FOV (Bell et al. 2019). While this particular field may be unique in its history and abundant diversity of FOV, farmers could face similar problems with multiple populations of FOV causing damage to their cotton.

The most prevalent races/genotypes found in this study including race 1, race 2, race 8, and LA-108 accounted for more than 93% of the samples collected. All of these races/genotypes, along with LA-110, had a positive correlation with DD60 accumulation. This means that as the season progressed and cotton matured, higher rates of FOV symptoms were documented. This trend to later symptom expression partially mitigates damage. Plants which develop symptoms after flowering are often still capable of producing bolls though they may be reduced in number, size, and lint quality (Davis et al. 2006). In contrast, plants that are symptomatic early in the season often die and therefore produce no cotton. This trend of later disease expression is in contrast to studies with FOV race 4, a race not found in this testing, which has often been associated with early season (4-10 node stage) infections in California (Hutmacher et al. 2011).

The 2019 cotton season proved to be a severe FOV infection year compared to the 2018 season. Of the 661 total FOV samples collected during this study, 81% (535 samples) were collected during the 2019 testing versus only 126 collected in 2018. While there were twice as many plots (two tests) sampled in 2019, this increase in the number of plots fails to fully account for such a substantial increase in the number of samples. Environmental conditions, especially temperature, have been found to have an effect on the incidence and severity of FOV infection (Zhang et al. 2020). One major difference between the 2018 and 2019 seasons was the date of planting the cotton test. In 2018 the test was planted on May 17, and in 2019 the tests were planted

on April 24. The earlier planting date resulted in cool and wet conditions during the early part of the season during 2019, unlike the 2018 test. During the first 30 days of the growing season in 2018, the cotton received 10.6 cm of rain, and 551 DD60's had accumulated. During this same amount of time in 2019, the crop received nearly three centimeters more rain (13.0 cm), and only 350 DD60's had accumulated. This difference in environmental conditions between the two seasons could have significantly affected the disease pressure throughout the year as has been observed with Fusarium wilt affecting many crops around the world (Scott et al. 2010; Li et al. 2017; Jelínek et al. 2019). Zhang et al. (2020) documented increased disease severity with some races of FOV with lower temperatures in the early part of the growing season which resulted in reduced DD60 accumulation.

Only one cultivar, PhytoGen 800, sustained more damage from FOV in the 2018 season compared to the 2019 season. This cultivar had an AUDPC rating of 305.5 in 2018 and 294.7 in 2019. This higher disease progress curve rating in what was generally the less severe of the two years may have been a result of higher nematode pressure on the variety in the 2018 cotton season. This cultivar had an average *M. incognita* root population density of 2,069 eggs/g of root during the 2018 season and 524 eggs/g of root during the 2019 season (data not shown). In both cases, this cultivar sustained the highest nematode root population density of any of the cultivars included in the test. It is unclear why the nematode population density was so high on this cultivar during the 2018 season. But as there is a strong interaction between *M. incognita* and FOV, this higher nematode population density could have increased the damage from the Fusarium wilt disease complex (DeVay et al. 1997; Garber et al. 1979).

Even though the disease complex of FOV and *M. incognita* has been recognized since the first reporting of Fusarium wilt, the specifics of this interaction are not fully understood. It is

commonly thought that the increase in Fusarium wilt is the result of wounds created by nematode penetration and feeding. However, evidence suggests there is a more complex interaction due to physiological changes in the cotton root apart from wounds caused by the nematode (Starr 1998). One of the driving forces behind this theory is the greenhouse observation that FOV infection is more severe when *M. incognita* is inoculated on the plants about four weeks before inoculation with FOV (Porter and Powell 1967). *Meloidogyne incognita* has also been found to cause little wounding to plant roots during the infection process suggesting that the interaction may rely on other mechanisms (Endo and Wergin 1973). Giant cells, which are established for nematode feeding, are more susceptible to fungal infections, including infection by FOV which could play a role in the interaction of these two pathogens (Meléndez and Powell 1967; Fattah and Webster 1983). While the mechanism of this interaction may not be understood, the effects have been well documented (Atkinson 1982; Roberts et al. 1985; Starr et al. 1989). The National Cotton Fusarium Wilt Evaluation field used for this study has been infected with both the nematode and FOV pathogens for at least the last 60 years, and both of the pathogens continue to cause damage at this location.

Often the FOV-*M. incognita* disease complex is managed by reducing nematode population density in the cotton field (Colyer 1997; Jorgenson 1978). Whereas there are measures, such as rotations, nematicides, and resistant cultivars that are partially effective against *M. incognita,* there is a general lack of effective control methods against FOV. Lowering nematode population density has been demonstrated to reduce the amount of FOV infection caused by vascular-competent races/genotypes (Colyer et al. 1997). While lowering the *M. incognita* population density in the field can reduce the damage from all races/genotypes of FOV found in the study field, this research demonstrated that plant resistance to the FOV as expressed in these

cultivars appears to provide better protection than did *M. incognita* resistance. The cultivar PhytoGen 72, which is known to be widely resistant to FOV races/genotypes (Hutmacher et al. 2013), except for FOV race 4, had low levels of FOV infection even though it sustained a similar *M. incognita* root population density to that of the Rowden susceptible control. While the *M. incognita* resistant cultivars of Deltapine 1558NR B2RF and PhytoGen 480 W3FE did support a lower nematode population density, FOV disease incidence was not reduced. No significant correlation was observed between FOV race/genotype and *M. incognita* population density. FOV races/genotypes did not vary significantly in their distribution between the nematode resistant and nematode suspectable cultivars. This suggest that the FOV races/genotypes found in this study all interact synergistically with *M. incognit*a to cause Fusarium wilt and no isolates of the root-rot pathotypes are present at detectable levels in the field.

Other species of plant-parasitic nematodes have also been associated with an increased incidence of FOV, including *Rotylenchulus reniformis* and *Belonolaimus longicaudatus* (Jones et al. 1959; Khadr et al. 1972; Yang et al. 1975; Silva et al. 2019). *Belonolaimus longicaudatus* has been associated with many fields incurring losses from Fusarium wilt in Georgia in the last few years and is speculated to play a larger role in the Fusarium wilt-nematode disease complex than previously thought (Silva er al. 2019). Neither *R. reniformis* nor *B. longicadutaus* were detected in the field location used in this study. This is most likely due to soil texture at the research location, which was a Kalmia loamy sand soil type consisting of 80% sand, 10% silt, and 10% clay. This soil texture is well suited for *M. incognita* but not for *R. reniformis* which is better suited to more finely textured soils or for *B. longicadutaus,* which require higher sand contents (Robbins and Barker 1974; Robinson et al. 1987). The Fusarium wilt-nematode disease complex in this study is believed to rely entirely upon the interaction between FOV and *M. incognita*, as neither of the

other two nematodes were found in the test field. A more in-depth understanding of the interactions of FOV races and genotypes and different plant-parasitic nematodes may help in the future management of this disease complex.

The Fusarium wilt-nematode disease complex of cotton is a multifaceted pathogenicity system made up of many races/genotypes of the FOV pathogen and numerous plant pathogenetic nematodes. The apparent diversity of FOV is increasing with new genotypes of the pathogen being discovered and new species of *Fusarium* appearing to cause symptoms similar to Fusarium wilt (Guo et al. 2015; Zhu et al. 2019a, b). In China, a new genotype of FOV has been found causing Fusarium wilt of cotton. These newly described genotypes are distinctly different from previously described genotypes and races FOV in China (Guo et al. 2015). Additional *Fusarium* species (*F. proliferatum* and *F. solani*) have been identified causing wilt of cotton in New Mexico, producing symptoms of leaf yellowing and wilting as well as discolored vascular tissue (Zhu et al. 2019a, b). With the genetic variation that exists within this disease complex, more in-depth studies are needed to understand the relationships between these races/genotypes and how the disease may be managed. Recent work in full genome sequencing (Seo et al. 2020) may help to provide some insight into the relationships of this diverse group of cotton pathogens. With the spread of highly virulent races of the pathogen such as FOV race 4, better understanding of the disease interactions and options for management are of increasing importance.

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

| **Cotton Cultivar** | **Reported Resistance Traits** |
|---|---|
| **Upland Cotton – *Gossypium hirsutum*** | |
| Rowden | FOV and RKN susceptible |
| M-315 | RKN resistant |
| PhytoGen 480 W3FE | RKN resistant |
| DeltaPine 1558NR B2RF | FOV susceptible and RKN resistant |
| Stoneville 4946 GLB2 | RKN moderate resistance |
| **Acala Cotton - *Gossypium hirsutum*** | |
| PhytoGen 72 | FOV resistant, RKN susceptible |
| **Pima Cotton – *Gossypium barbadense*** | |
| PhytoGen 800 | FOV race 4 resistant, RKN susceptible |
| Pima S-7 | FOV and RKN susceptible |

Table 2: Primers and thermocycler settings used for race identification of FOV isolates

| Primer name | 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°C 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°C 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°C 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°C for 30 sec, 59°C for 30 sec, and 72°C for 15 sec with a final extension of 72°C for 1 min |
| R4R | TGCTCATCGTGGAGCATAAC | | |
| **Multiplex PCR for detection of T*f*O1, MULE/ T*f*O1, and MITE/ T*f*O1 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°C 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°C for 5 min |
| NL11 | CTGAACGCCTCTAAGTCAG | | |
| iNL11 | AGGCTTCGGCTTAGCGTCTTAG | | Used for sequencing only |
| NLa | TCTAGGGTAGGCKRGTTTGTC | | |
| CNSa | TCTCATRTACCCTCCGAGACC | | |
| iCNS1 | TTTCGCAGTGAGGTCGGCAG | | |

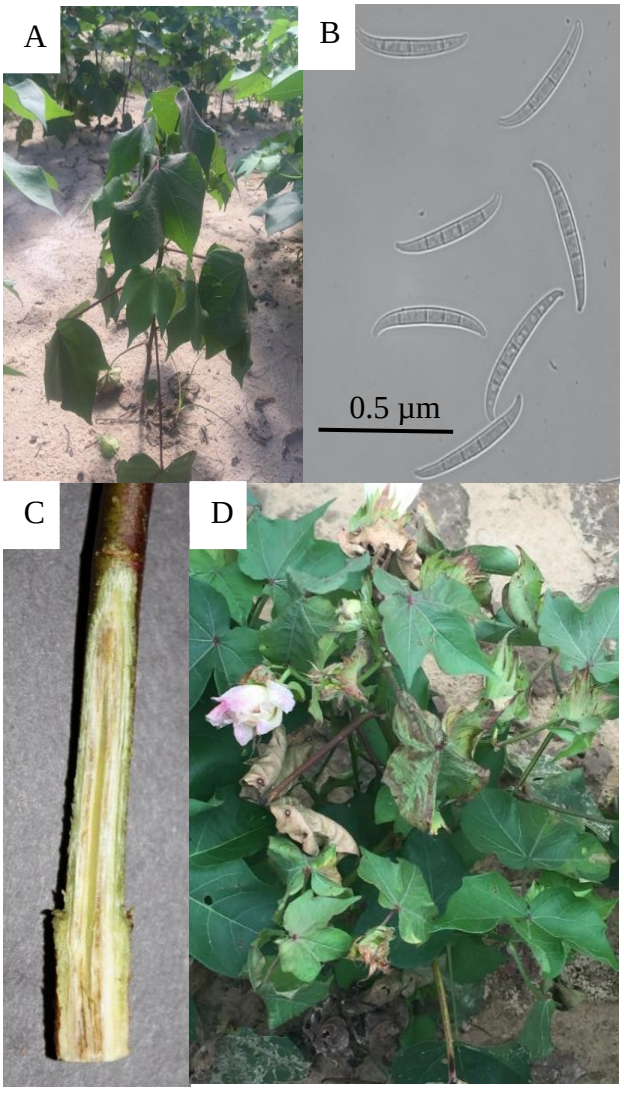
Table 3: *Meloidogyne incognita* race 3 average root population density by cotton cultivar from all *Fusarium* testing during 2018 and 2019 in the National Cotton Fusarium Wilt Evaluation field.

| **Cotton Cultivar** | ***M. incognita* eggs/g of root**[y] | ***M. incognita* Reported Resistance Traits** |
|---|---|---|
| Rowden | 230[z] cd | Susceptible |
| M-315 | 11 ab | Resistant |
| PHY 480 W3FE | 4 a | Resistant |
| DeltaPine 1558NR B2RF | 38 ab | Resistant |
| Stoneville 4946 GLB2 | 40 bc | Moderate Resistance |
| PhytoGen 72 | 125 cd | Susceptible |
| PhytoGen 800 | 1039 d | Susceptible |
| Pima S-7 | 790 d | Susceptible |

[y] *Meloidogyne incognita* eggs were extracted from two cotton roots collected at the end of each growing season just before defoliation of the crop. Values present are means taken all three site years of the testing.

[z] Values present are LS-means of *M. incognita* root population density across all three tests, separated using Tukey's HSD test at $P \leq 0.05$. Nematode egg data were log-transformed to satisfy the ANOVA assumption of normally distributed residuals values in the column followed by different letters differ significantly.

Figure 1: Signs and symptoms of Fusarium wilt on *Gossypium hirsutum*. A) Wilting of cotton plant caused by Fusarium wilt infection. B) *Fusarium oxysporum* f. sp. *vasinfectum* macroconidia at 400x magnification. C) Discoloration of the vascular tissue of a cotton stem. D) Leaf necrosis and wilting caused by Fusarium wilt.



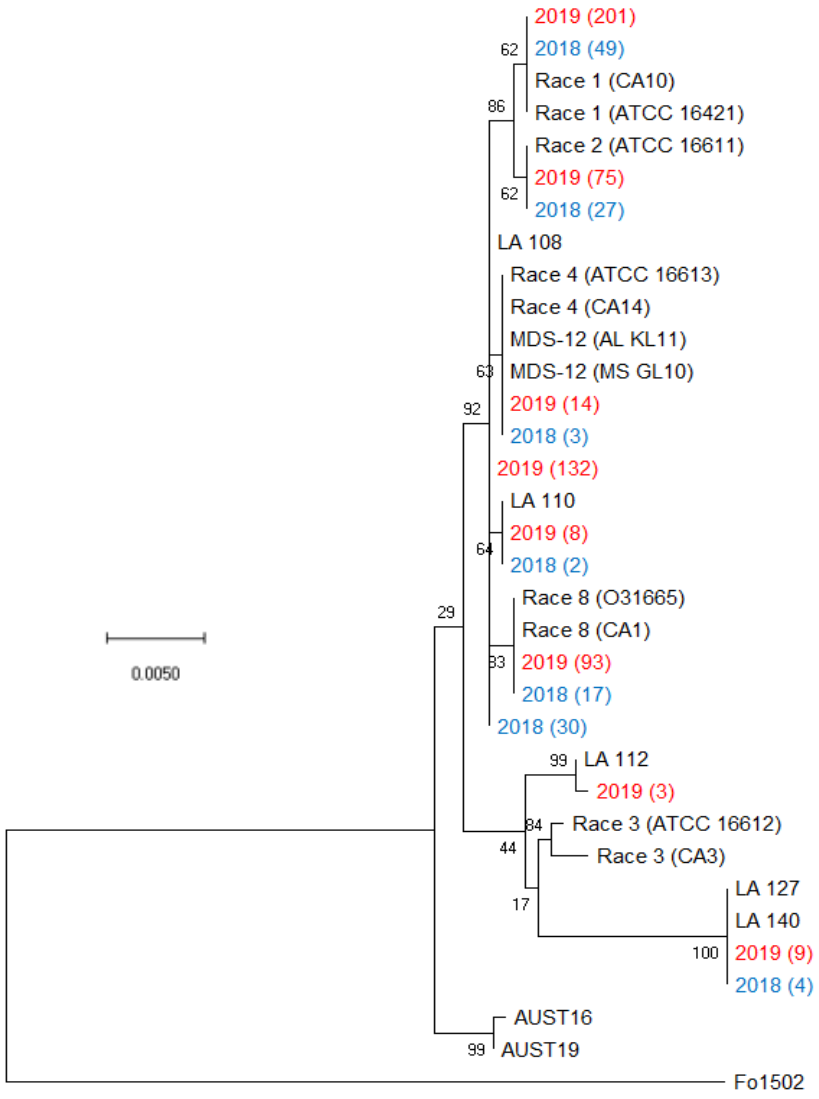



Figure 2: Condensed phylogenetic tree of FOV isolates collected during the 2018 and 2019 cotton seasons using a partial sequence analysis of the translation elongation factor, β-tubulin, and the phosphate permease genes. Tree was constructed in MEGAX using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2944.90) is shown. Bootstrap frequencies from 1,000 replications are noted next to every branch. Isolates with identical sequences collected during the study are represented 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 (201) represents 201 identical isolates that were collected in 2019. Isolates collected during the 2018 season are shown in blue and isolates collected during 2019 are shown in red. References isolates (in black) 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 non-pathogenic *Fusarium oxysporum* (isolate 1502) was used as an outgroup to root the tree.

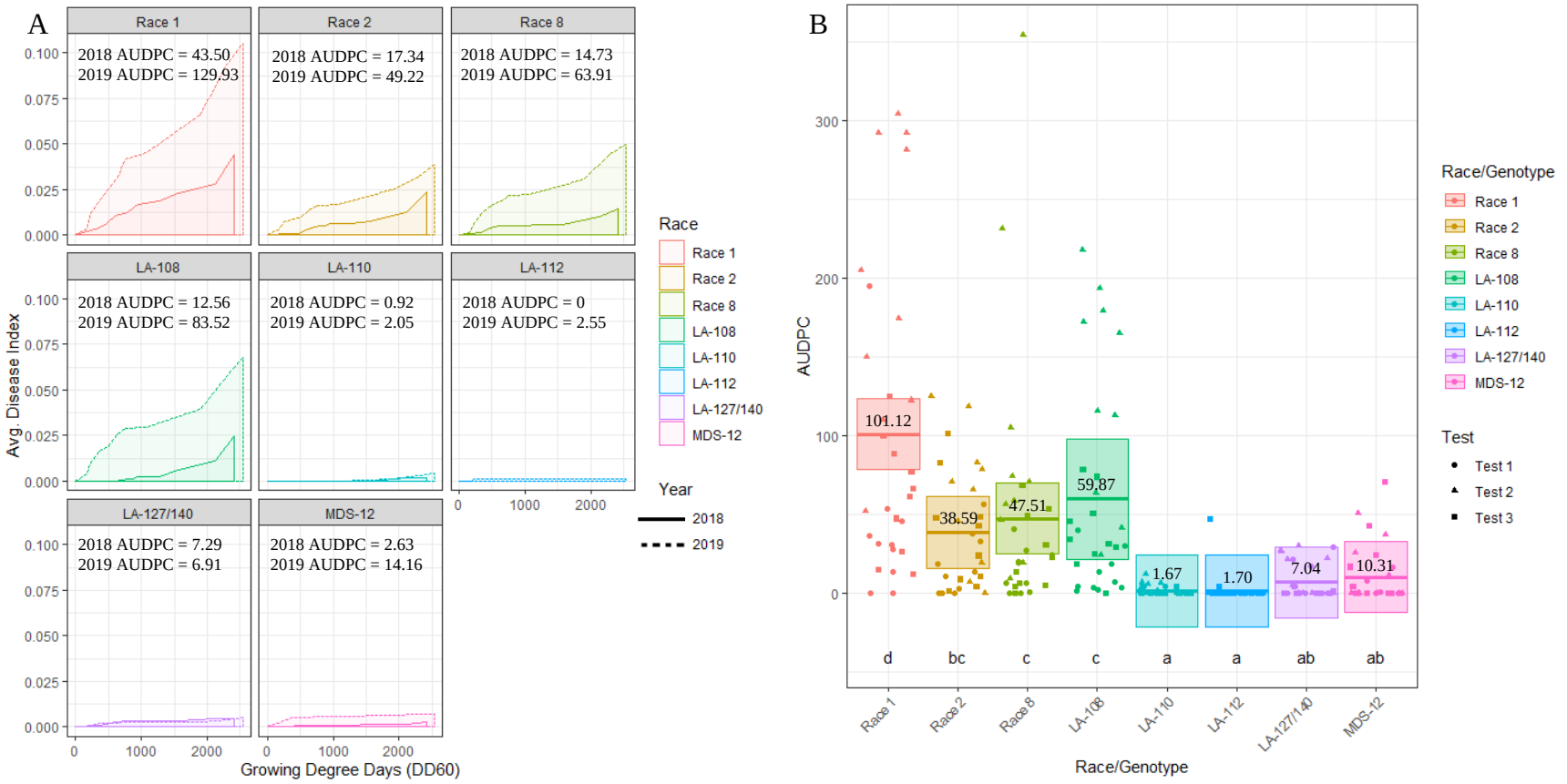



Figure 3: (A) Temporal disease progression curves for each race/genotype of FOV collected to show the distribution throughout the cotton seasons depicted as a solid line for 2018 testing and a dashed line for 2019 testing. Area under the disease progress curve (AUDPC) is shaded in a lighter color as indicated by the legend. (B) Box plots with means (indicated by thick horizontal lines) and 95% confidence intervals (shaded boxes) of the AUDPC values for each race/genotype of FOV as estimated by a linear mixed effect model shown as an average of all three test. Calculated AUDPC values for individual test plots are marked by symbols corresponding to the test from which they were collected. The mean AUDPC value is listed just above the mean line for each race/genotype of FOV and statistical significance is indicated by letters below the boxplots. Races/genotypes that share letters do not differ significantly.

1

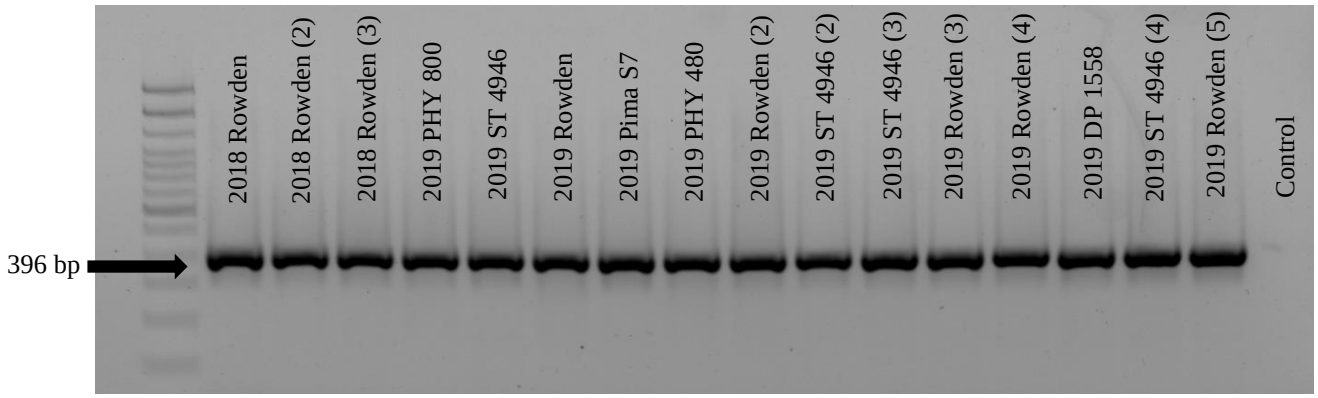



Figure 4: Gel electrophoresis image of a PCR analysis for the detection of T*fo*1, MITE/T*fo*1, MULE/T*fo*1 insertions into the PHO gene of FOV isolates which are commonly found in FOV race 4 isolates in the United States. Bands appear at 396 bp when no insertion is present, 583 bp when the T*fo*1 insertion is present, 426 bp when the MULE/T*fo*1 insertion is present, and 663 bp when the MITE/T*fo* insertion is present. Isolates shown are lane 1, 100 bp DNA ladder; lane 2-17, MDS-12 isolates of FOV; lane 18 water control. All isolates produced a band at 396 bp showing the lack of a T*fo* insertion into the PHO gene.

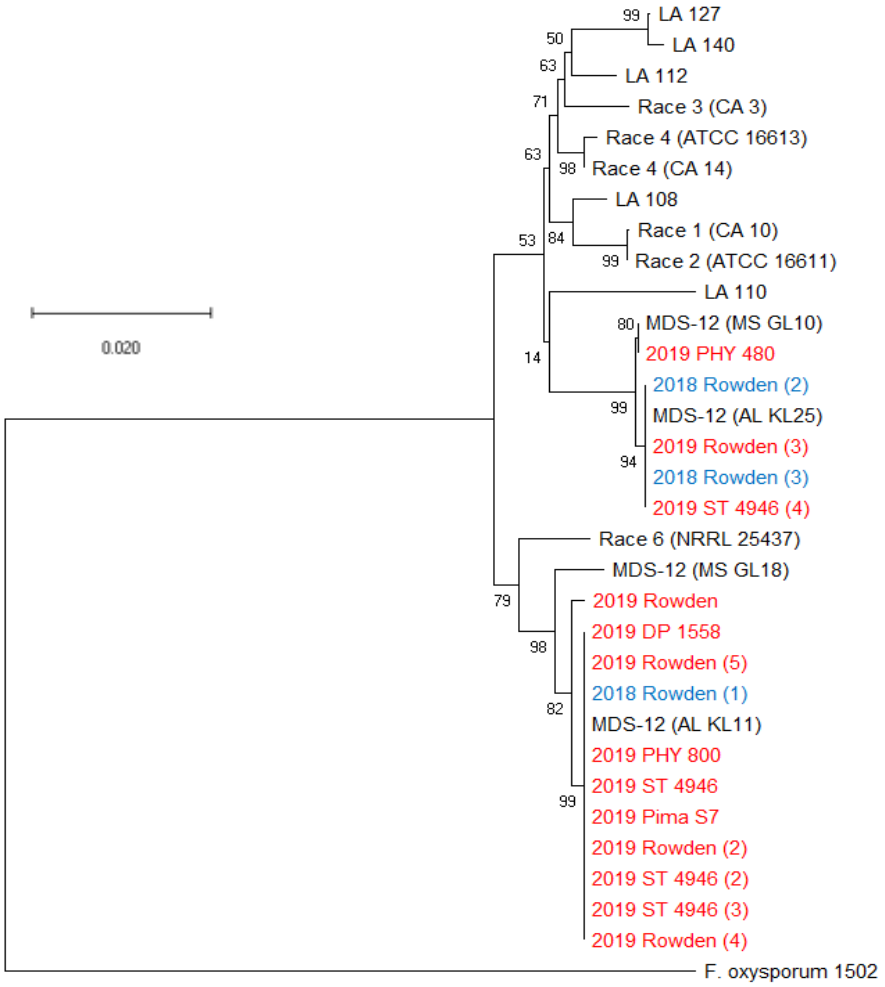



Figure 5: Phylogenetic tree of FOV isolates collected during the 2018 and 2019 cotton seasons using a partial sequence analysis of the translation elongation factor and nearly full-length sequences of the intergenic spacer region. This tree only shows isolates that were identified as FOV race 4 and MDS-12 when sequenced at the translation elongation factor, β-tubulin, and the phosphate permease gene regions, and thus required further sequencing for identification. Tree was constructed in MEGAX using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-7043.88) is shown. Bootstrap frequencies from 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, a number was added in parenthesis to separate isolates. For example, isolate 2018 Rowden (2) represents the second isolate collected from the Rowden cultivar in 2018. Isolates collected during the 2018 season are shown in blue and isolates collected during 2019 are shown in red. Reference isolates (in black) 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 non-pathogenic *Fusarium oxysporum* (isolate 1502) was used as an outgroup to root the tree.

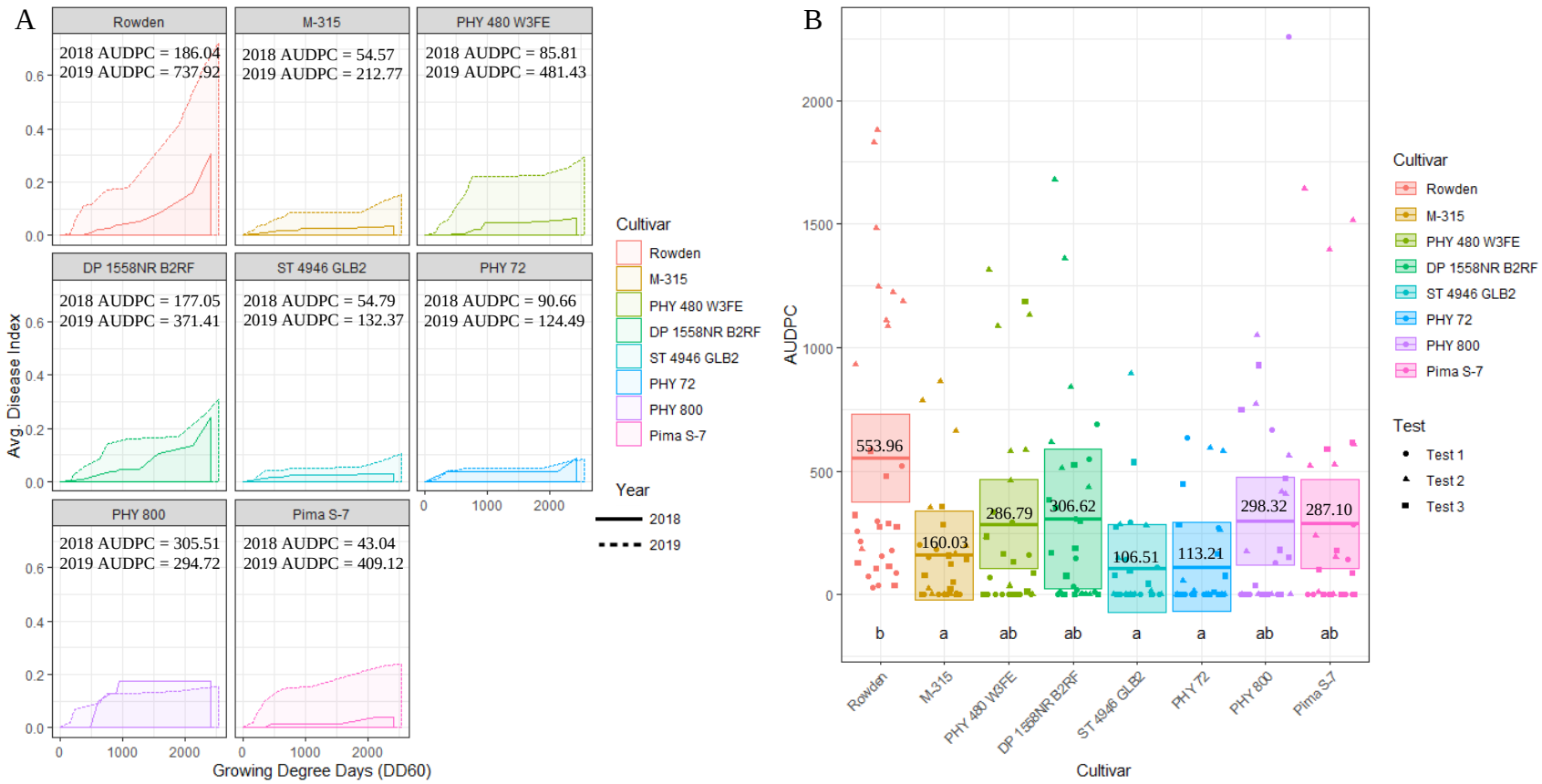



Figure 6: (A) Temporal disease progression curves for each cultivar included in the test to show the distribution throughout the cotton seasons demonstrated as a solid line for 2018 testing and a dashed line for 2019 testing. Area under the disease progress curve (AUDPC) is shaded in a lighter color as indicated by the legend. (B) Box plots means (indicated by thick horizontal lines) and 95% confidence intervals (shaded boxes) of the AUDPC values for each cotton cultivar as estimated by a linear mixed effect model shown as an average of all three test. Calculated AUDPC values for individual test plots are marked by symbols corresponding to the test from which they were collected. The mean AUDPC value is listed just above the mean line for each race/genotype of FOV and statistical significance is indicated by letters below the boxplots. Races/genotypes that share significance letters do not differ significantly.

1

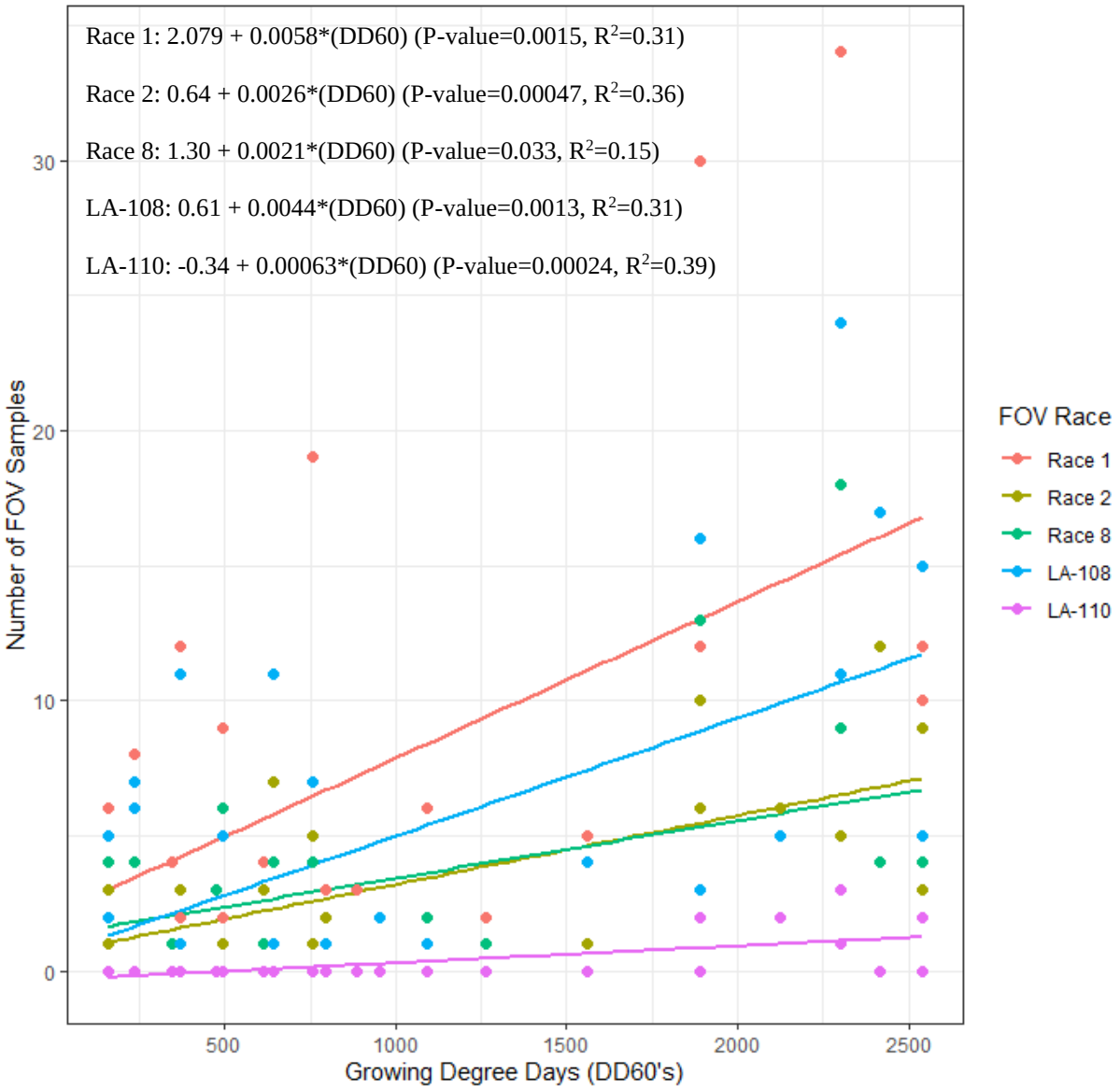



Figure 7: The 2018 and 2019 temporal distribution of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) throughout the cotton growing season. Graph shows the linear relationship between the number of FOV samples collected and the accumulation of growing degree days (DD60's). Linear relationships for race 1 race 2, race 8, LA-108, and LA-110 are shown. Other races and genotypes found in this study did not have a significant relationship with accumulation of DD60's and therefore are not shown in this figure.

## Chapter 3: Use of Reklemel™ for management of *Fusarium oxysporum* f. sp. *vasinfectum* and *Meloidogyne incognita* disease complex in cotton

**Abstract:** *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), the causal agent of cotton Fusarium wilt, can form a disease complex with *Meloidogyne incognita* to cause significant crop damage and losses. Multiple management strategies have been used to try to lessen the impact of this disease complex, several of which focus on reducing the nematode population density to minimize the impact of the overall disease complex. Reklemel™ is a new nematicide recently marketed for control of plant-parasitic nematodes. The objectives of this study were 1) evaluate the effects of Reklemel™ on the growth of FOV isolates *in vitro* and 2) assess crop growth, yield, and disease incidence with the application of Reklemel™ under greenhouse and field conditions. FOV races/genotypes collected from the field site were found to be impacted by Reklemel™ at varying levels in an *in vitro* assay. $EC_{50}$ values ranged from a low of 14.6 mg/L with a race 2 isolate to a high of 187.6 mg/L with an isolate of the genotype LA-108. In greenhouse tests, Reklemel™ was found to significantly reduce *M. incognita* population density compared to the untreated control but there was no significant effect on Fusarium wilt incidence. In field testing, Reklemel™ significantly reduced *M. incognita* population density and Fusarium wilt incidence compared to the control. A reduction in Fusarium wilt was not observed in treatments of Velum Total™, but reduced *M. incognita* population density was observed. No significant yield response was observed from treatment of Reklemel™ or Velum Total™.

## Introduction

*Fusarium oxysporum* f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hansen (FOV), the causal agent of cotton Fusarium wilt, was first identified infecting cotton in 1892 (Atkinson 1892). Since this time, the pathogen has been found infecting all four domesticated cotton species and in every major cotton-growing region around the world (Davis et al. 2006). Symptoms of the disease include wilting (Figure 1A), stunting, leaf chloroses and necrosis. Dark discoloration of the vascular tissue (Figure 1B) can be observed when the stem is sliced. Fusarium wilt of cotton is capable of causing large amounts of crop damage and yield loss. Fusarium wilt disease losses have been estimated over the last 55 years to cause an average annual yield loss of 62,391 bales valued at 29.9 million dollars at the cotton lint value of one dollar per pound (https://www.cotton.org/tech/pest/index.cfm).

A wide diversity of FOV race/genotypes are known to exist and cause Fusarium wilt on cotton. Past testing revealed that races 1, 2, 3, and 8 and genotypes LA-108, LA-110, LA-127/140, and MDS-12 were in Alabama (Scott 2012; Smith 2015; Cianchetta 2015). These races/genotypes of FOV are known to interact with nematodes to contribute to some of the yield losses associated with Fusarium wilt in the United States. Throughout the rest of the United States Cotton Belt and the world, other races/genotypes of FOV are also found to cause Fusarium wilt on cotton, including the highly virulent FOV race 4 and Australian biotypes (Armstrong and Armstrong, 1960; Davis et al. 1996). FOV race 4 and the Australian biotypes can cause high amounts of infection and damage even when nematodes are not present (Liu et al. 2011; Bell et al. 2017).

FOV has long been known to interact with several nematode genera to increase the incidence and severity of Fusarium wilt infection. One of the most well-known nematode-fungal interactions is between FOV and *Meloidogyne incognita* (Kofoid and White) Chitwood. The

interaction between these two pathogens was first observed in the original reporting of Fusarium wilt, which was known as Frenching at the time (Atkinson, 1892). During this report, Atkinson associated higher incidence and severity of Fusarium wilt with the presence of nematodes causing "knotty swellings" on the roots, which are assumed to be *M. incognita* (Atkinson, 1892). After this original report, other nematode species including *Rotylenchulus reniformis* (Linford and Oliveira) and *Belonolaimus longicaudatus* (Rau) have been found to share a similar synergistic interaction with FOV (Jones et al. 1959; Khadr et al. 1972; Yang et al. 1975; Silva et al. 2019a). The mechanisms of these interactions are poorly understood, but this increased susceptibility and severity of Fusarium wilt is believed to be based around the biochemical and physiological changes to the plant that are brought on by the nematode infection (Starr 1998).

Multiple strategies have been evaluated and implemented for the management of FOV with varying levels of success. Due to the FOV's ability to produce long-living chlamydospores and live on many plant species without causing disease, crop rotation has been relatively ineffective at managing the disease (Smith et al. 2001). Application of some fumigants have been effective in lowering FOV soil levels; however, the expense of application makes this impractical for many cotton farmers (Davis et al. 2006). The Fusarium wilt disease complex is often successfully and economically managed through the use of nematode control methods with the exception of fields which contain FOV race 4 or the Australian biotypes. Many different nematicides have been used for this purpose including in-furrow sprays, seed treatments, granular nematicides and, fumigants (Colyer et al. 1997; Jorgenson 1978; Jorgenson et al. 1978; Lawrence et al. 2007). It has even been speculated that the reduced use of some effective nematicides such as aldicarb may have resulted in increased Fusarium wilt incidence in Georgia (Silva et al. 2019a). Reklemel™ (Corteva Agriscience; Wilmington, DE) is a new sulfonamide compound that has been found effective at

reducing plant-parasitic nematodes such as *M. incognita* (Lahm et al. 2017; Thoden and Wiles 2019). The objectives of this study were to 1) evaluate the effects of Reklemel™ on the growth of FOV isolates *in vitro* and 2) assess crop growth, yield, and disease incidence with the application of Reklemel™.

## Materials and Methods

*In Vitro* Test

Eight isolates were collected from the National Cotton Fusarium Wilt Evaluation Field located at the Plant Breeding Unit of Auburn University's E. V. Smith Research Center in Tallassee, AL (latitude 32° 29'20.68" N longitude 85° 52'59.04" W) during the 2018 and 2019 cotton seasons. Isolate collection was conducted by removing plants from the field that were exhibiting foliar symptoms of Fusarium wilt and transporting these plants to the lab. Fungal isolations were accomplished in the lab by splitting the lower stem and upper taproot of the cotton plants using a scalpel and removing three small sections of the vascular tissue. These sections were surfaced sterilized in 95% ethanol for 30 seconds and then in a 0.625% NaOCl solution for one minute. After surface sterilization, the sections of vascular tissue were rinsed in sterile water and placed onto a Petri dish containing half-strength acidified potato dextrose agar (APDA). After Petri dishes were incubated in light at room temperature for three to five days to allow for fungal growth, individual cultures, morphologically identified as *Fusarium* (Leslie and Summerell 2006), were transferred to new Petri dishes containing half-strength APDA.

The race/genotype of each FOV isolate collected was identified by sequencing fragments of the translational elongation factor (EF-1α), Beta-tubulin (BT), and phosphate permease (PHO) genes. For sequence analysis, fungal isolates were transferred to new half-strength APDA plates whose surface was covered in a sterile cellophane sheet (Bennett et al. 2013). After allowing for

five to ten days of fungal growth, the mycelium was removed from the Petri dish by scraping the surface of the cellophane with a scalpel. DNA was extracted from this harvested mycelium using a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research; Irvine, CA) following the manufacture's protocol. Samples of fungal DNA were stored at -20°C until further use. From the extracted DNA, PCR amplifications were conducted 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. Primers and thermocycler settings listed in Table 1 were used. 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. After identification culters were preserved in a 15% glycerol solution at -80°C until used for *in vitro* testing (Leslie and Summerell 2006).

*In vitro* tests were conducted to assess the sensitivity of FOV isolates to Reklemel™. Evaluations were made by measuring mycelial growth rates of FOV isolates on media amended with Reklemel™. The test was conducted using one isolate each from FOV races/genotypes 1, 2, 8, MDS-12, LA-108, LA-110, LA-112, and LA-127/140. Each of the eight FOV isolates were grown on PDA plates amended with one of six rates of Reklemel™ (0.01, 0.1, 0.5, 1, 10, 100 ppm of fluazaindolizine) and one control plate that contained no Reklemel™ to assess sensitivity to the compound. A stock suspension of Reklemel™ was made by mixing the chemical with sterile water at a rate of 1000 ppm of fluazaindolizine. This suspension was then added to the PDA media at the desired concentration just before poring media into Petri dishes. Before poring the media, the antibiotics streptomycin and kanamycin were also added to the media at a rate of 100 mg/L to minimize bacterial growth. The day following preparation of the Petri dishes with amended media, a 5 mm FOV mycelial plug was aseptically transferred to each Petri dish. Each Petri dish was then

incubated at 28°C (±2°C) with a 12-hour photoperiod for eight days. This assay contained three replicates and was repeated once. After eight days of incubation, the diameter of mycelial growth was measured in two perpendicular directions and the diameter of the mycelial plug was subtracted before calculating the mean diameter of the colony.

Greenhouse Test

Greenhouse testing was conducted to determine the efficiency of Reklemel™ for managing Fusarium wilt incidence and *M. incognita* population density. All greenhouse testing was performed at the Plant Science Research Center located in Auburn, Alabama. The soil used was a Kalmia loamy sand texture (80% sand, 10% silt, and 10% clay; 1% organic matter, pH 5.3). This soil was collected from the Plant Breeding Unit of Auburn University's E. V. Smith Research Center located near Tallassee, AL. Before use, the soil was pasteurized at 88°C for 12 hours then allowed to cool for 24 hours before the process was repeated. Fertilizer and lime were added to the soil at rates recommended by the Auburn University Soil Lab and mixed with sand at a 2:1 soil to sand ratio. This soil and sand mixture was used to fill 473.2-$cm^3$ polystyrene food containers (Dart Container corporation; Mason, Michigan), which were used as test pots. Prior to filling pots with soil, four 2 mm holes were made in the bottom of each pot to allow for drainage of excess water. Five seeds of the upland cotton cultivar 'Rowden' (USDA-ARS; Washington, DC), which is highly susceptible to both FOV and *M. incognita* (Doan and Davis 2014), were planted 2.5 cm deep in simulated furrows made in each pot. After planting seeds, furrows were left open for the application of simulated in-furrow spray treatments. During the testing, natural light was supplemented with 1,000-watt halide bulbs that produce 110,000 lumens for 14 hours per day and the temperatures ranged from 22-35° C.

FOV, *M. incognita*, as well as Reklemel™ and Velum Total™ nematicides were applied at planting. Pots were inoculated with a FOV race 1 spore suspension. FOV isolates were collected and identified as previously described. Spore suspensions of FOV were prepared by growing the isolate in 10-15 Petri dishes containing Spezieller Nährstoffarmer agar (SNA) (Leslie and Summerell 2006). After allowing for ten days of growth, plates of FOV were flooded with 5 ml of sterile water and the surface of the media was scraped with an inoculation loop to dislodge the spores. Water containing the spores was then decanted from the Petri dishes through four layers of sterile cheesecloth and collected in a 50 mL conical tube. Spore concentrations of the final suspension were measured using a hemocytometer and were diluted with sterile water to a concentration of 5 x $10^6$ spores per milliliter. The spore suspension was applied to the pots by inserting a pipette tip 1.3 cm into the soil and applying 1 ml of the spore suspension at three locations within the pot for a total of 3 ml per pot.

*Meloidogyne incognita* race 3 eggs were extracted from greenhouse cultures maintained on 'Mycogen 2H723' corn (Corteva Agriscience; Wilmington, DE) by a modified version of the methodology of Hussey and Barker (1973). The corn roots were placed in a 0.625% NaOCl solution and shaken for four minutes at 1 g force using a Barnstead Lab-Line Max Q 5000E class shaker (Conquer Scientific; San Diego, CA). *Meloidogyne incognita* eggs were washed from the roots with tap water and collected on a 25-µm pore sieve. Eggs were then separated from the soil by sucrose centrifugation-floatation at 240 g for 1 minute (Jenkins 1964). After extraction, nematode eggs were enumerated under a Nikon TSX 100 inverted microscope (Nikon; Tokyo, Japan) at 40 times magnification. The final nematode suspension was diluted to the concentration of 10,000 *M. incognita* eggs/ml. Test pots were inoculated with 1 ml of the nematode suspension to achieve an at planting soil population density of 21 nematode eggs per cubic centimeter of soil.

After planting the test and inoculation with both FOV and *M. incognita*, chemical treatments of Reklemel™ and Velum Total™ [active ingredient fluopyram and imidacloprid (Bayer CropScience; Leverkusen, Germany)] were applied in comparison to an untreated control. Reklemel™ was applied at rates of 250, 500, and 1000 g fluazaindolizine/ha in 1 ml of tap water. Velum Total™ was applied at a rate of 184 g fluopyram/ha in 1 ml of tap water. An untreated control was included in the testing for comparison and was treated with 1 ml of tap water. All chemical treatments were applied directly on top of the seeds using a pipette before the seed furrows were closed to simulate an in-furrow spray.

Greenhouse tests were terminated 45 days after the initiation of the trials. At this time, plant growth parameters, Fusarium wilt incidence, and nematode population density data were recorded. Each plant shoot was measured to obtain plant height data. Shoot fresh weight (SFW) and root fresh weight (RFW) were measured after soil was washed from the roots for nematode extraction. Data are presented as total plant biomass (SFW + RFW). Fusarium wilt ratings were made by slicing the cotton stems of all the plants within each test pot to look for the presence or absence of vascular discoloration (Figure 1B). The Fusarium wilt incidence reported is a percentage of the plants within each pot that showed vascular discoloration symptoms. *Meloidogyne incognita* eggs were extracted from the cotton root as previously described. Eggs per gram of cotton root (eggs/g of root) were calculated by taking the ratio of total eggs extracted per the RFW on a per pot basis.

Field Test

Testing was conducted at the Plant Breeding Unit of Auburn University's E. V. Smith Research Center in Tallassee, AL, as previously described. The trial field is a Kalmia loamy sand soil type consisting of 80% sand, 10% silt, and 10% clay with 1% organic matter and a pH of 5.3.

Past testing revealed this field had a diversity of FOV races and genotypes, and it is known to contain an established population of *Meloidogyne incognita* race 3 (Smith 2015; Groover et al. 2020). The tests were planted on April 23, 2019, and May 7, 2020. However, due to inclement weather shortly after planting in 2019, which resulted in insufficient germination of the cotton, the test was replanted on May 15, 2019. All planting was conducted using a John Deere MaxEmerge (John Deere; Moline, IL) planter with Almaco cone planters (Almaco; Nevada, IA) at a planting rate of 13.1 seeds/meter of row. The test was arranged in a randomized complete block design with five replications. Each test plot consisted of four rows that were 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 the cultivar Delta Pine 1558NR B2RF chosen for its Fusarium wilt susceptibility. Treatments in this field test included an untreated control as well as in-furrow sprays of Reklemel™ and Velum Total™. In-furrow sprays were made at the time of planting using 8003 flat fan nozzles angled perpendicular to the row just in front of the planter closing wheels at a spray volume 93.5 L/ha. Reklemel™ was applied at the rates of 250, 500, and 1000 g fluazaindolizine/ha. Velum Total™ treatments were made at the rate of 184 g fluopyram/ha.

During the season *M. incognita* population density, plant height, plant biomass, and Fusarium wilt incidence were evaluated for each test plot. Nematode samples and plant growth parameters were collected on June 22, 2019 (42 DAP), and June 8, 2020 (32 DAP). Samples were collected using shovels to remove four arbitrarily selected plants from the middle two rows of each trial plot. These plants were measured to obtain plant heights. Measurements of SFW and RFW were recorded, and these combined weights are presented as total plant biomass. *Meloidogyne incognita* eggs were extracted and enumerated as described previously and are given as eggs/g of root. Fusarium wilt ratings were collected at the end of the cotton season on September 24, 2019,

and October 2, 2020. Evaluations were made by slicing the cotton stems of all the plants within a 1.5-meter section of each row to look for the presence or absence of vascular discoloration (Figure 1B). The Fusarium wilt incidence is presented as a percentage of the plants within each 1.5-meter section of the row that showed vascular discoloration symptoms. At the completion of the test, seed cotton was harvested on October 24, 2019, and October 7, 2020. Fusarium wilt rating and seed cotton yield data have not been collected for the 2020 test as this test is still in progress.

Statistical analysis

Statistical analysis was conducted to determine the effect of Reklemel™ on FOV growth, Fusarium wilt incidence, and *M. incognita* population density. All statistical analyses were conducted in SAS version 9.4 (SAS Institute, INC.; Cary, NC). For the *in vitro* assay $EC_{50}$ values, the estimated chemical concentration required to inhibit 50% of the mycelial growth was calculated by logarithmic ($log_{10}$) regression analysis using Proc Reg. For greenhouse and field assays, data were analyzed by ANOVA using Proc Glimmix. Means were separated using Tukey-Kramer's test at the $P \leq 0.05$ level. Student panels were produced to determine the normality of the residuals. In the case of nematode eggs/g of root, the data were log-transformed to satisfy the normal assumption and are presented as means from all replications included in the test.

## Results

*In vitro* Test

*In vitro* testing revealed that Reklemel™ affected the mycelial growth of FOV isolates using Reklemel™ amended media. The impact of Reklemel™ varied among the eight races/genotypes of FOV that were evaluated in this assay. $EC_{50}$ values for Reklemel™ ranged from 14.6 to 187.6 mg/L among the isolates (Table 2). The genotype most affected by Reklemel™ was race 2 to which had an $EC_{50}$ value of 14.6. The $EC_{50}$ value of all other races/genotypes included

in the testing ranged between 17 and 36 mg/L, except for the genotype LA-108. This genotype of FOV was less affected by Reklemel™ than other races/genotypes. The $EC_{50}$ value for the genotype LA-108 was 187.6 mg/L.

Greenhouse Test

There was no statistical difference among the three greenhouse tests that were conducted. Therefore, data from all tests were analyzed as one, and results are presented in Table 3. No significant difference was observed for any of the treatments in RFW, SFW, or plant biomass when measured at 45 DAP. All rates of Reklemel™ and treatment with Velum Total™ resulted in significantly reduced *M. incognita* eggs/g of root when compared to the untreated control. On average, across the three treatments of Reklemel™ we observed a 98% reduction and with Velum Total™ a 99% reduction in *M. incognita* eggs/g root. No significant change was detected in percent of Fusarium wilt infection. However, a numerical decrease in FOV infection was observed for all treatments of Reklemel™ with the highest treatment rate (1000 g ai/ha) supporting nearly half the rate of infection as the control pots.

Field Test

In field testing, treatments showed no significant effect on the plant stand counted 14 DAP with all treatments ranging from 10.9-11.5 plants per meter row (data not shown). None of the nematicide treatments had a significant impact on early-season plant growth as measured by plant height or biomass as measured at 42 DAP in 2019 or 32 DAP in 2020. All rates of Reklemel™ and the Velum Total™ significantly reduced *M. incognita* eggs/g of root compared to the untreated control. Reklemel™ reduced *M. incognita* eggs/g of root by 67% as an average across the three rates. Velum Total™ reduced *M incognita* eggs/g of root by 55%.

Fusarium wilt incidence was also measured in each plot at 132 DAP in 2019 and 148 DAP in 2020 (Table 4). A significant reduction in Fusarium wilt incidence was observed for the medium and high rates of Reklemel™ (500 and 1000 g ai/ha). These treatments reduced Fusarium wilt incidence by 52.6% and 65.2% respectively. No reduction in Fusarium wilt incidence was observed with Velum Total™ or the low rate (250 g ai/ha) of Reklemel™. Upon completion and harvest of the tests at 162 DAP (2019) and 153 DAP (2020), no significant seed cotton yield differences were measured among the treatments.

## Discussion

In greenhouse testing, no significant reduction in FOV infection was observed for any of the treatments. However, overall FOV infection rates were lower for the greenhouse test than what was found in the natural field setting. FOV incidence rates were 12% in the greenhouse and 16% in the field setting. This reduced rate of FOV infection and lack of significant reductions in FOV incidence may have been a result of only inoculating greenhouse trials with FOV race 1. This race was used because it was the most predominate race/genotype of FOV that was found in the field where further testing was conducted. However, in a field setting it is common for cotton plants to be exposed to multiple races/genotypes of FOV (Scott 2012; Smith 2015; Bell et al. 2017; Silva et al. 2019). Along with this, not all races/genotypes of FOV were affected by Reklemel™ at equal rates through our *in vitro* assay, and FOV race 1 had a higher $EC_{50}$ value than some other races/genotypes. Had a more diverse inoculum of FOV been applied, greenhouse study results may have aligned more with what was observed in field trials. Even with the lower incidence of Fusarium wilt, the tests did follow the same numerical trends that were observed in the field testing. The highest rates of FOV infection were found on the control and Velum Total™ treated plots, and numerically reduced rates on all Reklemel™ treated plots.

The field in which field tests were conducted is known to have a sustained rate of *M. incognita* race 3 and a wide diversity of FOV races/genotypes (Smith 2015; Groover at al. 2019). A known synergistic interaction exists between these two pathogens that results in large amounts of disease losses to the cotton industry (Atkinson 1982; Roberts et al. 1985; Starr et al. 1989). In this field study, we observed a decrease in both *M. incognita* population density and Fusarium wilt incidence when plots were treated with an in-furrow spray of Reklemel™. However, in this testing an in-furrow spray of Velum Total™ reduced *M. incognita* at similar rates as that of Reklemel™ but had no significant effect on Fusarium wilt. The disease complex of FOV and *M. incognita* have been managed for many years through the application of a variety of nematicides (Colyer et al. 1997; Jorgenson 1978; Jorgenson et al. 1978; Lawrence et al. 2007). These management strategies rely on their control on the *M. incognita* portion to minimize the effect of the overall disease complex. Both Reklemel™ and Velum Total™ treatments were successful at reducing *M. incognita* at similar rates but, there was no reduction in Fusarium wilt incidence for Velum Total™ treated plots. More research is needed to determine if the observed reduction in FOV incidence with treatments of Reklemel™ was a result of the chemical acting directly on the fungal pathogen.

In field testing, Reklemel™ has been previously reported to reduce *Meloidogyne* spp. population density. Fluazaindolizine, the active ingredient in Reklemel™, was introduced in 2017 as a highly effective and selective compound to control plant-parasitic nematodes (Lahm et al. 2017). Previous studies have demonstrated the chemical's ability to lower nematode population density, reduce nematode damage, and increase crop production (Becker et al. 2019; Desaeger and Watson 2019; Hajihassani et al. 2019; Silva et al. 2019b; Thoden and Wiles 2019). Our findings agreed with these previous studies that Reklemel™ lowered nematode population density in field

trials. However, no yield increase was observed with the reduction in nematodes. Due to inclement weather (unpredicted heavy rains) shortly after planting (April 23, 2019) of the cotton test there was a very poor stand that required replanting. This replanting may have had an effect on the outcome of the trial, the prevalence of *M. incognita* or FOV, or final cotton yield measurements.

DeltaPine 1558NR B2RF was used for this study to take advantage of the Fusarium wilt susceptibility of this cotton cultivar. This cotton dose cultivar contains two genes for *M. incognita* resistance (Faske et al. 2018). Greater reductions in *M. incognita* population density and associated yield increases may have been observed if a cultivar with no nematode resistance was used. However, DeltaPine 1558NR B2RF increased susceptibility to FOV allowed for better evaluation of the effects of Reklemel$^{TM}$ on the entire Fusarium wilt disease complex.

In this study, Reklemel$^{TM}$ was found to have an impact on the growth of FOV; unlike many other chemical nematicides that have been used to manage Fusarium wilt by reducing the nematode population density (Colyer et al. 1997; Jorgenson 1978; Jorgenson et al. 1978; Lawrence et al. 2007). Reklemel$^{TM}$ reduced the growth of FOV in an *in vitro* assay using amended media. The chemical's $EC_{50}$ value varied between 14.6 to 187.6 mg/L depending on the FOV race/genotype. This result suggests that applications of Reklemel$^{TM}$ may have an effect directly on FOV in combination with the nematode reduction that the chemical also provided. A small number of other chemicals have been used with varying levels of success lowering Fusarium wilt damage by acting directly on FOV (Ben-Yephet et al. 1987; Bennett et al. 2011). However, these are fumigant chemicals which in many cases are not economically feasible for cotton farmers.

FOV races/genotypes can be grouped into two separate pathotypes based on pathogenicity traits; these include the vascular-competent and the root-rot pathotypes (Bell et al. 2017). The vascular-competent pathotype relies on the presence of nematodes to cause significant damage,

while the root-rot pathotype can cause large amounts of damage without the presence of nematodes (Bell et al. 2017). This testing was only conducted with FOV races/genotypes (Table 2) found in the test field in Alabama that fall into the vascular-competent pathotype of FOV. No data were collected from races/genotypes that fall into the category of the root-rot pathotype which includes race 3, race 4, and the Australian biotypes. It is unknown what the effects Reklemel$^{TM}$ may have on the root-rot pathotype of FOV. Testing with the races/genotypes that fall into the root-rot pathotype may provide further clarity on whether the effects observed in this study were due solely to the impacts of Rekemel$^{TM}$ on *M. incognita,* FOV, or an interaction.

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Table 1: Primers and thermocycler settings used for race identification of FOV isolates

| Primer name | 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° |
| PHO 6 | GATGTGGTTGTAAGCAAAGCCC | | |

Table 2: *In vitro* test to determine the effective concentration of Reklemel™ to reduce 50% of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) mycelial growth ($EC_{50}$) values after eight days of incubation at room temperature.

| **FOV Isolate[y]** | **$EC_{50}$[z]** |
|---|---|
| Race 1 | 27.41 |
| Race 2 | 14.60 |
| Race 8 | 31.50 |
| MDS-12 | 27.59 |
| LA-108 | 187.63 |
| LA-110 | 17.25 |
| LA-112 | 32.15 |
| LA-127/140 | 35.72 |

[y] Isolates of FOV were collected from the Plant Breeding Unit of Auburn University's E. V. Smith Research Center during the 2018 and 2019 cotton growing seasons.
[z] $EC_{50}$ values were calculated by logarithmic ($log_{10}$) regression analysis in using Proc Reg via SAS 9.4. Calculations were conducted using data from FOV isolates grown on potato dextrose agar amended with Reklemel™ (0.01, 0.1, 0.5, 1, 10, 100 ppm) as well as streptomycin and kanamycin (100mg/L) to inhibit bacterial growth. Values present are an average of six observations.

Table 3: Greenhouse evaluations of plant growth, *Meloidogyne incognita* population density, and Fusarium wilt incidence measured at 45 DAP[w] after exposure to different rates of Reklemel™ and Velum Total™.

| Treatment | Biomass[x] (g) | *M. incognita* eggs/g of root | FOV Incidence (%) |
|---|---|---|---|
| Control | 32.6[y] a | 200.8 a | 12.3 a |
| Reklemel™ 250 g ai/ha | 37.1 a | 4.3 b | 9.0 a |
| Reklemel™ 500 g ai/ha | 36.6 a | 2.4 b | 7.3 a |
| Reklemel™ 1000 g ai/ha | 32.3 a | 3.0 b | 6.5 a |
| Velum Total™ 184 g ai/ha[z] | 39.0 a | 1.3 b | 12.4 a |

[w] Days after planting

[x] Biomass measurement is a combination of the root and shoot fresh weights

[y] Values present are LS-means across all three tests, separated using Tukey's test at $P \leq 0.05$. Nematode egg data were log-transformed to satisfy the ANOVA assumption of normally distributed residuals values in the column followed by different letters differ significantly.

[z] Velum Total™ (15.4% fluopyram, 22.2% imidacloprid) application rate were calculated for the concentration of fluopyram.

Table 4: Field evaluation of Reklemel™ and Velum Total™ on plant height, biomass, *Meloidogyne incognita* population density, Fusarium wilt incidence, and seed cotton yield at E.V. Smith Research Center, Tallassee, AL 2019 and 2020.

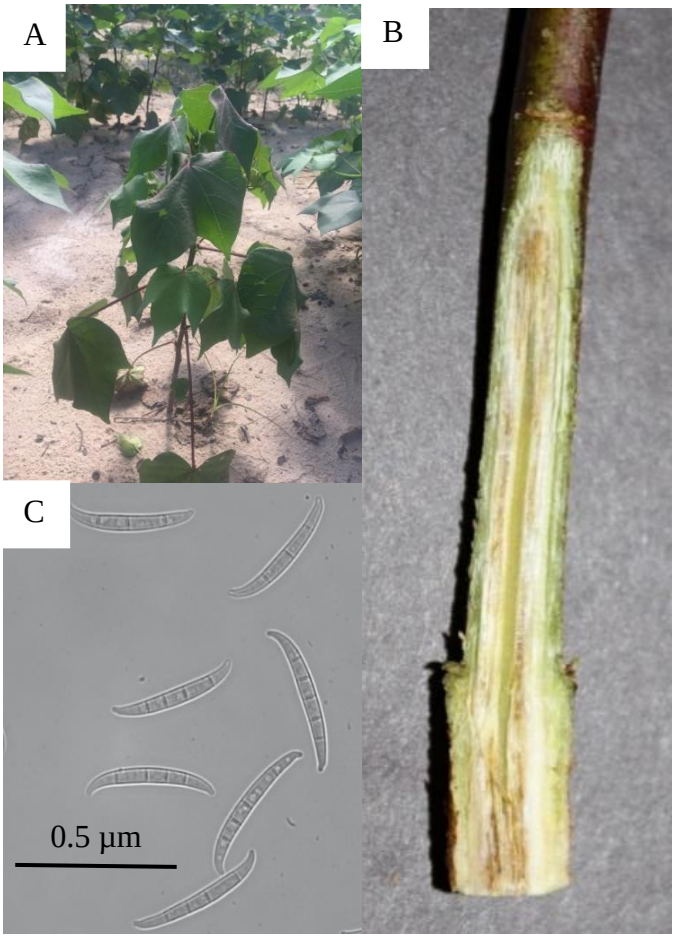
| Treatment | Plant Height (cm) | Biomass[x] (g) | *M. incognita* eggs/g of root | FOV Incidence (%) | Seed Cotton Yield (kg/ha) |
|---|---|---|---|---|---|
| Control | 19.9[y] a | 55.3 a | 895[z] A | 11.98 a | 3151 a |
| Reklemel™ 250 g ai/ha | 23.7 a | 89.6 a | 271 B | 7.87 ab | 3317 a |
| Reklemel™ 500 g ai/ha | 23.9 a | 100.9 a | 446 B | 5.68 b | 3213 a |
| Reklemel™ 1000 g ai/ha | 24.2 a | 97.0 a | 169 B | 4.17 b | 3167 a |
| Velum Total™ 184 g ai/ha[z] | 21.4 a | 65.4 a | 405 b | 7.87 ab | 3395 a |

[x] Biomass measurement is a combination of the root and shoot fresh weights.
In-furrow sprays were made at the time of planting using 8003 flat fan nozzles angled perpendicular to the row just in front of the planter closing wheels at a spray volume 93.5 L/ha.
[y] Values present are LS-means across all five replications of the test, separated using Tukey's test at $P \leq 0.05$. Nematode egg data were log-transformed to satisfy the ANOVA assumption of normally distributed residuals values in the column followed by different letters differ significantly.
[z] Velum Total™ (15.4% fluopyram, 22.2% imidacloprid) application rate were calculated for the concentration of fluopyram.

0

1

Figure 1: Symptoms and signs of Fusarium wilt on *Gossypium hirsutum*. A) Wilting of cotton plant caused by Fusarium wilt infection. B) Discoloration of the vascular tissue of a cotton stem. C) *Fusarium oxysporum* f. sp. *vasinfectum* macroconidia at 400x magnification.



## Appendix 1: Yield loss of cotton cultivars due to *Rotylenchulus reniformis* and the added benefit of a nematicide

**Abstract:** The objective was to determine cotton yield loss due to *Rotylenchulus reniformis* and document any yield benefit from the addition of a nematicide. Field trials were established in two adjacent fields, one was infested with *R. reniformis* and one where *R. reniformis* was not detected. In both fields, seven cotton cultivars were planted with and without Velum Total™ (1.02 L/ha). In 2017, *R. reniformis* reduced cultivar yields by an average of 59% between the non-infested and the *R. reniformis*-infested field. The nematicide application increased seed cotton yields in the *R. reniformis* field by 55% and no yield increase was observed in the non-infested field. In 2018, *R. reniformis* reduced seed cotton yields by an average of 42% between the non-infested field and the *R. reniformis*-infested field. Across the cultivars addition of the nematicide increased seed cotton yields by an average of 6% in the *R. reniformis*-infested field and an average of 8% in the non-infested field. The nematicide reduced *R. reniformis* eggs per gram of root by an average of 92% in 2017 and 78% in 2018 across all cotton cultivars. Overall *R. reniformis* reduced seed cotton yields by 50% which was equivalent to 2,225 kg/ha.

First described by Linford and Oliveira (1940) in Hawaii, the reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira is found in tropical, subtropical, and warm temperate regions around the world (Robinson et al. 1997). In the United States, the nematode quickly became one of the most commonly found plant pathogenic nematodes on cotton in the mid-south and southeast cotton regions. According to Robinson (2007), the reniform nematode has surpassed the root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood, as the major nematode affecting cotton in Mississippi, Alabama, and Louisiana. In 2018, it was estimated that more than 1.2% of the cotton crop, equivalent to 204,700 bales, was lost due to *R. reniformis* across the United States Cotton Belt (Lawrence et al. 2019). In Alabama, the impact was more severe, with an estimated 3% yield loss or approximately 26,000 bales of cotton (Lawrence et al. 2019).

The *R. reniformis* nematode is described as a semi-endoparasitic sedentary nematode with a host range of more than 314 plant species, including the regionally important agronomic crops of cotton and soybean (Wang 2013). In most situations, *R. reniformis* can be found in soil types with a high content of silt or clay (Moore and Lawrence 2013). In cotton fields, *R. reniformis* can spread via contaminated equipment and by the movement of water (Moore et al. 2010). Areas infested with *R. reniformis* typically have a high population density of the nematode due to a high potential rate of population increase compared to other nematodes due to a short life cycle which can be as little as 17 days under optimal conditions (Robinson 2007). Economic threshold data for *R. reniformis* in cotton is scarce; however, Luc et al. (2005) suggests that the tolerance value is around 100 nematodes/100 g of soil.

Symptoms of *R. reniformis* damage in cotton include reduced yield and stunting of the plant that can be described as a wave-type pattern of the canopy due to varying nematode

population density throughout the field. However, given time, population density of the nematode will become more evenly distributed throughout the field and this wave-type pattern will become less apparent (Lawrence and McLean 2001). In some soils, interveinal chlorosis, commonly referred to as “tiger striping”, has been observed in the foliage as a result of the nematode pathogen (Land et al. 2015). Nematode infection also results in a reduced root mass with fewer feeder roots to supply the plant with water and nutrients leading to poor growth (Lawrence and McLean 2001).

In general, nematode management consists of combining resistant cultivars, crop rotation, and application of nematicides. However, in cotton some of these management strategies are limited. Currently, no *R. reniformis* resistant cotton cultivars are available to farmers although breeding efforts to develop resistant cultivars are ongoing (Khanal et al. 2018). Crop rotation can be effective with crops such as corn, peanuts, sorghum, and other crops. However, these types of rotation crops are not always economically feasible for farmers. When crop rotations are effectively used, *R. reniformis* population density has been shown to rebound in as little as one year when a field is planted back in cotton (Stetina et al. 2007, Davis et al. 2003). Currently, applications of nematicides in the form of seed treatments, in-furrow sprays, or granular applications are the most common form of nematode management in cotton. For this trial, the nematicide Velum Total™ [a.i. fluopyram + imidacloprid (Bayer CropScience; Raleigh, NC)] was selected. Fluopyram is a nematistatic chemical documented to reduce *R. reniformis* infection at low concentrations (Faske and Hurd 2015). Imidacloprid is an insecticide commonly used as a cotton seed treatment to protect against early-season insect pressure. This study was conducted to evaluate the performance of seven commonly grown cotton cultivars with and without the application of Velum Total™ in the presence and absence of *R. reniformis*.

Establishment of field trials for evaluation of yield loss due to *Rotylenchulus reniformis*

2017 Trials

Seven commercially available cotton cultivars, those planted on a large amount of cotton acreage of the Southeastern Cotton Belt and representing four commercial seed companies, were planted in the presence and absence of *R. reniformis.* The trial was planted in two adjacent fields one of which had a high population density of *R. reniformis* (latitude 34°41'10.27" N longitude 86°53'1.17" W) and one where the presence of the nematode was not detected (latitude 34°41'10.27" N longitude 86°53'16.35" W). The nematode-infested field used in this study was infested with *R. reniformis* in 1997 and the population density increased in the first 10 years (Moore et al. 2008). At the initiation of this trial, two composite soil samples were collected: one from the *R. reniformis*-infested field and one from the adjacent non-infested field. From these samples, fields were determined to have initial *R. reniformis* soil population density of 5005 and 0 vermiform life stages /100 $cm^3$ of soil, respectively. In each field, the seven cotton cultivars (Table 1) were planted with and without an application of Velum Total™ (fluopyram, 179.7 g/L + fmidacloprid, 260.0 g/L) which was applied as an in-furrow spray at the time of planting. The applications were made using 8003 flat fan nozzles angled perpendicular to the row just in front of the planter closing wheels, at a rate of 1.02L/ha with a spray volume of 93.5 L/ha.

The trial was planted at the Tennessee Valley Research and Extension Center near Belle Mina, AL, on May 9, 2017. In both fields, the trial was arranged in a randomized complete block design with five replications, and plots were set up as four rows 7.6-meter long with 0.9-meter row spacing. A 1.9-meter wide alley separated each replication. The four-row plots had the left two rows untreated and the right two rows received the in-furrow spray with Velum Total™ at

planting forming subplots (Figure 1). The seven cotton cultivars were planted at a rate of 13.1 seeds per meter of row with a John Deere MaxEmerge planter (John Deere; Moline, IL) with Almaco cone planters (Almaco; Nevada, IA). Both the nematode-infested and the adjacent non-infested field have a Decatur silt loam (24% sand, 49% silt, and 28% clay) soil type with less than 1% organic matter and a pH of 6.5. The trial was irrigated as needed using a lateral irrigation system. Harvest occurred on November 10, 2017; 185 days after planting (DAP), using a 2-row cotton picker to harvest each subplot separately. Seed cotton yields obtained from each plot were weighed and recorded.

2018 Trials

This trial was a repeat of the 2017 trial, which was planted in the same nematode-infested, and non-infested field. This trial was identical to the 2017 trial in experimental design, plot layout, planting methods, and harvest. The trials only differed in the planting and harvest dates which were May 8 and October 5, 2018 respectively, 150 DAP. At the initiation of this trial, the *R. reniformis*-infested field and adjacent non-infested field had an initial soil population density of 4620 and 0 vermiform life stages /100 $cm^3$ of soil, respectively

**Nematode Sampling**

Nematode samples were collected by using shovels to remove the roots of eight arbitrarily selected plants from each plot. In each plot, four plants were removed from the Velum Total™ treated rows and four were removed from the non-treated rows. Sampling was conducted on June 22, 2017 (44 DAP) and on June 13, 2018 (36 DAP). These sampling dates had similar cotton growth stage and the amount of growing degree days (DD60's) that had accumulated since planting on May 9, 2017, and May 8, 2018, were similar (2017 trial: 682 and 2018 trial: 646). *Rotylenchulus reniformis* eggs were extracted from the cotton roots by a

modification of Hussey and Barker (1973). The roots were placed in a 0.625% NaOCl solution and shaken for four minutes at 120 rpm and 1 g force on a Barnstead Lab-Line Max Q 5000E class shaker (Conquer Scientific; San Diego, CA). Nematode eggs were rinsed from the roots with tap water and collected on a 25-µm-pore sieve. Nematode eggs were then processed by sucrose centrifugation-flotation at 240 g for 1 minute (Jenkins 1964). Samples of *R. reniformis* eggs were then enumerated under a Nikon TSX 100 inverted microscope (Nikon; Tokyo, Japan) at 40-x magnification. Eggs per gram of root were calculated by taking the ratio of total eggs extracted per the root fresh weight by subplot and averaged across replications by treatment.

## Statistical Analysis

Data were analyzed by ANOVA using Proc Glimmix via SAS 9.4 (SAS Institute, INC; Cary, NC), with 280 experimental units per parameter measured. Means were separated using Tukey HSD test at the $P \leq 0.05$ level. Student panels were produced to determine the normality of the residuals. In the case of nematode eggs/g of root, the data were log-transformed to satisfy the ANOVA assumptions of normally distributed residuals. Correlations and regression were analyzed using Proc Corr and Proc Reg. A four-way interaction analysis was conducted with the year, presence of the nematode, application of the nematicide, and cotton cultivar as the four variables. Trial results differed greatly between 2017 and 2018 so analysis was separated by year as a three-way interaction; source of variation in the data can be seen in Table 2.

## Cotton Yields and Nematode Population Density

2017 Trial

In this trial, a significant reduction in cotton yield was observed in the presence of *R. reniformis* ($P<0.0001$) as well as an associated increase in yield with the application of Velum Total™ ($P<0.0001$) in the nematode-infested field. A significant interaction of the presence of

the nematode and the application of Velum Total™ ($P$<0.0001) was also observed (Table 2) as the seed cotton yield was increased by the application of Velum Total™ only in the field with *R. reniformis* present. *Rotylenchulus reniformis* significantly reduced seed cotton yield averaged over all cultivars in the infested field by 2,429 kg/ha (59%) compared to the non-infested field (Table 3). In the *R. reniformis*-infested field an initial soil population density of 5,005 vermiform life stages per 100 $cm^3$ of soil was recorded. This resulted in an eggs/g of root population of 5,023 in the untreated plots at 44 DAP; however, an in-furrow spray of Velum Total™ provided early season nematode protection and reduced root population density by 91% at 44 DAP (Table 3). This reduction in the nematode root population density increased early season plant growth measured in plant height and plant fresh weights by 20% and 70% respectively (data not shown) which can be seen in figure 1. This increase in early season growth resulted in an increase in seed cotton yields in the field by 1389 kg/ha. However, this yield increase due to the addition of the nematicide was not equivalent to the yield of the non-infested field (4077 kg/ha averaged over all cotton cultivars). In the non-infested field, no yield effect was observed in response to the application of Velum Total™. All cultivars produced statistically similar seed cotton yields in both fields. PhytoGen 444 WRF produced the greatest yields when grown in the non-infested field; however, this cultivar's yield potential was reduced 39% when grown in the presence of the nematode (Table 4). In the *R. reniformis*-infested field, the highest yielding cultivar was Deltapine 1522 B2XF and this cultivar's yield potential was only reduced 16% when grown in the presence of *R. reniformis* compared to the non-infested field (Table 4).

2018 Trial

In this year a significant effect on yield was observed from the presence of *R. reniformis* ($P$<0.0001) and the application of Velum Total™ ($P$ < 0.0001), and a significant interaction was

observed between the presence of the nematode and the cultivar planted ($P$<0.0001) (Table 2). In 2018 similarly to 2017, yield reductions were measured in the *R. reniformis*-infested field compared to the non-infested fields. The presence of *R. reniformis* in the field at an initial soil population density of 4,620 vermiform life stages per 100 $cm^3$of soil resulted in a yield loss of 42% (2020 kg/ha) when compared to the non-infested field (Table 3). *Rotylenchulus reniformis* eggs per gram of root observed at 36 DAP were 78% lower with the application of Velum Total™. However, the overall *R. reniformis* population density of the field was much lower when samples were collected compared to 2017. With this lower population density, we did not observe an increase in early season plant growth (data not shown) or final seed cotton yields that we observed in the previous year of testing. There was no significant ($P$≤0.05) variation in nematode root population density among the seven cotton cultivars tested. In this trial significant differences were observed ($P$≤0.05) in seed cotton yield among the cotton cultivars (Table 5). In the non-infested field, Deltapine 1522 B2XF and Deltapine 1646 B2XF were the highest yielding cultivars. Deltapine 1522 B2XF was also the highest performing cultivar in the *R. reniformis* field, producing significantly ($P$≤0.05) increased seed cotton yield over PhytoGen 333 WRF and PhytoGen 444 WRF in both the control and Velum Total™ treated plots.

**Impacts on Nematode Management and Cotton Production**

Through this research, we were able to quantify the actual yield loss attributed to *R. reniformis* in a cotton field as well as potential benefit from the application of a nematicide (Velum Total™) with modern cotton cultivars. Overall, the seed cotton yield was negatively correlated with the *R. reniformis* root population density at 35-40 DAP. We observed a 25.8 kg/ha (±5.6; ±95% confidence interval) reduction in seed cotton yeild for every 100 eggs/g of root increase in nematode root population density ($P$<0.0001, $R^2$ = 0.23). In this model, the

nematode root population density accounted for 23% of the variation in the seed cotton yield. The yield loss caused by *R. reniformis* averaged 50% between the two years when all cultivars and treatments were compared. Yield loss due to *R. reniformis* observed in this set of trials was higher than previous reports. We were able to determine actual yield losses utilizing the paired field one where *R. reniformis* was present and one where it was not. Previous reports estimated yield losses ranging from 38-45% (Jones et al 1959; Robinson et al. 2005). By using the non-infested field, we were able to measure the yield potential of each cotton cultivar so that the yield loss attributed to the nematode could be more accurately estimated.

Between the two trials, we observed variable results in the performance of Velum Total™, which produced a 55% yield increase over untreated plots in 2017 and only a 6% increase in 2018. This is could be due to the effects of soil moisture at the time of planting and early part of the cotton-growing season. Through the first 45 days after planting, the 2017 trial received more than double the rain of the 2018 trial, 19.7 cm in 2017 and 8.7 cm in 2018 (Figures 2 and 3). This low rainfall resulted in unusually dry conditions at the time of planting and the time of nematode sampling and lack of moisture could have affected the nematicide efficacy. This issue of lack of yield response following a chemical application has been reported with other nematicides (Gazaway et al. 2001). Though no significant effect from the application of Velum Total™ was observed in seed cotton yield in 2018, a small, numerical increase was observed in both the nematode-infested and non-infested field, 6% and 8% respectively.

The use of nematicides to manage *R. reniformis* in cotton is essential due to limitations in other control measures such as the lack of cotton cultivars with resistance. All cotton cultivars in this trial supported a similar nematode root population density demonstrating that at least until the release of resistant cotton cultivars, *R. reniformis* management in cotton will continue to rely

on the use of chemical nematicides. In this study, Velum Total™ was useful for both lowering nematode root population density and under good growing conditions, this resulted in an increased yield.

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Table 1. Commercial cotton cultivars planted and their maturity in the 2017 and 2018 field trials conducted at the Tennessee Valley Research & Extension Center near Belle Mina, AL.

| Cotton Cultivar | Relative Maturity |
|---|---|
| Cropland 3885 B2XF | Mid-Full |
| Deltapine 1522 B2XF | Early-Mid |
| Deltapine 1646 B2XF | Mid-Full |
| PhytoGen 333 WRF | Early-mid |
| PhytoGen 444 WRF | Mid |
| PhytoGen 487 WRF | Mid |
| Stoneville 6182 GLT | Full |

Table 2. Source of variation for seed cotton yield (kg/ha) and *Rotylenchulus reniformis* eggs/g of root in 2017 and 2018 conducted at the Tennessee Valley Research & Extension Center near Belle Mina, AL.

| | 2017 | | 2018 | |
|---|---|---|---|---|
| Source of variation (F statistic) | Seed cotton yield 185 DAP[w] | *R. reniformis* eggs/g of root 44 DAP | Seed cotton yield 150 DAP | *R. reniformis* eggs/g of root 36 DAP |
| *R. reniformis* | 156.61$^{****}$[x] | - | 730.32$^{****}$ | - |
| Velum Total[y] | 24.09$^{****}$ | 88.02$^{****}$ | 12.64$^{***}$ | 23.62$^{****}$ |
| Cultivar[z] | 1.32 | 0.32 | 8.25 | 2.03$^{*}$ |
| *R. reniformis* x Velum Total™ | 27.55$^{****}$ | - | 2.09 | - |
| *R. reniformis* x Cultivar | 1.62 | - | 5.07$^{****}$ | - |
| Velum Total™ x Cultivar | 0.19 | 1.18 | 0.81 | 0.25 |
| *R. reniformis* x Velum Total™ x Cultivar | 0.32 | - | 0.27 | - |

[w] Days after planting

[x] Significance at the $P \leq 0.1$, 0.05, 0.01, and 0.001 level is indicated by *, **, ***, and **** respectively.

[y] Velum Total™ (Fluopyram + Imidacloprid) was applied at planting as an in-furrow spray at 1.02 L/ha.

[z] Cultivars included in this study were Cropland 3885 B2XF, Deltapine 1522 B2XF, Deltapine 1646 B2XF, PhytoGen 333 WRF, PhytoGen 444 WRF, PhytoGen 487 WRF, and Stoneville 6182 GLT.

Table 3. Seed cotton yields and average population density of *Rotylenchulus reniformis* eggs/g of root in the non-infested and *R. reniformis*-infested fields for both the 2017 and 2018 growing season at the Tennessee Valley Research and Extension Center near Belle Mina, AL.

| | Non-*R. reniformis* field | *R. reniformis*-infested field | |
|---|---|---|---|
| | | 2017 | |
| | Yield (kg/ha) | Yield (kg/ha) | Eggs/g root[x] |
| Untreated | 4125 (±749)[y] | 1696 (±766) b | 5032 (±4871) a |
| Velum Total[z] | 4078 (±911) | 3885 (±758) a | 435 (±1264) b |
| | | 2018 | |
| | Yield (kg/ha) | Yield (kg/ha) | Eggs/g root |
| Untreated | 4824 (±558) | 2804 (±669) | 337 (±375) a |
| Velum Total™ | 5220 (±579) | 2971 (±584) | 75 (±109) b |

[x] *Rotylenchulus reniformis* nematode eggs/g root were present in the *R. reniformis*-infested field, and not the non-infested field. Data present are LS-means and significant values are based on log-transformed data to satisfy the assumptions of normality.

[y] Values present are LS-means separated using Tukey's HSD test at $P \leq 0.05$ as well as standard deviations values in parenthesis, and values followed by different letters differ significantly. No letters present means that no significant difference was observed.

[z] Velum Total™ (Fluopyram + Imidacloprid) was applied at planting as an in-furrow spray at 1.02 L/ha.

Table 4. Seed cotton yield (kg/ha) harvested 185 DAP by cotton cultivar in the non-infested and *Rotylenchulus reniformis*-infested fields at the Tennessee Valley Research and Extension Center, 2017 near Belle Mina, AL.

| | Non-*R. reniformis* field | | *R. reniformis*-infested field | |
|---|---|---|---|---|
| | No nematicide | Nematicide[y] | No nematicide | Nematicide |
| Cotton Cultivar | kg/ha | | kg/ha | |
| Croplan 3885 B2XF | 3709[z] (±742) | 3821 (±903) | 2060 (±901) | 3182 (±871) |
| Deltapine 1522 B2XF | 4120 (±877) | 4249 (±1128) | 2097 (±654) | 3547 (±1121) |
| Deltapine 1646 B2XF | 4305 (±476) | 3525 (±305) | 1130 (±347) | 2670 (±143) |
| PhytoGen 333 WRF | 3912 (±1031) | 4073 (±1014) | 1417 (±380) | 2989 (±544) |
| PhytoGen 444 WRF | 4702 (±167) | 4762 (±650) | 1529 (±1200) | 2883 (±911) |
| PhytoGen 487 WRF | 3994 (±924) | 4064 (±1321) | 1892 (±906) | 3258 (±986) |
| Stoneville 6182 GLT | 4128 (±714) | 4047 (±721) | 1745 (±518) | 3061 (±395) |

[y] Velum Total™ (Fluopyram + Imidacloprid) was applied at planting as an in-furrow spray at 1.02 L/ha.

[z] Values present are LS-means separated using Tukey's HSD test at $P \leq 0.05$ as well as standard deviations values in parenthesis, and values in the same column followed by different letters differ significantly. Lack of letters present indicates no significant difference was observed.

Table 5. Seed cotton yield (kg/ha) harvested 150 DAP by cotton cultivars in the non-infested and *Rotylenchulus reniformis*-infested fields at the Tennessee Valley Research and Extension Center, 2018 near Belle Mina, AL.

| | Non-*R. reniformis* field | | *R. reniformis*-infested field | |
|---|---|---|---|---|
| | No Nematicide | Nematicide[y] | No Nematicide | Nematicide |
| Cotton Cultivar | kg/ha | | kg/ha | |
| Croplan 3885 B2XF | 4480[z] (±351) bc | 4919 (±544) ab | 2859 (±359) ab | 3049 (±438) ab |
| Deltapine 1522 B2XF | 5323 (±536) a | 5457 (±680) a | 3628 (±375) a | 3701 (±615) a |
| Deltapine 1646 B2XF | 5218 (±407) ab | 5628 (±349) a | 2535 (±692) b | 2941 (±314) ab |
| PhytoGen 333 WRF | 4996 (±351) abc | 5441 (±485) a | 2544 (±921) b | 2740 (±577) b |
| PhytoGen 444 WRF | 4778 (±595) abc | 5388 (±522) a | 2162 (±211) b | 2733 (±291) b |
| PhytoGen 487 WRF | 4731 (±567) abc | 5107 (±583) ab | 3036 (±601) ab | 3034 (±795) ab |
| Stoneville 6182 GLT | 4246 (±395) c | 4597 (±365) b | 2865 (±425) ab | 2599 (±411) b |

[y] Velum Total™ (Fluopyram + Imidacloprid) was applied at planting as an in-furrow spray at 1.02 L/ha.
[z] Values present are LS-means separated using Tukey's HSD test at $P \leq 0.05$ as well as standard deviations values in parenthesis, and values in the same column followed by different letters differ significantly.

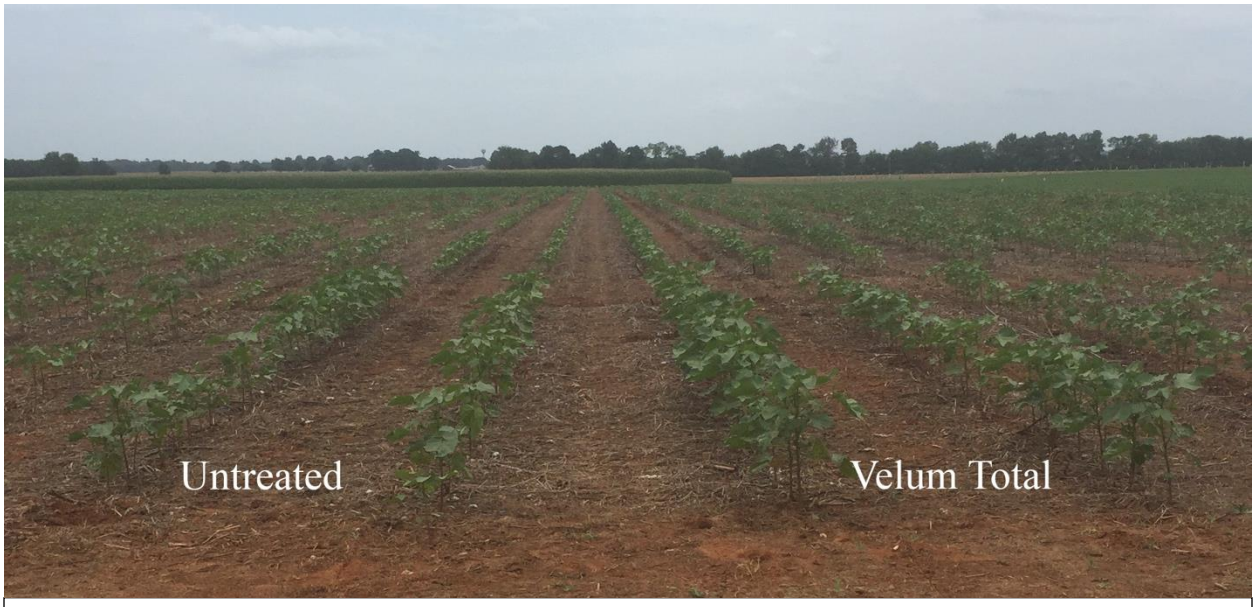


Figure 1. Trial plot containing Croplan 3885 B2XF cultivar taken 43 days after planting in 2018. Left two rows shown in the picture are the cultivar planted without the application of a nematicide the right two rows were treated with an in-furrow spray of Velum Total™ (1.02 L/ha) at the time of planting.

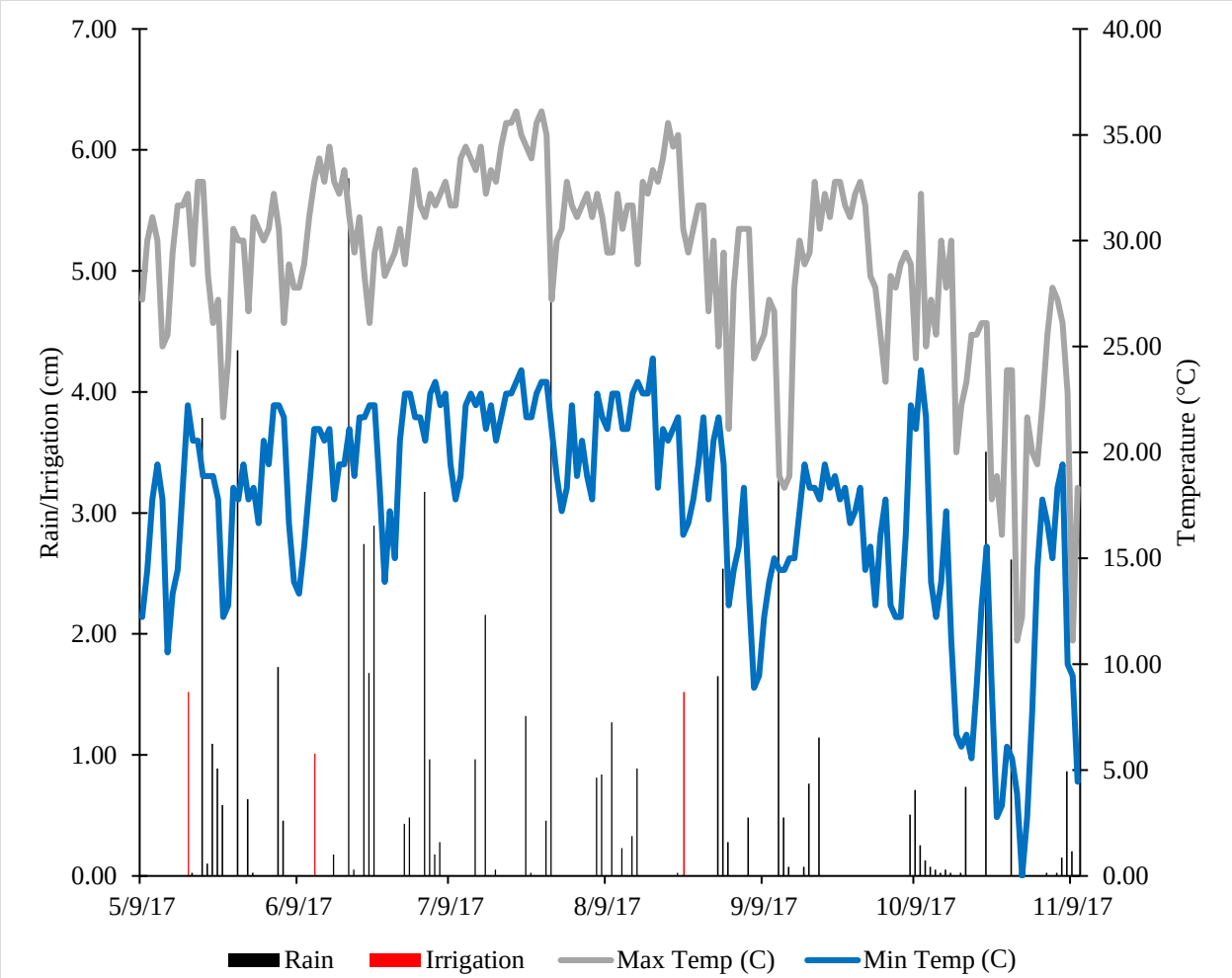


Figure 2. Rain, irrigation events, and temperatures from time of planting until harvest at the Tennessee Valley Research and Extension Center, 2017 near Belle Mina, AL.

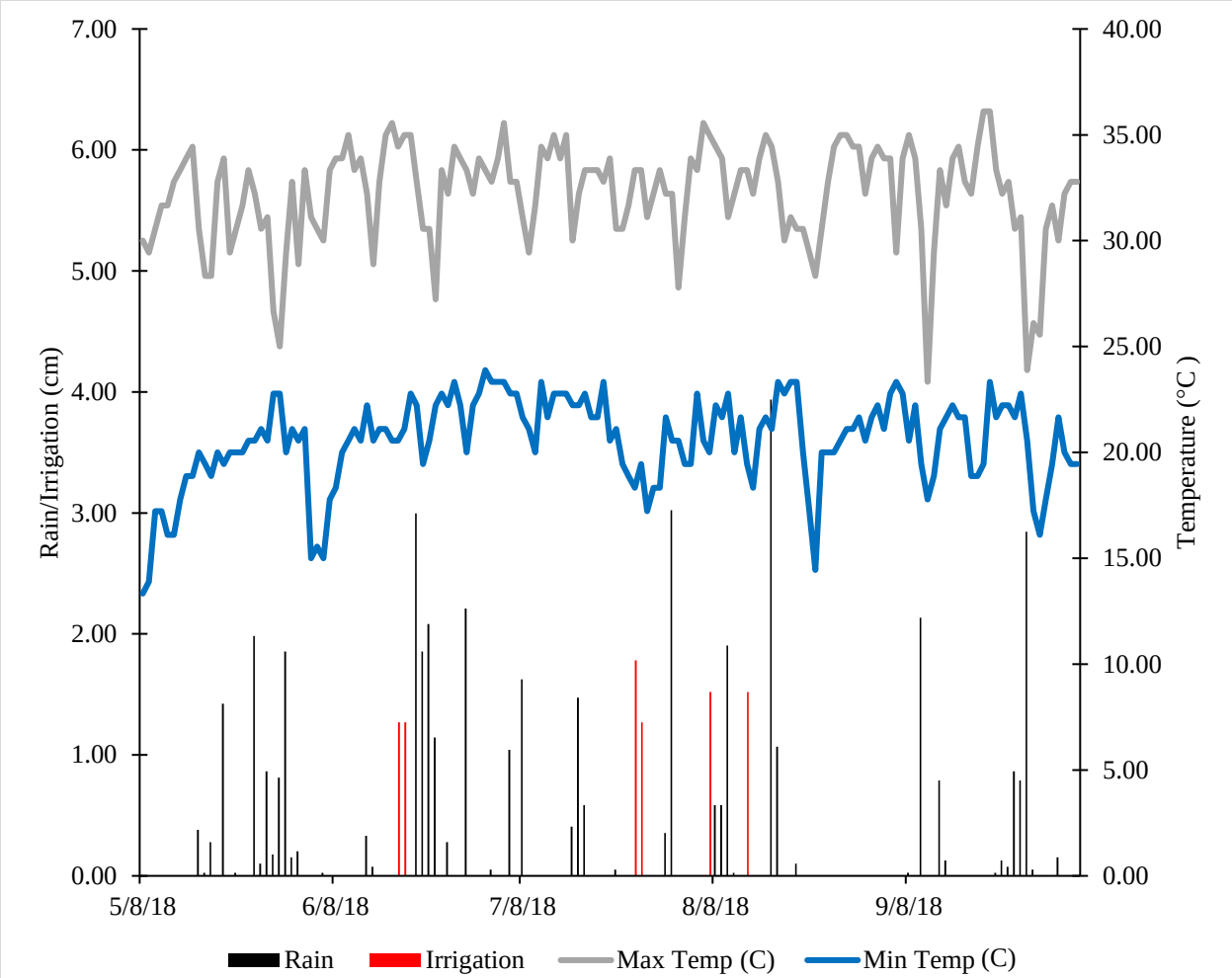


Figure 3. Rain, irrigation events, and temperatures from time of planting until harvest at the Tennessee Valley Research and Extension Center, 2018 near Belle Mina, AL.