BIOLOGY AND DIVERSITY OF *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* IN THE SOUTHEASTERN UNITED STATES

by

APARNA PETKAR

(Under the Direction of Pingsheng Ji)

ABSTRACT

Fusarium wilt of watermelon, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *niveum* (FON), is the most severe soilborne disease in watermelon worldwide and has been a serious threat to watermelon production in the United States. The goal of my proposed research is to determine fungicide resistance, mode of FON seed infestation, and genetic and phenotypic diversity of FON in the southeastern United States. Assessment of sensitivity of the isolates to fungicides indicated that all isolates were sensitive to prothioconazole at 10 µg/ml, while some isolates were resistant to thiophanate-methyl at 100 µg/ml. Sequencing a portion of the β-tubulin gene of resistant or sensitive isolates to thiophanate-methyl indicated that fungicide resistance was associated with a point mutation at nucleotide position 200 resulting in a substitution of phenylalanine by tyrosine. To determine how seeds in watermelon fruit can be infested by FON during the watermelon growing season, greenhouse and field experiments were conducted in 2014 and 2015 where watermelon flowers and immature fruit were inoculated with FON. Inoculation of the pericarp of immature fruit, pistil or peduncle resulted in both internal and external seed infestation under both greenhouse and field conditions. Simple sequence repeats (SSR) markers

were used for analysis of genetic diversity of the FON isolates. Discriminant analysis of principal components (DAPC) of 99 isolates grouped the isolates into eight major clusters with two prominent clusters (1 and 8). Cluster 1 consists of a total of 14 isolates out of which 85.7% of the isolates came from FL. However, the majority of isolates (92.4%) in cluster 8 came from GA. Both DAPC and population structure analysis demonstrated that the phylogenetic groups are closely associated with geographical location of pathogen isolation. The majority of the isolates in cluster 1 and cluster 8 either belonged to race 2 (35.6%) or race 3 (45.8%). Additionally, no relation between phylogenetic groups and races identified was observed. Overall, information gained from this dissertation research will add to basic and applied information in FON-watermelon pathosystem. In time, the findings can be incorporated into the integrated disease management options against this disease.

INDEX WORDS: Fusarium wilt of watermelon, *Fusarium oxysporum* f. sp. *niveum*, SSR, Fungicide resistance, Infection courts, Genetic and phenotypic diversity

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

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DEDICATION

I would like to dedicate this thesis work to my husband, Dr. Bhabesh Dutta, who has been a constant source of support and encouragement during the challenges of graduate school life. I am truly thankful for having you in my life. This work is also dedicated to my parents (Dr. Subhash Petkar and Mrs. Mangla Petkar) who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

ACKNOWLEDGEMENTS

I would like to express sincere appreciation to my major advisor Dr. Pingsheng Ji for his support and guidance while working on the research projects. I would also like to acknowledge my committee members Dr. David Langston, Dr. James Buck, Dr. Marin Brewer and Dr. Cecilia McGregor for their guidance and suggestions. I am grateful to the all the staff of Department of Plant Pathology, Tifton, GA, for providing technical assistance.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Since 1890s, Fusarium wilt of watermelon [*Citrullus lanatus* (Thumb.) Matsum. & Nakai)] has been a problem in the southern United States, causing significant damage (Atkinson, 1892). E.F. Smith (1899) conducted detailed work on pathogen description, host specificity, inoculation and histological studies. In histological studies, he described a phenomenon of microconidial transportation within the xylem and the formation of "embolisms in the vascular system" (tyloses). During the same year (1899), similar wilt diseases of muskmelon and cucumbers were reported from Ohio and Connecticut. The name for watermelon wilt fungus as *Fusarium niveum* E.F.S. was coined by Smith. He suggested the fungus to be an asexual stage of the perithecial ascomycete fungus, *Neocosmospora vasinfectum* Atk., which was named for the cotton wilt pathogen by Atkinson. In fact, Smith misidentified this pathogen to be a perfect stage of *Neocosmospora*. Later, W.A. Orton demonstrated the pathogenicity of *F. vasinfectum* on cotton and reported that the *Neocosmospora* was not the perfect stage of the fungus (Orton, 1907). Gradually, over the years with a number of detailed studies on Fusarium genera, the Fusarium section Elegans containing 10 species was reduced to one, *F. oxysporum*, which included numerous pathogenic formae speciales (Snyder & Hansen, 1940). Hence, the watermelon wilt pathogen was officially renamed as *F. oxysporum* Schlechtend.: Fr. f. sp. *niveum* (E.F. Sm.) W. C. Synder & H. N. Hans.

Pathogen Biology

Fusarium oxysporum f. sp. *niveum* (FON) has only an asexual reproductive stage and a sexual stage is not known. Asexual reproductive structures are micro- and macroconidia that are formed in an asexual fruiting body, the sporodochium. Microconidia are one-celled, formed on short conidiophores, oval to kidney-shaped, and are generally abundant. Macroconidia are fusiform (canoe-shaped) with three to five cells, often produced in clumps in sporodochia. Both microconidia and macroconidia can infect roots; however, the primary role of macroconidia is to aid in fungal survival as they possess the ability to form chlamydospores (asexual resting structures). Chlamydospores are thick walled survival spores that provide protection against unfavorable conditions. These resting survival spores are the primary means of FON survival and are typically formed under suboptimal fungal growth conditions or death of the host plant. There are two types of chlamydospores known for FON; one forms within the macroconidium and one forms within the mycelium. The chlamydospores which are formed from mycelia tend to occur singly or in pairs and may be either intercalary (within the mycelium) or terminal (occurring at the ends).

Symptoms

Symptoms of Fusarium wilt include damping-off and wilting during the seedling stage and vine wilting in mature plants. However, the specific symptom exhibited is dependent on the environmental conditions, age of plants during infection, pathogen density in soil and aggressiveness of FON (Kleczewski and Egel 2011; Martyn 1985, 1987; Martyn and Bruton 1989). In mature plants, symptoms can be seen after fruit-set. The symptoms include gradual change of crown leaf appearance from a dull gray-green to yellow, permanent wilting of vines,

and eventual death. Fusarium wilt also causes vine decline and can be easily confused with other vine decline diseases of watermelon (Martyn and McLaughlin 1983; Martyn and Vakalounakis 2012). In the field, symptoms typically occur within 3 to 4 weeks, starting with a graying of foliage followed by foliar chlorosis and wilt. Fusarium wilt is characterized by unilateral stem necrosis, which is easily visualized when runners or stems are sectioned. Under wet conditions white to pink fungal hyphae may be visualized emerging from necrotic tissues (Kleczewski and Egel 2011).

Physiological specialization and genetic relatedness

Formae speciales

The formae speciales of *Fusarium oxysporum* generally cause vascular wilts of cucurbits in a host-specific manner. However, cross pathogenicity has been observed in laboratory and greenhouse settings (Davis 1966; Gerlagh and Blok 1988; McMillan 1986; Martyn and McLaughlin 1983a; Owen 1955). In contrast, cross pathogenicity of different formae specialis has never been observed in the field, rather the interaction is highly host-specific. Although seven different formae speciales are known to cause vascular wilts in cucurbits, *F. oxysporum* f. sp. *melonis* (FOM; melon), f. sp. *cucumerinum* (FOC; cucumber), and f. sp. *niveum* (FON; watermelon) are economically important worldwide. Additionally, several physiological races have been recognized within each formae specialis. The genetic relatedness of *F. oxysporum* isolates within and between formae specialis are diverse (Baayen et al., 2000; Kistler 1997) and several techniques have been used to study the genetic diversity among isolates from different formae speciales. To date, none of the published techniques or

combination of techniques is able to completely unravel the genetic relatedness among formae speciales and races. Both monophyletic and polyphyletic origins of isolates belonging to different formae speciales were reported using molecular techniques such as mtDNA (mitochondrial DNA) and rDNA RFLPs (restriction fragment length polymorphism) and DNA fingerprinting with nuclear repetitive DNA. While research indicated FON to be monophyletic in origin (Kim et al. 1993a), the origin of FOM appeared to be polyphyletic (Jacobson and Gordon 1990b; Namiki et al. 1994). However, distinct similarities were observed between the mtDNA RFLP maps of FON and FOM (Jacobson and Gordon 1990b; Kim et al. 1992a) discrediting previous claims of divergence in origin.

Vegetative compatibility groups

Vegetative compatibility groups (VCGs) are generally used to distinguish isolates among formae speciales, which is based on the ability of different isolates to anastomose. It was observed that pathogenic subgroups are categorized in one or a few VCGs and isolates within a VCG tend to be more similar than isolates in different VCGs. More importantly, isolates within same VCGs may also be clonal lineages (Gordon and Martyn 1997; Leslie 1993). The relationship between forma specialis, race and VCG can be simple and direct as in case of FON (Larkin et al. 1990). In some cases, the relationship can be highly complex as in FOM (Jacobson and Gordon 1988, 1990a). Hence, a combination of phenotypic, genetic and nuclear markers along with vegetative compatibility and pathogenicity tests should be employed to distinguish *F. oxysporum* isolates from different formae speciales.

Detection and identification of FON

Traditionally, the identification of FON or any forma specialis is based on the isolation of the pathogen from symptomatic plants followed by inoculation of healthy plants of the same and/or related species. This method is highly reliable as most of the formae speciales are strictly host specific. However, this assay has many drawbacks which include requirement of growth chamber or greenhouse space, time consuming where symptom development may take up to several weeks to conclude, costly and more often depends on the environmental factors. The formae speciales of *F. oxysporum* cannot be distinguished morphologically as they are identical in culture. Hence, over the years, multiple molecular techniques have been employed to detect formae specialis; among them the polymerase chain reaction (PCR) has shown promise (Henson et al. 1993; Saikia and Kadoo 2010). The genomic ribosomal DNA (rDNA) and the internal transcribed spacer (ITS) regions of fungi have been widely recognized to differentiate a wide range of fungi. Using the sequences from the ITS region, Zhang et al. (2005) developed a species-specific PCR detection assay for FON which was able to detect and distinguish the fungus from *Stagonosporopsis* spp., causal agent of gummy stem blight of melons. The PCR assay formed a single 320 bp amplicon specific for FON and a single 420 bp amplicon specific for *M. melonis*. Using this assay, the researchers could detect all FON (n = 24) and *M. melonis* (n = 22) isolates from China. Lin et al. (2010) also designed PCR primers from a novel RAPD fragment of a FON isolate from Taiwan (OP-M12411). This PCR assay was able to detect and differentiate FON isolates from other formae speciales. The primer set was designated as Fon-1/Fon-2, which amplified a single 174 bp fragment specific to all FON isolates from Taiwan and the U.S. Importantly, the DNAs from isolates belonging to 13 other formae speciales, including those infecting other cucurbits (f. sp. *melonis*, *cucumerinum*, *luffae* and *momordicae*)

were not amplified.

Genetic diversity and physiological races

The races for pathogens are named by corresponding resistance genes in the host they defeat. For example, in cucurbits, race designation of formae speciales of *F. oxysporum* follows the protocol as suggested by Risser et al. (1976) for FOM. This method has been commonly used to designate races for other cucurbits (Martyn and Vakalounakis 2012). Physiological races are generally identified by specific disease reactions produced after inoculation of a reference, and unidentified FON races on a differential host set. The host differentials may vary in a specific and different resistance gene. This method is reliable when the resistance is conferred by a single dominant gene. However, when the resistance is conferred by recessive genes or set of multiple genes or genes with epistatic affects, the method becomes unreliable.

Currently, there are four races of FON, which have been described in the literature (race 0, 1, 2, & 3); however, there are some discrepancies in scientific community on the validity of four races (Martyn and Vakalounakis 2012). The FON races (race 0 and 1) were first identified in Italy (Cirulli 1972). While FON race 1 is most prevalent around the world and was originally identified by Smith in South Carolina, FON race 0 was first reported from Florida (Crall 1963). The FON race 0 was differentiated from race 1 by its ability to cause disease reactions (wilting symptoms) only in varieties that did not carry any resistance genes. Consequently, over the years, race 0 has become of minor economic importance as most of the commercial cultivars possess a resistance gene, Fo-1. However, the researchers have speculated that the distinction between race 0 and race 1 may not be qualitative rather quantitative. As a result, race 0 and race 1 may all be strains of race 1, varying in aggressiveness (Larkin et al. 1990; Martyn, 1987).

FON race 2 was identified in commercial watermelon fields in Israel (Netzer 1976). In

the US, race 2 was first identified in Texas (Martyn 1985, 1987). FON race 2 can overcome wilt resistance in all commercial cultivars evaluated. Following a survey by Martyn and Bruton (1989) in the US, race 2 was identified in three states: Texas, Oklahoma and Florida. Currently, race 2 has been reported from eight states in the US, including Georgia and in many countries (Bruton et al., 2008; Egel and Martyn 2013). Unlike race 1, which is widespread throughout the world, race 2 has a limited geographical distribution. However, occurrence of race 2 is of great concern for its ability to attack all current commercial varieties and hybrids. Contrastingly, the watermelon pollinizer varieties developed by Syngenta Seeds, Inc., Super Pollinizer 5 and 6 have shown some promise as race 2 wilt resistance differentials. The wilt resistance in these pollinizers was derived from PI-296341-FR (Egel and Martyn 2013).

The three races of FON (race 0, 1, and 2) were also found in commercial fields in Maryland and Delaware (Zhou and Everts 2003). Zhou and Everts (2003) observed that race 2 occurred with one or both the other races in the same fields. While 57% of the identified FON isolates belonged to race 1, race 0 and 2 accounted for 21 and 24% of the isolates identified, respectively. The majority of watermelon (80% by area) in the US is produced where the occurrence of race 2 has already been reported. Race 3 was recently identified from Maryland and has not been reported elsewhere in the world. This race is pathogenic on all cultivars, including the differential PI 296341-FR (Zhou et al. 2010).

Recently in a study published by Niu et al. (2016), the SIX6 (secreted in xylem protein 6) effector gene was identified in FON races 0 and 1 but was not identified in FON race 2. It was observed that, disrupting the FONSIX6 gene in race 1did not affect the growth rate or sporulation of the fungus but significantly enhanced FON virulence in watermelon. This suggests that mutant ΔFon1SIX6 allowed evasion of R protein mediated host resistance.

Contrastingly, when the wild type race 2 was complemented with FONSIX6 resulted in reduced virulence. This advocates that FONSIX6 is an avirulence gene and the fact that FONSIX6 gene was not identified in FON race 2, FON race 2 may have emerged from FON race 1 either resulting from loss of entire FONSIX6 gene or mutation that impaired the function of FONSIX6 gene.

Races and vegetative compatibility

A correlation between vegetative compatibility group (VCG) and virulence (race) was observed in the case of FON (Larkin et al. 1990). The FON isolates belonging to race 1 and race 2 were compatible with isolates of the same race but incompatible with the opposite race. The authors reported that the isolates from race 1 belonged to a single VCG (0081) while all race 2 isolates grouped in VCG 0082. Furthermore, all pathogenic isolates were incompatible (vegetative compatibility was not observed) with the non-pathogenic isolates of FON. The relationship between VCGs and races were more complicated in other forma specialis of *F. oxysporum*. For example, in case of f. sp. *melonis*, f. sp. *cucumerinum* and f. sp. *radicus-cucumerinum* the isolates of a single race may belong to multiple VCG or multiple races may belong to a single VCG or isolates may also belong to two different VCGs (bridging isolates) (Jacobson and Gordon 1988, 1990a; Valalounakis and Fragkiadakis 1999). In a study by Zhou and Everts (2007), three VCG groups were identified from a population of FON from Maryland and Delaware. Among the VCGs identified, VCG 0080 and 0082 were the same as previously reported by Larkin et al. (1996). A new VCG group (0083) was also identified in this study.

While Larkin et al. reported a good correlation between VCG and FON races, Zhou and Everts (2007) did not observe good correlation in the FON populations of Maryland and Delaware. The

FON isolates belonging to three races (0, 1 and 2) were associated with both VCG 0080 and 0082. In addition, six isolates which belonged to a new VCG 0083 were incompatible with other race 2 isolates; however, they were classified as race 2. Later studies showed that these isolates belonged to race 3. In a genetic study, Kim et al. (1995) transformed a FON race 2 isolate using a genomic library from a race 0 isolate. Except for two transformants, virulence was not affected for most of the transformants. The two transformants displayed lack of virulence in the cultivars 'Calhoun Gray' and 'Charleston Gray' but retained pathogenicity to 'Black Diamond'. Such characteristic was atypical for a race 0 phenotype. Interestingly, despite the change in pathogenicity and virulence of these transformants, vegetative compatibility was unaffected. The two transformants retained compatibility with the race 2-tester strain and were incompatible with race 0 (Kim et al. 1992b). Although the genetic makeup of FON is not fully understood, a study by Kim et al. (1993b) showed the fungus to possess 5 to 10 putative chromosomes ranging in size from approximately 900 to 4,400 kb. The minimum size of FON genome was reported as 15.8 to 26.0 Mb. These findings were also corroborated by other researchers (Migheli et al. 1993; Min 1995).

The genetics of resistance

The resistance against *F. oxysporum* was earlier believed to be controlled by recessive genes (Walker 1941); however, over the years, it was demonstrated that the trait was in fact conferred by a single dominant gene. For example in FON, the inheritance of resistance to race 1 was reported to be controlled by a single, dominant gene, Fo-1 (Henderson 1970; Netzer and Weintall 1980). In Fusarium wilt of cucumber (*F. o.* f. sp. *cucumerinum*), the resistance trait against race 1 and race 2 was also conferred by a dominant gene, FOC (syn. Fcu-1). Likewise,

in Fusarium wilt of melon (*F. o.* f. sp. *melonis*), resistance to races 0, 1 and 2 is controlled by two dominant genes, Fom-1 and Fom-2 (Martyn and Vakalounakis 2012). Diener and Ausubel (2005) studied the mechanism of Fusarium wilt resistance using *Arabidopsis thaliana* and *F. o.* f. sp. *matthioli* system. Using *Arabidopsis thaliana* ecotype Col-0, the resistance trait against *F. o.* f. sp. *matthioli* was observed to be controlled by six dominant loci (RFO). Among them, one locus (RFO1) had the strongest effect. Later, it was observed that the RFO1 mediated resistance was not race-specific as resistance to *F. o.* f. sp. *raphani* in ecotype Ty-0 was also conferred by the same locus. Further gene mapping studies showed that RFO1Col-0 is identical to an Arabidopsis gene WAKL22 (Wall-Associated Kinase-Like Kinase 22). This gene encodes a receptor-like kinase, which is devoid of an extracellular leucine-rich repeat domain.

Tremendous progress has been made with respect to mapping resistance genes for FON race 1, Xu (2014) used an F3 population developed from a cross between Calhoun Gray (resistant) and Black Diamond (susceptible) where he identified several single nucleotide polymorphism (SNP) makers which were linked to FON race 1 resistance on chromosome 1 of the draft genome sequence (Guo et al. 2013). In another study done by Lambel et al. (2014), F3 population derived from a cross between HMw017 (resistant) and HMw013 (susceptible) was used, seven quantitative trait loci (QTL) associated with resistance to FON race 1 was identified which included a major QTL (FO-1.1) on chromosome 1. Similarly, Ren et al. (2015) used an F8 population derived from the cross between cultivar 97103 and wild accession PI 296341-FR and found one major QTL associated with FON race 1 resistance on chromosome1 which explained 48.1% of phenotypic variation. Furthermore, similar results were observed when Meru and McGregor (2016) used F3 population derived from a cross between Calhoun Gray and Sugar Baby, where a major QTL for FON race 1 resistance was identified on chromosome

1 explaining 38.4% of the phenotypic variation.

The prevalence of FON race 2 over the last two decades led to the initiation of a screening program for resistance genes in PI lines. As such, by 1991, an inbred PI line (PI 296341-FR) displaying high resistance to race 2 was released (Martyn and Netzer 1991). Although this PI line had high resistance to races 0, 1 and 2, a serious drawback of segregating traits was observed later. This made the resistance trait to be highly variable where the traits segregated with a resistance: susceptibility ratio of approximately 95:5. This problem was mainly observed for inheritance of resistance against race 2 than race 0 and 1. The reasons behind such disparity of inheritance were implicated to the complicated inheritance characteristics of race 2 traits. The race 2 resistance in PI 296341-FR was conferred by one or more major dominant and recessive genes interacting with some minor genes (Hawkins et al. 2001; Zhang and Rhodes 1993; Zou et al. 2011). The resistance to FON race 0 in PI 296341-FR is conferred by a dominant gene, which is affected by number of non-allelic modifier genes (Zhang and Rhodes 1993). Such characteristics were not desirable for maintaining durable resistance. Similar to race 0 resistant traits, the resistance is controlled by a dominant gene which is influenced by a number of modifier genes (Netzer and Weintall 1980). The resistance traits against FON race 2 are controlled by at least one recessive pair of genes; however, an epistatic effect of a dominant gene from a susceptible parent over the recessive gene for resistance in PI 296341-FR proved undesirable. Consequently, the transfer of high level of resistance from PI 296341-FR into commercial cultivars has been difficult.

Recent advances in mapping resistance for FON race 2 include a study done by Ren et al. (2015), used an F8 population developed from a cross between 97103 and PI-296341 FR where they identified two QTL's related to FON race 2 resistance on chromosomes 9 and 10.

Additionally, Meru and Mcgregor (2016) developed an F2 population from a cross between UGA147 (resistant) and Charleston Gray (susceptible) and identified an intermediate QTL associated with FON race 2 resistance on chromosome 11 (*Qfon11*). Furthermore, Branham et al (2016) generated the first linkage map *Citrullus lanatus* var. *citroides* (Clc) through genotyping by sequencing and identified one major and four minor QTL associated with FON race 2 resistance. The major QTL was able to explain 43% of the phenotypic variation and was limited to 1.2 Mb interval on chromosome 9.

Infection, colonization and survival

Histopathology of fungal infection and colonization

Fusarium oxysporum is a common saprophyte in the roots of many crops but they lack ability to enter the vascular tissue and cause wilting symptoms (Gao et al. 1995). The pathogenic isolates of Fusarium possess the ability to enter the root tissues and invade the xylem elements (space-0) resulting in vascular wilt (Beckman and Roberts 1995). In the xylem vessels, Fusarium produces numerous microconidia where they are translocated through transpiration stream to outervessel leading to physical clogging. This process of inter vessel translocation of microconidia is repeated every 2 -3 days until the fungus has colonized considerable area of the host's xylem vessels. The infection process also involves the production of extracellular cell wall degrading enzymes (endopolygalacturonases, cellulases, hemicellulases, pectinases and others) of both host and pathogen origin resulting in the formation of gums and gels that block the vessels, preventing water transport. As host response to vascular invasion of Fusarium infection, tyloses may be induced resulting in blockage of xylem vessels. This process

consequently results in permanent wilting and plant death.

Beckman et al. (1995) correlated Fusarium resistance to the rate of xylem colonization of the host tissue by the fungus, or, the rate at which the host can block the spread of the pathogen.

In the *F. o.* f. sp. *lycopersici*-tomato pathosystem, a MAP kinase gene was involved in root penetration and subsequent pathogenesis by the fungus. In a similar study, F-box protein, Frp1 was identified as a critical protein for colonization and invasion of tomato roots (Jonkers et al. 2009). Further mutation studies showed that the fungal strains lacking this protein (Δ*frp1*) were limited in their ability to colonize and invade tomato roots. Lü et al. (2011) demonstrated that the FON race 1 strain could attach and colonize the root surface of the resistant watermelon line (PI 296341-FR) by 12 hours post inoculation (hpi). Further, by 24 hpi, more than 50% of conidia attached on the root surface germinated and colonized along the root axis. By 3 dpi, infection process was further observed as penetration of hyphae into the epidermal cells and formation of appressoria at the penetration sites. By 5 dpi, root surface was covered with mycelium and sporulation on the root surface was observed by 8 dpi. While hyphae colonized the central cylinder of the roots and invaded into the xylem vessels leading to destruction of roots by 7 dpi in a susceptible watermelon 'Black Diamond' variety, apart from attachment and colonization of root surface, the fungus failed to grow into the xylem vessels in PI 296341-FR line. Consequently, FON race 1 did not cause wilting symptoms in PI 296341-FR line. Likewise, in a separate study by Zhou and Everts (2004b), significantly less colonization of the roots and stems of resistant watermelon cultivars as compared to susceptible cultivars by FON was observed. A positive correlation was observed between percent wilt and FON colonization in the roots and lower stems (Zhou and Everts 2004b).

The level of cultivar resistance and a reduced rate of FON spread in the stems were also positively correlated (Chang et al. 2008).

Contrastingly, in a recent study done by Sun et al. (2017) in *Fusarium oxysporum* f. sp. *niveum* pathosystem, it was observed that there is a non-causal relationship between fusarium wilt and water transport blockage. Also, it was noticed that the wilt associated with fusarium infection does not result from the water shortage but from transport of toxins. Furthermore, markers for water stress, abscisic acid, and proline content did not increase under infection. Indicating that the wilting associated with disease is not caused by water shortage.

Survival

Seed-borne nature of FON: The seed-borne nature of *F. oxysporum* formae speciales (watermelon, melon and cucumber) is widely known (Martyn and Vakalounakis 2012). The first report of the seed-borne nature of FON was by Fulton and Winston (1915). The authors recovered the pathogen from the testa (seed coats) of watermelon seeds following seed washing. Further confirmation of seed-borne nature of FON was demonstrated by Porter (1928). The author recovered FON isolates from seeds that developed in fruits whose vines were infected with the Fusarium wilt pathogen. In addition, FON isolates were also recovered from commercial watermelon seedlots in Georgia and Texas (Porter 1928). In another study, it was observed that the highly aggressive FON race 2 was seed-borne in Texas (Martyn 1987). Apparently, these seeds were used for the production of hybrid seedless watermelon. In a recent study, the mechanism of seed infection was studied (Egel personal communication). Using nit-mutants of FON, it was demonstrated that the pathogen can invade flesh and seeds of

watermelon when inoculated through the peduncle; however, a detailed description of mode of pathogen invasion in seeds is lacking.

Survival in the soil: The long-term survival of Fusarium in the soil is aided by resistant resting spores called chlamydospores. The chlamydospores can either form directly in the hyphae or by the morphological modification of macroconidia in sporodochia. The saprophytic survival of *F. oxysporum* in the soil occurs rarely, rather, it is short-lived. This is due to the limited capabilities of the hyphae to survive periods of stress. Fungal hyphae can also survive in organic rich soil; however, survival aided with chlamydospores is more efficient. The impact of long-term survival of inoculum in soil was documented by Porter (1928). The researcher documented a scenario of a watermelon field in Iowa that remained fallow for 16 years after initial report of Fusarium wilt outbreak, when re-planted, experienced an extensive disease outbreak. In studies by McKeen and Wensley (1961), it was observed that *Fusarium* species *lycopersici*, *melonis*, and *niveum* could survive and remain pathogenic for 11 years or more in soil tube cultures stored at 3 - 4 °C. Among the formae specialis, f. sp. *melonis* remained viable after 17 years of storage. Consequently, crop rotation scheme of 5-7 years is routinely used as one of the management options for Fusarium wilt of watermelon.

Management of Fusarium wilt

Watermelon genomics and resistance to Fusarium wilt

Use of Marker Assisted Selection (MAS) has helped in the progress of identification of important genetic traits in watermelon, including disease resistance genes. A PCR protocol was

recently developed to differentiate watermelon lines resistant to FON (Lin et al. 2009). The authors utilized the sequence characterized amplified region (SCAR) markers that amplified a single 898 bp fragment in FON-resistant watermelon lines; however, these fragments were not present in susceptible lines. Recently, a genetic map with relatively high marker coverage consisting of 698 SSR, 219 insertion deletion and 36 structure variation markers was constructed by Ren et al. (2012). Genotyping by sequencing (GBS) is a next generation sequencing tool that have allowed sequencing of DNA fragments tagged with short sequences of DNA. It can detect novel plant single nucleotide polymorphisms (SNPs) in performing genotyping studies. Lambel et al. (2014) generated thousands of SNPs for linkage analysis in watermelon by using GBS. Additionally, Nimmakayala et al. (2014) estimated genome wide linkage disequilibrium decay in watermelon and identified selection sweeps resulting from watermelon domestication by using GBS. Irrespective of the ability of GBS to generate thousands of SNP markers, low genetic diversity in cultivated watermelons have limited the number of polymorphic markers that are available to study elite populations.

In a revolutionary development in transgenic watermelon, researchers used pollen-tube pathway to introduce FON resistant traits to susceptible watermelon cultivar (Chen et al. 1998). The authors injected a genomic fragment of squash DNA fused with the GUS marker into the pollinated ovaries. The resultant transformants displayed FON resistant traits obtained from squash DNA in approximately 5% of the transgenic plants. However, the exact reason for the transmissible traits in squash DNA fragment responsible for the resistance is unknown.

A transcriptome profile analysis was performed in watermelon by Lu et al. (2011) with response to FON inoculation both during compatible and incompatible situations. In an incompatible reaction between PI 296341-FR and FON race 1, a significant differential

expression of 24 to 592 genes in roots was observed. The genes like pathogenesis-related (PR) genes, transcription factors, signaling/regulatory genes and cell wall modification genes were differentially induced only during incompatible reactions but not in the compatible reactions. Genes regulating jasmonic acid (JA) and shikimate-phenylpropanoid-ligin biosynthesis were highly induced for longer periods in the incompatible reaction compared to the compatible reaction. On the contrary, genes for transporter proteins such as aquaporins were down-regulated in the incompatible reaction compared to the compatible reactions. The authors indicated that such phenomenon could be due to the role transporter genes play during in the development of wilt symptoms (Lu et al. 2011).

Induced resistance

Induced resistance has been explored in cucurbits against both soilborne and foliar pathogens (Biles and Martyn 1989; Mas et al., 1981). It is defined as the ability of one pathogen or strain to protect a plant from a similar or related pathogen or strain by inducing resistance of the host plant. Several researchers demonstrated the phenomenon of induced resistance when susceptible cucurbit plants were pre- inoculated (induced) with either a non-host forma speciales or an avirulent race. Induction of induced resistance provided protection against Fusarium wilt or a foliar pathogen such as *Colletotrichum lagenarium*. It was observed that the effectiveness of induced resistance was stronger when the inducing organism was closely related to the challenge organism (e.g., formae speciales in the same family or avirulent races). In addition, effectiveness of induced resistance was also higher when challenge inoculation was performed 24-h post induction with inducible organism. Although these studies showed the potential of induced resistance in disease management, this management option in FON and watermelon

system remains unexplored.

Grafting watermelon onto cucurbit rootstock

Grafting of watermelon on cucurbita rootstock has been a common practice to control Fusarium wilt. This was first done in Japan (Ashita 1927) where watermelon was grafted onto *Cucurbita moschata* rootstock against FON. This was effective due to the fact that formae speciales of *F. oxysporum* are host specific and hence, FON infection to rootstock of other cucurbit hosts would not occur. Bottle gourd (*L. siceraria*) and bitter gourd (*Momordica charantia*) were also used as root stocks in Japan and East Asia but the identification of new forma speciales *F. o.* f. sp. *lagenariae* and *F. o.* f. sp. *momordicae*, respectively, has limited *C. moschata* to be used for root stock in grafting (Sun and Huang 1983). Recently, six watermelon rootstocks (Ojakkyo, Shintosa Camel, Strong Tosa, Emphasis, Macis and WMXP3945) were tested for frequency of infection by two races of FON (Keinath and Hassell 2014). It was observed that the grafted watermelon plants produced greater weights and number of fruits than the non-grafted plants. Additionally, rootstocks restricted the movement of FON to watermelon scions, suppressed wilt symptoms and increased the watermelon yield in infested field.

Disease suppressive soil and biological control

A soil is regarded as disease suppressive where disease severity remains limited despite of high pathogen populations. In case of Fusarium wilt of cucurbit, disease suppressive soils have been well described especially for the Chateaurenard soils in southern France (Alabouvette et al. 2009). The soil of this particular region was suppressive against Fusarium wilt of melon. In watermelon, detailed studies on soil suppressiveness against FON were done in Florida. A monoculture of watermelon for 7 years in particular experimental site resulted in induction of

soil suppressiveness against FON (Hopkins et al. 1987). With the exception for two cultivars, 'Smokylee' and 'Crimson Sweet', wilt severity increased among all cultivars irrespective of the resistance level. Interestingly, when a susceptible cultivar was grown in soil from Crimson Sweet plot, disease incidence did not occur indicating FON suppressiveness is transmissible. The effectiveness of suppressive soils was reduced upon moist heating to 70˚C or methyl bromide treatment. The authors concluded that the monoculture of 'Crimson Sweet' resulted in increased populations of actinomycetes, fluorescent pseudomonads and overall bacteria along with *F. oxysporum* populations in the suppressive soils (Larkin et al. 1993a). In fact, further investigations revealed that the suppressive nature of this soil was due to indigenous *F. oxysporum* populations. The mechanism of suppressiveness was not attributable to competitive exclusion or antibiosis against FON rather induced resistance in the host (Larkin et al. 1996). The potential of mycoparasite against FON was demonstrated by Harveson & Kimbrough (2002). The authors isolated a novel mycoparasitic fungus, *Sphaerodes retispora* var. *retispora* from roots of watermelons infected with FON. The mycoparasite, when applied in encapsulated form to FON-infested soil, disease incidence was significantly reduced as compared to inoculated plants without the mycoparasite.

Cultural control

Hairy vetch (*Vicia villosa* Roth), a green manure, has been demonstrated to be a potential management tool for Fusarium wilt of watermelon. However, the control would be ineffective when watermelon, especially triploid watermelon, is produced in severely infested soils. In a field experiment the efficacy of hairy vetch green manure alone and in combination with a moderately wilt-resistant (MR) triploid watermelon cultivar was evaluated against

FON. These treatments were also compared with pre-plant soil fumigants. The results showed the reduction in wilt incidence for both the treatments; however, they failed to produce an acceptable level of marketable yield. Interestingly, the authors observed an additive effect when both treatments were combined. The resultant additive treatment was significantly greater than that observed with the fumigants methyl bromide or metam sodium. The researchers also observed reduced stem colonization by FON with hairy vetch green manure treatment alone as compared to the fallow treatment. Stem colonization was significantly reduced and was lowest in treatments where the MR cultivar was grown in green-manure plots. These results demonstrated the potential of combined use of hairy vetch green manure and a MR cultivar in the suppression of Fusarium wilt in triploid watermelon (Zhou and Everts 2006).

Chemical control

Chemical fungicide application continues to be a significant component in developing effective disease management options for Fusarium wilt of watermelon. Three chemical fungicides, Quadris (a.i. azoxystrobin), Proline (a.i. prothioconazole), and Topsin (a. i. thiophanate-methyl) have been demonstrated to be effective against FON (Sanders and Langston, 2011). Azoxystrobin acts as qionone outside inhibitor (QoI) and affects mitrochondrial respiration whereas prothioconazole belongs to demethylation inhibitor (DMI) that affects sterol biosynthesis in fungi. Thiophanate-methyl belongs to the benzimidazoles group that affects β- tubulin and suppresses the respiration enzyme cytochrome c oxidase. Topsin and Quadris have been registered to be used for control of diseases on watermelon, and Proline was recently labeled for Fusarium wilt of watermelon.

Justification for research and objectives

The fungicides prothioconazole and thiophanate-methyl have different modes of action but they all target single sites. Development of resistance to fungicides targeting single sites is common in populations of fungal pathogens, and it is possible that isolates of FON may develop resistance to the fungicides. However, current status of resistance in FON populations prevalent in watermelon fields to the above mentioned fungicides is largely unknown. In addition, the relative effectiveness of the fungicides in suppression of mycelial growth and sporulation of FON has not been documented. Determining the sensitivity and resistance of FON populations to these chemicals will provide a useful guide for developing and implementing effective disease management programs involving these fungicides.

As mentioned earlier, four races of FON have been identified so far according to their aggressiveness on different watermelon cultivars. Race 0 does not cause significant losses since most watermelon cultivars are resistant to this race. Race 1 occurs throughout watermelon production areas of the United States and seeded, diploid-hybrid cultivars usually have good resistance to Race 1 (Kleczewski and Egel 2011). Race 2 was reported in Georgia in 2008 and has been found in a few other states and is aggressive on both seeded and seedless watermelons (Bruton et al. 2008; Kleczewski and Egel 2011). More recently, a new race (race 3) was found in Maryland that is more aggressive than the other three races (Zhou and Everts 2010). It is unknown if race 3 exists in the southeastern states and what is the current status of prevalent races in this region. In addition, genetic diversity of FON populations in the southeastern US has not be studied and variability in FON populations is a challenge to management of Fusarium wilt. Determining races and genetic diversity of this pathogen prevalent in watermelon production in this area will contribute to a more comprehensive understanding of the nature of the pathogen

and help develop more effective disease management programs.

Fusarium wilt of watermelon is a seed-borne and seed transmitted disease (Porter,1928). Infested seeds can be one of the important sources of pathogen introduction to new areas; however, knowledge about the seed infection process of FON is still unknown. There is considerable knowledge gap with respect to detection of seed-borne inoculum, time of seed infection, and potential infection courts for seed infection on disease transmission. Specific knowledge gained from these studies will improve our understanding of the role of FON inoculum in the development of Fusarium wilt of watermelon.

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CHAPTER 2

SENITIVITY OF *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* TO PROTHIOCONAZOLE AND THIOPHANATE-METHYL AND GENE MUTATION CONFERRING RESISTANCE TO THIOPHANATE-METHYL[1]

[1]Petkar, A., Langston, D. B., Buck, J. W., Stevenson, K. L., and Ji, P. 2017. Plant Dis. 101: 366-371. Reprinted here with permission of publisher.

ABSTRACT

Fusarium wilt incited by the fungus *Fusarium oxysporum* f. sp. *niveum* (FON) is a soilborne disease that affects watermelon production worldwide. Approaches for effective management of Fusarium wilt in watermelon are limited. Studies conducted in recent years indicated that prothioconazole and thiophanate-methyl reduced the disease significantly under field conditions. However, effects of the fungicides on different life stages of FON and potential existence of fungicide resistance in FON populations are unknown. In the present study, effects of prothioconazole and thiophanate-methyl on mycelium growth and spore germination of FON isolates collected in watermelon fields in Georgia were determined. In vitro mycelium growth studies indicated that all 100 isolates evaluated were sensitive to prothioconazole; EC_{50} values ranged from 0.75 to 5.69 μg/ml (averaged 1.62 μg/ml). In contrast, 33% and 4% of the isolates were resistant to thiophanate-methyl at 10 and 100 μg/ml, respectively. Microconidial germination assays showed that 36% and 64% of the isolates tested were sensitive or intermediately sensitive to prothioconazole at 100 μg/ml, but the fungicide did not inhibit spore germination at 10 μg/ml. Sequencing a portion of the β-tubulin gene of eight isolates resistant or sensitive to thiophanate-methyl indicated that fungicide resistance was associated with a point mutation at nucleotide position 200 resulting in a substitution of phenylalanine by tyrosine. This is the first report of isolates of *F. oxysporum* resistant to thiophanate-methyl. Results of the research suggest that prothioconazole may be a viable option for management of Fusarium wilt of watermelon while thiophanate-methyl should be used judiciously due to the existence of isolates resistant to the pathogen.

INTRODUCTION

Fusarium wilt, incited by *Fusarium oxysporum* f. sp. *niveum* (FON), is among the most severe diseases in watermelon (*Citrullus lanatus*) worldwide. In the southeastern United States, the disease caused increasing damage in watermelon production in recent years. Both seedless and seeded watermelon cultivars can be affected by the disease. In the field, Fusarium wilt may appear early in the watermelon growing season and a characteristic symptom of the disease is wilting of one or two vines while other vines appear healthy. Internal plant tissue discoloration can be seen when lower stems are sectioned. Eventually plants infected may become wilted completely especially if young plants get infected. Under conditions favorable for disease development, yield losses of more than 80% have been reported. This disease is known to have a wide distribution in Asia, Americas, and other watermelon growing continents (Egel and Martyn 2013).

Four races of FON have been identified so far based on their aggressiveness on different watermelon cultivars. Race 0 does not cause significant losses since most watermelon cultivars are resistant to this race. Race 1 is probably the most widely occurring race in the U.S. which is usually aggressive on seedless watermelon cultivars (Kleczewski and Egel 2011). Race 2 is aggressive on both seeded and seedless watermelons and is known to be established in several watermelon-producing states including Georgia (Bruton et al. 2008; Kleczewski and Egel 2011). More recently, a new race (race 3) was reported in Maryland, which is more aggressive than the other three races (Zhou et al. 2010).

In the U.S., Fusarium wilt has been managed through use of host resistance, such as seeded diploid cultivars of watermelon that are resistant to FON race 1 (Egel and Martyn 2013). However, an increase in losses caused by the disease has resulted from increasing production of seedless cultivars that are not resistant to race 1. Fusarium wilt incidence has also risen due to the

prevalence of race 2 of the pathogen, which is more aggressive on diploid cultivars than race 1. Additionally, an increasing concern to farmers is the emergence of the more aggressive race 3 identified in Maryland (Zhou et al. 2010). So far, reliable resistance to all races of FON is not available, especially in commercial triploid cultivars of watermelon.

Other management strategies for FON include grafting and cultural practices. Utilization of cover crops such as vetch (*Vicia villosa*) and crimson clover (*Trifolium incarnatum*) prior to planting of watermelon reduced Fusarium wilt in some field studies (Himmelstein et al. 2014; Keinath et al. 2010; Zhou and Everts 2004, 2006). Grafting, using non-host root stocks reduced Fusarium wilt of watermelon; however, the high labor cost incurred with this practice may limit widespread adoption in the U.S. (Davis et al. 2008; Keinath and Hassell 2014).

Proline (a.i. prothioconazole; Bayer CropScience, Research Triangle Park, NC) is the only fungicide registered on watermelon in the U.S. for managing Fusarium wilt. Studies conducted in Georgia indicated that Proline and Topsin (a.i. thiophanate-methyl; United Phosphorus Inc., King of Prussia, PA) reduced the disease significantly under field conditions (Sanders and Langston 2011). More recently, a series of greenhouse and field studies were conducted on watermelon in Maryland, Indiana, Delaware, and Georgia. These studies indicated that thiophanate-methyl and prothioconazole provided the greatest reduction of Fusarium wilt, and prothioconazole in combination with thiophanate-methyl showed a tendency to enhance disease reduction (Everts et al. 2014).

The effects of prothioconazole and thiophanate-methyl on different life stages of FON have not been reported. In addition, it is unknown if FON populations have developed resistance to the fungicides. Prothioconazole and thiophanate-methyl have different modes of action but they both target single sites (Brent and Holloman 2007), hence there is the risk that isolates of FON may

develop resistance to one or both fungicides. Resistance to thiophanate-methyl by plant pathogens has been reported. Thiophanate-methyl belongs to the methyl benzimidazole carbamate (MBC) fungicide group and acts by binding to tubulin, thereby blocking mitosis (FRAC 2016). The most common mechanism of resistance to MBC fungicides is a change in the amino acid composition of the target molecule, β-tubulin, often conferred by a single point mutation at specific sites in the gene that encodes the protein (FRAC 2016). Studies on resistance to thiophanate-methyl have focused on pathogens other than *Fusarium* spp., and resistance has been documented in populations of pathogens such as *Botrytis cinerea* and *Monilinia fructicola* (Amiri et al. 2014; Chen et al. 2013; Zhu et al. 2010). Resistance to thiophanate-methyl was due to a glutamic acid to alanine substitution at codon 198 (E198A) in β-tubulin in *M. fructicola* (Zhu et al. 2010). Resistance to thiophanate-methyl was also reported due to other mutations in β-tubulin E198Q or F200Y in *M. fructicola* (Chen et al. 2013).

The objectives of this research were to: 1) determine the effects of prothioconazole and thiophanate-methyl on mycelial growth and microconidial germination of FON isolates infecting watermelon in Georgia, and 2) identify mutations in the β-tubulin gene of FON isolates that were determined as thiophanate-methyl resistant in this study. This research would provide information for better utilization of the fungicides in designing effective disease management programs.

MATERIALS AND METHODS

***Fusarium oxysporum* f. sp. *niveum* isolates.** One hundred FON strains isolated from watermelon in commercial fields in Georgia in 2012 and 2013 were used (Fig. 2-1). Stem sections (3-5 cm) of symptomatic watermelon plants removed from the hypocotyl region were surface-disinfested in sodium hypochlorite (0.6%) for 2 min. Plant tissues were rinsed 3 times in sterile distilled water

(SDW), and after drying on sterile paper towels tissues were cut into small pieces and placed on semiselective peptone pentachloronitrobenzene agar (PPA; Nash and Snyder 1962). After 7-day incubation in darkness at 25°C, the plates were examined microscopically and fungal isolates were identified based on morphological criteria for hyphae, microconidia and macroconidia described previously (Nelson et al. 1983). Putative FON isolates were grown on potato dextrose agar (PDA), and after 5-7 day incubation at 25°C, single spore cultures were produced from all of the isolates as described previously (Leslie and Summerell 2006). For long-term storage, a loopful of mycelium of single spore isolates was placed in 2-ml Microbank vials (Pro-Lab Diagnostics Inc., Round Rock, TX) that are designed for fungal storage and contain treated beads and a cryopreservative solution. The vials were stored at -80°C.

Molecular identification. One hundred isolates grown on PDA (25°C, 7 days) were used for extraction of DNA using DNeasy Plant Mini kit (Qiagen, Valencia, CA). Polymerase chain reaction (PCR) amplification was conducted using FON-specific primers FON1 and FON2 (Lin et al. 2010). The PCR reaction mixture (25 µl) contained PCR reaction buffer (1×), 200 µM each dNTP, each primer (0.5 µM), *Taq* DNA polymerase (0.5 U), and 10 ng of DNA. PCR conditions were as reported by Lin et al. (2010), which included: denaturing at 94°C for 90 s, 30 cycles of denaturing at 94°C for 30 s, annealing at 62°C for 30 s, and polymerization at 72°C for 60 s, with a final extension (72°C) for 10 min. The PCR product was evaluated by electrophoresing in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 0.5× Tris-borate-EDTA buffer.

Pathogenicity tests. Watermelon seedlings (cv. Sugar Baby) were grown in 9-cm diameter pots containing sand:peat:vermiculite mixture (4:1:1, v:v:v). One hundred isolates were grown on PDA

at 25°C for 7 days. Five 7-mm diameter agar plugs from the edge of a growing colony were aseptically transferred to a flask (500 ml) with 200 ml of liquid mineral salts medium (Esposito and Fletcher 1961). The liquid culture was incubated for 2 weeks at 23±1°C with shaking (150 rpm). Microconidia were harvested by filtering the liquid culture through three layers of sterile cheesecloth. The concentration of the microconidial suspension was determined using a hemocytometer and adjusted to 10^6 spores/ml by adding an appropriate amount of SDW. Watermelon seedlings at the first true leaf stage were inoculated by applying 5 ml of conidial suspension to the base of each seedling (Latin and Snell 1986), 10 plants for each isolate. Ten plants received SDW treatment served as control. All plants were placed in a greenhouse (28ºC day and 20ºC evening, 70-80% relative humidity), and Fusarium wilt incidence was recorded at 3 weeks after inoculation. Diseased plants were sampled for isolation and confirmation of the causal agent using the methods described above. The experiment was conducted twice under similar conditions.

Percentage of fungicide-resistant isolates. A collection of 100 FON isolates from Georgia was used in the study. Resistance of the isolates to prothioconazole and thiophanate-methyl was determined based on mycelium growth on PDA amended with the fungicides. Technical grade prothioconazole and thiophanate-methyl (Sigma-Aldrich, St. Louis, MO) were dissolved in acetone to make stock solutions of 10 mg/ml. After autoclaving and cooling to 48ºC, PDA medium was amended using the fungicide stock solutions to final concentrations of 0 (control), 10, and 100 µg/ml. The isolates were grown on PDA at 25°C for 7 days, and an agar plug (7 mm diameter) was excised from the edge of the colony and placed upside down at the center of the plates amended with the fungicides. Three plates were used for each treatment

(fungicide/concentration/isolate). The plates were incubated at 25°C for 7 days, and colony diameters on fungicide amended and non-amended control plates were measured and used to determine resistance to the respective fungicides as reported previously (Jackson et al. 2012). The percentage of resistant (colony size > 90% of the control), intermediately sensitive (30 to 90% of the control), and sensitive (< 30% of the control) isolates was calculated. The experiment was conducted twice under similar conditions.

Sensitivity of mycelial growth to prothioconazole. None of the 100 isolates were found to be resistant to prothioconazole at 10 or 100 µg/ml, hence, a subset of isolates was selected arbitrarily for determining sensitivity to the fungicide. Eighteen isolates were grown on PDA at 25°C in the dark for 7 days. An agar plug was taken as described above and placed on PDA amended with prothioconazole at 0, 0.1, 0.5, 1, 3, 5 and 10 µg/ml. Three plates were prepared for each concentration and isolate. Colony diameters were measured after incubating at 25°C for 7 days, and relative growth (RG) was quantified as the ratio of colony diameter (with diameter of the original plug subtracted) on fungicide-amended medium to that on non-amended medium. The effective concentration that suppressed mycelium growth by 50% (EC_{50}) was calculated for each isolate based on linear regression of probit-transformed relative inhibition (1- RG) on $\log_{10}$-transformed fungicide concentration. The assay was repeated and data from the two experiments were combined. The Shapiro-Wilk test (PROC UNIVARIATE) was used to determine if frequency distribution of EC_{50} values followed normality in SAS (version 9.2, SAS Institute Inc., Cary, NC). Paired t-test was conducted in order to compare the mean $\log_{10}$-transformed EC_{50} values among the two experiments.

Effect of prothioconazole on microconidial germination. Fourteen isolates were grown on PDA at 25°C in the dark for 7 days. SDW (5 ml) was added to each plate and spores were harvested by gently rubbing the surface of the mycelium with a sterile glass spreader. Spore suspensions were collected in 15-ml sterile centrifuge tubes, and each plate was washed with an additional 5 ml of SDW for spore collection. The concentration of microconidia in each suspension was determined using a hemocytometer and adjusted to approximately 5×10^3 spores/ml with SDW. Fifty microliters of each suspension was spread-plated onto water agar amended with prothioconazole at 0, 10, and 100 µg/ml. Three plates were used for each fungicide concentration and isolate. After incubation (25°C) for 18 h in darkness, microconidia were stained with lactophenol cotton blue stain (Smith and MacHardy 1982), and 100 germinated and non-germinated microconidia on each plate were counted. Isolates were classified as resistant (germination rate > 90% of the control), intermediately sensitive (30 to 90% of the control), and sensitive (< 30% of the control). The experiment was conducted twice, and data from the two experiments were combined after insuring that there was no significant effect of experiment by treatment interactions ($P = 0.36$).

Determining the β-tubulin gene mutation. Eight isolates, 4 resistant and 4 sensitive to thiophanate-methyl at 100 µg/ml, were arbitrarily selected and used to sequence partial β-tubulin gene for possible mutations. Total DNA of each isolate was extracted using a DNeasy Plant Mini kit (Qiagen). A partial fragment of the β-tubulin gene was amplified using the primer sets FU-tubulin2 and FU-tubulin3 (Chung et al. 2009). PCR was performed in 25 µl of reaction mixture containing one unit of HotStarTaq DNA polymerase (Qiagen), 250 µM of each dNTP, 0.2 µM of each primer, and reaction buffer (2 mM $MgCl_2$). The amplification was performed using the protocol reported previously (Chung et al. 2009). QIAquick PCR purification kit (Qiagen) was

used to purify PCR product, and PCR product sequencing was conducted by Eurofins MWG operon LLC (Louisville, KY). The amino acid sequences were aligned and analyzed using an alignment tool, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

RESULTS

Identification of FON isolates. The 100 isolates were identified as FON based on morphological characteristics and PCR analysis using FON-specific primers FON1 and FON2 that produced a 174-bp DNA fragment. In addition, all isolates were tested for pathogenicity by inoculating watermelon seedlings under greenhouse conditions. All isolates caused typical Fusarium wilt symptoms (wilting of stem and leaves) and disease incidence was 100% three weeks after inoculation. Seedlings treated with SDW remained asymptomatic.

Percentage of isolates resistant to thiophanate-methyl and prothioconazole. All 100 isolates were sensitive to prothioconazole at 10 or 100 µg/ml (Fig. 2-2A and 2-2B). The percentage of isolates resistant, intermediately sensitive, and sensitive to thiophanate-methyl (100 µg/ml) was 4, 84, and 12, respectively. At 10 µg/ml of thiophanate-methyl, 33 and 67% of the isolates were resistant and intermediately sensitive, respectively (Fig. 2-2A, Table 2-1). Only isolates from Berrien county (n = 4) were resistant to 100 µg/ml of thiophanate-methyl (Table 2-1). All isolates from Telfair and Ben Hill counties were sensitive, and isolates from Irwin, Crisp, Worth, Sumter, and Wheeler counties were intermediately sensitive to 100 µg/ml of thiophanate-methyl (Table 2-1).

Sensitivity of mycelial growth to prothioconazole. EC_{50} values of prothioconazole for suppressing mycelium growth of the isolates were lognormally distributed. EC_{50} values ranged from 0.75 to 5.69 μg/ml, with a mean of 1.62 μg/ml (Fig. 2-3). The minimum inhibitory concentrations that inhibited mycelium growth of the isolates completely ranged from 3 to 5 μg/ml.

Effects of prothioconazole on microconidial germination. At 100 μg/ml of prothioconazole, 30 and 70% of the 14 tested isolates were sensitive and intermediately sensitive. Spore germination was not inhibited at 10 μg/ml and germination rates of all isolates on fungicide amended plates were >90% compared to the non-amended control plates.

β-tubulin gene mutation. Differences in the amino acid sequence spanning from 190-250 were detected between isolates sensitive and resistant to thiophanate-methyl at 100 μg/ml. The four isolates sensitive to thiophanate-methyl had phenylalanine at position 200 (TTC) and the four isolates resistant to the fungicide had tyrosine at the position (TAC). The nucleotide changes at amino acid position 200 from TTC (phenylalanine) to TAC (tyrosine) were correlated with resistance of the isolates to the fungicide (Fig. 2-4).

DISCUSSION

Fusarium wilt is a troublesome disease for watermelon production in the southeastern U.S. Due to limited options for managing the disease, identification of effective fungicides and monitoring development of fungicide resistance in the pathogen populations are highly desirable. Fungicides currently labeled for managing Fusarium wilt on watermelon in the US are limited; however, field studies conducted in recent years indicated that prothioconazole and thiophanate-methyl were

effective in reducing Fusarium wilt on watermelon (Everts et al. 2014; Sanders and Langston 2011). In the present study, prothioconazole was more effective than thiophanate-methyl in suppressing mycelial growth of FON isolates in vitro, and no isolate was resistant to prothioconazole while isolates resistant to thiophanate-methyl were identified based on *in vitro* mycelial growth assays. This is the first report documenting existence of FON isolates resistant to thiophanate-methyl in field populations of the pathogen and association of resistance with an amino acid substitution in the β-tubulin target resulting from a point mutation in the gene.

Thiophanate-methyl has been reported to be effective in reducing diseases caused by *Fusarium* spp. such as *F. oxysporum* f. sp. *vasinfectum* in cotton and *F. o.* f. sp. *niveum* (Doan and Davis 2015; Everts et al. 2014; Sanders and Langston 2011). Information regarding resistance of isolates of *Fusarium* spp. to this fungicide is limited. In a study by Suga et al. (2011), isolates of *F. asiaticum* resistant to thiophanate-methyl were documented and the resistant isolates had a F167Y or F200Y mutation in the β-tubulin gene. Chung et al. (2009) reported that a few isolates of *F. oxysporum* f. sp. *gladioli* and *F. oxysporum* f.sp. *lilii* were resistant to thiophanate-methyl. However, sequencing partial β-tubulin gene of the resistant isolates did not identify mutations in either 198 or 200 position, suggesting other mechanisms might be involved in resistance of the isolates. In the present study, 4 and 33% of the FON isolates from watermelon were resistant to thiophanate-methyl at 100 and 10 µg/ml, respectively, and sequencing β-tubulin gene showed that mutation occurred at codon 200 with tyrosine substituting phenylalanine in the resistant isolates. No other point mutation in β-tubulin gene was found between sensitive and resistant isolates (data not shown).

Prothioconazole is in the demethylation inhibitor (DMI) fungicide class that affects sterol biosynthesis, thereby disrupting membrane structure (FRAC 2016). It has been shown to be

effective in reduction of diseases caused by *Fusarium* spp. under field conditions, including Fusarium wilt of watermelon (Everts et al. 2014; Sanders and Langston 2011) and Fusarium head blight of wheat (*F. graminearum*) (Freije and Wise 2015). Effects of prothioconazole on the different life stages of FON and resistance to this fungicide in populations of *Fusarium* spp. have not been reported. In a study on *F. graminearum* in Brazil, prothioconazole inhibited mycelial growth of the pathogen at a concentration of 0.1 µg/ml (Avozani et al. 2014). In the present study, all the 100 FON isolates were sensitive to the compound based on a mycelial growth assay, with an average EC_{50} value of 1.62 µg/ml. Prothioconazole was also active in suppressing microconidial germination at 100 µg/ml with no resistant FON isolates identified. When tested at a lower concentration (10 µg/ml), microconidial germination of the isolates evaluated was not effectively inhibited. Prothioconazole is suggested to be applied at 200 g/ha (label rate) for managing Fusarium wilt on watermelon. This application rate is generally higher than 100 µg/ml so should be effective for suppression of mycelial growth and microconidial germination, though it is difficult to accurately estimate concentrations of fungicides on the plants after they are applied.

In summary, prothioconazole was shown to be effective in suppressing mycelial growth of FON isolates in vitro and no isolate was found to be resistant to this fungicide. It was also effective in inhibiting microconidial germination when used at 100 µg/ml. Given the limited tools for managing Fusarium wilt of watermelon and the importance of this disease, prothioconazole can be a viable option to be recommended to growers. Thiophanate-methyl was less effective than prothioconazole in inhibiting mycelial growth of the FON isolates, and isolates resistant to thiophanate-methyl were documented based on in vitro assays and analysis of β-tubulin gene sequences. Hence, thiophanate-methyl should be used judiciously if recommended for managing Fusarium wilt of watermelon. Further studies can be directed at identification of new effective

fungicides, monitoring potential development of resistance to prothioconazole in the pathogen population, and development of integrated disease management programs incorporating prothioconazole with cultural practices such as host plant resistance and effective cover crops. These studies have the promise to reduce the risk of fungicide resistance development and may improve efficacy for managing this important disease.

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Table 2-1. Distribution of *Fusarium oxysporum* f. sp. *niveum* isolates with different levels of resistance to thiophanate-methyl in different counties in Georgia

County	Sensitive (%) *		Intermediate (%) *		Resistant (%) *	
	10 ppm	100 ppm	10 ppm	100 ppm	10 ppm	100 ppm
Ben Hill	0	100	100	0	0	0
Berrien	0	13.6	52.3	77.3	47.7	9.1
Cook	0	5	80	95	20	0
Crisp	0	0	50	100	50	0
Irwin	0	0	0	100	100	0
Sumter	0	0	0	100	100	0
Telfair	0	100	100	0	0	0
Tift	0	37.5	62.5	62.5	37.5	0
Wheeler	0	0	100	100	0	0
Worth	0	0	0	100	100	0

* Sensitivity to the fungicide was determined based on mycelium growth on PDA with or without the fungicide. Sensitive, intermediately sensitive and resistant represent < 30%, 30 to 90%, and > 90% of growth compared to the control, respectively.

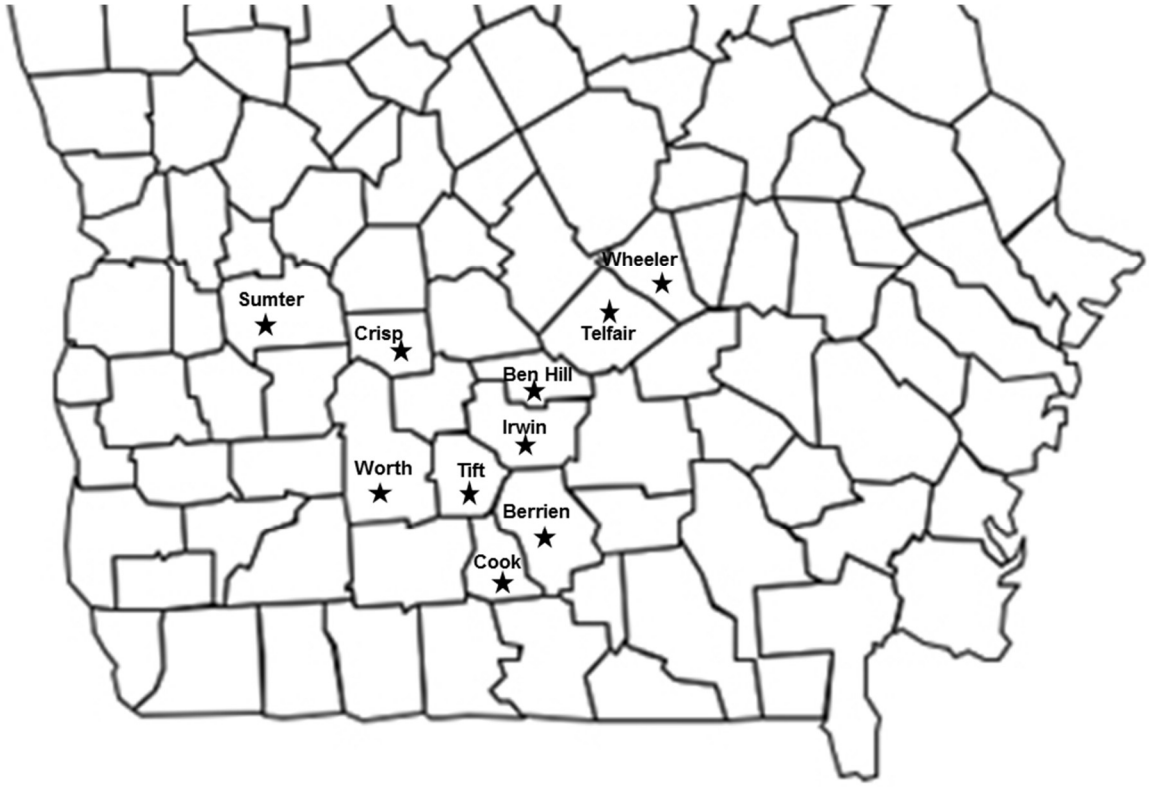

Fig. 2-1. Locations of watermelon fields in south Georgia for collection of isolates of *Fusarium oxysporum* f. sp. *niveum*. The isolates were collected from the counties marked with star symbol.

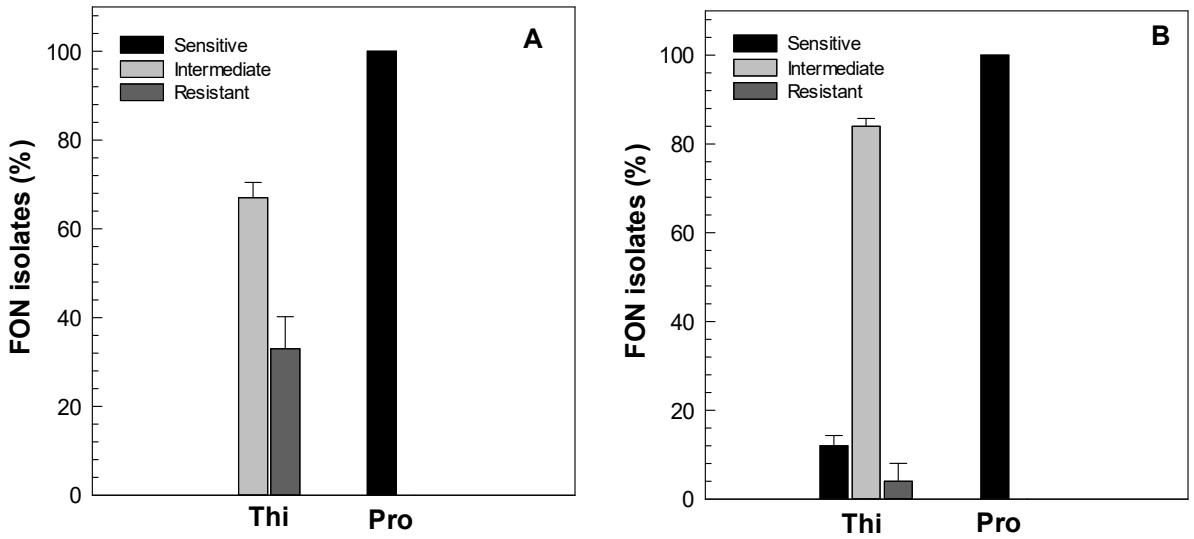

Fig. 2-2. Percentage of *Fusarium oxysporum* f. sp. *niveum* (FON) isolates that were sensitive, intermediately sensitive or resistant to 10 μg/ml (A) and 100 μg/ml (B) of thiophanate-methyl and prothioconazole based on mycelial growth of 100 isolates. Thi = thiophanate-methyl, and Pro = prothioconazole. Error bars indicate standard errors of the means of two repeated experiments.

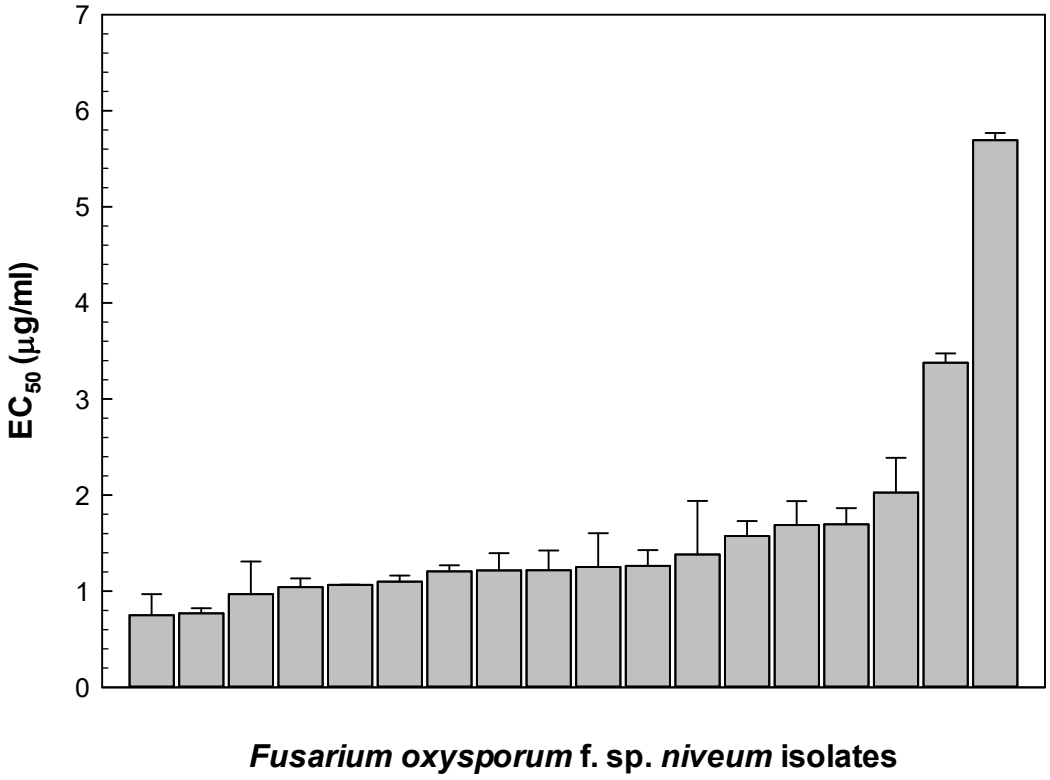

Fig. 2-3. Concentrations of prothioconazole that were 50% effective (EC_{50}) for inhibiting mycelial growth of *Fusarium oxysporum* f. sp. *niveum* isolates. Error bars indicate standard errors of the means of two repeated experiments.

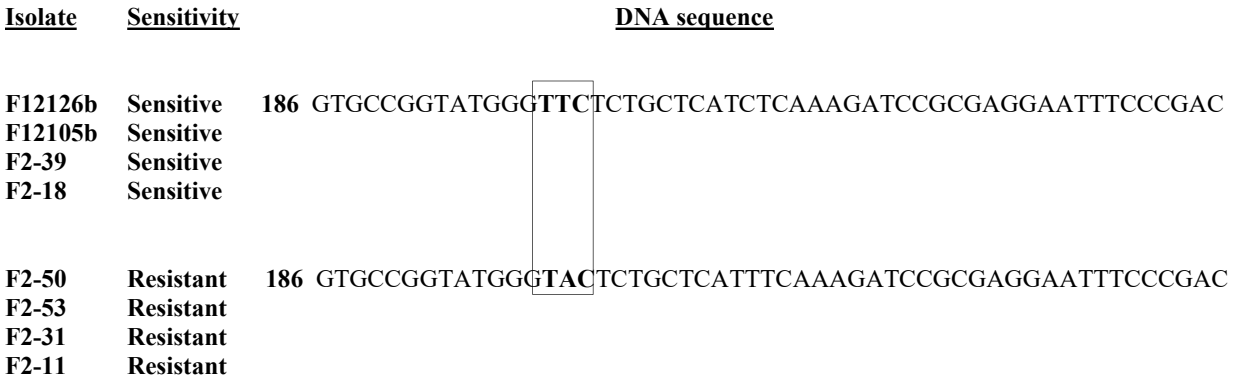

Fig. 2-4. Partial nucleotide sequence of β-tubulin gene of *Fusarium oxysporum* f. sp. *niveum* (FON) isolates sensitive or resistant to thiophanate-methyl. Isolates F2-50, F2-53, F2-31 and F2-11are resistant to thiophanate-methyl, and isolates F12126b, F12105b, F2-39 and F2-18 are sensitive to the fungicide. The box shows the nucleotide change from TTC to TAC at position 200 that confers resistance to thiophanate-methyl.

CHAPTER 3

INFECTION COURTS IN WATERMELON PLANTS LEADING TO SEED INFESTATION BY *FUSARIUM OXYSPORUM* F. SP. *NIVEUM*[1]

[1]Petkar, A., and Ji, P. 2017. Phytopathology 107:828-833. Reprinted here with permission of publisher.

ABSTRACT

Fusarium wilt incited by *Fusarium oxysporum* f. sp. *niveum* (FON) is a seed-transmitted disease that causes significant yield loss in watermelon production. The pathogen may infect watermelon seeds latently, which can be an important inoculum source and contribute to severe disease outbreak. However, information regarding infection courts of FON leading to infestation of watermelon seeds is limited. To determine how seeds in watermelon fruit can be infested by FON during the watermelon growing season, greenhouse and field experiments were conducted in 2014 and 2015 where watermelon flowers and immature fruit were inoculated with FON. Seeds were extracted from mature watermelon fruit, and infestation of watermelon seeds was determined by isolation of FON and further confirmed by real-time polymerase chain reaction (PCR) analysis. Inoculation of the pericarp of immature fruit resulted in 17.8 to 54.4% of infested seeds under field conditions and 0.6 to 12.8% of infested seeds under greenhouse conditions when seeds were not surface disinfested prior to isolation. Seed infestation was also detected in 0 to 4.5% of the seeds when seeds were surface disinfested prior to isolation. Inoculation of pistil resulted in 0-7.2% and 0-18.3% of infested seeds under greenhouse and field conditions when seeds were surface disinfested and not disinfested before isolation, respectively. Inoculation of the peduncle resulted in 0.6- 6.1% and 0-10.0% of infested seeds in the greenhouse and field experiments when seeds were surface disinfested and not disinfested before isolation, respectively. Seed infestation was also detected in all the experiments using real-time PCR assay when pericarp or pistil was inoculated, and in 3 of 4 experiments when peduncle was inoculated, regardless of whether seeds were surface disinfested or not disinfested. Pericarp and peduncle of immature watermelon fruit and pistil of watermelon flowers could be potential infection courts for *F. oxysporum* f. sp. *niveum* leading to infestation of seeds in asymptomatic watermelon fruit.

INTRODUCTION

Fusarium wilt incited by *Fusarium oxysporum* f. sp. *niveum* (FON) is among the most economically important diseases on watermelon occurring in almost all watermelon-producing regions of the world. Occurrence of the disease was first reported by E. F. Smith in the southeastern U.S. (Smith 1894). Symptoms of the disease include damping-off and wilt of watermelon vines at any stages of plant growth (Egel and Martyn 2007). A common symptom of this disease is wilting of some vines of the plant while other plant parts remain symptomless. Internal vascular discoloration is apparent when the root or lower stems are cross sectioned.

Southeastern states in the U.S., including Georgia, Florida, South Carolina and North Carolina, are major producers of watermelon in the nation with more than 50% of national watermelon production in this region. Increasing damage in commercial watermelon fields in the southeastern U.S. caused by Fusarium wilt has been observed in recent years with disease incidence over 50% in many fields (Ji and Petkar, unpublished). The disease is favored by cool wet conditions that are common in the southeastern states especially during the spring growing season. With the phase-out of methyl bromide, limited options are available for effective management of Fusarium wilt of watermelon. The only fungicide available, prothioconazole, was recently registered for the disease, and development of resistance by the pathogen to fungicides with single targeting site is a concern (Petkar et al. 2017). Using cover crops as soil amendments is a promising tactic for managing the disease (Keinath et al. 2010; Zhou and Everts 2004), though the approach has not been commonly adopted by growers in the southeastern U.S.

Another challenge in managing Fusarium wilt of watermelon is the seedborne nature of FON that facilitates long distance dispersal of the pathogen and disease outbreaks. The seedborne nature of FON has long been known. Fulton and Winston (1915) were the first to isolate FON

from watermelon seeds. Porter (1928) recovered FON isolates from seeds of watermelon fruit developed on FON infected vines and from commercial watermelon seedlots in Georgia and Texas. Later, Martyn (1987) isolated FON from watermelon seeds used for the production of hybrid seedless watermelon in Texas. In a more recent study, Michail et al. (2002) obtained FON isolates from 5 cultivars of watermelon and studied the relationship between level of seed infestation by FON and Fusarium wilt development on watermelon plants grown from the seeds. Disease incidence was more than 45% when using seeds with moderate (8.5-9.5%) and high (23-31.5%) percentages of infestation, but less than 5-10% when seeds with a lower percentage of infestation (1.5-2.5%) were grown. These studies indicate that watermelon seeds latently infected by FON play a significant role in development of Fusarium wilt.

Although it has long been demonstrated that infested watermelon seeds can be a significant source of FON inoculum, knowledge about the mode of FON infection leading to watermelon seed infestation by the pathogen is limited. Hence, the objective of this work was to determine potential infection courts of FON leading to infestation of watermelon seeds. Information about FON infection for seed infestation will not only advance our knowledge about pathogenicity and epidemiology of the pathogen, but also facilitate development of effective disease management approaches by protection of the infection courts to reduce seed infestation.

MATERIALS AND METHODS

FON isolate and inoculum preparation. The FON isolate (race 1) used in the study was provided by Hunt Sanders at the University of Georgia. Single spore cultures were generated, and pathogenicity on watermelon seedlings was verified as described previously (Petkar et al. 2017). Mycelia of the single-spore isolates were transferred to fungal storage vials (Pro-Lab Diagnostics Inc., Round Rock, TX) and kept at -80°C for long-term storage. A single-spore isolate FON R1 was used for all the greenhouse and field experiments. To prepare FON inoculum, the isolate was grown on PDA at 25°C for 7 days. Five mycelial plugs (7-mm diameter) from the edge of a growing colony were transferred to a flask (500-ml) containing 200 ml liquid mineral salts medium (Esposito and Fletcher 1961). After incubating at 23±1°C for 2 weeks with shaking (150 rpm), the liquid culture was filtered through sterile cheesecloth (3 layers). Concentration of microconidia in the liquid culture was calculated using a hemocytometer, and sterile distilled water (SDW) was added to adjust the concentration to 10^6 spores/ml to be used in the study.

Greenhouse experiments. Two greenhouse experiments were conducted, one in fall 2014 and one in spring 2015. Watermelon seeds (cv. Sugar Baby) were sown in seedling trays (with 3.5 x 3.5 cm cells) containing a commercial potting mix (Scotts Miracle-Gro, Marysville, OH). Seedlings were transplanted to 7.5-liter pots containing the same potting mix 3 weeks after seeding, one plant per pot. After anthesis, female watermelon blossoms (20/inoculation method) were hand-pollinated and tagged for inoculation with FON. Three potential infection courts were inoculated on separate plants: a) swabbing of pericarp of immature fruit (approximately 1 x 2.5 cm in size); b) inoculation of peduncle of immature fruit; and c) inoculation of pistil. For pericarp inoculation, the pericarp of an immature watermelon fruit was swabbed with a microconidial

suspension (10^6 spores/ml). For peduncle inoculation, the peduncle of an immature watermelon fruit was injected with a microconidial suspension (10^6 spores/ml, 10 µl/ peduncle). This was to determine if FON entering vascular tissues, through means like wounds that may occur during the growing season, might lead to seed infestation. For pistil inoculation, a female flower was inoculated by applying 10 µl microconidial suspension (10^6 spores/ml) to the stigma (Fig. 3-1). A randomized complete block design was used with five replicates and 15 plants for each inoculation site (pistil, pericarp, and peduncle). The same numbers of plants treated with SDW were used as controls. The plants were maintained under greenhouse conditions with 22/30ºC and 20/28ºC night/day temperature for the experiments in fall 2014 and spring 2015, respectively. At maturity, fruit were harvested and surface sterilized with 70% ethanol, and seeds were extracted. To prevent cross contamination from seed to seed, watermelon fruit was cut with a sterilized knife and seeds were taken out individually using a sterile spatula. Seeds were dried on sterilized filter paper and evaluated for FON infestation by plating as described below. For detection by a real-time polymerase chain reaction (PCR) assay, seeds from the same inoculation method in each replicate were pooled, maintained as a separate lot, and stored at 4ºC until processed.

Field experiments. Two field experiments were conducted at University of Georgia Coastal Plain Experiment Station experimental farm in Tifton, GA, in fall 2014 and spring 2015. Field soil was loamy sand with no history of infestation by FON. Raised beds (15-cm high, 76-cm wide) were prepared and centered 1.8-m apart. N-P-K (5-10-15) was applied (840 kg/ha) prior to laying plastic mulch, and the beds were covered by white or black plastic mulch in the fall and spring experiments, respectively. A single drip irrigation tape was installed approximately 2.5 cm below the surface in the center of the beds as the plastic mulch was applied. The drip tape used was Ro-

Drip (John Deere Water, San Marcos, CA) with 30 cm spacing of emitters and a flow rate of 56 ml/m/min.

Watermelon plants (cv. Sugar Baby) were grown in seedling trays as in the greenhouse study. Four-week old seedlings were transplanted at 61-cm spacing within a row in the field beds. The experimental plots consisted of a single row that was 3-m long with 2.5-m buffer zones without plants maintained between plots. A randomized complete block design with five replicates for each inoculation site (pistil, pericarp, and peduncle) was employed. As described in the greenhouse experiments above, female watermelon blossoms (20/inoculation method) were hand-pollinated after anthesis and inoculated at one of three sites. Mature fruit were harvested and surface sterilized with 70% ethanol, seeds were extracted and FON was detected using plating and PCR analysis as in the greenhouse experiments.

Assessment of FON seed infestation by plating assay. Watermelon seeds were tested for internal and external infestation by FON. Sixty seeds were used per replicate and inoculation method to detect internal and external infestation, respectively. For internal infestation, seeds were soaked in 0.6% NaOCl solution for 3 min, followed by rinsing with SDW. The seeds were dried on sterile filter paper and placed on peptone PCNB agar (PPA) plates (Leslie and Summerell 2006), 10 seeds per plate. The plates were incubated for two weeks at 25°C and checked periodically for putative FON colonies. For external infestation, seeds were rinsed with SDW, dried on sterile filter paper and placed on PPA plates. Putative FON colonies were purified by subculturing hyphal tips on PPA plates and then transferring onto potato dextrose agar. The cultures were identified by morphological characteristics (Leslie and Summerell 2006) and PCR analysis using FON-specific primers Fn-1/Fn-2 (Zhang et al. 2005) and Fon-1/Fon-2 (Lin et al. 2010). Total fungal genomic

DNA was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA). PCR was performed in 25 µl of reaction mixture containing 10 ng of DNA, 200 µM each dNTP, 0.5 µM each primer, 0.5 U of *Taq* DNA polymerase, and 10× PCR reaction buffer. The amplification was performed in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the methods reported earlier (Lin et al. 2010; Zhang et al. 2005). PCR products were electrophoresed on agarose gel (1.5%) and visualized as described previously (Petkar et al. 2017). External and internal FON seed infestation was quantified by percentage of seeds infested by FON. To determine the effect of inoculation site on FON seed infestation, data were analyzed with the PROC GLM procedure using the Statistical Analysis System (SAS Institute, Cary, NC) and Fischer's least significant difference (LSD) test was used for mean separation at P=0.05.

To further confirm the identity of the isolates, 11 representative isolates (one from each inoculation in the greenhouse and field experiments) were tested for pathogenicity on watermelon seedlings under greenhouse conditions. The isolates were grown and microconidial suspensions were prepared as described above. Watermelon seedlings (cv. Sugar Baby) were grown in 9-cm diameter pots containing sand:peat:vermiculite mixture (4:1:1, v:v:v). At the first true leaf stage, seedlings were inoculated by applying 5 ml of conidial suspension (10^6 spores/ml) to the base of each plant. Nine plants for each isolate, and 9 plants treated with SDW were used as controls. A randomized complete block design was used with 3 replicates. The plants were incubated in a greenhouse (28/20ºC day/night), and Fusarium wilt incidence was recorded three weeks after inoculation. Diseased plants were sampled for identification of the causal agent by isolation on PPA and PCR analysis using primer sets Fn-1/Fn-2 and Fon-1/Fon-2 as described above. The experiment was conducted twice under similar conditions.

Assessment of FON seed infestation by real-time PCR assay. Watermelon seeds from the three inoculation sites (60 seeds/replicate/inoculation site) were either surface disinfested with 0.6% NaOCl or non-disinfested and crushed in a coffee grinder (Hamilton Beach Inc., Southern Pines, NC). Crushed seeds were transferred to 10 microcentrifuge tubes (1 ml powder/tube), and the grinder was rinsed with SDW for three times after each sample processing. Crushed seed in each tube was weighted, and total genomic DNA was extracted using DNeasy Plant Mini kit. DNA of crushed seeds was assayed by real-time PCR with primers Fn-1 and Fn-2. For real-time PCR, 5 µl DNA was amplified in a 25 µl volume of PCR master mix containing 25 µM each primer and 12.5 µl of Sso Fast EvaGreen Supermix (Bio-Rad Laboratories). Each DNA sample (from 1 ml of crushed seeds in one tube) was amplified in three repeated PCR reactions. Amplification was performed in a Cepheid SmartCycler (Cepheid, Sunnyvale, CA) using the protocol by Zhang et al. (2005).

To quantify FON DNA from the seeds, genomic DNA of isolate FON R1 was extracted as described above. A standard curve was established by plotting the log of known concentrations of DNA of FON R1 from 10 ng to 100 fg, with 10-fold dilutions, against the cycle threshold (Ct) values. Ct values resulting from DNA samples of the watermelon seeds were plotted onto this curve, and the concentrations of FON DNA and amount of FON DNA per gram of seeds were calculated. Samples with Ct values larger than 35 were considered negative for FON. Quantities of FON DNA from seeds generated from the three inoculations were analyzed using SAS as described above.

RESULTS

FON seed infestation detection by plating assay. All isolates from seeds identified to be FON by PCR using primers Fn-1/Fn-2 gave positive results when analyzed by PCR using primers Fon-1/Fon-2. In the greenhouse study in 2014, external infestation by FON was detected in 0.6% of the seeds for both pericarp and pistil inoculations, and internal seed infestation was not detected (Fig. 3-2A). For peduncle inoculation, 0.6% of the seeds were positive for internal infestation by FON, and external infestation by FON was not detected. In the 2015 greenhouse experiment, external seed infestation was detected for all the three inoculation sites, and the percentage of infested seeds was significantly higher (P=0.02) for pistil inoculation (18.3%) than pericarp (12.8%) and peduncle (6.1%) inoculation (Fig. 3-2B). For internal infestation, pericarp inoculation resulted in 4.5% seed infestation, which was significantly higher (P<0.01) compared to peduncle (1.1%) and pistil (1.7%) inoculation. FON was not detected from seeds, externally or internally, from watermelon treated with SDW using the three inoculation sites in both 2014 and 2015 experiments.

In the field experiment conducted in 2014, external seed infestation was only detected from pericarp inoculation (Fig. 3-3A). A higher level of internal seed infestation (P=0.02) was detected for pericarp (2.8%) as compared to peduncle (0.6%) inoculation. Neither external nor internal seed infestation was detected from pistil inoculation. In the field study in 2015, external and internal seed infestation was detected from watermelon inoculated at the three sites (Fig. 3-3B). Pericarp inoculation resulted in a significantly higher (P<0.01) percentage of external seed infestation than pistil and peduncle inoculation. For internal infestation, significantly higher infestation was detected for pistil inoculation compared to peduncle and pericarp inoculation. External or internal seed infestation by FON was not detected from watermelon treated with SDW

using the three inoculation sites in either the 2014 or 2015 field experiments. In the pathogenicity assays, 11 representative isolates from the field and greenhouse experiments caused disease on watermelon seedlings with 100% disease incidence three weeks after inoculation. The non-inoculated plants remained symptomless. FON was re-isolated from the inoculated plants but not the control plants.

FON seed infestation detection by real-time PCR assay. In the 2014 greenhouse experiment, FON seed infestation was detected from all pericarp and pistil inoculated seedlots, but not from peduncle inoculated seedlots when the seeds were surface disinfested or not disinfested. More FON DNA was detected in seeds from pericarp inoculation than seeds from pistil inoculation when seeds were not surface disinfested, and there was no significant difference in FON DNA detected in seeds from pericarp and pistil inoculation when seeds were surface disinfested (Fig. 3-4A). In 2015 greenhouse study, seed infestation was detected from all seedlots using the three inoculations when seeds were surface disinfested or not disinfested. Higher amount of FON DNA was detected in seeds from pistil (P=0.03) and peduncle (P=0.02) inoculation compared to seeds from pericarp inoculation when seeds were not disinfested (Fig. 3-4B). For surface disinfested seeds, significantly higher amount of FON DNA was detected in seeds from pericarp (P=0.01) and pistil (P<0.01) inoculation than seeds from peduncle inoculation. FON infestation was not detected by real-time PCR assay from seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

In the field study conducted in 2014, seed infestation by FON was detected in all seedlots from the three inoculated sites. There was no significant difference (P=0.4) in amount of FON DNA detected in seeds from the three inoculated sites regardless of whether seeds were surface

disinfested or not disinfested (Fig. 3-5A). In the 2015 experiment, FON was detected from seeds generated through all three inoculations, when seeds were surface disinfested or not disinfested. Amount of FON DNA was significantly higher in seeds from pericarp inoculation compared to pistil and peduncle inoculation when seeds were not disinfested (Fig. 3-5B). Similarly, higher amount of FON DNA was detected in seeds from pericarp inoculation compared to pistil and peduncle inoculation when seeds were surface disinfested. FON infested seeds were not detected from watermelon treated with SDW with the three inoculations.

DISCUSSION

Infestation of watermelon seeds by FON has been reported in a number of studies (Boughalleb and El-Mahjoub 2006; Fulton and Winston 1915; Martyn 1987; Porter 1928); however, none of the studies investigated potential infection courts for FON seed infestation. In the present study, inoculation of pistil, pericarp and peduncle with FON did not cause Fusarium wilt symptoms on watermelon under greenhouse and field conditions. FON was detected on watermelon seeds, internally and externally, using plating and real-time PCR assays, suggesting pistil as well as pericarp and peduncle of immature fruit can be potential infection courts for FON leading to infestation of seeds in asymptomatic watermelon fruit.

Inoculation of pericarp resulted in seed infestation in all the greenhouse and field studies, and internal seed infestation was detected in 3 of the 4 experiments. Inoculation of peduncle resulted in internal seed infestation in all the experiments. Inoculation of pistil resulted in seed infestation in 3 of the 4 experiments, and internal seed infestation was detected in 2 of the 4 experiments. Across the experiments, inoculation of peduncle, pistil and pericarp resulted in 0.6-10%, 0-18.3%, and 0.6-54.4% of infested seeds, respectively, when assayed by plating. Detection

using real-time PCR further confirmed that seeds collected from watermelon plants inoculated with FON were infested with the pathogen, regardless of the inoculation sites used. Unlike the plating assay, real-time PCR could amplify DNA of viable or non-viable FON cells, so the PCR assay could not determine if FON from the seeds were dead or alive. This could explain the discrepancy between the plating and PCR results. Limited information is available regarding potential infection courts of FON leading to infestation of watermelon seeds. In a recent review, Martyn (2014) stated that nit mutants of FON were isolated from watermelon seeds following inoculation of peduncles, though details were not reported.

Infection courts for seed infestation have been studied in other fungal pathosystems. Menzies and Jarvis (1994) reported that inoculation of peduncles with *F. oxysporum* f. sp. *radicis-lycopersici* resulted in tomato fruit rot and infested seeds. A series of studies conducted by Kuniyasu (1981) and Kuniyasu and Kishi (1977) demonstrated that the pathogen of Fusarium wilt of bottle gourd (*F. oxysporum* f. sp. *lagenariae*) could infest bottle gourd seeds by direct invasion through vascular bundles of mature fruit. *Alternaria brassicae* and *A. brassicicola* (causal agents of Alternaria black spot of cabbage and canola) can infest cabbage and canola seeds through invasion of ovary walls (Domsch 1957). Seed infestation through pistil invasion has been studied in *Ustilago* spp., causal agent of smut in cereal crops. It was reported that chlamydospores of *Ustilago* spp. of barley and wheat germinated on style surfaces and the resulting hyphae gained entry into the embryo through pollen tubes (Lang 1917). Studies indicated that conidia of *Alternaria alternata* can germinate on stigmas of pepper flowers, ingress the ovary through the style in the form of hyphae, and establish in pepper seeds (Meiri and Rilsky 1983). In other studies, infection of pearl millet by *Claviceps fusiform* occurred through the stigma (Luttrell 1977; Thakur

and Williams 1980). To our knowledge, seed infestation by invasion through pistil or pericarp has never been reported in *Fusarium* spp.

Variability in the level of FON seed infestation occurred in the field and greenhouse studies. Potential reasons for variability in seed infestation in the experiments could be due to a variety of factors. Infection of plants by pathogens is a complicated process, and minor changes in physiological status of host plants as well as biotic and abiotic environments associated with the plants may affect pathogen invasion and seed infestation. In addition, distribution of infested seeds in a seedlot may not be uniform and some seeds may have a level of inoculum below the detection threshold, which also contribute to variability in seed infestation detection when assayed by plating. Such variability has been observed with detection of infested seeds in other pathosystems. It was reported (Dutta et al. 2014) that detection of bacterial pathogens from seeds was non-uniform, and the observed variability was due to a number of factors including host susceptibility, distribution of inoculum within a seedlot, pathogen population per seed, and environmental conditions. Variation in seed infestation was also reported with other pathogens due to environmental factors (Jordan et al. 1992; Schuh 1992).

Results in our study were consistent with previous findings that FON can be present on watermelon seeds externally or internally. Boughalleb and El-Mahjoub (2006) reported that FON was isolated externally and internally from watermelon seeds. FON was isolated from the seed coat of watermelon or the interior of the seed obtained from infected watermelon fruit (Martyn 1987; Taubenhaus 1935). In a study by Michail et al. (2002), FON was isolated from the testa, embryo axis and cotyledons of watermelon seeds with the pathogen most frequently from the testa. In the present study, FON was isolated from watermelon seeds treated or not treated with NaOCl, and seed infestation was further confirmed by real-time PCR assay using FON-specific primers.

However, we did not determine in what internal tissues of the seed FON was present, which could be interesting to be investigated in future studies.

In summary, this study provides evidence that pistil, pericarp and peduncle of watermelon can be sites of FON infection leading to subsequent seed infestation in symptomless fruit. In considering the importance of seedborne inoculum in development and epidemics of the disease, protection of these infection courts by chemicals or biocontrol agents may contribute to reduction of Fusarium wilt in watermelon. Further studies can also be conducted to elucidate how inoculation of pistil, pericarp and peduncle with FON results in seed infestation, such as colonization, multiplication and migration of the pathogen in different plant tissues associated with watermelon fruit. Information gained from these studies will help develop more effective management strategies to reduce loss caused by the disease through reduced seed infestation in watermelon production.

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Fig. 3-1. Inoculation of watermelon plants with *Fusarium oxysporum* f. sp. *niveum*. A) Inoculation of pericarp; B) Inoculation of peduncle; and C) Inoculation of pistil.

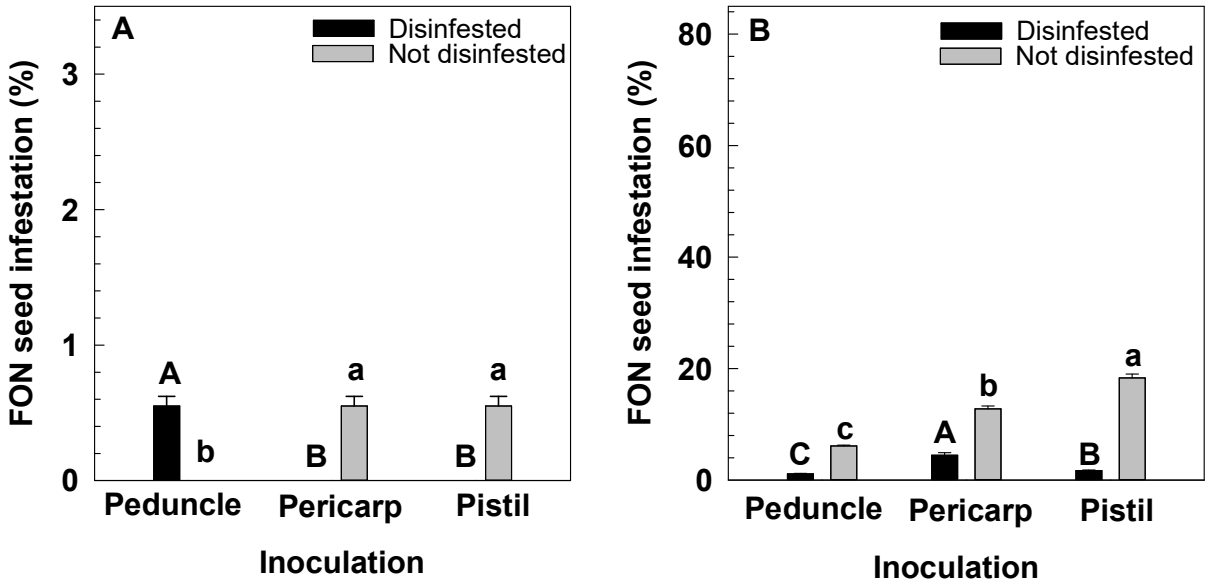

Fig. 3-2. Percentage of seed infestation by *Fusarium oxysporum* f. sp. *niveum* (FON) following peduncle, pericarp and pistil inoculation under greenhouse conditions when detected by plating assay. A) Greenhouse study in 2014; and B) Greenhouse study in 2015. "Disinfested" and "Not disinfested" indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before isolation by plating. Error bars indicate standard errors of the means. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test (P=0.05). FON was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

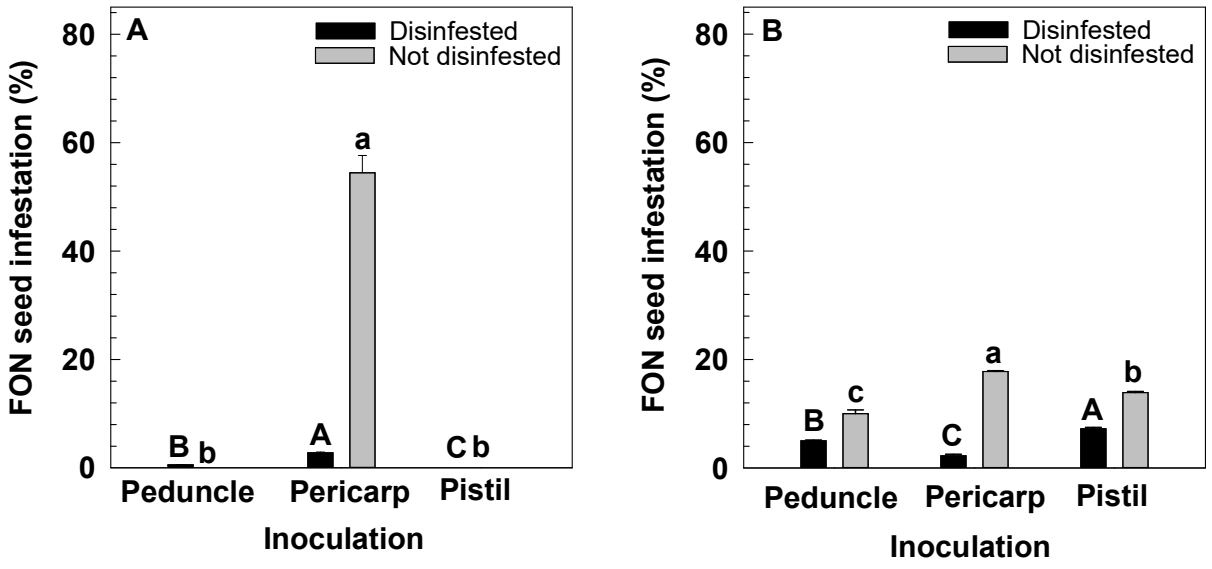

Fig. 3-3. Percentage of seed infestation by *Fusarium oxysporum* f. sp. *niveum* (FON) following peduncle, pericarp and pistil inoculation under field conditions when detected by plating assay. A) Field study in 2014; and B) Field study in 2015. "Disinfested" and "Not disinfested" indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before isolation by plating. Error bars indicate standard errors of the means. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test (P=0.05). FON was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

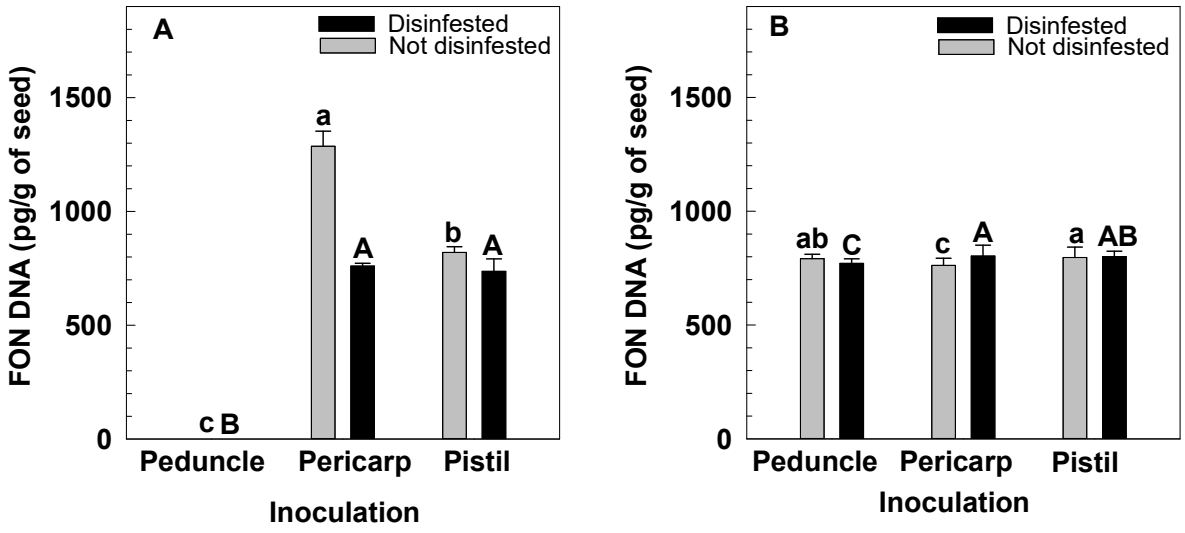

Fig. 3-4. Amount of DNA of *Fusarium oxysporum* f. sp. *niveum* (FON) detected in watermelon seeds following peduncle, pericarp and pistil inoculation under greenhouse conditions when detected by real-time PCR assay. A) Greenhouse study in 2014; and B) Greenhouse study in 2015. "Disinfested" and "Not disinfested" indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before used for real-time PCR assay. Error bars indicate standard errors of the means. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test (P=0.05). FON was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

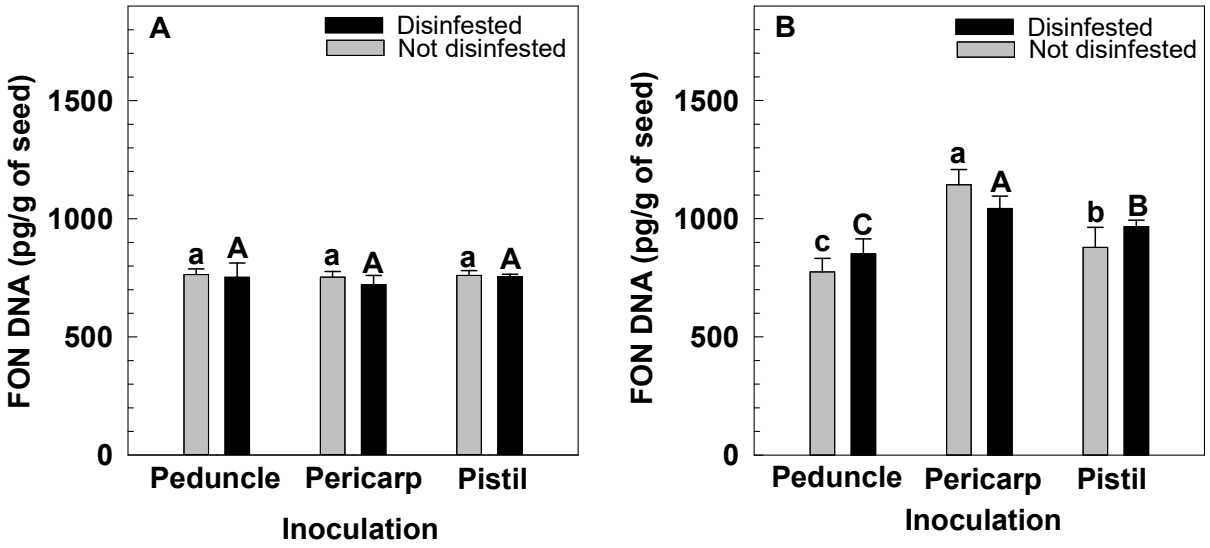

Fig. 3-5. Amount of DNA of *Fusarium oxysporum* f. sp. *niveum* (FON) in watermelon seeds detected following peduncle, pericarp and pistil inoculation under field conditions when detected by real-time PCR assay. A) Field study in 2014; and B) Field study in 2015. "Disinfested" and "Not disinfested" indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before used for real-time PCR assay. Error bars indicate standard errors of the means. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test (P=0.05). FON was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

CHAPTER 4

GENETIC AND PHENOTYPIC DIVERSITY OF *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* ISOLATES COLLECTED FROM WATERMELON IN THE SOUTHEASTERN UNITED STATES[1]

[1]Petkar et al. To be submitted to Plant Dis.

ABSTRACT

Fusarium wilt of watermelon, caused by *Fusarium oxysporum* f. sp. *niveum* (FON) occurs worldwide and is responsible for substantial yield loss in watermelon producing areas of the southeastern United States. Management of this disease largely relies on the use of integrated pest management (i.e., fungicides, resistant cultivars, crop rotation, etc.). Knowledge about race structure and genetic diversity of FON in the southeastern US is limited. To determine the genetic diversity of FON, simple sequence repeat (SSR) markers were used. Discriminant analysis of principal components (DAPC) of 99 isolates using SSR markers grouped the isolates in eight distinct clusters with two prominent clusters (clusters 1 and 8). Cluster 1 consists of a total of 14 isolates, out of which 85.7% of the isolates were collected in Florida. However, most of the isolates (92.4%) in cluster 8 were collected in Georgia. Both DAPC and population structure analysis revealed that the genetic groups are closely associated with geographical locations of pathogen collection. Three races of FON (races 0, 2 and 3) were identified in the phenotypic analysis; with race 3 identified for the first time in Georgia. Overall, 5.1%, 38.9% and 55.9% of the isolates were identified as race 0, race 2 and race 3. Majority of the isolates in cluster 1 and cluster 8 belonged to either race 2 (35.6%) or race 3 (45.8%). Additionally, no relationship between phylogenetic groups and races of the isolates was observed. Information obtained on genotypic and phenotypic diversity of FON in the southeastern US will help in development of effective disease management programs to combat Fusarium wilt.

INTRODUCTION

Watermelon (*Citrullus lanatus*) is an economically important crop belonging to the Cucurbitaceae family, with a farm gate value of $124 million in GA (Wolfe and Stubbs 2016). Watermelon production can be severely affected by a vascular disease, Fusarium wilt (Boyhan et al. 2003; Egel and Martyn 2007; Martyn and McLaughlin 1983; Robinson and Decker-Walters 1997; Zhou et al. 2010). Fusarium wilt of watermelon is caused by a soilborne fungal pathogen, *Fusarium oxysporum* f. sp. *niveum* (FON), which is host specific to watermelon. FON was first identified by E. F. Smith in South Carolina and Georgia (Smith 1894). Four races of FON (0, 1, 2 and 3) have been identified based on their aggressiveness to overcome specific resistance in a set of differential cultivars; Sugar Baby, Charleston Gray, Calhoun Gray and PI-296341-FR (Bruton and Damicone 1999; Cirulli 1972; Martyn 1996; Netzer 1976; Zhou and Everts 2001).

FON race 0 was first reported in Florida in 1963, which is not of high economic importance as most of the commercial watermelon cultivars possess the resistant *Fo-1* gene to the pathogen (Martyn 2014). FON race 1 is widespread throughout the watermelon producing areas in the US and many diploid (seeded) and a few newly released triploid (seedless) varieties possess resistance towards race 0 and 1 (Egel and Martyn 2007). FON race 2 was first reported from Israel (1976) and later reported in the US (1981) (Martyn 2014). It is aggressive on both seeded as well as seedless watermelon cultivars and a high level of race 2 resistance is not available in commercial watermelon varieties making it an economically important issue for the watermelon growers in the US. Contrastingly, the watermelon pollinizer varieties developed by Syngenta Seeds, Inc., Super Pollinizer 5 and 6, have shown some promise as race 2 wilt resistance differentials. The wilt resistance in these pollinizers was derived from PI-296341-FR (Egel and Martyn 2013). Race 3 of FON was first identified in Maryland and later in Florida

and is more aggressive than the other three races (0, 1 and 2) (Zhou et al. 2010; Amardasa et al., 2018).

Management of Fusarium wilt is challenging both due to long term survival of chlamydospores in soil and evolution of new races (Martyn 1996). Current management options for this disease include the use of seed treatments, crop rotation, soil fumigation, and chemical management, but in most cases, they are inadequate to manage FON epidemics (Martyn and Vakalounakis 2012). In terms of chemical management, only prothioconazole (Proline, Bayer Crop Science) is registered for use against this pathogen (Petkar et al. 2017). Limited benefits can be observed in fields with low levels of infestation; however, in heavily infested fields, current chemical management practices are not effective. Phasing out of methyl-bromide has left even fewer options for managing this disease (Wechter et al. 2012). Because of the destructive nature of this disease, watermelon growers in the southeastern US often prefer resistant cultivars as the primary management option for Fusarium wilt (Bruton 1998; Zhou and Everts, 2004). However, resistant commercial triploid cultivars are available only against race 0 and 1; and widespread distribution of FON race 2 renders this strategy ineffective (Egel and Martyn 2007; Martyn and Netzer 1991).

It has long been known that FON isolates from watermelon are highly diversified with different races, but little is known about the genetic diversity of FON and the potential relationship between genetic diversity and other traits such as races or geographical locations. Furthermore, no recent studies have been carried out to examine the genetic diversity of FON in the US. In addition, phenotypic diversity of FON populations in the southeastern US has not been studied and hence, this knowledge is critical in devising effective management strategies to combat Fusarium wilt of watermelon. Thus, the objectives of this study were to examine the genetic and phenotypic (race)

diversity of FON isolates collected from watermelon in the southeastern US. We hypothesize to find low genetic and phenotypic diversity among FON isolates. We also hypothesize to find a relationship among races and isolates.

MATERIALS AND METHODS

FON isolation. FON isolates were collected from different watermelon producing counties in GA and FL in 2012 and 2013 (Table 4-1). Isolates from Tift County, GA were collected in 2012 and isolates from Berrien County, GA; Cook County, GA and FL were collected in 2013. Fungal individual from a single Fusarium wilt infected plant was defined as an isolate. Isolates from same field location were assigned to the same population. For example, all the isolates from Berrien County, GA were assigned as population 1; all the isolates from Cook County, GA were assigned as population 2. FON was isolated from symptomatic watermelon plants which were further identified morphologically (Leslie and Summerell 2006) and using FON-specific primers Fn-1/Fn-2 (Zhang et al. 2005) and Fon-1/Fon-2 (Lin et al. 2010). Briefly, total fungal genomic DNA was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA) and 10 ng of DNA was amplified in a 25 µl of reaction mixture containing 200 µM each dNTP, 0.5 µM each primer, 0.5 U of *Taq* DNA polymerase, and 10× PCR reaction buffer. The amplification was performed in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the protocol described by Lin et al. (2010). The thermal cycler PCR conditions included: denaturing at 94°C for 90 s, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 62°C for 30 s, and polymerization at 72°C for 60 s, with a final extension at 72°C for 10 min. The amplified products were electrophoresed on agarose gel (1.5%) and visualized using Gel DOC XR+ system (Bio-Rad Laboratories). Upon confirming the isolates to be FON, single spore isolates were prepared as described previously

(Leslie and Summerell 2006). These single spore FON isolates were further used in genetic and phenotypic diversity studies.

Determining genetic diversity of FON isolates. Ninety-nine single spore FON isolates were genotyped using 15 published SSR markers for *F. oxysporum* (Bogale et al. 2005; Mahfooz et al. 2015)(Table 4-2).

For DNA extraction, isolates were grown on potato dextrose agar (PDA) for 7 days at 25ºC. Fungal DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. A NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to quantify DNA concentration. To determine the best annealing temperature, a gradient polymerase chain reaction (PCR) was performed at annealing temperatures ranging from 50 to 60ºC using 15 pairs of primers for SSR markers and two isolates (F1-4, F4-3). An annealing temperature of 55ºC generated the best result and was then used for subsequent studies. PCR was performed in a 10 µl of reaction mixture containing 2 µl of 5x Clear GoTaq Flexi Buffer (Promega Corp., Madison, WI), 1 µl of 25mM $MgCl_2$, 0.8 µl of 2.5 mM dNTP, 0.5 µl of 1 µM M13 tagged forward primer, 2 µl of 1 µM reverse primer, 1.8 µl of 1 µM of M13 primer (M13-TGTAAAACGACGGCCAGT) fluorescently labeled with IRD Dye 700 CW fluorophore (Eurofins MWG Operon, Huntsville, AL), 0.04 µl of GoTaq Flexi DNA polymerase, 0.86 µl of PCR grade water (Teknova, Hollister, CA) and 1 µl of FON DNA (2.5 ng/µl). The amplification was performed in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA) using a protocol as described by Li et al. (2017). Briefly, the PCR conditions included: initial denaturing at 94°C for 3 min, followed by 39 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 1 min, and polymerization at 72°C for 70 s, with a final extension step at 72°C for 10 min. The PCR product

(2 µl) was combined with 5 µl of Blue Stop (LI-COR Biosciences, Lincoln, NE) and 0.35 µl of this mixture was loaded on an acrylamide gel (6.5%) using a LI-COR Biosciences 4300 DNA analyzer. Alleles were scored based on allele size.

Population genetic analyses. For manual data analysis, each FON isolate was visually scored based on allele/fragment size. Data was then used for further analyses.

Identification of clonal genotypes. FON is an asexual fungus with no sexual stages yet identified. Clones are genetically identical individuals that arise due to asexual reproduction. A multilocus genotype (MLG) is an exclusive combination of alleles across two or more loci. For fungal organisms that often reproduce clonally, MLG is valuable in identifying both the mode and spread of an organism. However, based on the sample size, the number of markers used, the number of alleles for each marker, and the allele frequencies, the same MLG may arise by recombination or outcrossing, and not necessarily be indicative of a clone. To confirm that the repeated genotypes are clones and not arising from an outcrossing event, we used MLGsim v2.0 (Stenberg et al. 2003) to calculate the probability (ρ_{sex}) that each MLG occurring more than once is not arising from an outcrossing event (Parks and Werth 1993). A significantly lower ρ_{sex} value indicates that the repeated MLG likely arose from clonal reproduction. The P values for ρ_{sex} were estimated by 1000 random permutations of the data.

Analyses of population structure. To determine the genetic structure among FON populations, Genalex v.6.5 (Peakall and Smouse 2012) was used to estimate the number of alleles and unbiased gene diversity (*h*) for each microsatellite locus. Likewise, analysis of molecular variance (AMOVA) was used to calculate genetic differentiation (Φ_{PT}). To estimate major patterns of variation within and among populations from different geographical locations, a principal

coordinate analysis (PCoA) was conducted with Genalex v.6.5. AMOVA was also used to calculate variation within and among different FON races. To determine if two individuals taken at random have unique genotypes, genotypic diversity ($\hat{G}$) was estimated in R package adegenet v 1.4-1 (Jombart 2008; Jombart and Ahmed 2011). In order to understand FON population structure without a priori assignment of individuals to populations, the number of clusters among FON sampled from different locations in southeastern US and the assignment of FON isolates to each cluster; discriminant analysis of principal components (DAPC) was used by applying R package adegenet v 1.4-1 (Jombart 2008; Jombart and Ahmed 2011). DAPC clusters were determined by K-means clustering of principal components, where K was inferred as the number of cluster where the Bayesian information criteria (BIC) increases or decreases by a negligible amount.

Phenotypic diversity (race typing). Fifty-nine single spore FON isolates were selected for race typing. The set of differential cultivars used to determine FON races under greenhouse conditions included: Sugar Baby (no resistance to any race), Charleston Gray (resistant to race 0), Calhoun Gray (resistant to race 1), and PI-296341-FR (resistant to race 2) (Zhou et al. 2010). Reference FON isolates for race 0, race 1 (race 0 and race 1 received from Hunt Sanders, University of Georgia, Tifton, GA) and race 2 (received from Dr. Anthony Keinath, Clemson University, Charleston, SC) were included in each experiment as controls.

To prepare FON inoculum, isolates were grown on PDA at 25°C for 7 days. Five mycelial plugs (5 mm in diameter) from the edge of a growing colony were transferred aseptically to a 250-ml flask containing 200 ml quarter strength potato dextrose broth (PDB). The liquid culture was incubated for 14 days at 23±1°C on an orbital shaker (G10 Gyrotory Shaker, New Brunswick Scientific Company, NJ) at 150 rpm. Colonized liquid PDB was filtered through three layers of

sterile cheesecloth and the concentration of spore suspension (>95% microconidia) was adjusted to 1×10^6 spores/ml by adding an adequate amount of sterile distilled water (SDW).

For greenhouse evaluation, watermelon seedlings of four differentials were grown in pots (9-cm diameter) containing a mixture of sand:peat:vermiculite (4:1:1, v: v: v). Seedlings were inoculated at first true leaf stage by pipetting 5 ml conidial suspension (1×10^6 spores/ml) near the base of each watermelon seedling. Ten plants/isolate/cultivar in separate pots were inoculated and the same number of plants treated with SDW served as a negative control. After inoculation, seedlings were maintained at 28ºC day and 20ºC evening with 70-80% relative humidity in the greenhouse. The percentage of seedlings showing characteristic symptoms of Fusarium wilt such as yellowing, stunting, or wilting were evaluated weekly and disease incidence was recorded after 4 weeks. Additionally, plants were also rated for disease severity at the end of 4 weeks using a scale from 0 to 9 with a score of 0 representing asymptomatic plants, 3 for plants with cotyledon lesion, 5 for plants showing slight wilting and stunting, 7 for plants with severe wilting and stunting and 9 for dead plants. Plants rated as 0 were classified as resistant, 1 or 3 as intermediate resistant and 5, 7 or 9 as susceptible (Williams and Palmer 1996). For assessing disease reactions on each differential and isolate treatment, differential with ≥33% wilt incidence was considered as susceptible whereas differential with <33% wilt incidence was considered as resistant (Martyn and Bruton 1989). A total of two independent greenhouse experiments were conducted.

In the greenhouse studies, three symptomatic seedlings/isolate/differential were used to confirm if the symptoms observed were due to FON infection using the method previously described (Petkar et al. 2017). Briefly, seedlings were surface sterilized and placed on peptone PCNB agar (PPA) plates (Leslie and Summerell 2006). The plates were incubated for two weeks at 25°C and checked periodically for putative FON colonies. Later, colonies were purified by sub-

culturing hyphal tips on PPA plates and then transferring onto potato dextrose agar. The cultures were identified to be FON based on morphological characteristics (Leslie and Summerell 2006) and PCR analysis using FON-specific primers Fn-1/Fn-2 (Zhang et al. 2005) and Fon-1/Fon-2 (Lin et al. 2010) as described above.

RESULTS

Genetic diversity of FON isolates. For the population genetics analyses of FON out the 15 primer pairs used, five of the microsatellite loci were identified as monomorphic (MB9, MB10, MB13, MB18, and Fo310) and one locus (MB5) did not amplify majority of the isolates. Hence, FON population genetic analyses were based on 9 microsatellite loci. The 9 primer pairs used in this study created polymorphic profiles for the 99 isolates. The number of alleles for the 9 loci varied from 3 to 16. For the 99 FON isolates, 14 MLG were identified. It was observed that around 75% of the genotypes were repeated and hence the genotypic diversity for the 99 isolates was very low ($\hat{G}$=0.423). Additionally, for each population, the genotypic diversity was very low ($\hat{G}$ <0.423) except for FL population ($\hat{G}$ =0.600) (Table 4-3). Rarefied allelic richness for all the populations ranged from 2.90 to 4.85 indicating that most loci for populations showed over 4 alleles when standardized for sample size. Contrastingly, allelic evenness, which is the measure of the distribution of genotype abundances ranged from 0.391 to 0.706. All the GA populations had allelic evenness values of ≤0.50 and FL population had allelic evenness value of 0.706, indicating that all the MLG's observed in FL population are closer to equal abundance than those in GA population (Table 4-3). Higher genotypic diversity value ($\hat{G}$ =0.600) with a higher allelic evenness value (0.706) for FL population suggests that FL population is more diverse than GA population.

Overall, for all the four populations compared, 6 repeated MLG's (g_2) were found across all the population from southeastern US (Table 4-4). The ρ_{sex} value associated with all the repeated MLG's was highly significant indicating that these repeated MLG's did not likely arise from recombination, and were the result of clonal reproduction (Table 4-4). Moreover, within each sampled population there were many clonal MLG's containing multiple members. For instance, GA1 contained MLG's with 36 members while GA2, GA3 and FL contained around 17 members, respectively. Also, MLG (11) was shared among all the four populations (Table 4-3, labeled as x_{11}) and MLG (14) was shared among 2 populations (Table 4-4, labeled as y_{14}).

Population structure of FON. Pairwise population statistics (Φ_{PT}) was performed on all the four populations. Significant differences were observed between FL population and GA1, GA2, GA3 population ($P \leq 0.002$) (Table 4-5), indicating geographical differences between populations. Among GA populations, significant differences were observed only among GA3 and GA1 populations ($P \leq 0.01$) (Table 4-5). The PCoA analysis revealed that GA isolates belonging to three distinct populations were clustered together and less diverse from each other. The Florida population on other hand was more spread out and diverse. In addition, it was also observed that the first and second principal coordinates explained 80.0% and 5.6% of the variance, respectively (Fig. 4-1).

Φ_{PT} analysis was performed on the three different FON races (0, 2 and 3). Significant partitioning of the genetic variation was not observed between any of the FON races (Table 4-6). Moreover, molecular variance among races accounted for 0% of the variation, while 100% of the variation was observed within the races indicating high diversity within each race (Table 4-7).

DAPC. DAPC supported K=8 clusters. None of the clusters in DAPC overlapped with each other (Fig. 4-2). Clusters 2 through 7 each represented a single FON isolate whereas cluster 1 and 8

represented 14 and 79 FON isolates, respectively (Fig. 4-3). Additionally, we identified some clusters which represented isolates that belong to a dominant or prevalent clone, clones with genotypes containing similar alleles and unique genotypes with dominant clone. It was also seen that, some clusters were distributed across multiple populations. For instance, cluster 1 contained one of the dominant clones (14 FON isolates), detected mostly in the FL population. On the other hand, most of the GA isolates belonged to cluster 8 (represented by navy blue) containing 79 members from all the three GA locations (Fig. 4-3). The other clusters represented different MLG's containing very few members.

Phenotypic diversity. Fifty-nine FON isolates from the eight genetic clusters identified above (K=8) (Fig. 4-3) were selected for race determination. Overall, 5.1% (3/59), 38.9 (23/59), and 55.9% (33/59) of the isolates were identified as race 0, race 2 and race 3, respectively. The majority of the isolates used for race identification were from cluster 1 (14/59) and cluster 8 (39/59). Among cluster 1, majority of the isolates were collected from FL (85.7%) (12/14) and likewise a majority of cluster 8 isolates were collected from GA (36/39). Among the isolates in cluster 1, 7.1% (1/14), 42.8% (6/14) and 50% (7/14) were identified as race 0, race 2 and race 3, respectively (Table 4-1). Among the isolates in cluster 8, 2.6% (1/39), 38.5% (15/39) and 58.9% (23/39) were identified as race 0, race 2 and race 3, respectively. None of the isolates in clusters 1 or 8 were identified as race 1.

With respect to geographical location, GA isolates accounted for 14.3% (2/14) of cluster 1, out of which 7.1% (1/14) each were identified as race 0 and race 3, respectively. In contrast, FL isolates from the same cluster accounted for 85.7% (12/14), and 42.8% (6/14) of the isolates were identified as race 2 and race 3, respectively. Cluster 8 includes 66.1% (39/59) of the race identified isolates, out of which 61% (36/59) were collected in GA, where 2.6% (1/39), 38.5% (15/39) and

51.3% (20/39) identified as race 0, 2 and 3, respectively. Florida isolates in the same cluster accounted for only 7.7% (3/39) of the race identified isolates, all of which belonged to race 3 only. Other clusters (2, 3, 4, 5, 6 and 7) included only one isolate each either identified as race 0, race 2 or race 3 (Table 4-1). For instance, cluster 3, 4, 5 and 6 contained one isolate each from GA identified as race 0, race 3, and race 2 respectively. However, cluster 2 and cluster 7 contained one isolate each from FL identified as race 3, respectively (Table 4-1). Disease severity of watermelon differentials inoculated with FON isolates belonging to either race 2 or race 3 ranged from 5 to 9, respectively. Seedlings treated with SDW remained asymptomatic. One hundred percent of the seedlings assayed for putative FON symptoms in the greenhouse studies were confirmed as FON by specific PCR assays as described above.

DISCUSSION

The population genetic structure of FON in southeastern US were investigated using nine published microsatellite markers (Bogale et al. 2005; Mahfooz et al. 2015). In the current study, we identified eight genetic clusters of FON isolates collected from watermelon fields in GA and FL. The DAPC analysis identified eight distinct FON clusters with the majority of isolates belonging to two of the clusters (1 and 8) (Fig. 4-3). Cluster 1 consists of isolates that belonged to FL (85.7%) whereas most of the isolates (92.4%) in cluster 8 belonged to GA. The observations may suggest that the populations from GA and FL are different. However, it is not clear as to why couple of isolates from GA were clustered in FL group and vice versa. It is possible that there could be potential movement of FON between two states through infested seeds or transplants. Hence, further population based studies of FON isolates from seed and/or transplant may shed some light on this observation.

Among the FON populations collected throughout the southeastern US, we observed clones that occurred at a high frequency and were widespread at the regional level. Overall six clones were distributed across the four populations obtained from GA and FL. Additionally, most of the isolates were associated with only few MLG's (11 and 14). One clone was distributed across all the four populations (GA1-GA3 and FL) whereas another clone was only distributed across two populations (GA3 and FL). Among the four FON populations, clones represented 87 of the FON isolates (Table 4-4). Similar observations of clonal populations with limited genotypic diversity were reported in other *Fusarium oxysporum* pathosystems. For example, in Fusarium wilt of banana [causal organism: *Fusarium oxysporum* f. sp. *cubense* (FOC)], fungal populations from different geographical locations were clonal. Ten clonal lineages were identified, and among them two largest lineages had pantropical distribution (Koenig et al. 1997). Similarly, in *Fusarium oxysporum* f. sp. *albedinis* (causal agent of Fusarium wilt of date palm) isolates collected from different geographical locations in Morocco were found to be less diverse and clonal (Tantaoui et al. 1996)

The diversity among GA populations was lower (allelic evenness≤0.50); however, FL population had a higher diversity than GA populations (allelic evenness=0.706) (Table 4-3). The reason behind prevalence and extensive presence of clones is unclear. However, one of the possible reasons could be related to the presence of asexual propagules like chlamydospores, which are reported to survive in the soil for up to 16 years (Martyn 1996). These propagules occupy small discrete territories and can be spatially rearranged when the soil is cultivated or irrigated. The propagules may propagate clonal lineages for a brief period of time or for many seasons either locally or over a wide geographical area (Anderson and Kohn 1995). These asexually produced chlamydospores can disperse clonal lineages through movement of infested seeds, transplants,

irrigation water and agricultural machinery. Prevalent and widespread clones were also reported in case of *Phytophthora infestans* (Goodwin et al. 1994) and *Sclerotinia sclerotiorum* (Anderson and Kohn 1995) and *Stagonosporopsis* sp. (Brewer et al. 2015)

Genetic structure of FON population was identified at the regional level based on both Φ_{PT} statistics and analysis of variance of principal components. Significant differences were observed between GA and FL populations (Table 4-5). Also, among GA population differences were observed only between GA3 and GA1 populations (Table 4-5). This indicates that gene flow is occurring among fields from either of the states rather than between two states. Additionally, population structure study using DAPC revealed that populations are geographically structured. The majority of isolates in cluster 8 belonged to GA (92.4%) whereas majority of isolates in cluster 1 belonged to FL (85.7%).

Phenotypic diversity of representative isolates from eight clusters revealed that race 2 and race 3 are the predominant races in both states. More than 90% of the FON isolates from the 8 clusters belonged to race 2 or race 3. Less than 5% of the isolates belonged to race 0. This is the second instance where FON race 3 was identified in the southeastern US. Recently, FON race 3 was identified in three different counties of Florida (Madison, Levy, and Lee) (Amardasa et al 2018). Prior to FL, race 3 was also identified in Maryland (Zhou et al. 2010). In GA, FON race 2 was identified by Bruton et al. in 2008; however, race 3 had never been reported prior to this study. The detection of race 3 in GA could possibly be due to introduction and establishment of pathogen through contaminated seeds or transplants harboring FON race 3. Perhaps, race typing of FON isolates from contaminated seed source may provide some light on this hypothesis. Alternatively, it can also be hypothesized that extensive use of watermelon with race 2 resistant pollinizers in infested soil could pose high selection pressure for the evolution of race 3 isolates.

Phenotypic study revealed the presence of lower percentages of race 0 isolates. In addition, none of the isolates was identified as race 1. It is plausible that race 0 and 1 may be an ancestral race and that races 2 and 3 may be of more recent origin (Martyn 2014). Additionally, when molecular variance among different races was calculated, 100% of the variation was observed within each race rather than among different races. It is possible that isolates from different races have the same genotype and are an aggressive form of another as observed by Nui et al (2016). The researchers observed that an effector gene, *FONSIX6* is present in race 0 and race 1 but absent in race 2. Further they speculated that race 0 can be a less aggressive form of race 1. In *Fusarium oxysporum* f. sp. *lycopersici*, *SIX* genes (avirulence genes) are located in chromosome 14 (supernumerary chromosome or pathogenicity chromosome) and are responsible for virulence/aggressiveness in different races of the pathogen. The researchers claim that evolution of new races can be attributed to the horizontal transfer of this supernumerary chromosome. Although, *FONSIX6* was determined in FON race 0 and race 1, its location in the chromosome is still undetermined. It can be speculated if *FONSIX6* is located in a supernumerary chromosome, FON race 1 could have horizontally acquired this avirulence gene during the evolutionary process. Effector genes specific in FON race 2 and race 3 if any, are yet to be discovered. Further, it will be interesting to investigate the effector repertoire and their locations in chromosome. Hopefully, these future investigations may shed some light in the evolution of FON race 2 and race 3.

Comparable results were observed in a previous study by Bruton et al. (2008), where lower percentage of isolates in GA were identified as race 2, e.g., out of eight isolates tested two belonged to race 2. It may be possible that race 0 is serving as the base population for development of other FON races due to widespread use of race 0 or 1 resistant watermelon cultivars. FON race 0 was first reported in Florida in 1963 (Martyn 2014) whereas FON race 2 was not reported until 1985

in Texas (Martyn 1985, 1987). It is proposed that with the advancement in evolution, the populations of race 0 will continue to decline whereas those of race 2 and race 3 will continue to rise. This can be illustrated from distribution frequency of different races in Georgia (Table 4-1) where out of the 59 isolates tested only 5% of the isolates belonged to race 0, and higher percentages of the isolates belonged to race 2 (38.9%) and race 3 (55.9%). Related results were also observed by Zhou and Everts (2007) who reported more isolates of FON race 2 as compared to race 1 or 0 from a field in Maryland.

In our study, a strong correlation of FON genotypes with geographical locations as compared to pathological races was observed. Previous studies had similar observations where FON races did not correlate with the genetic diversity. Kim et al. (1992) studied the genetic variation of 50 FON isolates encompassing three known races (0, 1 and 2) collected from different geographical locations using restriction fragment length polymorphisms (RFLP) and observed that there was no relationship between RFLP haplotypes, races or geographical locations. Additionally, they also observed that all the races were closely related and shared common sequences of chromosomal DNA (Kim et al. 1993).

In other *Fusarium oxysporum* pathosystem, similar observations were made. Castano et al. (2014) studied the genetic and phenotypic diversity of *Fusarium oxysporum* f. sp. *dianthi* in southern Spain using random amplified polymorphic DNA (RAPD)-PCR, DNA sequence analysis of the *TEF1-α* gene, and race-specific molecular markers. It was found that high genetic homogeneity within the races in the investigated population was observed, which was consistent with earlier reported studies. Additionally, Alves-Santos et al. (1999) studied 128 *F. oxysporum* f. sp. *phaseoli* strains in Spain using intergenic spacer (IGS) region polymorphism of ribosomal DNA, electrophoretic karyotype patterns, vegetative

compatibility and pathogenicity analyses. No correlation between pathogenicity and VCG, IGS restriction fragment length polymorphism, or electrophoretic karyotype was observed. Contrastingly, in *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) (causal agent of Fusarium wilt of cotton) random amplified polymorphic DNA (RAPD) markers clustered the 46 FOV isolates into three groups based on geographical locations and pathological reactions. Three distinct virulence groups were identified that corresponded with three races 3, 4 and A (Assigbetse et al. 1994).

In summary, this study provides new insights into the current status of genetic and phenotypic diversity of FON in the southeastern US. A new race of FON, race 3, was identified for the first time in GA and the SSR analysis proved to be a useful tool in determining the genetic diversity of FON isolates, which divided the FON isolates from GA and FL into eight distinct clusters with two prominent clusters (cluster 1 from FL and cluster 8 from GA). Determining races and genetic diversity of FON prevalent in watermelon production in this region will contribute to a more comprehensive understanding of the nature of the pathogen and help develop more effective disease management programs.

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Table 4-1. Location of isolation, phylogenetic groups, and races of *Fusarium oxysporum* f. sp. *niveum* isolates from watermelon in Georgia and Florida.

Isolate	Location	Genetic cluster[1]	Disease incidence (%)[2]				Race Identified[3]
			Sugar Baby	Charleston Gray	Calhoun Gray	PI-296341-FR	
F1-2[a]	Berrien, GA	8	100	100	100	90	3
F1-4[a]	Berrien, GA	8	100	100	70	0	2
F1-5a[a]	Berrien, GA	8	100	100	100	70	3
F1-5b[a]	Berrien, GA	8	100	100	100	70	3
F1-7[a]	Berrien, GA	8	100	100	70	0	2
F1-8[a]	Berrien, GA	8	100	100	100	80	3
F1-9[a]	Berrien, GA	8	100	100	100	80	3
F1-12[a]	Berrien, GA	8	100	100	90	0	2
F1-13[a]	Berrien, GA	8	100	90	90	0	2
F1-14[a]	Berrien, GA	8	100	100	90	0	2
F1-15[a]	Berrien, GA	8	100	100	90	0	2
F1-16[a]	Berrien, GA	8	95	5	0	0	0
F1-17[a]	Berrien, GA	8	100	100	70	0	2
F1-18[a]	Berrien, GA	8	100	100	100	90	3
F1-19[a]	Berrien, GA	8	100	100	100	80	3
F1-20[a]	Berrien, GA	8	100	100	80	0	2
F2-3[b]	Berrien, GA	8	100	100	90	0	2
F2-6[b]	Berrien, GA	8	100	100	80	0	2
F2-10[b]	Berrien, GA	8	100	100	90	0	2

F2-28 [b]	Berrien, GA	8	100	100	100	70	3
F2-34 [b]	Berrien, GA	8	95	100	90	85	3
F2-39 [b]	Berrien, GA	8	100	100	100	70	3
F3-1 [c]	Cook, GA	8	100	100	80	0	2
F3-2 [c]	Cook, GA	8	100	100	100	70	3
F4-3 [d]	Cook, GA	8	100	100	100	90	3
F3-7 [c]	Cook, GA	8	100	100	100	80	3
F3-20 [c]	Cook, GA	8	100	100	100	80	3
F3-22 [c]	Cook, GA	8	100	100	100	90	3
F3-23 [c]	Cook, GA	8	100	100	100	80	3
F3-24 [c]	Cook, GA	8	100	100	100	80	3
F3-26 [c]	Cook, GA	8	100	100	100	90	3
F3-29 [c]	Cook, GA	8	95	100	80	85	3
F3-34 [c]	Cook, GA	8	100	100	100	80	3
122e [e]	Tift, GA	8	100	100	90	0	2
122g [e]	Tift, GA	8	90	85	90	10	2
12105b [e]	Tift, GA	8	85	85	90	0	2
13-101	FL	8	100	100	100	90	3
13-119	FL	8	100	100	90	95	3
13-134	FL	8	100	100	90	75	3
13-124	FL	7	95	85	85	90	3
12065 [e]	Tift, GA	6	90	95	95	10	2
122a [e]	Tift, GA	5	90	85	80	0	2
12126b [e]	Tift, GA	4	80	80	80	80	3

F2-18 [b]	Berrien, GA	3	100	0	0	0	0
13-115	FL	2	90	80	100	70	3
123a [e]	Tift, GA	1	90	0	0	0	0
12105a [e]	Tift, GA	1	90	90	90	70	3
13-102	FL	1	85	80	95	85	3
13-111	FL	1	85	90	85	80	3
13-112	FL	1	100	100	80	0	2
13-113	FL	1	90	85	90	90	3
13-114	FL	1	100	90	90	90	3
13-117	FL	1	100	100	80	0	2
13-118	FL	1	100	100	90	0	2
13-132	FL	1	90	90	80	85	3
13-133	FL	1	90	80	80	10	2
13-135	FL	1	90	90	100	80	3
13-136	FL	1	100	100	70	0	2
13-159	FL	1	95	100	85	5	2
*Race 0	GA		95	0	0	0	0
*Race1	GA		90	85	0	0	1
*Race 2	SC		95	90	90	0	2

[1]Phylogenetic group determined by simple sequence repeat markers.

[2]Mean disease incidence was recorded as the number of seedlings showing Fusarium wilt symptoms to the total number of seedlings planted (n =10 seedlings/isolate/cultivar/experiment).

[3]Seedlings with ≥33% wilt incidence was considered as susceptible whereas seedlings with < 33% wilt incidence was considered as resistant (Martyn and Bruton, 1989).

[a-c]Isolates with same letters indicate isolates collected from same field.

Berrien, GA; Cook, GA and FL isolates were collected in 2013; Tift, GA isolates were collected in 2012.

[*]Reference isolates used for race 0, race 1 and race 2.

Table 4-2. Primer sequences, SSR motifs and allele size used in the simple sequence repeat (SSR) analysis.

Primer	Primer sequence (5'–3')	T_a*	SSR motif	Allele size
MB2	F: TGCTGTGTATGGATGGATGG R: CATGGTCGATAGCTTGTCTCAG	57	$(GT)_{11}(GA)_6$	234, 237,238, 240, 242, 246, 248, 250, 252, 254,260, 264, 271, 275
MB5	F: ACTTGGAGGAAATGGGCTTC R: GGATGGCGTTTAATAAATCTGG	54	$(TG)_9$	252, 254, 256, 267, 274, 344
MB9	F: TGGCTGGGATACTGTGTAATTG R: TTAGCTTCAGAGCCCTTTGG	51	$(CA)_9$	126, 105, 130, 141, 234, 237, 240, 254
MB10	F: TATCGAGTCCGGCTTCCAGAAC R: TTGCAATTACCTCCGATACCAC	48	$(AAC)_6$	206, 208
MB11	F: GTGGACGAACACCTGCATC R: AGATCCTCCACCTCCACCTC	68	$(GGC)_7$	172, 175, 177, 180, 182, 186
MB13	F: GGAGGATGAGCTCGATGAAG R: CTAAGCCTGCTACACCCTCG	68	$(CTTGGAAGTGGTAG CGG)_{14}$	144, 264, 296, 376, 382, 345, 395, 400, 422, 476, 483, 492, 500
MB14	F: CGTCTCTGAACCACCTTCATC R: TTCCTCCGTCCATCCTGAC	57	$(CCA)_5$	183, 184,186
MB17	F: ACTGATTCACCGATCCTTGG R: GCTGGCCTGACTTGTTATCG	57	$(CA)_{21}$	299, 301, 303, 308, 312, 317, 319, 320, 321, 331, 334, 337, 339
MB18	F: GGTAGGAAATGACGAAGCTGAC R: TGAGCACTCTAGCACTCCAAAC	57	$(CAACA)_6$	284, 289, 293
Fo9	F: GGCAGAAAAGATACTGAACG R: TTGAATTGCCAACTCTTCTT	55	$(GGA)_{11}$	204
Fo120	F: GAAAGTGGATGGAAGAAAGA R: GATAGGCTGTTGTTGTGGTT	54.8	$(CAG)_7$	300
Fo310	F: CATTGCAGCAGGAATTAGAT R: CTAGGTAGGCATACGAGGGT	55.6	$(CA)_7$	318
Fo314	F: GAAAAGGAGAGACTGCAAAA R: CTCTTCTTCCTCGTGTTGAC	54.8	$(GAA)_4$ $(GAG)_9$ $(GAG)_4$	297

Fo671	F: TGTTCCCTGAGTTGGTAGTC R: CTCCCAATCAGATCCTTCT	55.0	(CAG)8	111
Fo1513	F: TGTTCCCTGAGTTGGTAGTC R: CTCCCAATCAGATCCTTCT	55.0	(GGA)9	125

* PCR annealing temperature.

Table 4-3. Estimates of genetic diversity for *Fusarium oxysporum* f. sp. *niveum* population from the southeastern United States.

Population[a]	No. of isolates (N)	No. of MLG (g)	Genotypic diversity ($\hat{G}$)[b]	Allelic richness[c]	Allelic evenness[d]
GA1	40	5	0.188	2.90	0.391
GA2	19	3	0.194	3.00	0.475
GA3	20	5	0.420	4.85	0.500
FL	20	5	0.600	4.85	0.706
Total	99	14	0.423	4.25	0.400

[a] Four populations were sampled: Berrien county, Georgia (GA1), Cook county, Georgia (GA2), Tift county, Georgia (GA3), Florida (FL).

[b] $\hat{G}$ is the genotypic diversity that represents the probability of two randomly selected individuals having unique multilocus genotypes (MLG), and was calculated using R package adegenet v 1.4-1 (Jombart 2008; Jombart and Ahmed 2011).

[c] Allelic richness measure of number of observed MLGs.

[d] Allelic evenness measure of distribution of genotype abundances.

Table 4-4. Clonal composition of *Fusarium oxysporum* f. sp. *niveum* populations from the southeastern United Sates.

Population[a]	N	g[b]	g_2[c]	Number of isolates in each repeated MLG[d]
GA1	40	5	1	36^{x11*}
GA2	19	3	1	17^{x11*}
GA3	20	5	2	15^{x11*} , 2^{y14*}
FL	20	5	2	6^{x11*} , 11^{y14*}
Total	99	14	6	74^{x11*}, 13^{y14*}

[a] Four populations were sampled: Berrien county, Georgia (GA1), Cook county, Georgia (GA2), different counties, Georgia (GA3), Florida (FL).

g[b] Number of MLG in each population.

g_2[c] Number of MLG represented by more than one member (repeated MLG).

[d] Superscripts of "x" or "y" indicate MLG that were present in more than one population, with "X" representing MLG present in three or more different populations and "y" indicating repeated MLG in two different populations.

[*] Highly significant $\rho_{sex.}$

Superscript number indicates the repeated MLG to which the isolates belong.

Table 4-5. Geographical population structure measured by Φ_{PT} between *Fusarium oxysporum* f. sp. *niveum* populations in the southeastern United States.

	Population[a]			
Population	GA1	GA2	GA3	FL
GA1	…			
GA2	0.00 ^{ns}	…		
GA3	0.122 ^{**b}	0.096 ^{ns}	…	
FL	0.651 ***	0.585 ***	0.321 ***	…

[a] Four populations were sampled: Berrien county, Georgia (GA1), Cook county, Georgia (GA2), Tift county, Georgia (GA3), Florida (FL).

[b] Pairwise population differentiation was measured by Excoffier's Φ_{PT} (Excoffier et al.1992), an analog of F_{st} analysis measured via analysis of molecular variance, using GenAlEx v.6.503 (Peakall and Smouse 2006).

Significance levels (ns=not significant, * = P≤0.05, ** = P≤0.01, *** = P≤0.002) are based on P values determined by 1000 permutations of the data.

Table 4-6. Population structure measured by Φ_{PT} between *Fusarium oxysporum* f. sp. *niveum* races in the southeastern United States.

	Race[a]		
Race	0	2	3
0	…		
2	0.129 [ns]	…	
3	0.083[ns]	0.000 [ns]	…

[a] Three races of *Fusarium oxysporum* f. sp. *niveum* from GA and FL were identified.

[b] Pairwise population differentiation was measured by Excoffier's Φ_{PT} (Excoffier et al.1992), an analog of F_{st} analysis measured via analysis of molecular variance, using GenAlEx v.6.503 (Peakall and Smouse 2006).

Significance levels (ns=not significant, * = P≤0.05, ** = P≤0.01, *** = P≤0.002) are based on P values determined by 1000 permutations of the data.

Table 4-7. Analysis of molecular variance for three *Fusarium oxysporum* f. sp. *niveum* races collected from four different locations in the southeastern United States.

Source of variation	d.f.	S.S	M.S.	Variance%
Among races	2	3.167	1.583	0%
Within races	53	81.869	1.545	100%
Total	55	85.036	3.128	

d.f., Degree of freedom; S.S, Sum of squared differences; M.S., Mean square

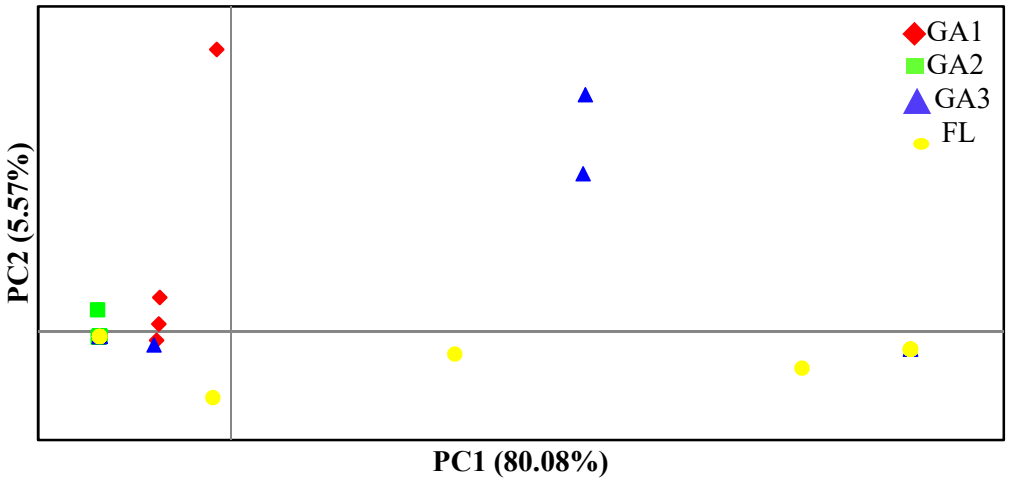

Fig.4-1. Principal coordinate analysis of four populations of *Fusarium oxysporum* f. sp. *niveum* isolates collected from watermelon fields in southeastern United States. Sampling locations include Berrien County, Georgia (GA1), Cook County, Georgia (GA2), Tift County, Georgia (GA3), and Florida (FL). PC1 (80.08%) and PC2 (5.57%) refer to the first and the second coordinate.

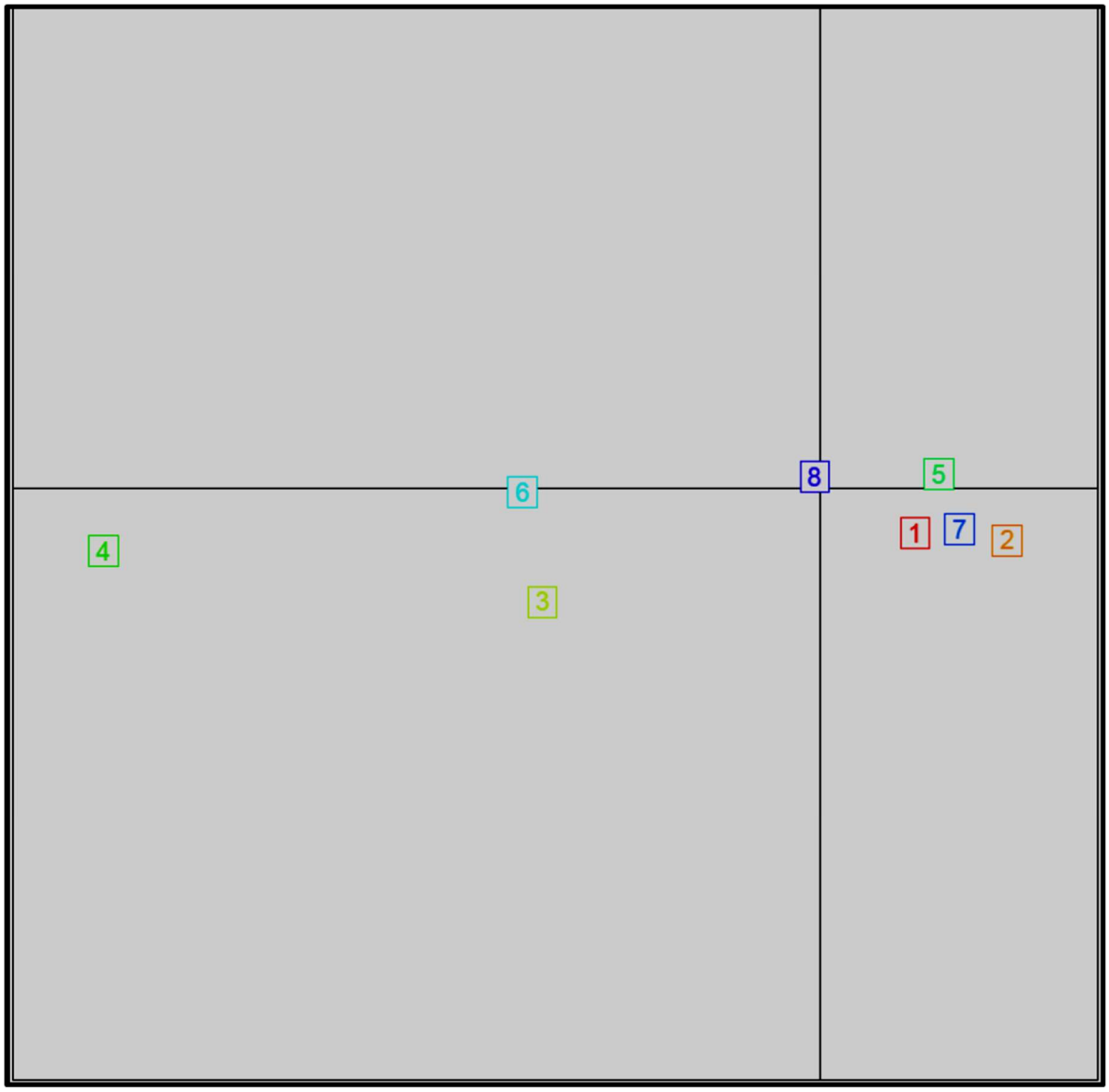

Fig.4-2. Discriminant analysis of principal components for *Fusarium oxysporum* f. sp. *niveum* (FON) from the southeastern United States showing scatterplot of the 8 assigned clusters based on Bayesian information criterion. The four populations include: Berrien County, Georgia (GA1), Cook County, Georgia (GA2), Tift County, Georgia (GA3), and Florida (FL).

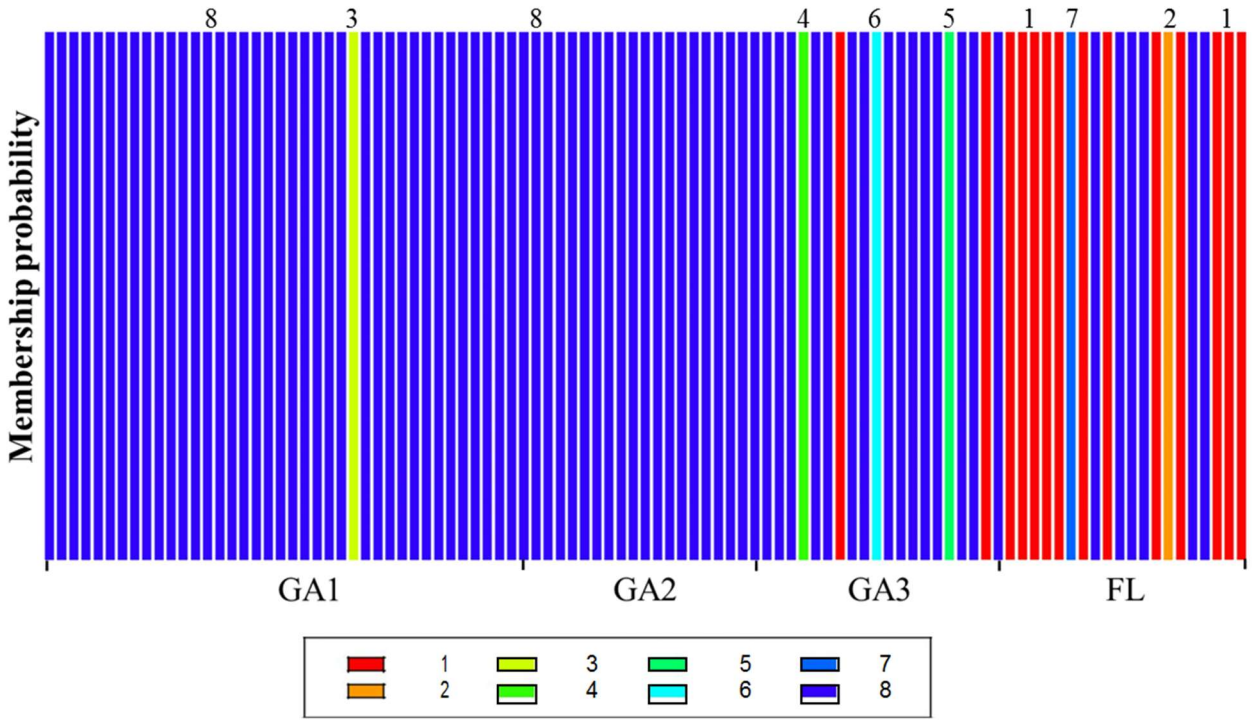

Fig.4-3. Discriminant analysis of principal components for *Fusarium oxysporum* f. sp. *niveum* (FON) from the southeastern United States showing a histogram of assignment probability of 99 FON isolates from 4 field populations into eight genetic clusters. The four populations include: Berrien County, Georgia (GA1), Cook County, Georgia (GA2), Tift County, Georgia (GA3), and Florida (FL).

CHAPTER 5

CONCLUSION

Fusarium wilt of watermelon, caused by a fungal pathogen *Fusarium oxysporum* f. sp. *niveum* (FON), is the most severe soilborne disease in watermelon worldwide and has been a serious threat to watermelon production in the United States. Initial symptom of the disease includes loss in turgor pressure of the leaves and vines. As the disease progresses, the infected plants turn dull green to yellow and eventually become necrotic (Kleczewski and Egel 2011, Martyn 2014). The pathogen can also cause pre-or post-emergence damping off in young seedlings and can lead to wilting of mature plants in the field (Martyn and Vakalounaki 2012). A characteristic symptom of this disease is unilateral stem necrosis, which can be easily visualized when runners or stems are sectioned. Under favorable conditions, yield losses of more than 80% can be experienced. Recent outbreaks and associated losses due to FON can be attributed to the following reasons: (1) emergence and distribution of new FON races (example: race 3) to which no commercial resistant varieties are available (Egel et al. 2005); (2) seedborne nature of Fusarium wilt facilitating long distance dispersal of the pathogen and resulting in outbreaks; (3) phasing out of effective soil fumigant like methyl bromide; (4) inefficacy of crop rotation due to long-term survival of resilient spores in absence of host and lack of suitable land. The $520 million watermelon industry in the United States is threatened by Fusarium wilt, for which there are limited management options. Furthermore, population structure of the pathogen and status of fungicide sensitivity are not well explored especially in the southeastern U.S. **Hence, the overall goal of the proposed research was to determine fungicide resistance, infection courts in watermelon leading to seed**

infestation, genetic and phenotypic diversity of FON in the southeastern United States. The specific objectives of this research were:

1. Determine the effect of fungicides prothioconazole and thiophanate-methyl, on different asexual life stages of FON isolates from watermelon.

2. Determine infection courts in watermelon plants for FON leading to seed infection.

3. Determine genetic and phenotypic diversity of FON in the southeastern US.

Fungicide sensitivity was evaluated for FON isolates collected in the southeastern US. Based on an in vitro mycelial growth assay, all 100 isolates evaluated were sensitive to prothioconazole at both 10 and 100 µg/ml; EC_{50} values ranged from 0.75 to 5.69 µg/ml with a mean EC_{50} of 1.62 µg/ml. In contrast, 33% and 4% of the isolates were resistant to thiophanate-methyl at 10 and 100 µg/ml, respectively. Microconidial germination assays showed that 36% and 64% of the isolates tested were sensitive or intermediately sensitive to prothioconazole at 100 µg/ml, but the fungicide did not inhibit spore germination at 10 µg/ml. Sequencing a portion of the β-tubulin gene of eight isolates resistant or sensitive to thiophanate-methyl indicated that fungicide resistance was associated with a point mutation at nucleotide position 200 resulting in a substitution of phenylalanine by tyrosine. This is the first report of isolates of *F. oxysporum* resistant to thiophanate-methyl. Results of the study suggest that prothioconazole may be a viable option for managing Fusarium wilt of watermelon while thiophanate-methyl should be used judiciously due to the presence of resistant isolates in field populations of the pathogen.

Investigation on the potential infection court leading to FON infection was evaluated in greenhouse and field conditions. Inoculation of pericarp of immature fruit resulted in 17.8 to 54.4% of infested seeds under field conditions and 0.6 to 12.8% of infested seeds under greenhouse conditions when seeds were not surface disinfested before isolation. Seed infestation was also detected in 0 to 4.5% of the seeds when seeds were surface disinfested before isolation. Inoculation of pistil resulted in 0-7.2% and 0-18.3% of infested seeds under greenhouse and field conditions when seeds were surface disinfested or not disinfested before isolation, respectively. Inoculation of peduncle resulted in 0.6-6.1% and 0-10.0% of infested seeds in the greenhouse and field experiments when seeds were surface disinfested or not disinfested before isolation, respectively. Seed infestation was also detected using real-time PCR assay in all the experiments when pericarp or pistil was inoculated and in 3 out of 4 experiments when peduncle was inoculated, irrespective of surface disinfestation. The results suggest that watermelon tissues including pericarp of immature fruit, pistil, and peduncle could be potential infection courts of FON leading to seed infestation by the pathogen.

Genetic and phenotypic diversity of FON isolates collected from the southeastern US was determined. Simple sequence repeat (SSR) markers were used for genetic diversity determination. Discriminant component analysis of principal components (DAPC) of 99 isolates using simple sequence repeat (SSR) markers grouped the isolates into eight major clusters with two prominent clusters (1 and 8). Cluster 1 consists a total of 14 isolates out of which 85.7% of the isolates belong to FL. However, the majority of isolates (92.4%) in cluster 8 belonged to GA. Both DAPC and population structure analysis revealed that the phylogenetic groups are closely associated with geographical location of isolation. Three races of FON (races 0, 2 and 3) were identified from the

phenotypic analysis; with race 3 identified for the first time in GA. Majority of the isolates in cluster 1 and cluster 8 either belonged to race 2 (35.6%) or race 3 (45.8%). Additionally, no relation between phylogenetic groups and races identified was observed. Information obtained on genotypic and phenotypic diversity of FON in the southeastern U.S diversity will help to develop effective disease management programs to combat this disease.

In summary, this study provides new insights into the current status of fungicide resistance, potential infection courts in watermelon leading to FON seed infestation, genetic and phenotypic diversity of FON in the southeastern US. New race of FON, race 3 was identified for the first time in GA and the SSR maker analysis proved to be a useful tool in determining the genetic diversity, which divided the FON isolates from Georgia and Florida in two major clusters. In addition, some FON isolates were reported to be resistant to thiophanate-methyl whereas majority of the isolates were sensitive to prothioconazole. Subsequently, peduncle, pistil, and pericarp of female watermelon plant were found to be potential infection courts for both external and internal seed infestation. Overall, information gained from this dissertation research will add to basic and applied information in FON-watermelon pathosystem. In time, the findings can be incorporated into the integrated disease management options against this disease.

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