DEVELOPMENT OF NOVEL DIAGNOSTIC ASSAYS AND DETERMINATION OF FUNGICIDE RESISTANCE MECHANISMS FOR *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* USING MOLECULAR ANALYSIS

by

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(Under the Direction of Emran Ali and Pingsheng Ji)

ABSTRACT

Fusarium wilt, caused by the soilborne ascomycete fungus, *Fusarium oxysporum* forma specialis *niveum* (FON), is one of the most damaging diseases of watermelons worldwide. Management of FON is difficult due to the presence of four races (0, 1, 2, and 3), which are increasingly virulent on resistant watermelon cultivars. Additionally, there is only one fungicide (Proline) available to manage FON on watermelon. A loop mediated isothermal amplification assay was developed for rapid and specific molecular detection of FON. Through whole genome sequencing, a polymerase chain reaction (PCR) assay was developed to differentiate FON race 3 from races 1 and 2. Additionally, FON isolates sensitive to Proline (a.i. prothioconazole) were mutagenized to generate fungicide resistant mutants. Sanger sequencing and an expression analysis via qPCR amplification of *CYP51* showed 2 point mutations in the coding region and a statistically significant increase in gene expression in resistant mutants compared to the sensitive isolates.

INDEX WORDS: Fusarium wilt of watermelon, *Fusarium oxysporum* f. sp. *niveum,* pathogen diagnosis, race differentiation, whole genome sequencing, molecular analysis, Proline, prothioconazole, fungicide resistance, gene expression

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DEDICATION

For Allina Bennett

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Watermelon Production. Watermelon (*Citrullus lanatus*) is a flowering plant species in the family Cucurbitaceae that is cultivated for its fruit in many forms across the world. Nutritionally, watermelons are 91% water, however, they also provide a significant source of Vitamin C, Vitamin A, Vitamin B6, and antioxidants [1].

While the majority of global watermelon production occurs in China, the United States ranks 7th worldwide, and over 75% of American watermelon consumption comes from domestically grown watermelon [1, 2]. Although a majority of watermelons eaten in the US come from the US, the percentage of watermelon imports has seen a steady climb since 1990, when imports were only 7%, to over 30% in 2016 [3]. While watermelon can be produced in most states in the US, California, Georgia, Florida, and Texas grow 70% of all watermelons due to an extended growing season and higher temperatures. In 2017, production in the US reached roughly 115,000 acres, totaling 40 million pounds. Acreage has decreased almost 50% since 1990, but production has not suffered due to improved cultivation and production techniques. According to the USDA ERS, watermelons sold in the United States totaled $580 million in 2016 [4]. Sales for seedless watermelons were 85% of all watermelons sold in the United States in 2016, up 34% from 2003 [3].

Georgia is continually a national leader in watermelon production, ranking second or third each year. The crop's total acreage of 20,000 produced a farm gate value of $123 million in

2018 [5, 6]. The majority of the crop is grown in the southern half of the state, and while locally grown watermelons can be bought locally, the majority are exported to northern states and Canada [6]. Per acre, Georgia growers can produce a yield of between 35,000-50,000 pounds, but various factors, such as poor weather or disease, can prevent maximum production. As is the trend across the United States, the production of seedless watermelons is increasing every year in acreage but seeded watermelons continue to be grown adjacent to seedless watermelons for pollination purposes. The most popular seedless varieties include Cotton Candy, Honey Heart, Queen of Hearts, and King of Hearts. Other popular varieties are Sugar Baby, Golden Crown, Charleston Grey, Crimson Sweet, and Jubilee [7]. Recently, the use of plastic mulch has increased and become widespread in Georgia and the Southeast in general on multiple crops, and watermelons in particular benefit from this practice. As the plastic mulch raises the temperature of the soil, watermelons grown on plastic can be harvested earlier - meeting a higher demand and therefore price per melon. Additionally, the plastic can conserve moisture and nutrients, saving growers significant input costs.

Diseases of Watermelon. Watermelon production in Georgia faces a number of problems due to the favorable conditions for diseases that occur within the state. The traditional warm and humid climate allows for several stem and foliar diseases, and the history of growing watermelons in Georgia has allowed for the further accumulation of pests and diseases [8]. Seedling pathogens such as *Pythium* spp., *Rhizoctonia* spp., and *Fusarium* spp. (together causing disease commonly known as damping off) are common pathogens of watermelon due to the favorable conditions that are prevalent when watermelons are being planted (cool and moist in early spring). Management strategies such as crop rotation and resistant varieties are in use, but if no control is used, over 50% losses have been seen in the field [9]. Root-knot nematodes are

also a consistent source of loss in the watermelon industry by causing damage to the root system which prevents proper uptake of water and nutrients. Root damage can also provide an ingress site for soil-borne pathogens to more easily cause disease [10, 11]. Other foliar and stem diseases of watermelon include gummy stem blight caused by *Stagonosporopsis* spp. (which can also cause damping off in seedlings), and anthracnose, a disease caused by *Colletotrichum orbiculare* which affects all above ground parts of the watermelon plant. Later in the season, other diseases occur that cause fruit rot when climatic factors favor them. Those known include rind necrosis (pathogen unknown but in association with *Erwinia* spp.), *Acidovorax avenae* subsp. *citrulli* causing fruit blotch on both seedlings and fruit, and *Phytophthora capsici* causing Phytophthora root and fruit rot [12]. Cucurbit downy mildew, whose causal agent is *Pseudoperonospora cubensis*, is limited to the foliage of the plant, but can affect a number of cucurbit crops after overwintering in Florida before being spread northward during the growing season [12, 13]. The viruses that affect watermelon production include the *Watermelon mosaic virus* (also known as *Papaya ringspot virus*) which can be transmitted by aphids at any point during the growing season, and *Cucurbit leaf crumple virus* (CuLCrv) which is vectored by whiteflies which are more common in fall. Finally, often viewed as the most important pathogen of watermelon globally, is Fusarium wilt of watermelon [14].

Fusarium Wilt of Watermelon. Fusarium wilt of watermelon is caused by the pathogen *Fusarium oxysporum* forma specialis *niveum* (FON). FON was first discovered in 1894 in the southeastern United States and is now present in every watermelon growing region in the US and on all six continents in the world that cultivate watermelon [15]. While the fungal species *Fusarium oxysporum* (FO) has over 100 formae speciales, many are host specific, which is the case for FON. Many formae speciales of *Fusarium oxysporum* are morphologically similar and

many more are indistinguishable even with the use of advanced microscopy. The diversity of species within the genus *Fusarium* includes plant pathogens, human pathogens, and many non-pathogenic strains. In addition to the species level complexity, a single species and dozens of formae speciales may be subdivided into races for their cultivar specificity [16]. In the Cucurbitaceae, six different formae speciales have been described, with *niveum* and *melonis* being the two most economically important. And while cross infection has been seen in greenhouse and laboratory conditions, this is a rare occurrence in the field and is therefore described as insignificant [17]. FON is a soil-borne pathogen, but it can be transmitted for long distances through the transport of contaminated soil or infected tissue.

Fusarium wilt is most commonly characterized by a loss of turgor pressure (wilting) of all aboveground tissues which can begin in new growth or at the crown. Shortly thereafter wilted leaves turn yellow and then necrotic under favorable conditions. When low levels of inoculum are present in the ground, a single runner or vine can show signs of wilting and necrosis – rather than the entire plant. Infected plants that do not die from Fusarium wilt are often stunted and see reduced yields [15]. Wilting is due to the colonization of the xylem of the plant by the pathogen, blocking the flow of nutrients and water to the aboveground parts of the plant. Cutting into the crown of a diseased plant will often reveal brown streaks in the xylem that can assist in primary diagnosis. Plants in the field commonly show initial symptoms within the first 4 weeks after planting, however, the disease occurs whenever conditions are favorable and can infect as late as post-fruit set [9, 17].

Morphologically, FON is similar to other formae speciales in the *Fusarium oxysporum* species complex (FOSC); however, no known sexual stage has been observed. Much of the spread of FON during the season occurs through asexual spores known as macroconidia. Macroconidia are

3-5 cells in length, fusiform in shape, and are generated in large numbers. Macroconidia are responsible for some infection of the plant's roots and the generation of chlamydospores. Chlamydospores are asexual resting spores that can live in the soil for up to 15 years and are responsible for the majority of initial infections [18]. Chlamydospores are the main survival spores for the pathogen and are generated when the host plant is dead or when there are not sufficient nutrients for continued pathogen growth. When chlamydospores germinate, they produce hyphae that penetrate the cortex of the plant root and continue to produce mycelium and microconidia within the xylem. Alternatively, microconidia consist of only a single cell, are quite short lived, and are smaller than both macroconidia and chlamydospores.. Microconidia are most likely not responsible for initial infection but instead are generated on conidiophores that are within the xylem cells and further systemic fungal infection. Host plant defense is the cause of the symptomatic wilting, and as the pathogen infects watermelon tissue, tyloses (an extension of parenchyma cells) are produced which block water and infective propagules from moving in the xylem. In resistant cultivars of watermelon, these tyloses will successfully block the spread of the pathogen, limiting the damage to a single isolated location. In non-resistant cultivars, the pathogen breaks down tyloses causing gumming in the xylem and continued spread of FON. Once the host is dead, mycelium moves to the exterior of the plant, producing macroconidia and eventually chlamydospores [14, 15].

Methods of diagnosis: Overview of traditional, PCR, LAMP techniques for FON detection. Identification of the pathogen often begins in the field and moves to the lab for confirmation. As described previously, in-field diagnostics are limited to observation of wilting vines and brown streaking in the xylem when the crown is cut. In moist or highly humid conditions, mycelium can be observed at the base of a highly necrotic crown [8, 15, 19]. Unfortunately for diagnostic

purposes, other pathogens as well as other formae speciales of *Fusarium* show similar symptoms as FON. In watermelon, *Verticillium dahliae* (causing Verticillium wilt) leads to identical xylem browning and wilting symptoms and further investigation must be done to confirm the diagnosis. Further identification is done by microscopic analysis of spore shape and size. Spore morphology is often enough to obtain the level of *Fusarium oxysporum*; however, it is not sufficient when detecting or differentiating to the forma specialis level or when differentiating between races. Bioassays have been used in the past to determine a species level diagnosis which involves testing the isolate in question on many different plant hosts to determine the pathogen specificity. Because the *Fusarium oxysporum* species complex (FOSC) contains many species/formae speciales, bioassays are now insufficient or impractical. Thus, molecular techniques and assays are used for diagnostic confirmation.

Currently, there are three potential assays available for FON diagnosis. The first is both a qPCR and PCR primer, Fn-1/Fn-2, for FON specific detection designed by Zhang et al. (2005) [20]. Issues of accuracy arose when using this primer set for FON specificity on samples from the United States. A new PCR primer set, Fon-1/Fon-2, was developed by Lin et al. (2010) for FON differentiation based on an RAPD sequence [21]. To improve on the ability to detect FON in soil, a loop mediated isothermal amplification (LAMP) assay was developed by Peng et al. (2013), however, again when tested on FON isolates from the Southeast, it did not result in successful amplification [22].

FON races and methods of race differentiation. Globally, watermelon losses have recently increased due to FON, traditionally in those locations where resources are limited and that have a history of watermelon production [23]. Furthermore, a notable increase in outbreaks of the disease has been reported in regions that heretofore have not detected it. This, in part, is

suspected to be due to the spread of the highly virulent and resistance-breaking race 2. FON is currently divided into four separate races: 0, 1, 2, and 3. Race 1 was originally described in 1963 in Florida when it was differentiated from race 0 which is only pathogenic to cultivars that lack all resistance genes. Therefore, it does not pose a significant economic threat, as most of these cultivars are not in common use. One exception is the cultivar Sugar Baby, which is still in use and can be used for traditional differentiation methods which will be discussed further. Race 1 is the most widespread of all the races of FON and has been identified in all parts of the world where watermelon is grown. Breeders have accordingly bred resistance against FON races 0 and 1 into multiple popular watermelon cultivar lines, decreasing the severity of the problem in areas where resistant seed can be planted., as some investigators believe that races 0 and 1 are all the same race with variations in their virulence [14]. Crucially, race 2 can overcome the resistance of all commercially grown lines of watermelon, and the disease has seen a significant spread in the last three decades. Race 2 was first identified as a new race in Texas in 1985, shortly followed by Oklahoma in 1988, Florida in 1989, Maryland and Delaware in 2001, Indiana in 2005, and Georgia and South Carolina in 2008 [24-30]. Internationally, race 2 has been detected in China, Cyprus, Greece, Israel, Korea, Spain, Tunisia, and Turkey, and [30-35]. In response to the spread of race 2 internationally, breeders have developed a PI line (PI-296341-FR) that shows resistance to race 2; however, resistance from this line is not yet available for commercial production because the PI line does not have commercial qualities (small fruit with many seeds and white flesh). While PI-296341 may not have commercial value, it does have scientific value, as it allows for the differentiation of FON race 2 from new more virulent, and pathogenic races such as race 3. Race 3 was reported in Maryland in 2009 as the first race able to infect the PI line which was confirmed in 2010 [36], followed by similar reports of race 3 in Florida and Georgia

in 2018 and 2019 [37, 38]. Work done by Zhou et al. (2012) confirmed the selective pathogenicity of FON, as it was nonpathogenic on other cucurbit hosts validating its status as a forma specialis [39]. Additionally, races found in Maryland were vegetatively compatible with each other, but not with reference isolates -demonstrating genetic differences based on location. When a genetic study was done by Petkar et al., the variation within the southeastern US (GA and FL) showed a strong correlation depending upon location, and the majority of the isolates taken from both states were of races 2 and 3 [38]. In 2020, researchers from South Carolina did a similar survey study but included isolates that had been gathered since 2005 [40]. A majority of isolates were found to be race 2, and no race 3 isolates were found; however, this was evaluated entirely based on the bioassay results.

As discussed, differentiation between races of FON is traditionally done by inoculating resistant or susceptible cultivars with FON isolates and evaluating their pathogenicity based on disease development. The traditional cultivar pattern used is viewed most accurately in Table 1.1 which was developed and used by other authors [8, 14, 23, 36]. Although the theoretical results displayed in the table are binary and seem to be easy to differentiate, the results are scored using a different method. The various cultivars are grown in identical soil and climate conditions and a conidial suspension of FON is delivered to the germinating seed. After 4 weeks of growth, all plants are scored from 0-9 based on the reaction, 0 being asymptomatic, 3 having cotyledon lesions, 5 for symptoms of slight wilting or stunting, 7 for severe wilting and growth stunting, and 9 for dead plants. A score of 0 is viewed as resistant, scores of 1-4 as intermediately resistant, and 5-9 as susceptible [25, 28, 30, 38]. Serious issues arise quickly when performing this method of race differentiation, beginning with the assessment of the scores being subjective and creating errors when deciding between susceptible and resistant results. Experimental

conditions such as temperature, humidity, soil type, and propagule concentration must be standardized but these are difficult to replicate exactly between laboratories. The virulence of isolates and the resulting infection score may also change over time, possibly an effect of long term storage. Some cultivars listed above have alternatives that are similar in degree of resistance, but the literature is contradictory and confusing [39, 41, 42,]. In order to confirm the inaccuracies in this method, the same FON isolate was sent to multiple diagnostic labs which study FON, and each returned a different race result. Molecular and genetic factors may also contribute to the confusion, as members of the FOSC are known to do horizontal gene and chromosomal transfer from one isolate to another [9, 19, 30, 43]. Pathogenicity chromosomes have also been recorded as able to transfer a pathogenic status to an otherwise non-infectious strain [44-47].

To develop watermelon lines resistant to FON, researchers determined that the absence of the *Secreted in Xylem 6* (*SIX6*) gene was necessary for race 2 level pathogenicity. A new marker was created to amplify *SIX6* to distinguish race 2 from races 0 and 1, as race 3 was not compared [48]. Subsequent questions have arisen about the correlation between the traditional method of identifying race 2 isolates using the bioassay and the molecular absence of *SIX6,* as the results are not well aligned [40]. Even still, due to the variable and transferrable nature of *Fusarium* genomes, identifiable markers remain in question if targeted to proteins that have been linked to a specific function other than pathogenicity [44,45]. Molecular methods that target conserved genes responsible for pathogenicity are needed going forward for enhanced FON race differentiation.

Methods of Control: Fungicides. The only registered fungicide for the control of FON on watermelon is Proline 480 SC (Bayer CropScience, Research Triangle Park, NC), which is a

demethylation inhibitor (DMI) fungicide with the active ingredient prothioconazole. DMI fungicides work by inhibiting the biosynthesis of ergosterol which functions as part of the plasma membrane of some fungi, thus inhibiting growth and development. DMI fungicides are a widely used class of fungicides and reports of resistance in fungal pathogens are equally widespread [49-51]. While DMI fungicides are only under a medium risk of developing resistance, FON specifically is assumed to have a higher likelihood of resistance development due to the fact that only one fungicide is registered and used for control [52].

In the literature, FON is reported to be sensitive to prothioconazole, but growers have reported insensitivity in local populations. In addition to growers reporting insensitivity to the fungicide, FON develops chlamydospores which last much longer in the soil compared to the macro and microconidia and are highly resistant to heat and chemicals [14]. Since the phasing out of methyl bromide, growers have struggled to find a way to inhibit FON breakouts [53]. Other fungicides (pydiflumetofen) are beginning to appear for FON but are not yet approved [54-56].

No mechanisms for the resistance of FON to prothioconazole have been documented; however, three mechanisms of resistance to DMI fungicides have been characterized in other fungal organisms. The first is single nucleotide polymorphisms (SNPs) or InDel modifications in the coding sequence of the target gene, *Sterol 14α-Demethylase Cytochrome P450* (*CYP51*). These SNPs confer small amino acid changes (point mutations) that can alter the structure of the protein and subsequently change the binding of the fungicide to the protein [57]. Other mutations can arise but will be silent, meaning that although there is a SNP, the amino acid code remains the same as the sensitive form. These mutations are detected by sequencing each copy (if there are multiple copies) of *CYP51* in both sensitive and resistant isolates and analyzing the resulting

gene sequences. The second resistance mechanism is gene overexpression [58]. This is often caused by an alteration of the promoter region which can include insertions, deletions, and other transposable elements that alter the affinity for binding with the ribosome for translation [51, 59]. While sequencing the promoter region can provide information about altered gene expression, expression levels are detected using RNA sequencing and quantitative PCR (qPCR) followed by comparing the level of *CYP51* in resistant isolates versus that of sensitive isolates [60]. The third mechanism of resistance involves changes in efficiency/activity of efflux transporters – molecular machinery that expels the fungicide before it can have its desired inhibitory effect or over-expression of ATP-binding cassette (ABC) transporters [61-63]. Efflux transporters can be effective at conferring fungicide resistance not only to DMIs but to other classes of fungicides as well. Often, detection and determination of whether efflux transporters causing resistance comes by inhibiting the transporter via added chemicals and comparing resistance levels.

Justification and Objectives

Improved detection of *Fusarium oxysporum* f. sp. *niveum*. Accurate disease detection and identification are necessary for the implementation of appropriate disease management. FON is difficult to detect in the field until an infection takes place, and even when FON is suspected, time and resources must be spent to confirm the diagnosis. Race differentiation is often either impossible or impractical for growers and researchers due to resource restrictions on materials required for the bioassay. The challenges of FON diagnosis and additional race differentiation require modern molecular assays for exact and rapid results. Genomic resources for FON are not widely available for researchers either, so improvements of current methods require novel sequence generation. The main goal of this research project was to improve the current methods

of molecular detection and develop new markers for race differentiation. The direct objectives of this study were as follows:

1. Develop a real-time portable detection method (LAMP assay) to differentiate FON from other formae speciales.
2. Develop a real time LAMP assay that amplifies *SIX6* and allows for rapid differentiation of race 2 isolates.
3. Carry out whole genome sequencing (WGS) of each FON race to allow for marker development.
4. Develop a race-specific PCR primer set, based upon WGS results, that can successfully differentiate race 3 isolates.
5. Survey the prevalence of FON races within a collection of 150 southeastern US FON isolates using the newly developed PCR primer set and compare the molecular survey results to the bioassay results for each isolate.

Determine mechanisms of fungicide resistance. As FON is currently controlled with a single DMI fungicide chemistry, prothioconazole, the risk of fungicide resistance development is medium to high. Especially due to the spread of more pathogenic and virulent races for which there is no host resistance, chemical management has become even more important. Because no resistance has been found in field isolates, no mechanisms of resistance for this pathogen have been documented. Hence, the second goal for this research was to determine a mechanism for possible fungicide resistance in FON isolates to the fungicide "Proline" (a.i. prothioconazole). The specific goals were as follows:

1. Using UV radiation and high concentrations of prothioconazole, generate fungicide resistant FON mutants.
2. Select fungicide resistant isolates from within the population of generated FON mutants following multiple (10-12) rounds of growth over 15 weeks.
3. Characterize possible mechanisms of resistance in fungicide resistant FON mutants via sequencing of the cytochrome P450 gene (*CYP51*) and expression analysis.

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Tables

Table 1.1. Cultivar and FON race table used in the bioassay for race differentiation.

Genotype/Cultivar	Race 0	Race 1	Race 2	Race 3
Sugar Baby	Susceptible	Susceptible	Susceptible	Susceptible
Charleston Gray	Resistant	Susceptible	Susceptible	Susceptible
Calhoun Gray	Resistant	Resistant	Susceptible	Susceptible
PI-296341-FR	Resistant	Resistant	Resistant	Susceptible

CHAPTER 2

DEVLEOPMENT OF TWO NOVEL LAMP ASSAYS FOR THE IDENTIFICATION AND RACE 2 DIFFERENTIATION OF *FUSARIUM OXYSPORUM* F. SP. *NIVEUM*[1]

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ABSTRACT

Fusarium oxysporum f. sp. *niveum* (FON) is the causal agent of Fusarium wilt of watermelon, a leading limiting factor of watermelon production internationally. Traditional methods of detection and differentiation of this pathogen from other formae speciales are impossible due to the identical morphology and level of variation in culture morphology and microscopic characteristics. Other methods such as bioassays are resource heavy and time consuming, so molecular methods of detection have become necessary for proper management of this pathogen and other formae speciales within the *F. oxysporum* species complex. FON consists of four races (0, 1, 2, and 3) which vary in their pathogenicity and virulence on various watermelon cultivars. Race differentiation relies upon a bioassay that contains several shortcomings. To overcome these limitations, loop mediated isothermal amplification (LAMP) assays were designed to improve current molecular methods of detection and differentiation. In this study, two LAMP assays were developed, one for the specific detection of *F. oxysporum* f. sp. *niveum* and the other for differentiation of FON race 2 from the other races. The LAMP assays presented here can be used in the field with real-time amplification.

INTRODUCTION

Fusarium wilt of watermelon is known as one of the most economically important diseases of watermelon internationally and is caused by the fungal pathogen *Fusarium oxysporum* f. sp. *niveum* (FON) [1-3]. FON is pathogenic only on watermelon, but other formae speciales have a larger host range which include watermelon (*F. oxysporum* f. sp. *melonis*), contributing to confusion around specific pathogen diagnosis [4]. FON is characterized in the field by a single stem or runner of the plant wilting while the other vines remain healthy [3, 5]. This characteristic wilting is caused by a buildup of microconidia that are produced within the xylem of the plant and causes another identifiable feature of vascular discoloration [6]. In the field, infected plants have a red to brown color in the vasculature of infected plants [3]. Disease pressure increases with extended periods of cool and wet weather, usually in the spring when plants are seedlings and susceptible to disease [7].

First discovered in 1894 by EF Smith in the southeastern US [8], FON continues to cause damage and limit production due to its ability to survive for long periods in the soil. It produces three types of reproductive bodies: microconidia, macroconidia, and chlamydospores. Microconidia, as discussed before, develop within the plant host but also provide a source of secondary inoculum; macroconidia are multi-celled and are the main source of secondary inoculum; and chlamydospores are the primary source of inoculum and can survive in the soil for decades [2, 9]. This is true of all the formae speciales within the *F. oxysporum* species complex (FOSC) which prevents specific identification based on morphology [2, 3, 10]. There is a single polymerase chain reaction (PCR) assay designed by Lin et al. (2010) (Fon-1, Fon-2) which is able to specifically amplify FON and no other tested *F. oxsyporum* formae speciales [11].

Four races of FON (0, 1, 2, and 3) have evolved to become pathogenic on previously resistant watermelon cultivars [3, 8]. While race 1 is the most internationally widespread, the more aggressive race 2 has increased in its range over the last two decades. No commercially viable watermelon cultivars exist that are resistant to race 2 isolates of FON [5, 12, 13]. A plant introduction line (PI-296341-FR) is resistant to race 2 but only able to be used for race differentiation [14]. Race 3 overcomes resistance in the PI line and no cultivars are known to have resistance to race 3 [8]. To differentiate these races, a bioassay is used which evaluates the infection of an individual isolate on a particular set of watermelon cultivars whose resistance level is known. This bioassay requires particular and specific experimental conditions and resources difficult to obtain in order to get an accurate result [3, 8]. Seeds of certain cultivars are challenging to source, time and money are required to run multiple greenhouse assays, and experimental conditions such as temperature and humidity are difficult to control in many locations. Ranking levels or degrees of infection is also somewhat arbitrary, although more specific requirements (such as the number of lesions or number of leaves wilted) are used for better accuracy [15]. These challenges provide inconsistent or incorrect results quite often, so molecular methods are needed for proper and consistent differentiation. A single PCR assay has been developed by Niu et al (2016), which identifies the absence of the avirulence gene *Secreted in Xylem 6* (*SIX6*) in race 2 isolates while present in the other races [16]. Amplification of this gene can be used for race 2 differentiation in a more rapid and accurate manner than the bioassay but is still limited to the tools required for PCR in a lab environment.

Both PCR and quantitative PCR (qPCR) reduce the time required to detect the pathogen and increase the specificity of the assay, however, both require expensive and large equipment operated by experienced researchers to properly function. Loop mediated isothermal

amplification (LAMP) has been developed as a new method of molecular detection that requires only a single temperature for successful amplification, allowing for an increased degree of portability for the assay. LAMP assays are also often more sensitive and require less time when compared to conventional PCR [17, 18]. LAMP primer sets consist of a minimum of 4 primers (F3, B3, FIP, BIP) that recognize and bind to 6 different regions on the target DNA, and one can increase the speed and accuracy of the assay by using 2 additional primers (LB and LF) [19]. LAMP assays can additionally be used for real-time detection using the Genie III amplification instrument.

In this study, two LAMP primer sets were developed. One for the specific amplification of FON isolates and the second for the differentiation of race 2 isolates from the other races. These new LAMP assays provide additional rapid molecular assays for the improvement of FON detection and management.

MATERIALS AND METHODS

Sample acquisition and DNA extraction. Samples of FON were taken from watermelon fields across the southern United States over the past 10 years or donated from other labs. The fungal isolates were grown on potato dextrose agar (PDA) plates at 26°C for 10-14 days in the dark before being removed for DNA extraction. Mycelial tissue (100-150 mg) was scraped from the plate with a sterile scalpel, then placed into a 1.5 mL tube with 4-5 sterile 3.2 mm steel beads. The tissue was lysed in a FastPrep FP120 cell disruptor (ThermoSavant). DNA was extracted using a DNeasy extraction kit (Qiagen, MD, USA) according to the manufacturer's instructions. DNA concentrations were quantified using a NanoDrop LITE (Thermo Scientific, Waltham, MA, USA).

PCR amplification and species determination. To confirm the identity of each isolate/DNA sample the previously published primer set Fon-1/Fon-2[12] was used (Table 2.2). PCR reactions contained: EconoTaq® PLUS Green 2X Master Mix (Lucigen, Middleton, WI, USA) (12.5 µL), forward primer (1 µL), reverse primer (1 µL), target DNA (1 µL), and ddH2O (9.5 µL) totaling 25 µL per reaction. PCR was performed using the following cycling conditions: 95˚C for 5 min, followed by 35 cycles of 95˚C for 30 s, 56˚C for 30 s, and 72˚C for 30 s, with a final extension of 72˚C for 7 min. All primers were synthesized by Sigma Aldrich. PCR products were visualized using a 1% agarose gel in SYBR safe stain (Invitrogen, Waltham, MA, USA) and a UV gel doc (Analytik Jena, Jena, Germany).

LAMP primer design. Primers were designed with Primerexplorer V5 software (Eiken Chemical Co., Toyko, Japan) using the sequences "*Fusarium oxysporum* f. sp. *niveum* isolate Fon-H0103 RAPD marker genomic sequence" (GenBank accession: EU603504.1), and "*Fusarium oxysporum* f. sp. *niveum* partial Fonsix6 gene for secreted in xylem protein, strain race 1" for FONL1 and FON6L, respectively [16]. All primers were synthesized by Sigma Aldrich (St. Louis, MI, USA) and stored at -20°C. Five primers were constructed for the FON specific LAMP reaction: two outer primers (F3 and B3), two inner primers (FIP and BIP), and a loop forward primer (LF) (Table 2.2). Six primers were used for the FON race 2 differentiation assay which included an additional loop backward primer (LF and LB).

LAMP optimization: LAMP assays were optimized using the Genie III amplification instrument (Optigene, Horsham, England) to determine what temperature resulted in the fastest reaction and the highest florescence by running them in variable temperatures (60-70°C) and observing the florescence graph (Figure 2.5&2.6). Variable LAMP primer concentrations were not tested due to previous optimization studies [20] and were as follows: F3 and B3, 0.2 µM

each; FIP and BIP, 1.6 µM each; and LF and LB at 0.8 µM. Each reaction totaled 25 µL, and in addition to the primer set contained: LavaLAMP DNA master mix (Lucigen, Middleton, WI, USA) (12.5 µL), Green florescent dye (0.5 µL), genomic DNA (1 µL), and ddH2O (8.5 µL). LAMP reactions were performed at 95˚C for 3 min then the optimized temperature (66˚C or 65˚C) for up to 60 min.

LAMP specificity. To test FONL1, DNA was extracted as described previously from other formae speciales and isolates representing all four races and tested using the LAMP assays to determine specificity alongside positive and negative controls. To test the specificity of FON6L, all races were used but no other formae speciales. The reactions were done with the optimized temperature and run for a full 60 minutes. All tests were done in triplicate.

LAMP sensitivity. To determine the sensitivity of the LAMP assays, a tenfold serial dilution was made with the extracted DNA of a race 1 isolate from 10 ng μL^{-1} to 0.01 pg μL^{-1}. The dilutions were done in triplicate and viewed using both methods: agarose gel and real-time amplification graph. Samples were considered negative if undetectable on an agarose gel to the naked eye. The PCR assay sensitivity was tested alongside each respective LAMP assay using the same serial dilutions to compare.

RESULTS

LAMP assay specificity. To determine the specificity of the FON specific LAMP primer set, FONL1, an isolate representing each race was used alongside the closely related species *F. oxysporum* f. sp. *lycopersici* (FOL) and *F. oxysporum* f. sp. *vasinfectum* (FOV). LAMP results showed positive amplification for all races of FON and were negative for FOL and FOV (Figure 2.1). Specificity was determined for the race 2 specific primer set, FON6L, by testing all races

with the new marker to confirm only race 2 as negative. Only race 2 was negative for amplification showing the same specificity as the previously published PCR primer set (Figure 2.2).

LAMP assay sensitivity. The lowest detection level for FONL1 was 0.1 pg μL^{-1} (Figure 2.3A), while the lowest detection level for the FON specific PCR primer set (Fon-1, Fon-2) was 0.01 ng μL^{-1} (Figure 2.3C). When using the real-time amplification graph to determine the sensitivity level, it was seen that the highest concentration (10 ng μL^{-1}) was amplified at between 10-20 minutes, and the lowest level of sensitivity (0.1 pg μL^{-1}) amplified at 40-50 minutes (Figure 2.3 B). Primer set FON6L amplified as low as 1.0 pg of DNA which was the same sensitivity to that of the PCR, FONSIX6; however, the lowest amplifying concentration amplified before 20 minutes using the Genie III real-time amplification instrument (Figure 2.4).

LAMP assay optimization. The optimal temperature of FONL1 was determined to be 66°C by both of these metrics (Figure 2.5) and occurred between 12-15 minutes after assay start. The optimal temperature for FON6L was determined to be 65°C based on the speed of amplification and was seen between 12-15 minutes after assay start (Figure 2.6).

DISCUSSION

Rapid, sensitive, and accurate pathogen diagnosis and detection are crucial in plant disease management, and often preludes all other phases [21]. Traditional pathogen diagnosis based on morphological and microscopic characteristics is unable to distinguish between highly similar pathogens and properly diagnose them [22]. Species within the *Fusarium oxysporum* species complex (FOSC), most notable for causing various Fusarium wilts, are far too similar morphologically for exact identification, so infectious bioassays are typically used to have a

more accurate determination [2, 3, 8]. Races within FON are even more genetically similar and the bioassay used for race differentiation has multiple variables that decrease the accuracy of the assay. For both bioassays, there are issues of accuracy, cost, and timeliness when compared to modern molecular detection methods. Currently, the best method available for specific detection of FON is a conventional PCR by Lin et. al. (2010), and the only race that can be differentiated is race 2 using a separate PCR assay by Niu et al. (2016) [11, 16]. Although PCR is a clear improvement on the morphologic or bioassay methods, it is limited in portability, speed, and equipment needed [19, 23].

Loop mediated isothermal amplification (LAMP) assays require less time (less than one hour) and only one temperature (rather than thermal cycling) which can be provided by a portable heating block for sample detection in the field [17-20]. Additional ability to save time and in-field application comes from using a Warmstart intercalated dye or the Genie III real-time amplification instrument which is also portable.

To improve on the current methods of detection and race differentiation, two new LAMP primer sets were designed to improve the ability of the FON specific and race 2 specific PCR assays in a more rapid and specific manner. Sequences adopted from PCR primer sets were used for the design of new LAMP primer sets “FONL1 and “FON6L”. FONL1 is able to specifically amplify all FON races (0, 1, 2, and 3) selectively, without amplifying closely related formae speciales. FON6L amplifies races 0, 1, and 3, allowing for the absence of amplification to differentiate race 2 isolates (Figures 2.1&2.2). These LAMP assays were compared to their respective conventional PCR primer sets developed for specific FON amplification in both sensitivity, cost per reaction, and time required to complete the assay. The FONL1 LAMP assay was able to detect the presence of FON at a concentration as low as 0.1 pg μL^{-1} of genomic

DNA, which was determined to be 100 times more sensitive than the conventional PCR (Figure 2.3) and was able to positively amplify the sample in less than 20 minutes using the Genie III real-time amplification instrument (not including DNA extraction). While FON6L could amplify as fast as 10 minutes after heating, it could detect a concentration as low as 1.0 pg which was as sensitive as the FONSIX6 PCR assay (Figure 2.4). The cost per reaction was slightly higher than conventional PCR but has potential field applications and was significantly faster. Both primer sets were tested using a temperature gradient to determine the optimal temperature which was determined to be 66°C for FONL1 and 65°C for FON6L (Figure 2.3&2.4). FON6L is not specific to FON, as it amplifies samples that contain the *SIX6* gene which includes *F. oxysporum* f. sp. *lycopersici*, so both primer sets must be used in unison to confirm first the specific identity as FON.

These LAMP assays were done in triplicate and used multiple methods of visualization to confirm the results and test the possibility of a field application. Both assays can be adapted for on site or in field specific diagnosis of FON, provided a DNA extraction method that is also field ready. LAMP assays generally have become more popular recently for the increased speed and sensitivity compared to the more common conventional PCR and these assays have the potential to increase the ability of growers and laboratories to diagnose FON going forward.

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Table 2.1. Comparison of molecular detection methods

Method	Time	Cost per sample	Sensitivity	Specialized equipment	Lab facility needed
PCR	2 hrs	$5.00	0.01 ng	Yes	Yes
qPCR	1.5 hrs	$7.00	0.1 pg	Yes	Yes
LAMP	0.5-1 hrs	$6.00	0.1 pg	No	No

Table 2.2. Primers used in this study

Assay	Primers	Sequence 5'-3'	Source
PCR	Fon-1	CGATTAGCGAAGACATTCACAAGACT	Lin et al.,
	Fon-2	ACGGTCAAGAAGATGCAGGGTAAAGGT	2010
FONL1	FONL1-F3	CGATTAGCGAAGACATTCA	This study
LAMP	FONL1-B3	ACCTTTACCCTGCATCTTCTTGACCGT	
	FONL1-FIP	CTGGGATACAGACGTGAAGTAGATTTT	
		CAAGACTAATGATGTCATG	
	FONL1-BIP	AACATATCATAAAGGGCCATCAAGTTT	
		TTCTAGGTGCGGCAGTAAATCCA	
	FONL1-LF	AGTAATGGATTTACTGCCGCAC	
FON6L	FON6L-F3	TGAAGCTCGCTCTTATCGCATCAATCT	This study
LAMP	FON6L-B3	GGAGCGGTCATAGGTCTGT	
	FON6L-FIP	CATCAGCAGATTCGGGTTCGGTTTTTAT	
		CAATCTTGGCTGCCGG	
	FON6L-BIP	TTGAACCACCGAAGGCGGATTTTTCTG	
		GACAGTTCGTGACGG	
	FON6L-LF	AAGGGGACCAGCTACGCAG	
	FON6L-LB	TATCATCTTTGGTCTCTCGAGACA	

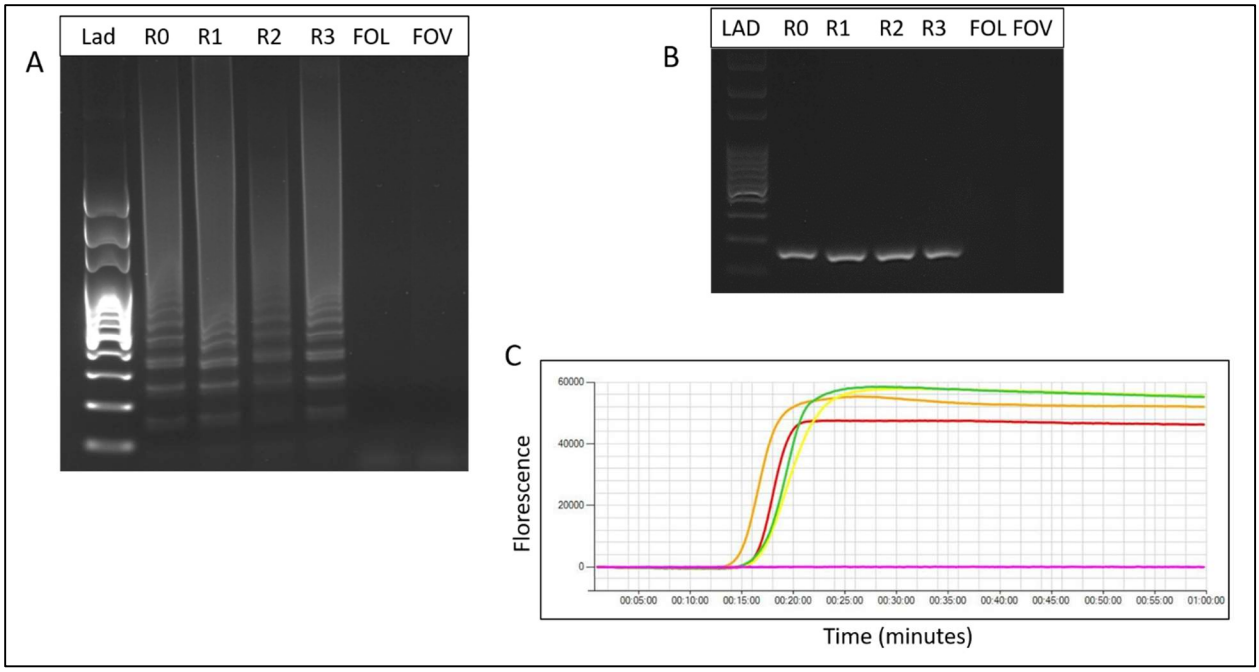

Figure 2.1. FONL1 assay results. A. Race specific LAMP amplification of all FON races, *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) were negative. B. PCR primer set Fon-1/Fon-2 amplification of the same samples. C. Genie III real-time amplification of FON races (R0: Red, R1: Orange, R2: Yellow, R3: Green, FOL: Pink).

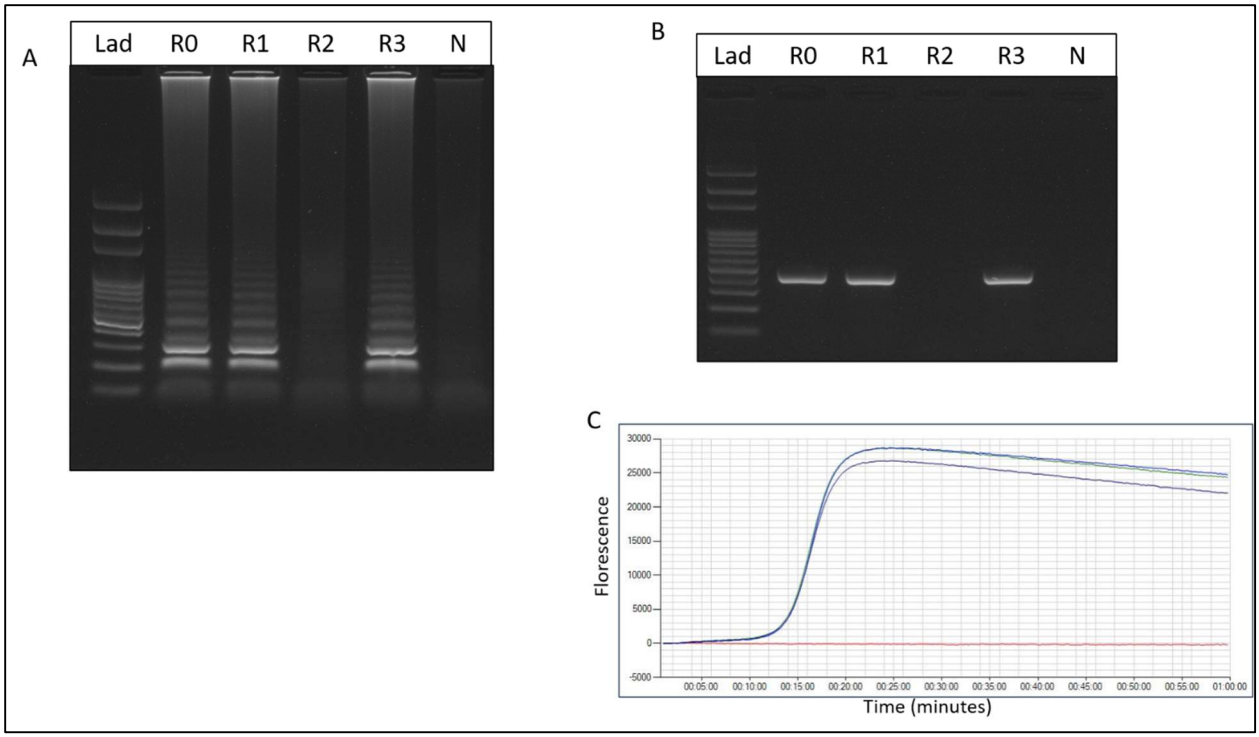

Figure 2.2. FON6L assay results. A. LAMP results of FON6L on races 0, 1, 2, and 3. B. FONSIX6 PCR results on races 0, 1, 2, and 3. C. Real-time amplification of FON6L. Blue = race 0, Purple = race 1, Red = race 2, and Green = race 3.

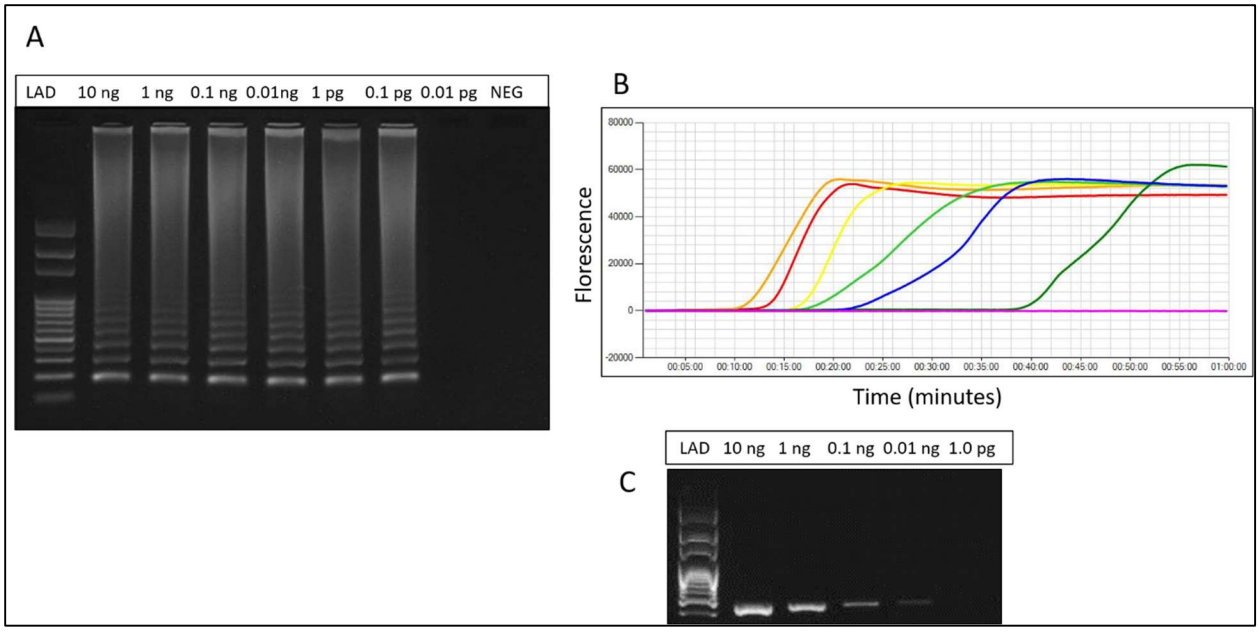

Figure 2.3. FONL1 sensitivity determination and comparison with PCR. A. FONL1 LAMP amplification of FON race 1serial dilution starting at 10 nanograms of genomic DNA. Lowest level of detection is 0.1 picograms of DNA. B. Genie III real-time amplification graph of serial dilution (Red: 10 ng, Orange 1.0 ng, Yellow 0.1 ng, Light Green 0.01 ng, Blue 1.0 pg, Dark Green 0.1 pg, Pink 0.01 pg, Purple negative (not seen below pink). C. PCR amplification of serial dilution of FON genomic DNA, the lowest PCR amplification level was 0.01 ng of DNA.

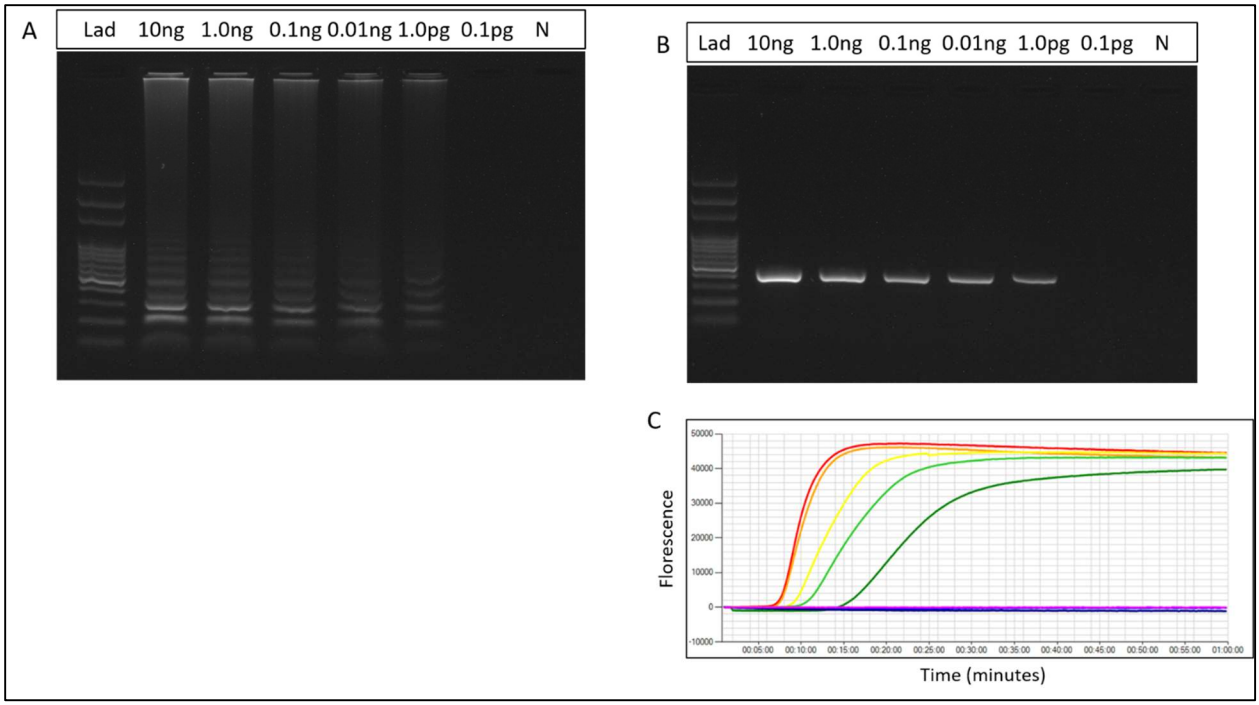

Figure 2.4. FON6L sensitivity determination and comparison with PCR. A. FONL1 LAMP amplification of FON race 1 serial dilution starting at 10 nanograms of genomic DNA. Lowest level of detection is 1.0 picograms of DNA. B. FONSIX6 PCR sensitivity with the same serial dilution starting at 10 ng of DNA. C. Genie III real-time amplification of FON6L. Red = 10 ng, orange = 1.0 ng, yellow = 0.1 ng, light green = 0.01 ng, dark green = 1.0 pg, and blue = 0.1 ng. Pink = negative.

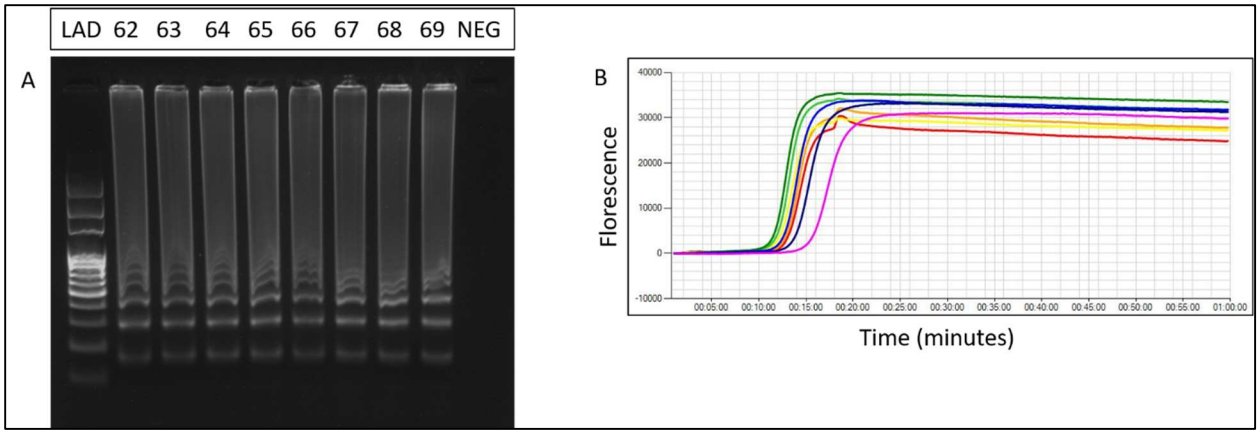

Figure 2.5. Optimization of temperature for FONL1. A. Gel image of temperature optimization of FONL1 LAMP primer set from 62°C to 69°C. There was no difference between any of the samples. B. Genie III real-time amplification graph of temperature optimization. Red: 62°C, Orange: 63°C, Yellow: 64°C, Light Green: 65°C, Dark Green: 66°C, Blue: 67°C, Purple: 68°C, Pink: 69°C. 66°C (Dark green) was the fastest to amplify and had the highest florescence level.

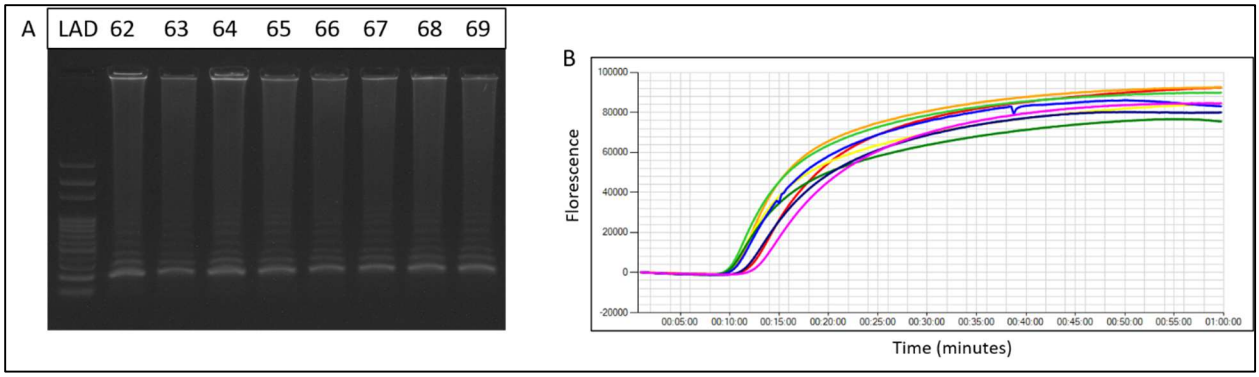

Figure 2.6. Optimization of temperature for FON6L. A. Gel image of temperature optimization from 62°C to 69°C. B. Real-time amplification of FON6L using the Genie III amplification instrument. Red = 62°C, Yellow = 63°C, Orange = 64°C, Light Green = 65°C, Dark Green = 66°C, Blue = 67°C, Purple = 68°C, Pink = 69°C.

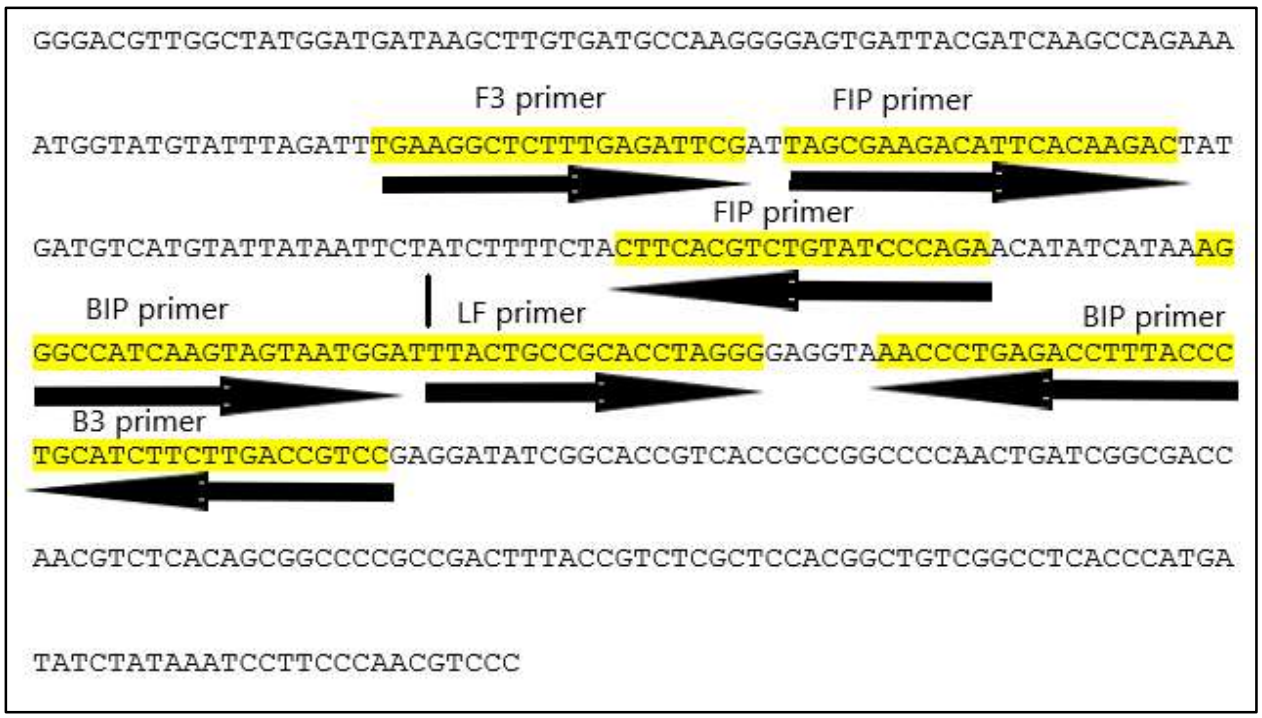

Figure 2.7. FONL1 primer design graphic. Highlighted regions specify nucleotides used for sequencing and arrows point to the direction in which primers were synthesized. Arrows pointing backward were reverse complimented before synthesis. Between BIP and LF primers, a black vertical line divides the two primers where highlighting did not specify the change. For specific sequences see Table 2.2.

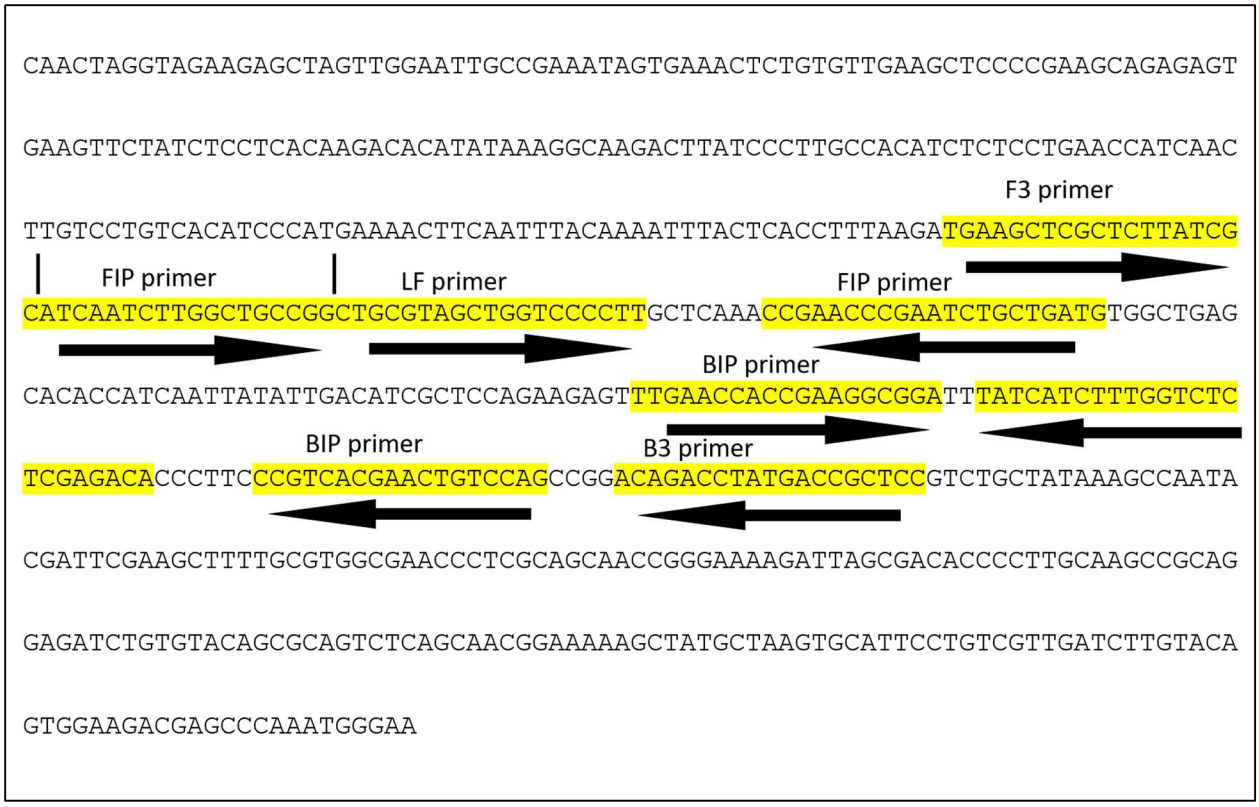

Figure 2.8. FON6L primer design graphic. Highlighted regions specify nucleotides used for sequencing and arrows point to the direction in which primers were synthesized. Arrows pointing backward were reverse complimented before synthesis. Between F3/FIP and FIP/LF primers, a black vertical line divides the primers where highlighting did not specify the change. For specific sequences see Table 2.2.

CHAPTER 3

DRAFT GENOME SEQUENCES OF THREE *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* ISOLATES USED IN DESIGNING MARKERS FOR RACE DIFFERENTIATION[2]

[2]Hudson, O., Hudson, D., Ji, P., & Ali, M. E. 2020. Accepted by *Microbiology Resource Announcements*. Reprinted here with permission of the publisher, American Society for Microbiology.

ABSTRACT

Here, we report the draft genome sequences of three *Fusarium oxysporum* f. sp. *niveum* isolates that were used to design markers for molecular race differentiation. The isolates were collected from watermelon fields in Georgia (USA) and were determined to be different races of *F. oxysporum* f. sp. *niveum* using a traditional bioassay.

ANNOUNCEMENT

Fusarium wilt in watermelon is caused by the fungal pathogen *Fusarium oxysporum* f. sp. *niveum*, which is one of the most impactful pathogens for watermelon production worldwide (https://edis.ifas.ufl.edu/PP352) (1–3). Three isolates of *F. oxysporum* f. sp. *niveum* were obtained from infected watermelon plants (*Citrullus lanatus*) with typical symptoms of *Fusarium* wilt in commercial fields in Georgia. Traditional diagnostic methods are unable to identify the pathogen as *F. oxysporum* f. sp. *niveum* with 100% confidence, so molecular assays are required to confirm the identity of an isolate (4–6). Additionally, it is currently recognized that *F. oxysporum* f. sp. *niveum* has 4 races (R0, R1, R2, and R3) which require a bioassay for differentiation, as at the date of this writing, only race 2 may be distinguished molecularly (https://edis.ifas.ufl.edu/PP352) (1, 3, 7).

The three isolates that were chosen for sequencing were isolated from the lower stem (hypocotyl region) of diseased watermelon plants. Samples were surface-disinfested in 0.6% NaOCl, rinsed in sterile distilled water, and cultured on semi selective peptone pentachloronitrobenzene agar plates (8). Fungal cultures grown from the samples were identified based on the morphological characteristics, and single-spore isolates were grown on potato dextrose agar (PDA) plates and incubated at 25°C for 7 days. The races of the isolates were identified by inoculating differential watermelon plants and evaluating the disease development as reported previously (5, 7). Three isolates, representing three races of *F. oxysporum* f. sp. *niveum*, were grown on PDA for 10 days; next, 100 mg of mycelia was used for DNA extraction. DNA was extracted using the DNeasy plant mini kit (Qiagen) and was concentrated and purified using Quantum Prep PCR Kleen Spin columns (Bio-Rad) according to the manufacturer's instructions. PCR was performed on all isolates using Fon-1/Fon-2, primers

specific to *F. oxysporum* f. sp. *niveum*, to confirm the identity of all *F. oxysporum* f. sp. *niveum* isolates, and the race 2-specific primer set FON*SIX*6F/R to confirm the race 2 isolate (9, 10). DNA was standardized at 200 ng/µL for each extraction and submitted to Novogene Co., Ltd. (Beijing, China) for whole-genome sequencing.

Libraries were prepped using an NEB Ultra II kit and sequenced using the paired-end strategy PE150 on an Illumina NovaSeq 6000 platform. The original optic data obtained by high-throughput sequencing were transformed into raw sequenced reads using Casava v.1.8 base calling and stored in FASTQ (fq) format. Quality control was performed using readfq v.10 (https://github.com/lh3/readfq) using default parameters, and low-quality sequences were removed (11, 12). Raw reads were assembled into scaffolds using SPAdes v.3.14.1 with the k-mer values 21, 33, 55, and 77 (13). Bowtie 2 v.2.4.1 was used to align the paired-end reads against the scaffolds produced by SPAdes, producing the SAM alignment files (14). SAMtools v.1.10 was used to convert the alignment files to BAM format and then sort and index the BAM files. Pilon v.1.23 (using - -frags mode) was used to polish the BAM files, yielding the final output of FASTA files (15, 16). Contigs shorter than 200 bp were removed from polished FASTA files using a novel Python script for contig filtration (17). The genome characteristics and accession numbers are given in Table 3.1.

DATA AVAILABILITY

All data for this whole-genome sequencing project were deposited under the GenBank BioProject accession number PRJNA656528. The raw reads of the genomic data for the isolates were deposited under the following SRA accession numbers: SRR12492378, SRR12492379,

and SRR12492380. The accession numbers for each isolate are as follows: SAMN15791673, SAMN15791674, and SAMN15791675.

ACKNOWLEDGEMENTS

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Table 3.1. FON assembly data. Summarized genome assembly data and accession numbers for sequenced *Fusarium oxysporum* f. sp. *niveum* (FON) isolates

Isolate	Genome size (bp)	No. of Contigs> 50000	N_{50} (bp)	Average coverage	G+C content	GenBank Biosample no.	GenBank Project no.	SRR accession no.
FON1	61207430	245	154443	28.65x	48.79	SAMN15791673	PRJNA656528	SRR12492378
FON2	54074873	217	161737	22.92x	47.53	SAMN15791674	PRJNA656528	SRR12492379
FON3	55220015	220	158709	22.38x	47.54	SAMN15791675	PRJNA656528	SRR12492380

CHAPTER 4

MARKER DEVELOPMENT FOR DIFFERENTIATION OF *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* RACE 3 FROM RACES 1 AND 2[3]

[3] Hudson, O., Waliullah, S., Fulton, J. C., Ji, P., Dufault, N. S., Keinath, A., & Ali, M. E. 2021. Accepted by the International Journal of Molecular Sciences. Reprinted here with permission from the publisher, the Multidisciplinary Digital Publishing Institute.

ABSTRACT

Fusarium wilt of watermelon, caused by *Fusarium oxysporum* f. sp. *niveum* (FON), is pathogenic only to watermelon and has become one of the main limiting factors in watermelon production internationally. Detection methods for this pathogen are limited, with few published molecular assays available to differentiate FON from other formae speciales of *F. oxysporum*. FON has four known races that vary in virulence but are difficult and costly to differentiate using traditional inoculation methods and only race 2 can be differentiated molecularly. In this study, genomic and chromosomal comparisons facilitated the development of a conventional polymerase chain reaction (PCR) assay that could differentiate race 3 from races 1 and 2, and by using two other published PCR markers in unison with the new marker, the three races could be differentiated. The new PCR marker, FNR3-F/FNR3-R, amplified a 511 bp region on the "pathogenicity chromosome" of the FON genome that is absent in race 3. FNR3-F/FNR3-R detected genomic DNA down to 2.0 pg/µL. This marker, along with two previously published FON markers, was successfully applied to test over 160 pathogenic FON isolates from Florida, Georgia, and South Carolina. Together, these three FON primer sets worked well for differentiating races 1, 2, and 3 of FON. For each marker, a greater proportion (60 to 90%) of molecular results agreed with the traditional bioassay method of race differentiation compared to those that did not. The new PCR marker should be useful to differentiate FON races and improve Fusarium wilt research.

INTRODUCTION

Fusarium oxysporum f. sp. *niveum* (FON) is the causal agent of Fusarium wilt of watermelon and a common limiting factor for watermelon production worldwide [1,2,3,4,5]. FON is a soilborne ascomycete fungus and one of at least 106 formae speciales within the *Fusarium oxysporum* species complex [6]. As with all formae speciales of *F. oxysporum*, FON has three types of spores: single-celled microconidia, multi-celled macroconidia, and over-wintering chlamydospores. The long-lasting survivability of chlamydospores demonstrates the challenge of combating this pathogen using crop rotation and chemical fumigants [5,7,8,9]. In addition, diagnosis based on spore morphology cannot be done to the forma specialis level due to the likeness of spores between formae speciales and other *Fusarium* species [10,11,12].

Fusarium wilt can be identified in the field by observing a single side or leader of the watermelon plant wilting with the rest of the plant being unaffected [13]. Wilting is due to the accumulation of microconidia in the plant's xylem tissue and the defense response of the plant to form tyloses, a mechanism by which the plant suppresses fungal growth [5,14,15]. The vasculature of the watermelon plant turns red/brown before the infection spreads to the rest of the plant which is a key diagnostic feature in the field [16,17,18,19]. Often, FON infections occur early in the growth stage and cause damping-off of seedlings, particularly when grown in the presence of nematodes whose damage from feeding may allow for the ingress of the pathogen [20,21]. Symptoms may vary (variable wilting, chlorosis, necrosis, and damping off), complicating the diagnosis of the disease. Variation is due to a number of factors such as environmental conditions, the concentration of pathogenic propagules, the race of the pathogen, age of the plant, and watermelon cultivar [1,14,22,23].

Although FON is a soilborne pathogen, it is internationally widespread with regions of historic watermelon production seeing the highest disease pressure [19,24,25]. Growers in the United States, particularly in Delaware, Florida, Georgia, Maryland, South Carolina, and Texas, continue to have difficulties controlling this disease with resistant cultivars [1,5,16,18,26,27,28]. Unfortunately, many previously resistant cultivars have become unsustainable as new populations of FON have emerged which can overcome the plant host resistance. These newly resistant populations have been designated as races that describe the unique interaction between pathogen and host [2,29,30]. While FON's pathogenicity is limited to watermelon, four races of the pathogen are currently identified, race 0, race 1, race 2, and race 3, each subsequent race being more virulent with wider pathogenicity with race 3 having the largest cultivar range [1,4,29,30,31,32,33]. The first two races were described in 1963 when the newly pathogenic race 1 was discovered in Florida, dividing FON into races 0 and 1 [14]. Since that time, the other two races emerged in the United States: race 2 in 1981 in TX [14,34] and race 3 in 2009 in MD [1,14]. Race 2 is aggressive on all commercial watermelon cultivars but not the PI line (PI-296341-FR), and race 3 is aggressive on all watermelon cultivars and PI lines. Until recently, it was believed that race 1 was the most widespread race, race 2 less well distributed, and race 3 limited to a very small geographic area. As of today, race 3 has been detected in 3 states: FL [31], GA [32], and MD [1],. Recent survey studies in SC, GA, and FL have shown race 2 to be widespread, and in the case of GA, race 3 is also widespread, demonstrating the previously unknown dissemination of these highly aggressive races of FON [18,32].

Molecular detection methods have been developed for the differentiation of FON from other closely related formae speciales in the *F. oxysporum* species complex, as traditional methods of morphological identification cannot identify the pathogen beyond the species level [5,7,14,19,25].

Race differentiation, however, is limited to a bioassay, which is a lengthy, expensive, and inaccurate method. Seedlings of multiple cultivars with different levels of susceptibility are grown and inoculated with the pathogen and disease development is then scored. Based on the average score, the pathogen is given a race determination. This method takes weeks to grow plants and evaluate disease, the seeds of certain cultivars are difficult to source, and the experimental and greenhouse conditions (e.g., temperature, humidity, spore concentration, etc.) can have a significant effect on the level of pathogen virulence leading to an incorrect result [1,5,14,35,36]. In addition, a single race may have isolates that have vastly differing virulence on a single cultivar, not only due to the aforementioned experimental conditions but also possibly due to other unknown molecular mechanisms. Multiple isolates of race 1 can infect susceptible cultivars at disease levels ranging from 5 to 100% [37]. Thus, races are difficult to determine correctly based on phenotypic observations. These shortcomings demonstrate the necessity for a molecular approach for differentiation of FON races.

Niu et al. (2016) reported that on chromosome 14 of FON (also known as the pathogenicity chromosome), race 2 isolates lack the avirulence gene known as "*secreted in xylem protein 6*" (*SIX6*). By using polymerase chain reaction (PCR) primers specific to *SIX6*, race 2 could be differentiated molecularly from races 0 and 1. Race 3 isolates were not tested by Niu et al., however, upon whole-genome analysis, *AVRSIX6* was identified in the race 3 isolate, showing that the *AVRSIX6* primer could differentiate race 2 isolates from all other currently recognized races.

The primary objective of this study was to develop a molecular method of differentiating FON race 3 isolates from race 1 and race 2 isolates to identify this new aggressive race rapidly and accurately. A comparison of the pathogenicity chromosome from published whole-genome sequences by Hudson et al. (2020) was used to find conserved and unique regions that could be

used to design markers for race 3 specific amplification [38]. In addition, a FON race-differentiation protocol was developed by using the new PCR-based race 3 marker along with two other previously published FON-specific PCR markers, which was validated in race identification of 161 FON isolates collected from Florida, Georgia, and South Carolina.

MATERIALS AND METHODS

FON Isolates and DNA Extraction. FON isolates were gathered from laboratories of Ji, Dufault, and Keinath at the University of Georgia, University of Florida, and Clemson University, respectively (Table A.2). Many of the isolates were previously identified to races using greenhouse bioassay by the respective labs. No bioassay testing was done in this study. The isolates were grown on potato dextrose agar (PDA) plates at 25°C for seven days, and fungal tissue (100 mg) was scraped into a 1.5-mL safe lock tube (Eppendorf Canada Ltd., Mississauga, ON, Canada) with steel beads for homogenization. Samples were homogenized in the FastPrep FP120 cell disruptor (Qbiogene, Carlsbad, CA, USA) for 30 s at speed 5, twice, or until there were no large pieces of mycelia. DNA was extracted using the DNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA was purified using Quantum Prep PCR Kleen Spin Columns (BIO-RAD, Hercules, CA, USA). Total DNA yield and purity were estimated by measuring OD at 260 nm and 260/280 nm with a NanoDrop LITE (Thermo Scientific, Waltham, MA, USA).

Sequence Alignment and Primer Design. Genomic sequences of FON isolates (BioProject number: PRJNA656528 and accession numbers SAMN15791673, SAMN15791674, and SAMN15791675) and the reference genome of FOL 4287 (NCBI: txid426428) were obtained from NCBI [38,49]. Sequences were aligned and visualized using the Interactive Genomics Viewer (IGV) (2013-2018 Broad institute and the Regents of the University of

California). Targets for primer design were searched on chromosome 14 (NC_030999.1), as it contained previously targeted gene candidates for race differentiation [40]. Regions absent in race 3 but present in races 1 and 2 were targeted specifically [40]. Using this approach, 21 different loci were isolated as candidates for differentiation based on their absence in the race 3 genome. To narrow the possible pool of target loci, BLAST was used to determine the likelihood of genetic conservation within the genome based on shared sequences with related species. In addition, sequences containing coding regions and hypothetical proteins were targeted specifically for primer design. After using BLAST, a further narrowing of candidates was done based on primer design properties such as GC content analysis and amplicon size. Consequently, seven primer pairs were then designed manually and checked for quality and content using the Integrated DNA Technologies PrimerQuest Tool. All primers were synthesized by Sigma Aldrich (St. Louis, MO, USA) and stored at −20°C. All primers are listed in Table 4.2.

Selection of Race-Specific Diagnostic Marker Primer Set and PCR Conditions. To select a marker that specifically amplified races 1 and 2 but not race 3, all primer sets were tested against the three isolates sequenced by Hudson et al. (2020), then on a larger pool of isolates from multiple states (20 from each state) [38]. Primers with weak signals, strong dimers, or double bands were removed. Finally, two primer sets remained and were used to amplify all FON isolate DNA. From these PCR tests, a final primer set was chosen after showing clear and consistent amplification of target isolates. The final primer set was a result of targeting an 1121-bp region in chromosome 14 that was identical in races 1 and 2, and entirely absent in race 3 (Figure 4.1). The primer, named FNR3-F/FNR3-R, amplified a 511 bp (555 bp in the FOL reference) region of the larger 1121-bp region that contained multiple hypothetical proteins depending on the codon frame viewed. PCR reactions were executed on a thermal cycler

(Biorad-96 well T100™, Bio-rad, Hercules, CA, USA) using EconoTaq® PLUS Green 2X Master Mix (Lucigen, Middleton, WI, USA) which included the following components: EconoTaq® PLUS Green 2X Master Mix (12.5 µL), forward primer (0.3 µM), reverse primer (0.3 µM), target DNA (1 µL), and ddH_2O to total 25 µL per reaction. PCR products (4 µL per sample) were run on a 1% agarose gel stained with SYBR safe stain (Invitrogen, Waltham, MA, USA) and then imaged on a UV gel doc (Analytik Jena, Jena, Germany). PCR was performed using the following conditions: denaturation at 95°C for 3 min followed by 35 cycles of amplification at 95°C for 30 s, annealing at 63°C for 30 s and 72°C for 40 s, and terminated by a final elongation at 72°C for 6 min.

Optimization of the Developed Marker. The new primer set was tested to determine the optimal annealing temperature, the level of specificity, and the detection limit of genomic DNA. To optimize the annealing temperature a gradient PCR was run from 60 to 70.5°C and the highest temperature was selected that did not diminish the brightness of the amplicon. To analyze the specificity, the FNR3-F/FNR3-R primer set was tested with a range of non-FON isolates. PCR samples were made to the same concentrations as described above with approximately 50 ng of DNA per reaction. A positive FON control and a negative water control were included with the non-target samples. Other pathogens tested were as follows: *Phytophthora capsici*, *Phytophthora sojae*, *Pseudoperonospora cubensis*, *Cucurbit leaf crumple virus*, *Rhizoctonia solani*, *Colletotrichum orbiculare*, *Fusarium solani*, *F. oxysporum* f. sp. *vasinfectum*, and *F. oxysporum* f. sp. *lycopersici*. To test the sensitivity levels of the newly designed PCR marker, the genomic DNA extracted from the race 1 standard isolate was standardized to 20 ng/µL, then underwent a tenfold serial dilution down to 0.2 pg/µL of DNA. These dilutions were amplified using the new primer set FNR3-F/FNR3-R to determine the

minimum sensitivity. PCR results (4 µL per sample) were imaged on a 1% agarose gel and the lowest successful amplification was determined to be the detection limit of the new primer set.

Development and Application of a Protocol for Race Differentiation. A protocol with three PCR primer sets was used to amplify and determine races of the FON isolates (Figure 4.2). The first primer set (Fon-1/Fon-2) was used to amplify FON isolates specifically, with no other pathogenic *F. oxysporum* formae speciales being amplified [19]. The second primer set (FON*SIX6*-F/ FON*SIX6*-R) was used to determine race 2 isolates based on the absence of the *SIX6* gene [3]. The final primer set "FNR3-F/FNR3-R" was developed in this study and selectively amplifies races 1 and 2 and has no amplicon for race 3 isolates. Two hundred one FON isolates from various field locations and labs in Florida, Georgia, and South Carolina were tested using the new protocol with the three primer sets to establish their identity: Fon-1/Fon-2 to confirm their identity as FON, FON*SIX6*F/FON*SIX6*R to differentiate race 2 from races 1 and 3, and FNR3-F/FNR3-R to differentiate race 3 isolates.

RESULTS

Sequence Analysis and Primer Design. Genomic analysis of the three FON races used in this research revealed that the pathogenicity chromosome yielded several regions that were absent in race 3 and present in races 1 and 2. After testing the race standards in a larger pool of isolates, a single primer set was chosen (FNR3-F/FNR3-R) that amplified a 511-bp region of a larger region (1121 bp) absent in race 3 (Figure 4.1). This region also was chosen because of BLAST results identifying high homology (>90% identity) with a hypothetical protein from an *F. oxysporum* f. sp. *lycopersici* sequence (accession: XM_018387901), a *F. odoratissimum* (formerly *F. oxysporum* f. sp. *cubense*) sequence (accession: XM_031202915), and an unnamed *F. oxysporum* isolate from Australia (accession CP053262) [39,40]. Within the

1121-bp region absent in race 3 isolates, two separate open reading frames (ORFs) consisting of 74 and 62 amino acids are present as predicted by ORFfinder from the National Center for Biotechnology Information (NCBI) and the Interactive Genomic viewer (IGV).

Specificity and Sensitivity of the Race 3 Marker. The race 3-specific marker, FNR3-F/FNR3-R, was optimized and tested for specificity and sensitivity (Figure 4.3). The specificity of the new marker was determined by testing additional cucurbit pathogens as well as other fungal and oomycete isolates: *Phytophthora capsici*, *Phytophthora sojae*, *Pseudoperonospora cubensis*, *Cucurbit leaf crumple virus*, *Rhizoctonia solani*, *Colletotrichum orbiculare*, *Fusarium solani*, *F. oxysporum* f. sp. *vasinfectum*, and *F. oxysporum* f. sp. *lycopersici*.

Besides FON races 1 and 2, only *F. oxysporum* f. sp. *lycopersici* (FOL) was amplified using the same primer set and conditions. However, a slightly larger band size (555 bp) was amplified in FOL due to the differences in the individual base pairs of the FOL vs. FON amplicons (Figure 4.3A). To determine the sensitivity of the new marker, a serial dilution of the race 1 standard isolate was made from 20 ng/µL to 0.02 pg/µL and tested using the same PCR conditions. PCR results demonstrated the lowest successful amplification occurring at 2.0 pg/µL of genomic DNA (Figure 4.3B). The annealing temperature was determined to be optimized at 63°C, showing the highest temperature with equally bright amplicons as lower temperatures (Figure 4.3C).

Development of a Protocol for Race Differentiation. A protocol was developed by using the new marker FNR3-F/FNR3-R in concert with the previously published Fon-1/Fon-2 and FON*SIX*6F/FON*SIX*6R markers, and differentiation of races 1, 2, and 3 from each other is possible by running them subsequently, as shown in Figure 4.2A. After DNA is isolated, samples are first confirmed with Fon-1/Fon-2 primers for their identity as FON isolates. Primer set

FON*SIX6*F/FON*SIX6*R is then run to determine if the isolate is race 2 based on the absence of the amplicon. If there is an amplicon, samples are then amplified using FNR3-F/FNR3-R primers to determine if the isolate is race 1 or race 3, based on the absence (race 3) or presence (race 1) of the amplicon. This process is demonstrated in Figure 4.2C with 10 isolates of unknown identity to show what possible reactions can occur. Based on the results of these 10 isolates, two were identified as race 2 (US-2, 5) shown by a positive reaction with Fon-1/Fon-2 and FNR3-F/FNR3-R, but a negative reaction for FON*SIX*6F/R; two were identified as race 3 (US-8, 9) shown by a positive reaction for Fon-1/Fon-2, positive for FON*SIX*6F/R, and negative for FNR3-F/FNR3-R, and six were identified as race 1 (US-1, 3, 4, 6, 7, 10) as all reactions were positive

Race Distribution of Experimental Samples. Two hundred one FON isolates from various field locations and labs in three states in the southeastern USA (FL, GA, and SC) were tested using the newly developed race differentiation protocol to establish their identity. FON samples that showed signs of contamination of any marker based on the gel images were removed or re-extracted and retested. All isolates were run with all markers in triplicate to confirm results.

The method of determining race 1 isolates came only after using all the primer sets, as only race 1 isolates would show positive amplification from all three primer sets, whereas the other two races would be determined by their absence in amplification for each respective marker. After the removal of race 0 isolates identified by bioassays and non-FON samples (negative for Fon-1/Fon-2), 161 isolates remained and are presented with their PCR results and resulting race determination (Table A.1). The race distribution of all isolates tested, regardless of state was: 53% race 1, 25% race 2, and 22% race 3. Of the 28 GA isolates, 50% (14) were race 1, 25% (7) were race 2, and 25% (7) were race 3. Of the 85 SC isolates, 44.7% (38) were race 1,

28.24% (24) were race 2, and 27.06% (23) were race 3. Of the 48 FL isolates, 66.6% (32) were race 1, 20.8% (10) were race 2, and 12.5% (6) were race 3 (Figure 4.4). Additionally, isolates race-typed using the bioassay were compared to molecular assay results. Of those isolates, 26 (89.65%) for race 1 matched between the assays, 33 (80.49%) for race 2, and 14 (60.87%) for race 3 (Table 4.1).

DISCUSSION

The concept of races in various pathosystems is established, however, the requirement of a specific avirulence gene being identified as necessary for pathogenicity in order to confirm a race has been a recent addition [41,42,43]. This confirmation-by-correlation of *AVR* gene(s) to race has not been established in *Fusarium oxysporum* f. sp. *niveum*, as no genes have been confirmed to confer pathogenicity by being transferred to a less-virulent strain via horizontal chromosome transfer, so a more general approach is needed for race identification. Due to this, the bioassay that is used for FON race differentiation has a number of problems. First, experimental conditions (temperature, humidity, soil type, etc.) of the bioassay are difficult to standardize from lab to lab, not to mention from season to season. Second, isolates of a given race can change in virulence over time (perhaps due to other conditions such as long-term storage) and the scoring method to determine race, of which there are multiple, relies on the virulence level of an isolate [44,45,46]. Third, the cultivars used have discrepancies in the literature and because seeds of some cultivars are difficult to source, others must be substituted. Some researchers claim a range of resistance among seven cultivars, others use only four or five cultivars with a distinct delineation between their reactions, and still others substitute certain cultivars for others possibly changing the level of resistance again [4,10,47,48]. To confirm these inaccuracies between methodologies, copies of a single isolate were sent to multiple labs with access to the bioassay and different race results were

returned. The genetic variability within a single race, or from isolate to isolate, is a known complexity in the *Fusarium oxysporum* species complex, characterized well by the lack of consistency when testing isolates with the traditional bioassay. Factors such as mobile pathogenicity chromosomes and horizontal chromosome transfer have been seen in other formae speciales and shed some light as to why certain markers do not remain successful over time, or in distinct geographic locations. Horizontal chromosome transfer presents the largest problem when maintaining consistency in evaluating virulence of isolates, as both the transfer from one forma specialis to another and from one *Fusarium* species to another have been documented [40,49,50,51].

As traditional methods for identification within the *F. oxysporum* species complex are known to be inadequate, molecular methods are necessary for accurate identification. Races complicate the process of molecular differentiation due to the highly conserved genomic content that they share (often < 1%) [32,42,52,53,54]. This can be seen through sequencing of traditional molecular marker genes such as *internal transcribed spacer* (*ITS*), heat shock proteins (HSP), *β-tubulin*, *intergenic spacer* (*IGS*), and *Cytochrome c oxidase* (*COX*), all of which were determined to have 100% conservation across all races sequenced. In addition, *F. oxysporum* is known to have the ability to transfer genes and chromosomes horizontally, both genes related and unrelated to pathogenicity [49,55,56,57]. As a result, a comparative genomics approach was used to identify genetic regions that would allow for consistent differentiation of race 3 from the other two races of economic impact: 1 and 2, which are currently increasing in presence across the world [1,24,31,58,59,60]. No race 0 isolate was available for analysis or comparison. The whole-genome sequencing results were published previously by Hudson et al. (2020) which contain more analysis and details of the WGS data [38]. It was noted that during testing, race 0 isolates identified by the

bioassay were most commonly negative for FON*SIX6*F/R, signifying race 2 as the identity, but other race 0 isolates had variable results including negative for both Fon-1/Fon-2 and FNR3 primer sets. Additional isolates tested in the bioassay were nonpathogenic on susceptible cultivars and pathogenic on the resistant PI line which did not allow for proper race differentiation (J. Fulton, personal communication). The variation of isolate pathogenicity and race 0 variability underlines the necessity for additional genes to be sequenced and correlated closely with pathogenicity.

In an attempt to neutralize some of the variations from the bioassay results, several steps were made to confirm that the genomic sequences of each race were accurate to the claimed race and the region in the genome chosen for marker development would be conserved. As the key importance between races is differential pathogenicity, and due to a high concentration of SNPs and InDels, we focused on chromosome 14, where previous studies on FON and other *F. oxysporum* formae speciales had identified avirulence genes [37,40,49,61,62]. Other chromosomes (3, 6, and 15) are additionally known to be involved in pathogenicity but were not used as fewer genetic changes were seen [14]. The primary group of these avirulence genes is the "secreted in xylem" or *SIX* genes [57,63,64]. One such gene, *SIX6*, was developed previously and used in this study to differentiate race 2 isolates based on the absence of *SIX6* [3,65,66]. The identity of the race 2 whole genome sequence was additionally confirmed through chromosomal analysis to lack the *SIX6* gene region, implying that the race 2 isolate was consistent with previous research. This method of genomic confirmation gave better confidence to molecular results as they correlate with race differentiation; both race 1 and race 3 have identical copies of *SIX6*, also suggesting separate origins for races 2 and 3.

In this study, the FNR3 marker was developed using comparative genomics in an attempt to provide stable locations in the FON genome that will rapidly determine a FON isolate as a highly

virulent one, currently characterized as race 3. This marker was designed based on a unique region on the "pathogenicity chromosome" of the FON genome, absent in race 3 but present in races 1 and 2. This marker was found to be effective to differentiate race 3 from other races and no false negatives or false positives were observed during the validation with other phytopathogens except *F. oxysporum* f. sp. *lycopersici* (FOL) (Figure 4.3). The sensitivity of this assay revealed the detection limit of the primer set to be 2.0 pg/μL and the optimized annealing temperature was 63 °C for 30 s (Figure 4.3). In order to determine the race (1, 2, or 3) of an isolate that is pathogenic on the least resistant cultivar, a protocol was developed that outlines all possible results from testing a FON isolate with the three primer sets (Figure 4.2A). Multiplex PCR was attempted for this study, but consistent results could not be obtained. It was probably because cycle conditions differed too greatly, or inhibition due to multiple PCR primers occurred. It is important to note that race 1 isolates must receive a positive reaction from all three primer sets to confirm race 1 as the identity, but the initial Fon-1/Fon-2 primer set is required for all FON identifications (Figure 4.2).

After race typing, isolates were rearranged to reflect their geographic state of origin and assessed on that basis. In this study, the races of available isolates we identified were as follows for all three states combined: race 1: 53.4%, race 2: 24.84%, and race 3: 21.74% (Figure 4). Of the three states, only two had previously reported the presence of race 3 FON isolates (Florida and Georgia) but South Carolina had not [1,18,31,32]. According to the molecular test results, this would be the first time race 3 has been detected in SC, however, previous studies have addressed the lack of a race 3 phenotype in SC based on testing of a number of cultivars of watermelon. While isolates and locations were not sampled randomly as in a survey, GA isolates differed in percentages from previously reported studies in which race 3 had been the most common race, instead of race 1 in this study [32]. The percent correlation (%) of race differentiation between

bioassay vs. molecular assay was determined based on a sample of 93 isolates that were tested for race determination by the two methods (Table A.2). The percentage (%) of molecular results agree with the bioassay results of race 1, 2, and 3 at 89.6%, 80.5%, and 60.9%, respectively (Table 4.1). A significant disagreement was observed between the two approaches for race identification, which may be due to the aforementioned experimental variation within the bioassay, long-term storage of some isolates, or inconsistencies with cultivar usage and reaction with the pathogen. Alternatively, race 3 isolates, as they were only recently recognized within the literature, could be a group of other, yet uncharacterized, races all with higher virulence than race 2 isolates. The additional possibility, specifically to *Fusarium* spp., of horizontal chromosome shifting would theoretically allow for variability of a single isolate, causing alterations in results of both bioassay and molecular assay.

Based on previously published reports on pathogenic races, it is hypothesized that due to loss of function or absence of avirulence (*AVR*) genes in specific races, the pathogen could circumvent the resistance of the plant host [3,67,68,69]. While the targeted region for race 3 differentiation is not a known *AVR* gene, similar processes of novel resistance could be occurring. This would be evidence against the thesis of sequential development of races in the order in which they have been detected but instead races 2 and 3 arising from a common origin such as race 0 or race 1. Alternatively, gene acquisition conferring quantitative disease resistance could play a significant role in the development of newly pathogenic races, as has been seen in other *Fusarium* spp. Further analysis of international FON samples of all races is necessary to increase the confidence of marker stability and determine the mechanisms of resistance. This means that the availability of more whole-genome sequences like the ones used in this study will allow easier marker design and comparison in the future. Specifically, multiple genomes of isolates that are at the extremes of

virulence should be analyzed and compared as should isolates from distinct geographic origins. In addition to the region selected for amplification with the FNR3-F/FNR3-R primer set, screening of possible effector protein-coding regions in the FON genome would be a reasonable next step for the development of knockout mutants to test pathogenicity on resistant watermelon cultivars and to connect the identity of a specific FON race to an *AVR* gene. The marker presented in this study should improve the speed and accuracy of the current diagnostic ability for FON and provide a jumping off point for other researchers to investigate similar regions involved in pathogenicity and race development.

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Table 4.1. Percentage of agreement for race differentiation using bioassay vs. molecular assay.

FON Race	Number of Isolate Tested Using Both Methods	Percentage (%) of Molecular Results Agree with the Bioassay Race Results
Race 1	29	89.65%
Race 2	41	80.49%
Race 3	23	60.87%

Table 4.2. List of primers used in this study

Assay	Primers	Sequence (5'-3')	Product size (bp)	Source
FON specific primer	Fon-1	CGATTAGCGAAGACATTCACAAGACT	174	Lin et al., 2010
	Fon-2	ACGGTCAAGAAGATGCAGGGTAAAGGT		
Race 2 differentiating primer	FONSIX6F	CGCTCTTATCGCATCAATCT	453	Niu et al., 2016
	FONSIX6R	GGGTTGACTGAGGTCGTGGT		
Race 3 differentiating primer	FNR3F	CGGCTTTCCTCTGTCAGATAGT	511	This study
	FNR3R	TAGTGAGGTCCATGCCACGAA		

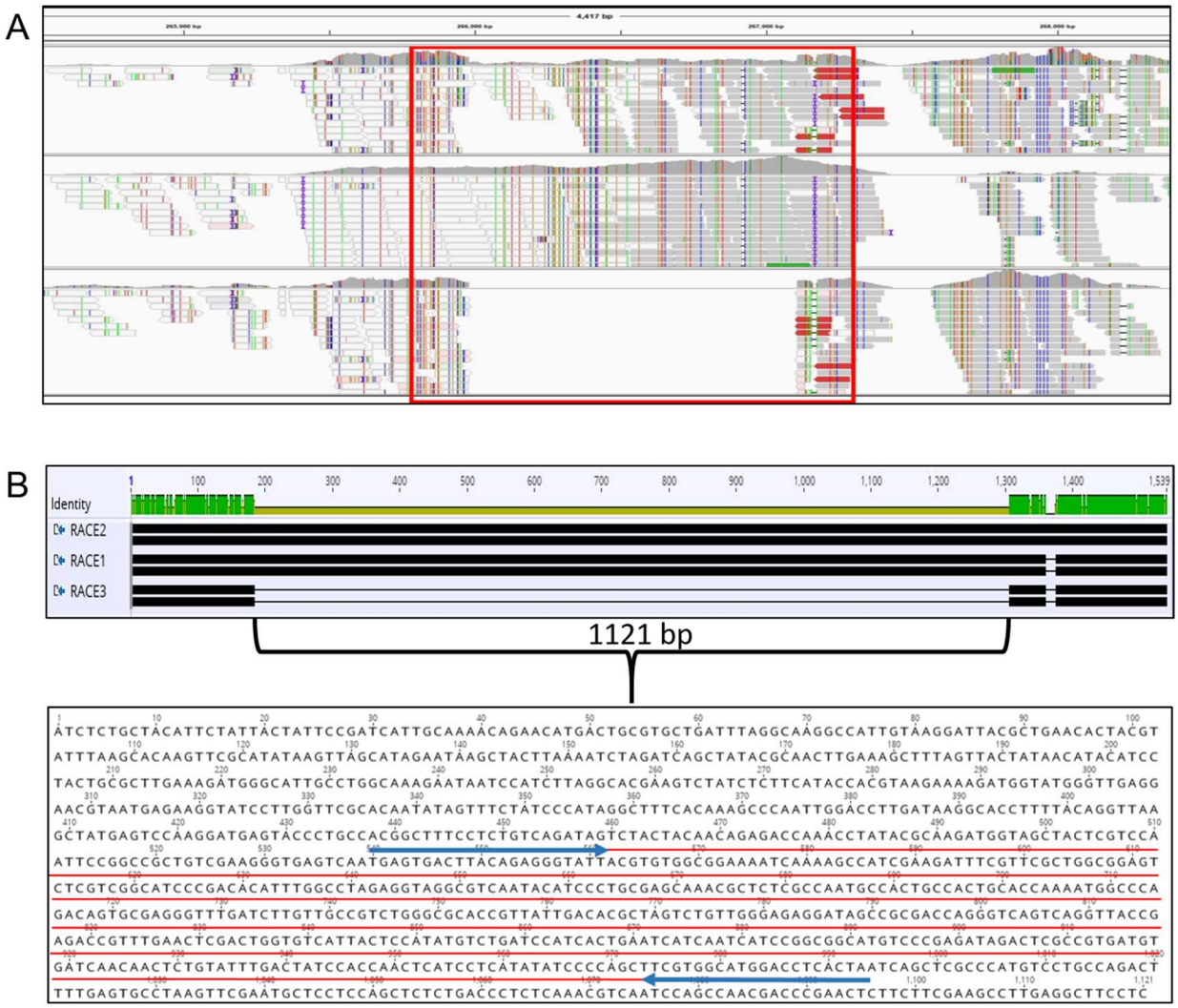

Figure 4.1. Region targeted for PCR amplification on chromosome 14 of *Fusarium oxysporum* f. sp. *niveum*. A: Interactive Genomics Viewer window of races 1, 2, and 3. Red box indicates the area investigated for amplification. B: Geneious alignment of races 1, 2, and 3, zoomed in on a 1540 bp section showing 1121 bp absent in race 3. C. 1121 bp sequence on Geneious. Amplified sequence is underlined in red, forward and reverse primers are underlined in blue.

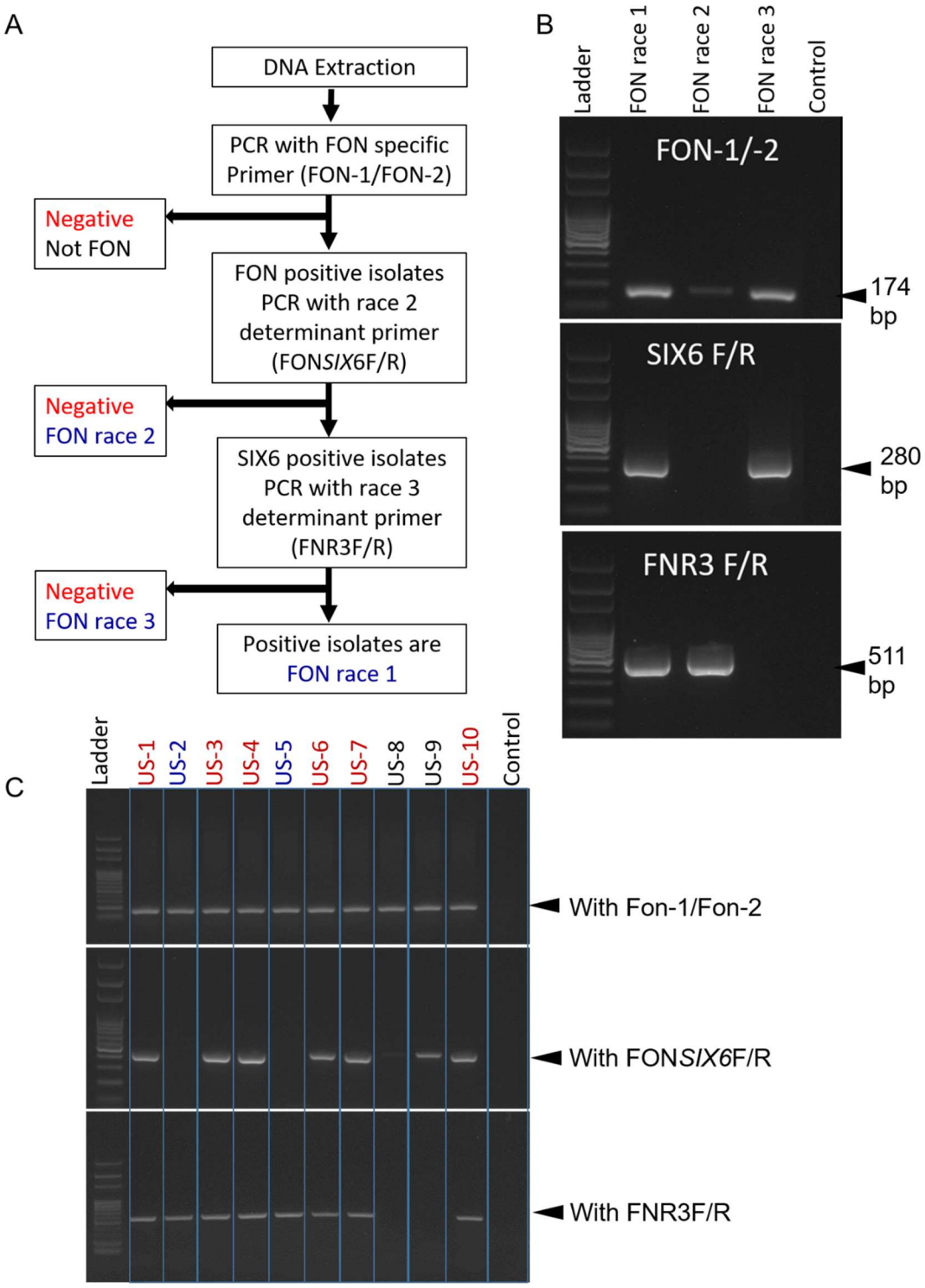

Figure 4.2. Utilization of all primers for FON race differentiation. A. Flowchart of FON isolate testing with all three marker sets to differentiate races 1, 2, and 3. Possible results are highlighted with blue. B. Example of a practical application on known isolates. C. Application on unknown field isolates. Absence of amplification of both FON*SIX6* F/R and FNR3-F/FNR3-R showed positive identity for races 2 and 3.

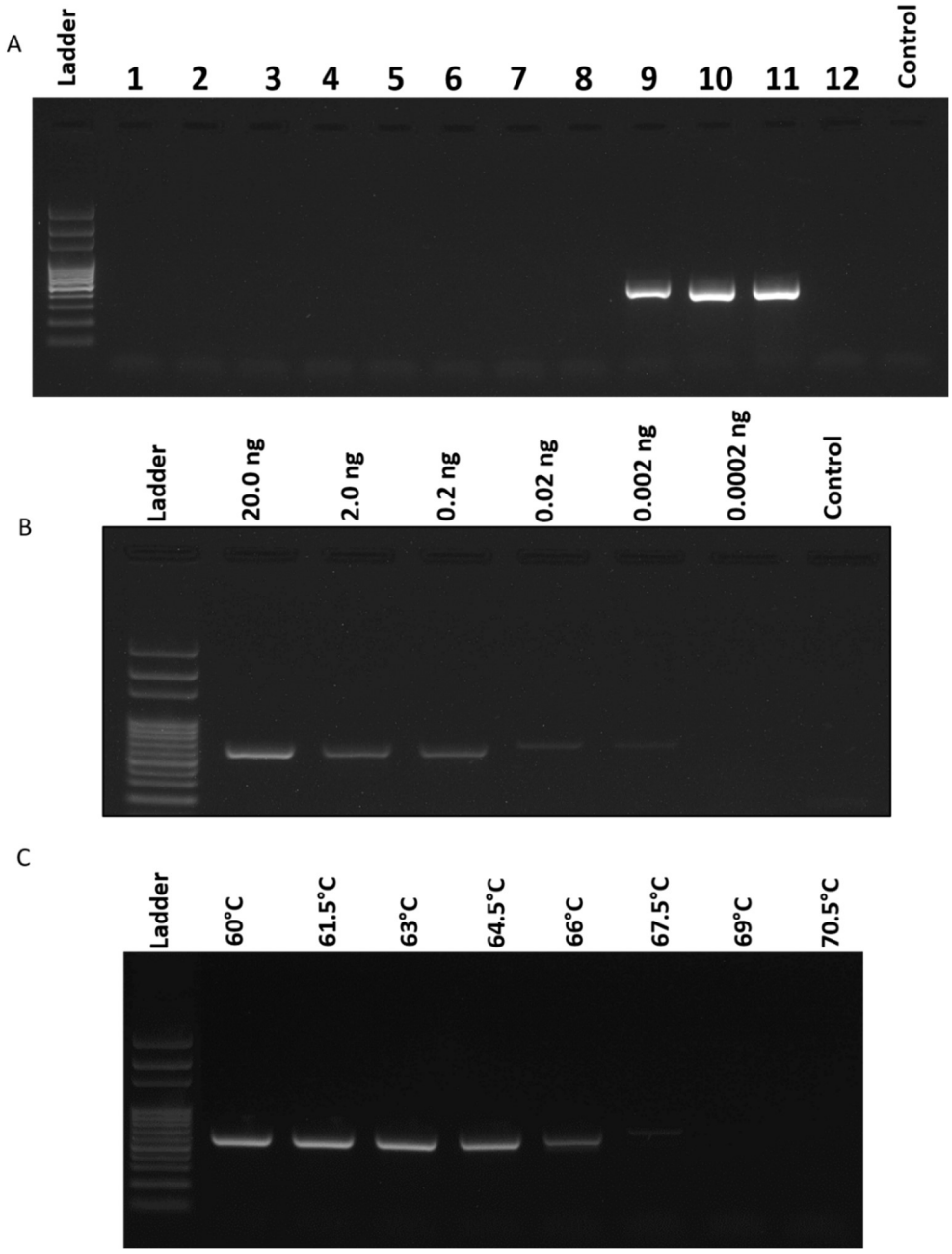

Figure 4.3. Temperature optimization and determinations of specificity and sensitivity for FNR3. A. Non-target amplification of FNR3-F/FNR3-R. Sample numbers correspond accordingly: 1. *Phytophthora capsici*, 2. *Phytophthora sojae*, 3. *Pseudoperonospora cubensis*, 4. Cucurbit leaf crumple virus, 5. *Rhizoctonia*, 6. *Colletotrichum orbiculare*, 7. *Fusarium solani*, 8. *F. oxysporum* f. sp. *vasinfectum*, 9. *F. oxysporum* f. sp. *lycopersici*, 10. FON race 1, 11. FON race 2, 12. FON race 3, and N = negative. B. Sensitivity determination of FNR3-F/FNR3-R using a serial dilution of FON DNA. DNA concentrations ranged from 20 ng/µL to 0.2 pg/µL. PCR positive bands amplified samples as low as 2.0 pg/µL. C. Gradient PCR results to optimize annealing temperature. Temperatures range from 60 to 70.5°C. The optimized temperature was 63°C.

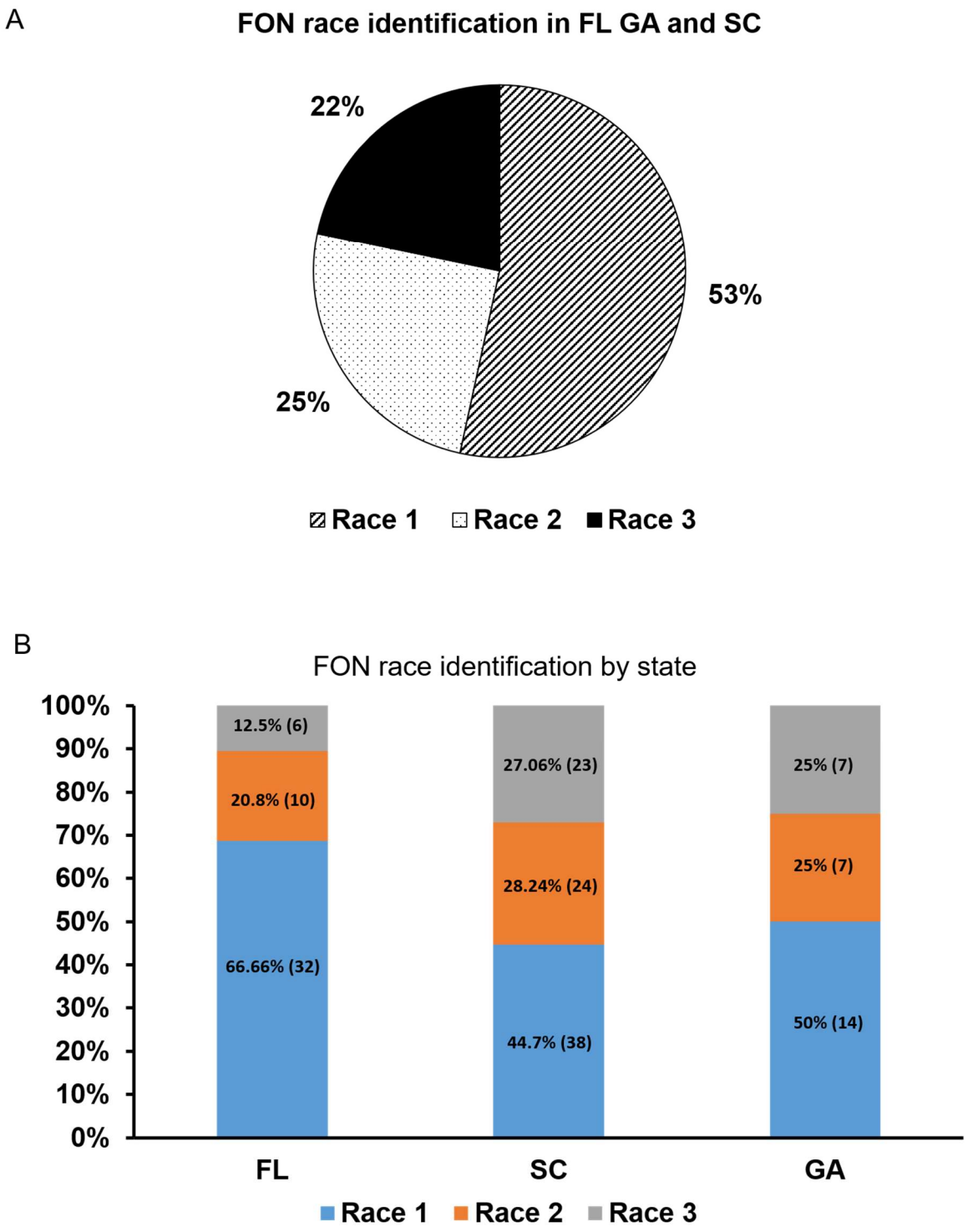

Figure 4.4. Molecular test results for race identification in states from which samples were taken. A: Race distribution of all states tested. B: Race distribution of individual state samples (Fl: Florida), (SC: South Carolina) (GA: Georgia). Race 1 = Blue, race 2 = Orange, race 3 = Grey. Number of each race are labeled in the colored section corresponding to the race and state.

CHAPTER 5

MECHANISMS OF FUNGICIDE RESISTANCE IN *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* TO THE FUNGICIDE PROTHIOCONAZOLE[4]

[4] Owen Hudson, Sumyya Waliullah, Pingsheng Ji, Md Emran Ali. Submitted to: *Scientific Reports* (Nature Research).

ABSTRACT

Fusarium oxysporum f. sp. *niveum* (FON) is the causal agent of Fusarium wilt in watermelon, a yield-limiting pathogen in watermelon production worldwide. A single demethylation inhibitor (DMI) fungicide, prothioconazole, is registered to control this pathogen so the risk of resistance arising in the field is high. To determine and predict the mechanism by which FON could develop resistance to prothioconazole, FON isolates were mutagenized using UV irradiation and subsequent fungicide exposure to create artificially resistant mutants. Isolates were then put into three groups based on the EC_{50} values: sensitive, intermediately resistant, and highly resistant. EC_{50} values were 4.98 µg/mL for the sensitive, 31.77 µg/mL for the intermediately resistant, and 108.33 µg/mL for the highly resistant isolates. Isolates were then sequenced and analyzed for differences in both the coding and promoter regions. Two mutations were found that conferred amino acid changes in the target gene, *CYP51A*, in both intermediately and highly resistant mutants. Expression analysis for the gene *CYP51A* also showed a significant increase of expression in highly resistant mutants compared to the sensitive controls. In this study, we were able to identify two potential mechanisms of resistance to the DMI fungicide, prothioconazole, in FON isolates gene overexpression and multiple point mutations. This research should improve the detection and management of fungicide resistant FON and related phytopathogens.

INTRODUCTION

Fusarium wilt of watermelon, caused by the ascomycete fungus *Fusarium oxysporum* f. sp. *niveum* (FON), is a significant factor limiting watermelon production worldwide [1-5]. Symptoms include single vine wilting, tip necrosis, dieback, and eventual plant death. This widespread pathogen is soil-borne and produces three different spore types: microconidia, macroconidia, and chlamydospores [3, 6]. Symptoms are caused by the host defense response to develop tyloses that attempt to block the vascular spread of the pathogen. Developing tyloses then clog the passage of water and nutrients within the plant causing loss of turgor pressure and wilting [7, 8]. While micro and macroconidia cause an in-season spread of FON and hyphal structures can overwinter, chlamydospores can survive in soils for up to 10 years and are resistant to extreme environmental conditions [9-11]. In addition to resistant spores, FON has evolved multiple races (0, 1, 2, 3), some of which are highly aggressive on all commercial watermelon cultivars [12, 13].

Management strategies have been reduced since the phasing out of methyl bromide as a soil fumigant due to its negative effect on the ozone [14]. Other soil fumigants have been used (chloropicrin and metam sodium), but they are not as effective as methyl bromide, so new chemistries and strategies are needed [2, 3, 15]. Crop rotation and nematode management have shown some success, but due to the prolonged survival of chlamydospores, these strategies have proven insufficient to halt their spread [16, 17]. Apart from fumigants, a single fungicide, prothioconazole (Proline 480 SC; Bayer CropScience, Research Triangle Park, NC, USA), is labeled for control of FON on watermelons [7]. Prothioconazole is a demethylation inhibitor (DMI) fungicide and has been tested in several studies to determine the sensitivity of FON populations [7, 18, 19]. To date, no reports of resistance have been made; however, management

continues to be problematic [7, 19, 20]. While other fungicides are being developed to control FON on watermelon, growers' options are limited and reports of ineffectiveness do occur when talking with growers [19-21]. Previous studies on FON sensitivity to prothioconazole determined that 10 µg/mL inhibited the growth of all isolates though spore germination was not inhibited greatly [7, 19].

DMI fungicides are at a medium risk of developing resistance, however, due to the single active ingredient registered for the pathogen, this likelihood is increased [22, 23]. DMI fungicides work by inhibiting the biosynthesis of ergosterol, a crucial component of fungal plasma membranes that is required for growth and development [22]. Specifically, DMI fungicides bind to the cytochrome P450 lanosterol 14α-demethylase (*CYP51*) to inhibit ergosterol biosynthesis [24].

There are three known mechanisms of fungicide resistance to DMI fungicides, each of which has variants of the specific mechanism that conveys the resistance [25]. The first mechanism is single nucleotide polymorphisms (SNPs) which alter the amino acid product and thus do not allow for proper binding of the fungicide to the gene product [26, 27]. There are a number of these SNPs reported to confer resistance, some are common across multiple genera, others are specific to species or even individuals [28]. The second mechanism is the overexpression of the *CYP51* gene, often due to insertions or deletions within the upstream promoter region of *CYP51* [29-31]. The third mechanism is the increased effectivity of drug efflux transporters such as ATP binding cassette (ABC) transporter genes [32-35]. In many *Fusarium* species, three copies of *CYP51* exist (*CYP51A*, *CYP51B*, and *CYP51C*), each with a different level of activity and the ability to "cover" for a separate copy [26, 28, 36]. As no mechanism for resistance has been determined for FON, the objective of this study was to

artificially mutate a FON isolate to become resistant to prothioconazole, then determine the mechanism by which the resistance had arisen. This study will provide a plausible mechanism for researchers to detect when resistance occurs naturally.

RESULTS

EC_{50} value and resistance factor determination. In total, nine FON mutants were generated using the UV irradiation method described above that showed resistance to prothioconazole. Both resistant and sensitive parental isolates were tested to determine their EC_{50} values using a mycelial growth inhibition assay (Figure 5.1). For sensitive isolates, the mean EC_{50} value was 4.98 µg/mL. Resistant isolates were separated into two groups, one as intermediately resistant (IR) and the other as highly resistant (HR). Intermediately resistant isolates had a mean EC_{50} value of 31.77 µg/mL and the highly resistant isolates had a mean EC_{50} value of 108.33 µg/mL (Figure 5.2). Resistance factor (RF) values were calculated from average EC_{50} values and determined to be 21.72 for the highly resistant isolate mean and 6.37 for the intermediately resistant isolate mean (Table B.2). Unpaired two-tailed student's t-tests showed a significant difference between sensitive isolates and intermediately resistant (P= 0.001) and highly resistant (P= 0.042) isolates.

Coding region and promoter sequence analysis. *CYP51A* is 1574 nucleotides long in FON (a total of 524 amino acids) and contains one intron of 53 bp. Three primers (FCypA1, FCypA2, FCypA3) successfully amplified this region (Figure 5.3). Several mutations were seen in the coding region sequence of the resistant isolates compared to the control sensitive isolates, all of which occurred in *CYP51A* and none in the other two copies, *CYP51*B and *CYP51*C. In *CYP51A*, three SNPs were present in the sequence of a highly resistant isolate and only two in

the intermediately resistant isolate (Figure 5.3). The first mutation, at nucleotide position 847, changed thymine to cytosine in both resistant isolates. This mutation conferred the amino acid change Y283H, changing a tyrosine to histidine at amino acid position 283. The second mutation occurred in only the highly resistant isolate at nucleotide position 1101 and changed an adenine to guanine. This mutation was silent, conferring no amino acid changes. The final mutation was observed at nucleotide position 1294 in both resistant isolates and changed thymine to adenine conferring the amino acid change S432T (serine to threonine). Three SNPs were seen in the highly resistant isolate sequenced and two resulted in changes in amino acid sequence, both of which were seen in the intermediately resistant isolate (Figure 5.3). The promoter region was sequenced until the first TATA box was found 747 bp upstream from the initial start codon. Promoter sequences did not differ in any nucleotide across any *CYP51* gene copy and resistant isolates were identical to the sensitive parental isolate.

Gene expression analysis. Evaluation of the relative expression (RE) of the *CYP51A* gene among the mutants revealed that it was increased two-fold among the intermediately resistant isolates and four-fold among highly resistant isolates from the sensitive isolates (Figure 5.5A). Differences in RE of *CYP51A* were statistically significant between both the sensitive and highly resistant isolates and the sensitive and intermediately resistant isolates. The sensitive isolate's mean RE was 8.39 whereas the highly and intermediately resistant isolates had RE's of 35.95 and 18.16, respectively. These results are 4.28 times (highly resistant) and 2.16 times (intermediately resistant) that of the sensitive isolate. $Log_{10}(RE)$ and $Log_{10}(EC_{50})$ values were positively and significantly correlated with an R^2 of 0.8652 ($Y = 1.8x - 0.7785$) (Figure 5.5B).

DISCUSSION

While watermelon cultivars resistant to some races of the Fusarium wilt pathogen have been developed, new races have evolved to overcome the resistance, so growers have to use other disease control methods such as chemical control. For control of *Fusarium oxysporum* f. sp. *niveum*, only prothioconazole (Proline 480 SC; Bayer CropScience, Research Triangle Park, NC, USA) is currently registered [7]. Although it is expected that other fungicides will be registered, repeated use of a single fungicide incurs a significant risk of developing resistance. It is currently unknown whether FON isolates resistant to DMI fungicides (to which prothioconazole belongs) exist, but this class has a medium risk of developing resistance. To better understand and predict how resistance might arise, we developed prothioconazole resistant FON mutants that could grow well on fungicide-amended media.

Two resistant groups were proposed based on the EC_{50} values of prothioconazole resistant mutants and subsequent resistant factors (RF): intermediately resistant (IR) and highly resistant (HR) isolates. Mean EC_{50} values of HR and IR groups compared to the sensitive (S) showed resistance factors of 6.37 for IR and 21.72 for HR. These groups were then analyzed with an unpaired two-tailed t-test for significance revealing a significant difference between sensitive and HR isolates.

Sequencing and analysis of *cytochrome P450 lanosterol 14α-demethylase* (*CYP51*) copies A, B, and C revealed that only *CYP51A* had mutations. While both the intermediately and highly resistant isolates had two mutations conferring amino acid changes, Y283H and S432T, the highly resistant isolate had an additional silent mutation at nucleotide position 1101. Of the two mutations conferring amino acid changes, changing a tyrosine to a histidine was previously reported by Qian et al. (2017) as a mechanism for resistance of *Fusarium graminearum* to a different demethylation inhibitor (DMI), tebuconazole. Although in that study the mutation was

seen at amino acid position 137 and occurred in the *CYP51*B copy, similar molecular binding alterations conferring resistance could be occurring in this study [26]. The second mutation, S432T, is not a well characterized mutation when investigating DMI resistance, although central serine amino acids have been found to be important to the molecular structure of the *CYP51A* protein [37]. The final mutation, which was silent, occurred only in the most resistant isolate as determined by the growth assay and changed an adenine to guanine at nucleotide position 1101. Silent mutations are not known to cause resistance to DMI fungicides, however, there is an increased presence of amino acid changes in resistant isolates of multiple phytopathogens, although often more than one [25, 38-40]. Due to the similarities between results in this study and the results from other studies mentioned previously, we believe it reasonable to consider these mutations to at least contribute to the fungicide resistance seen in the growth assays. Neither gene copies *CYP51*B or *CYP51*C had any nucleotide changes in either of the resistant isolates when compared to the sensitive parental isolate. No differences were seen across the sequenced 747 bp of the promoter regions in any of the three gene copies of *CYP51*.

As *CYP51A* incurred mutations from the irradiation, further investigation by way of an expression analysis took place and revealed a statistically significant difference between the highly resistant and sensitive isolates. The RE analysis revealed that the highly resistant isolate had an expression level 2.16 times that of the intermediately resistant isolate and 4.28 times that of the sensitive parental isolate (35.95 =HR, 18.16=IR). No mechanism was determined for the differences in expression when analyzing the promoter sequences, but it should be noted only 747 bp of the promoter were sequenced and additional aberrations could have occurred upstream of the first TATA box. Increases in *CYP51* gene expression have been correlated multiple times

to resistance in DMI fungicides due to the increased target gene availability, thus, it is reasonable to contribute a significant level of resistance to the differences in relative expression [34, 41, 42]. While definitive conclusions about the source of DMI resistance in field FON populations should not be drawn from these data, the detected mutations and differences in gene expression suggest two possible mechanisms. These changes were characterized to better predict possible mechanisms of resistance to the only class of fungicides registered for FON. In the case of DMI resistance in FON field isolates, we hope that this research can assist in detecting the mechanism rapidly, saving resources for researchers and growers. Further analysis of ABC transporters and other efflux transporters or expression of other gene copies, not studied here, should be additionally considered as they could also be contributing to resistance.

MATERIALS AND METHODS

FON isolates. Isolates of FON were obtained from commercial watermelon fields in Georgia by taking samples from infected plants and culturing them on semiselective peptone pentachloronitrobenzene agar plates [43]. To test in vitro fungicide sensitivity, isolates were grown on full strength potato dextrose agar (PDA) plates and subcultured on PDA plates amended with 10 µg/mL prothioconazole (pure product, Chem Service, West Chester, PA, USA) (Figure B.1). The value of 10 µg/mL was used to determine sensitivity since it completely inhibited the growth of FON isolates from Georgia as reported previously [19]. The isolate (B3-12) was chosen for mutagenesis because it was the most sensitive to the fungicide in order to compare the effects of resistance.

Generation of FON mutants resistant to prothioconazole. Mycelial plugs of isolate B3-12 from a PDA plate were transferred to ½-strength potato dextrose broth (PDB) and

incubated at room temperature under continuous light with shaking at 150 rpm. After 10 days, the liquid medium was filtered through sterilized cheesecloth. The spore concentration was quantified using a hemocytometer and reconcentrated to 10^5 spores/mL via centrifugation and decantation. A 100-µL aliquot of the spore suspension was then spread on the fungicide amended media and incubated for 5 hours in the dark at 26°C for spore germination. After 5 hours, plates were taken to a sterile hood and exposed to UV light at a distance of 20 cm for 30 seconds before being incubated again for 7 days in the dark at 26°C. This was replicated in 10 separate plates, and three plates were subjected to the same treatment without UV exposure. After 7 days, UV irradiated plates were inspected for growing colonies which were then transferred to PDA with no fungicide for another 7 days in the dark at 26°C. These isolates were then plated on PDA with 10 µg/mL prothioconazole before being transferred to plates with increased fungicide concentrations (+5 µg/mL every subsequent week) until reaching a concentration of 50 µg/mL, then repeated at 50 µg/mL for 3 weeks. Control isolates (not exposed to UV) were transferred to PDA with no fungicide each time mutants were transferred. This protocol was developed and modified based previously article by Ali et al(2018) [44].

EC_{50} value determination for sensitive and resistant isolates. After 20 weeks, resistant and sensitive isolates were plated on various concentrations of fungicide amended PDA to determine EC_{50} values. Based on the growth results, isolates were separated into three groups: sensitive, intermediately resistant, and highly resistant, to better categorize the EC_{50} values. The fungicide concentrations increased by a factor of ten starting with 0 µg/mL, then 0.1 µg/mL, 1.0 µg/mL, 10 µg/mL and finally 100 µg/mL. An additional concentration of 50 µg/mL was made for visualization of the mycelial growth inhibition but was not used in calculating EC_{50} values. After 14 days, five measurements per isolate were made from the center of the colony to the

growing edge (radius) and the average length was calculated. This was done for nine resistant and four sensitive isolates in duplicate and the means were again averaged for each concentration to obtain a mean value for each group. Using average EC_{50} values, the Resistance Factor (RF) was additionally calculated. RF values were calculated according to Lin et al. (2020) (using sensitive isolate EC_{50} mean value) and correlated to FON resistance levels in Table 5.1 [44, 45].

DNA and RNA extraction. After determining significant differences in growth between sensitive and resistant isolates, the isolates were grown on full strength PDA plates for two weeks before 100 mg of mycelium was scraped from the plate and placed in a 1.5 mL safe-lock tube (Eppendorf Canada Ltd, ON, Canada). Four steel balls were added to each tube and homogenized using a FastPrep FP120 cell disruptor (Qbiogene, Carlsbad, CA, USA) for three rounds of speed 4.0 for 30 seconds. Samples were then extracted using DNeasy (DNA) and RNeasy (RNA) Plant Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Total DNA and RNA were quantified, and purity was estimated by measuring OD 260 nm and OD 260 nm/280 nm using a NanoDrop spectrophotometer (NANODROP LITE, Thermo Scientific, Waltham, MA, USA).

Primer design. Primers used in this study are found in Table B.1 and contain a mix of previously published primers and new primers developed for this study specifically. The three primers from Zheng et al. (2018) (FOCYP51Bpyes2-F and FoCYP51Bpyes2-R) and Zhang et al. (2006) (Fn-1 and Fn-2) which were used in this study, overlapped with whole genome sequences (WGS) of FON obtained from Hudson et al. (2020) (BioProject PRJNA656528) and determined applicable for this research [36, 46, 47]. All novel primer sets used in concert with previously published primers were developed from and used the same whole genome sequences mapped to the *Fusarium oxysporum* f. sp. *lycopersici* 4287 (FOL) reference genome (BioProject

PRJNA342688) on the Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA). Each copy of the *CYP51* gene (A, B, and C) was identified from the FOL reference *CYP51* gene (XP_018249826.1) and aligned to the FON WGS. Primers were developed on the Integrated DNA Technologies Primer Quest™ Tool. Downstream primers were designed to overlap upstream primers to obtain full coverage of the gene sequence. PCR amplicons ranged in size from 336-bp to 712-bp to obtain high quality reads. Quantitative PCR (qPCR) primers used in the expression analysis were developed using the same method but for a product size of <200 bp.

Sequencing of coding and promoter regions of *CYP51*. Extracted DNA was amplified using polymerase chain reaction (PCR) with the primer sets specific to each gene copy. PCR solutions totaled 50 µL and consisted of *Taq* polymerase (25 µL), forward primer (20 µM), reverse primer (20 µM), 2 µL of 150 ng/µl genomic DNA, and rest was filled with PCR grade H_2O. Samples for amplification of both coding and promoter regions were then added to a thermal cycler with conditions listed in Table B.1. PCR amplicons were confirmed as positive without contamination by running them on a 1% agarose gel and imaging using a UV geldoc (Analytik Jena, Upland, CA, USA). Samples were then purified using a commercial cleanup column (BioRad Laboratories, Hercules, CA, USA), and submitted to Retrogen (Retrogen, San Diego, CA, USA) for Sanger sequencing.

Exon and promoter sequence analysis. Upon receipt of sequencing results, fasta files were downloaded and aligned on Geneious V 11.1.5 (https://www.geneious.com) to one another. The sequences were separated into individual gene copies (*CYP51A*, B, C), and then aligned to the reference genome sequences of each copy, using both Bioproject PRJNA342688 (FOL) and Bioproject PRJNA656528 (FON) for alignment. Introns were removed based on the alignment with FOL reference genome gene *CYP51* (ID 28952942). Isolate sequences were then compared

across each gene copy at the individual nucleotide level. Differences were identified when the sensitive (parental) isolate was compared with resistant isolates. Single nucleotide polymorphisms (SNPs) were determined to confer amino acid changes by translating the nucleotide sequence to the amino acid sequence on Geneious. Promoter sequences were submitted to this same process of alignment but without amino acid translation. Promoter regions were sequenced until the first TATA box, 747 bp upstream from the start codon of the first exon.

Gene expression analysis. To further investigate the effects of mutagenesis, gene expression analysis was performed to determine the relative expression levels of *CYP51A* in resistant and sensitive isolates of FON. For the expression analysis, total RNA extracted from fungal mycelium was converted into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction. A quantitative real-time PCR (qPCR) assay was performed on a BIORAD CFX connect real-time system (Bio-Rad Laboratories) in 10 µL reactions consisting of 5 µL SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories), 10 ng cDNA, 300 nM forward and reverse primers, and rest was filled with dH_2O. Newly developed primers specific to FON*CYP51A* were used to determine the expression of the candidate gene. Expression of FON from Zhang et al. (2006) was used as an endogenous control [47]. The recommended thermal cycling protocol for SsoAdvanced SYBR Green was used: activation/DNA denaturation at 95°C for 30 s, denaturation at 95°C for 10 s, and annealing/extension at 60°C for 30 s for 40 cycles. A melt curve analysis was included: 65 to 95 °C at 0.5-°C increments, 5 s per step. Samples were run in Bio-Rad plastics and sealed with optical adhesive seals (Bio-Rad Laboratories). All assays included reverse transcription–negative controls to check for genomic DNA contamination and no template controls to check for other contamination. Each reaction was run in technical triplicate. The $2^{-\Delta\Delta Ct}$ equation by

Livak and Schmittgen (2001) was used to determine the relative gene expression [48]. Three isolates of each resistance level were run in triplicate and averaged for each resistance grouping (highly resistant, intermediately resistant, and sensitive).

Statistical analysis. Data are represented as mean ± SEM. Graphs were prepared and all data were analyzed using GraphPad Prism 8. Statistical significance was determined by the two-tailed Student's t-test and Pearson's R. $P < 0.05$ was considered statistically significant.

Molecular modeling. Molecular models of the *CYP51A* gene product were created using SWISS-MODEL with the *CYP51* gene copy from *Aspergillus fumigatus* as the model reference [49]. Alignments of *FONCYP51A* were done after Intron removal using UniProtKB – A0A0D2Y5I9 on Geneious software to confirm the coding region as similar (Gene ID: 28952942). Zoomed-in regions highlight the impacts of point mutations on the molecular structure as determined from sequencing data and SNP determination.

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Table 5.1. Sensitivity grouping based on resistant phenotype (EC_{50}) values.

Resistance Factor (RF)	Resistance Level	Resistance Group
<3.0	Sensitive	Sensitive
3.0-5.0	Mild resistance	Intermediately resistant
5.1-10.0	Intermediate resistance	
10.1-15.0	Moderate resistance	
>15.1	High resistance	Highly resistant

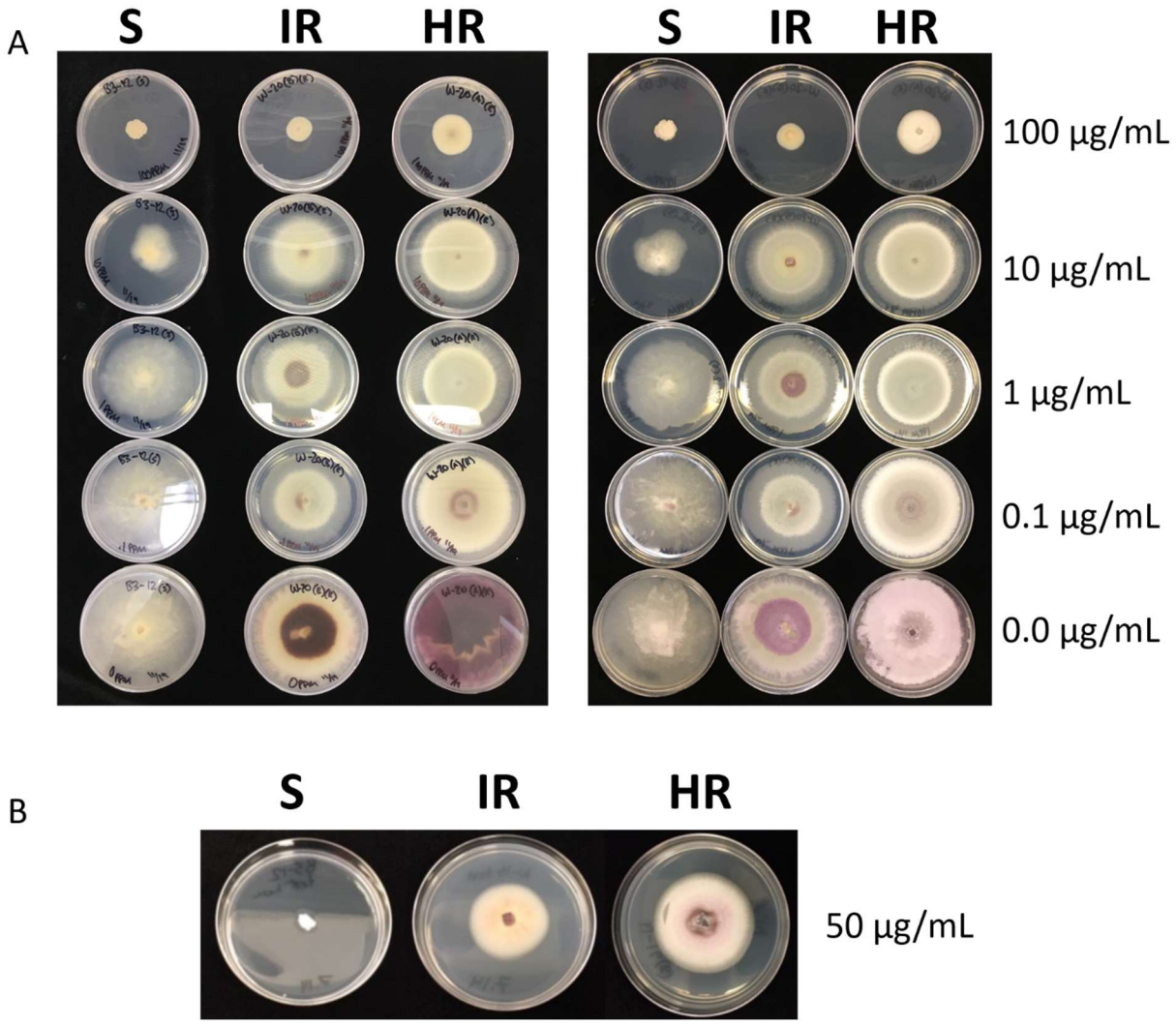


Figure 5.1. Phenotypic growth assay. A) Growth assay on decreasing levels of prothioconazole amended media from 100 to 0 µg/mL. Both sides of the media and fungal cultures are shown. S = Sensitive isolate, HR = Highly resistant isolate, IR = Intermediately resistant isolate. B) The same isolates were grown at 50 µg/mL prothioconazole to show the morphological differences. All plates are shown at 14 days post inoculation.

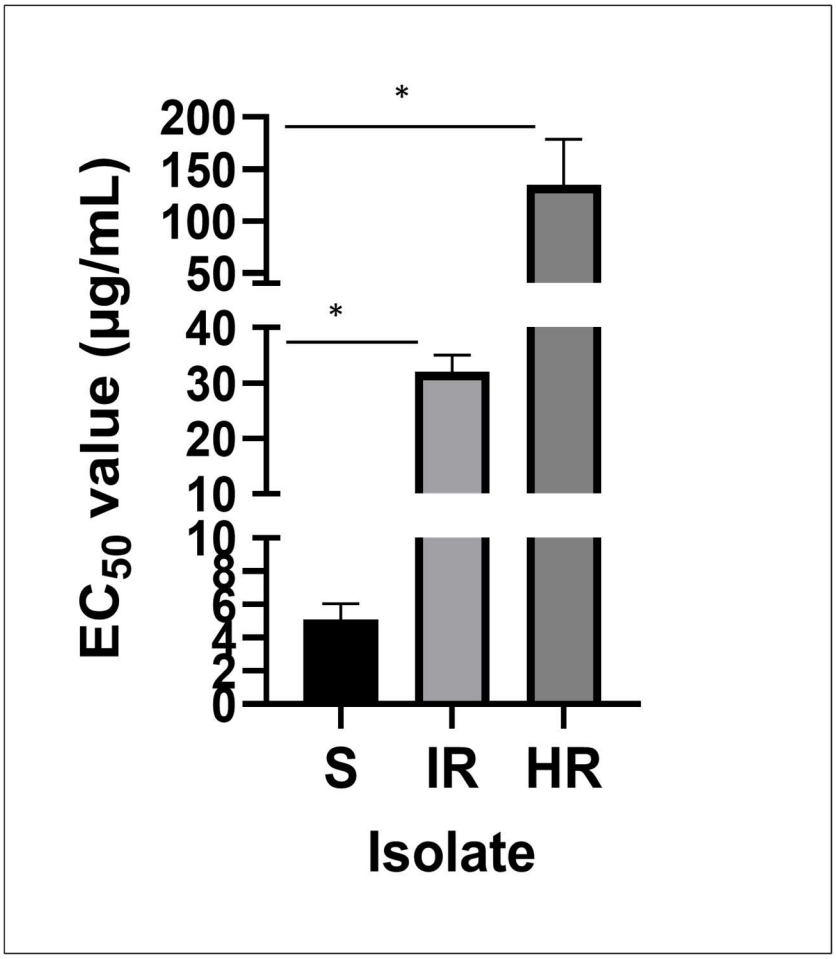
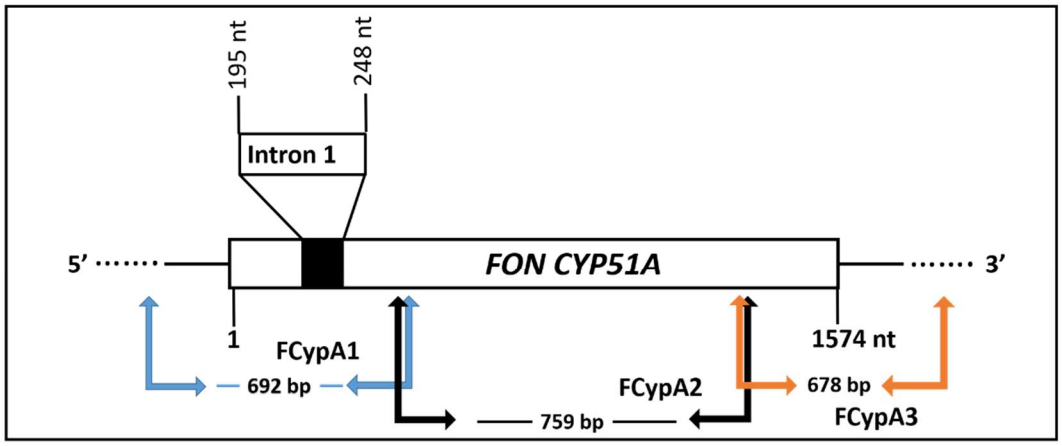


Figure 5.2. In vitro sensitivity of FON mutants to prothioconazole. The graph displays the comparison of mean EC_{50} values among mutant isolates. Asterix (*) signifies a significant difference ($P < 0.05$) between the isolate group and the sensitive parent group.

A



B

Isolate	EC_{50} (µg/ml)	Nucleotide (847)	Amino Acid (283)	Nucleotide (1294)	Amino Acid (432)
Sensitive 1	4.98	842-TCGCG**T**ACATG	279-KEIA**Y**MMIT	1289-CTGTT**T**CTAAA	428-YGAV**S**KGTK
Highly Resistant	108.33	842-TCGCG**C**ACATG	279-KEIA**H**MMIT	1289-CTGTT**A**CTAAA	428-YGAV**T**KGTK
Intermediately Resistant	31.77	842-TCGCG**C**ACATG	279-KEIA**H**MMIT	1289-CTGTT**A**CTAAA	428-YGAV**T**KGTK

Figure 5.3. *CYP51A* gene schematic and detected mutations. A) Intron and exon organization of *CYP51A*. Primers used for sequencing this gene are written in blue, black, and orange. B) Table with isolates' group mean EC_{50} values and mutation locations of each isolate group. Amino acids changed from sensitive to resistant are highlighted with colored text (blue = nucleotide changes, red = amino acid changes).

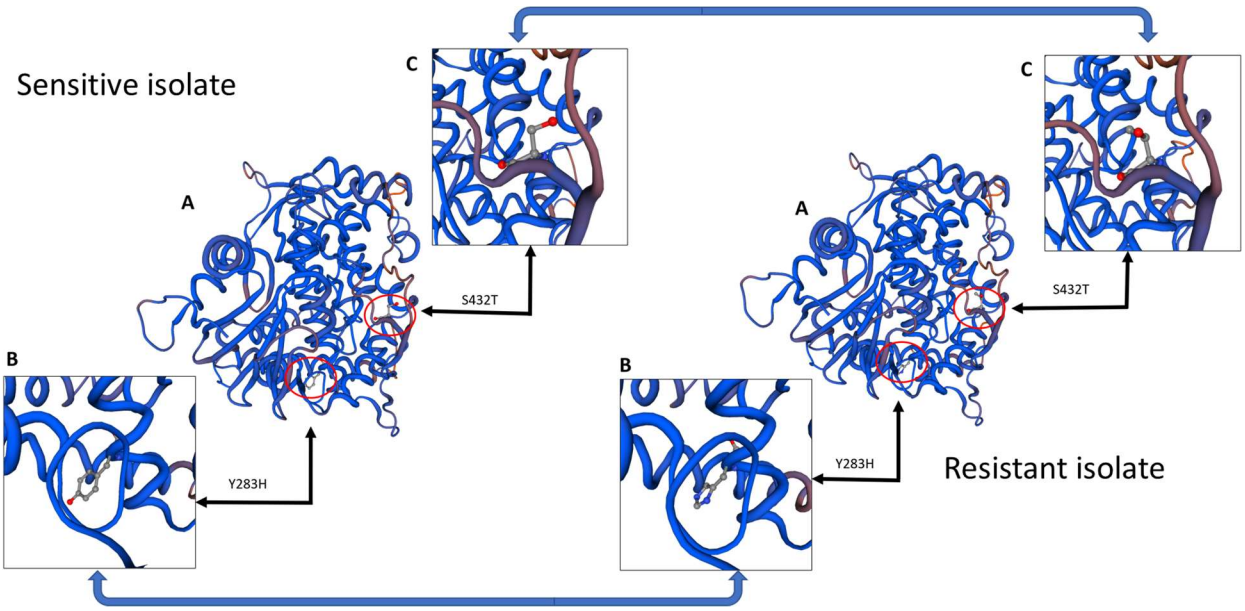


Figure 5.4. Molecular modeling for resistant and sensitive isolate *CYP51A* gene products. A) Molecular model of fungicide sensitive *CYP51A* with red circles highlighting the molecular arrangement of specific amino acids mutated in the resistant version. B) Zoomed in window at tyrosine 283 to show molecular structure and location. C) Zoomed in window at serine 432 to show molecular structure and location. D) Molecular model of highly fungicide resistant *CYP51A* with red circles highlighting the molecular arrangement of specific amino acids different from the sensitive version. E) Zoomed in window of histidine 283 (changed from tyrosine) to show molecular structure and location. F) Zoomed in window at threonine 432 (changed from serine) to show molecular structure and location.

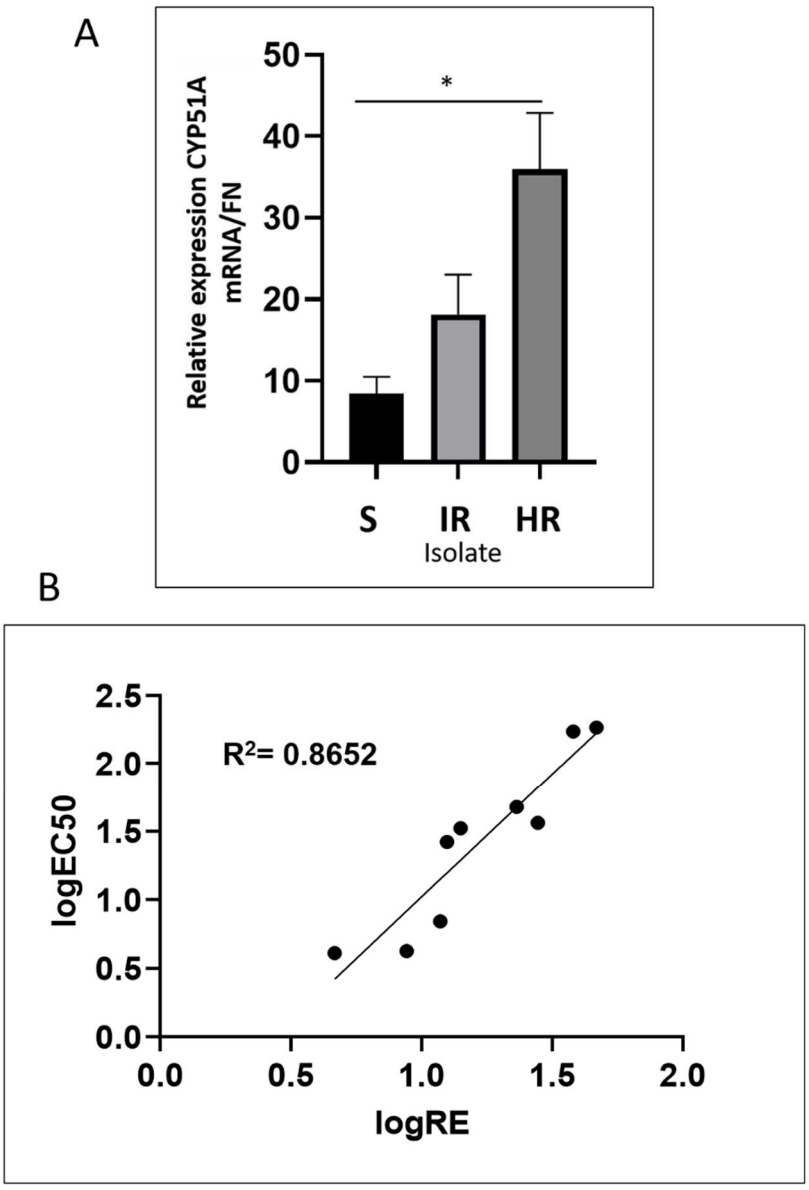


Figure 5.5. Relative expression (RE) of *CYP51* and correlation with isolate sensitivity. A) Graphical representation of the RE calculated with the reference FON gene using the $2^{-\Delta\Delta Ct}$ method. Asterix (*) indicates significant differences between sensitive and resistant groups. B) Correlation between RE and EC_{50} values of *Fusarium oxysporum* f. sp. *niveum* (FON). R^2 value = 0.8652 showing a positive and statistically significant correlation between gene expression and growth on fungicide.

CHAPTER 6

CONCLUSIONS

As one of the most significant yield-limiting pathogens afflicting watermelon, *Fusarium oxysporum* f. sp. *niveum* (FON) is the target for research on disease management around the world [1, 2]. FON is a member of the *Fusarium oxysporum* species complex (FOSC) which causes wilts on a broad range of plants [3]. Development of resistant cultivars has been the historical focus for watermelon breeders, however, several races (0, 1, 2, and 3) that can overcome the host resistance have begun to spread around the world and, more specifically, the east coast of the United States [2, 4-6]. Traditional methods to detect FON and determine the correct race are inefficient and time-consuming, as they require fungal culturing and isolation of the pathogen before conducting a bioassay which involves infecting seedlings of different watermelon cultivars and assessing the infection [7]. Additionally, the bioassay is known to produce inconsistant results from lab to lab and other experimental variables like money, time, and experimental materials make the bioassay an undesirable method [8-10]. Hence, a sensitive and quick diagnostic tool for identifying the disease and differentiation of FON races is needed to facilitate more effective management practices for growers, farmers, and researchers. Without knowledge of the pathogen ID and the race(s) predominant in a grower's field, cultivar usage and chemical control may be incorrect. FON specific molecular detection methods based on polymerase chain reaction (PCR) have been limited in the past with a previous PCR marker (Fn-1/Fn-2) being updated with a separate PCR

marker that is more specific (Fon-1/Fon-2) [8, 9]. Niu et al. (2016) was also able to identify a race 2 specific gene that is absent only in race 2 and can be used for race 2 differentiation [11].

To enhance identification abilities, two loop mediated isothermal amplification (LAMP) assays were developed for FON specific amplification and race 2 differentiation in this study. These LAMP assay primers were developed using the sequences used in the previously developed PCR assay as templates. LAMP assays have increased access to molecular identification by increasing sensitivity while decreasing the time and resources required compared relative to PCR.

To design a method of molecular differentiation of race 3 isolates from the other races of FON, an investigation into the variability between gene sequences of several races was done. Genes commonly used for fungal barcoding such as the interal transcribed spacer (ITS), translational elongation factor 1α (TEF1α), large ribosomal subunit RNA (LSU), RNA polymerase II (RPB1), Intergenic Spacer (IGS), cytochrome c oxyidase subunite I (COI), heat shock protein (HSP), and β-Tubulin (β-Tub), all were sequenced for multiple race isolates and all revealed near 100% conservation of sequences. With such a high degree of conservation between races, Illumina whole genome sequencing (WGS) and subsequent analysis using isolates of races 1, 2 and 3, to obtain regions of dissimilarity. While WGS analysis revealed a number of SNPs and InDels, larger regions (>1000 bp) were targeted for marker development to obtain consistent and significantly different results. Previous studies have focused on chromosome 14 of FON, or the pathogenicity chromosome, as many avirulence (AVR) genes have been located there, including *AVRSIX6* [11-13]. Because of its involvement in pathogenicity and virulence in multiple FOSC members, this chromosome was targeted in this study for analysis and subsequent primer design. A region of 1121-bp was located on chromosome 14 of both races 1 and 2 but was absent in race 3. A primer set was developed that amplifies a 511-bp region within the larger region in races 1 and 2 but not

in race 3. The region amplified by FNR3 was additionally chosen due to multiple hypothetical proteins being detected both by the ORF Finder on Geneious and when the region was submitted to NCBI BLAST. When used in tandem with the other previously published primer sets, a researcher can differentiate races 1, 2, and 3 from each other by running each primer set independently and following the simple flowchart in Figure 4.2A.

The new primer set, "FNR3F/R", amplified both races 1 and 2 in addition to the reference genome of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) but the amplicon was slightly larger (555-bp) in FOL. Once determined to successfully amplify only races 1 and 2 from the race standard isolates, 161 FON isolates were obtained from labs in Florida, Georgia, and South Carolina to test with the new primer set. Many of these isolates had previously been assigned races based on the bioassay results determined by the researchers that donated their samples, so the marker was then compared with those results. Of those isolates, 26 (89.65%) for race 1 showed the same results between the molecular assay and the bioassay, 33 (80.49%) for race 2, and 14 (60.87%) for race 3. These proportions were to be expected due to the inconsistent results of the bioassay and the unexpected presence of race 3 in South Carolina. Overall, the breakdown of races of all isolates sampled was 53% race 1, 25% race 2, and 22% race 3. In each state surveyed, race 1 was always the most common, while race 3 was either the least common or tied (in the case of GA isolates) with race 2. Our results reflect different proportions than surveys done in each state prior to this research, with race 3 isolates never before detected in South Carolina.

To continue this work, a greater number of FON isolates with high confidence race determinations should be submitted for whole genome sequencing including race 0 isolates. Previously studies have shown regional differences in addition to partial or full horizontal chromosome transfer, both of which could have impacts on consistent primer amplification of a

specific race [12, 13]. Regions used for differentiation should continue to be correlated with genes, but if possible, correlated with genes involved in pathogenicity and selected based on predicted gene function [14]. Confirmation of these markers by way of gene knockouts and reinfection with previously resistant cultivars could provide a system of differentiation by way of correlating specific races with genes that confer higher or lower levels of virulence [15]. This way if genes conferring pathogenicity on certain cultivars are transferred horizontally, their function and detection ability is also conferred [12, 13]. Special attention to FON's international distribution also should be made by researchers of FON; isolates from multiple countries/continents should be analyzed for the conservation of the primer targets [8, 9, 10].

Fungicides (Proline, a.i. prothioconazole) have been successful at controlling FON to a certain extent as no resistant isolates have been found in the field, however, the overwintering chlamydospores prevent total control of the disease [16-18]. Chlamydospores are difficult to remove with fumigants and their long term survivability in the soil prevents crop rotation and tillage from being successful methods for control [19-21]. In addition, prothioconazole, a single-site mode of action fungicide is at a medium to high risk of resistance development by FON populations. In general, any microorganism controlled with a single chemistry inherently has an increased risk of developing resistance [22, 23]. Thus, FON is of medium to high risk of developing resistance to prothioconazole because it is a DMI (at medium risk) and the only registered fungicide [17, 24]. DMI fungicides target the *Sterol 14α-Demethylase Cytochrome P450* (*CYP51*) gene, which is responsible for ergosterol biosynthesis, a large component of fungal cell walls [25]. Because no known resistant FON isolates have been documented and characterized, none of the three mechanisms of resistance to DMI fungicides have been documented either. To determine the mechanism by which FON could become resistant to the fungicide, mutants were

created via UV irradiation and repeated growth on fungicide amended media. Mutagenesis successfully resulted in a set of isolates with a range of decreased sensitivity to prothioconazole. EC_{50} values were determined using a serial dilution of the fungicide and measuring the average radial growth at 10 DPI. Resistance factors (RF), a ratio of EC_{50} values of the sensitive isolates compared to that of the resistant isolates, were then calculated for all isolates. Values then were used to group isolates into three different ranges of RF, one for sensitive (RF < 3; EC_{50} mean = 4.99), one for intermediately resistant (RF= 5.1-10; EC_{50} mean = 31.77), and one for highly resistant (RF > 15; EC_{50} mean = 108.33).

To determine the differences in each level of resistance, DNA and RNA were extracted and analyzed from two resistant mutants and the sensitive parental isolate. With access to whole genome sequences of several FON isolates aligned to the FOL reference (BioProject PRJNA342688) which is fully annotated, the homologous *CYP51* gene region in the FOL reference was identified in the FON genome via the Interactive Genomics Viewer (IGV) [26]. PCR primers specific to *FONCYP51* were made for amplification of all three copies (*CYP51A*, *B*, and *C*) and their promoter regions up to the first TATA box (~750-bp). Multiple primers were used on each gene copy to obtain full coverage of the 1574-bp (*CYP51A*) gene. An additional qPCR primer set was designed using the same methodology for a qPCR gene expression analysis. After alignment and intron removal, sequencing of each *FONCYP51* gene copy revealed SNPs present in only *CYP51A* of both the resistant isolates and not on the other two copies. In both the highly resistant and the intermediately resistant isolates, two SNPs were found to confer point mutations: Y283H, changing a tyrosine to a histidine at amino acid position 283, and S432T, changing a serine to a threonine at amino acid position 432. In the highly resistant isolate, there was an additional SNP, but it resulted in a silent mutation. That silent mutation was located at nucleotide position

1101 and changed an adenine to a guanine. A tyrosine to histidine point mutation (as seen in the resistant FON isolates) had been recorded previously in *Fusarium graminearum* conferring resistance to a different DMI fungicide, tebuconazole [27]. None of the *FONCYP51* gene copies contained any SNPs or InDels in their promoter sequences up to the first TATA box.

As only *CYP51A* was determined to have sequence mutations, it was used to quantify the gene expression level using a *CYP51A* specific qPCR primer set. Fn-1/Fn-2 qPCR primer set (REF) was used as a control [8]. Although no changes were detected in the promoter region of *CYP51A,* the expression was significantly higher in both intermediately and highly resistant isolates compared to the sensitive parental isolate. The gene expression of the highly resistant isolate was also significantly higher than that of the intermediately resistant isolate resulting in a strong positive correlation (R^2=0.8652) between gene expression and fungicide resistance. The strong correlation and the history of genetic overexpression conferring resistance to multiple groups of fungicides suggest it is reasonable to think that the overexpression of *FONCYP51A* is involved in decreased fungicide sensitivity [27-29]. That being said, one cannot confirm the mechanism of resistance without exploring all possible options. Efflux transporters, which have been involved in mediating cross-resistance to multiple fungicides including DMIs, were not examined here [30, 31]. The source of overexpression or increased copy number of the target gene, *CYP51*, is not known. Up to 750-bp of the promoter region was sequenced, however, it was not confirmed to be the entire promoter for *CYP51A*, it simply was the first TATA box found upstream of the start codon. It could be that the promoter region is longer and the change that conferred overexpression was not detected, or that a *downstream* aberration could have changed the expression. Other ways genes can become overexpressed include chromosomal duplication, although this is less likely via UV irradiation, or individual point mutations as seen in

FONCYP51A here have previously occurred with general genetic overexpression [32-35]. It is suggested that the observed increase in expression could be compensatory for the reduced enzyme efficiency originating from point mutations that hinder correct binding with normal metabolic function [36]. Other instances of *CYP51* overexpression that cannot be linked to promoter changes have been documented in other fungal pathogens [37, 38]

In-vivo studies are currently in place to determine if the mutant isolates with reduced fungicide sensitivity continue to be virulent to the same degree as the sensitive parental isolate, or if the reduced sensitivity comes with a fitness cost. If fungicide resistance does occur in FON populations, this research should provide a plausible starting point for further investigations into resistance mechanisms. Primers designed for promoter and coding region amplification are novel and can be applied for the aforementioned research, as could be methods for mutagenesis. Investigations into efflux transporters, particularly ATP-binding cassette (ABC) transporters, could be the next step for future research. Additional research could also include sequencing a greater number of highly and intermediately resistant isolates to determine if the point mutations seen in this study continue to appear in the same position, or if point mutations are linked to the overexpression of *CYP51A*. Determining the mechanism behind increased gene expression requires extensive research into proteomics, however, it could start with analyzing DNA methylation and histone modification of promoter regions [38].

Overall, the results presented in this thesis provide improvements on the current system for FON race differentiation and information about potential mechanisms for fungicide resistance in the same pathogen. Two loop mediated isothermal amplification (LAMP) assays, and a PCR primer set were developed to detect and differentiate FON and its races. Our new FNR3 PCR marker has the ability to differentiate what are currently known as race 3 isolates from races 1 and

2 using a region absent in chromosome 14 of the race 3 genomes. The whole genome sequences used for the development of FNR3 are published on NCBI and were the first available whole genomes of FON on the database. Finally, mutants resistant to the only registered fungicide, Proline (a.i. prothioconazole), were determined to have two different possible mechanisms accounting for the resistant phenotype: point mutations in the coding region and overexpression of the target gene, *FONCYP51A*. An increased number of whole genome sequences with documented pathogenicity studies will improve the correlative ability of researchers developing new resistant watermelon varieties and methods of detection and race differentiation. Studies attempting to correlate genetic function and molecular pathways related to pathogenicity will better characterize the variability within FON isolates. Additional characterization and sequencing of fungicide resistant mutants would increase understanding about the genetic effect on resistance in FON. Overall, the findings in this thesis could simplify disease diagnosis and provide information on the risk and mechanisms of resistance development to prothioconazole in chemical management. Information generated from these studies should be used in addition to the most up to date research of FON genomics to improve management of the disease going forward.

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APPENDIX A

APPENDIX TO CHAPTER 4

Table A.1. Race differentiation using PCR assays with all pathogenic FON isolates tested.

Sl. no	Isolate Name	Source	Tested primer			Race
			FON1-2	SIX6F/R	FNR3	
1	GA-1	GA	P	N	P	R2
2	GA-2	GA	P	N	P	R2
3	GA-3	GA	P	N	P	R2
4	GA-4	GA	P	P	N	R3
5	GA-5	GA	P	N	P	R2
6	GA-6	GA	P	P	N	R3
7	GA-7	GA	P	P	N	R3
8	GA-8	GA	P	P	P	R1
9	GA-9	GA	P	P	N	R3
10	GA-10	GA	P	P	P	R1
11	GA-11	GA	P	P	P	R1
12	GA-12	GA	P	N	P	R2
13	GA-13	GA	P	P	P	R1
14	GA-14	GA	P	P	P	R1
15	GA-15	GA	P	P	P	R1
16	GA-16	GA	P	P	P	R1
17	GA-17	GA	P	P	P	R1
18	GA-18	GA	P	P	P	R1
19	GA-19	GA	P	P	P	R1

20	GA-20	GA	P	P	N	R3
21	GA-21	GA	P	P	P	R1
22	GA-22	GA	P	P	P	R1
23	GA-23	GA	P	P	N	R3
24	GA-24	GA	P	P	N	R3
25	GA-25	GA	P	P	P	R1
26	GA-26	GA	P	P	P	R1
27	GA-27	GA	P	N	P	R2
28	GA-28	GA	P	N	P	R2
29	FL-1	FL	P	N	P	R2
30	FL-2	FL	P	P	P	R1
31	FL-3	FL	P	P	N	R3
32	FL-4	FL	P	N	P	R2
33	FL-5	FL	P	P	P	R1
34	FL-6	FL	P	P	P	R1
35	FL-7	FL	P	P	P	R1
36	FL-8	FL	P	P	P	R1
37	FL-9	FL	P	P	P	R1
38	FL-10	FL	P	P	P	R1
39	FL-11	FL	P	P	P	R1
40	FL-12	FL	P	P	N	R3
41	FL-13	FL	P	P	P	R1
42	FL-14	FL	P	P	P	R1
43	FL-15	FL	P	P	P	R1
44	FL-16	FL	P	P	P	R1
45	FL-17	FL	P	N	P	R2
46	FL-18	FL	P	P	P	R1
47	FL-19	FL	P	P	P	R1
48	FL-20	FL	P	P	P	R1

49	FL-21	FL	P	P	P	R1
50	FL-22	FL	P	N	P	R2
51	FL-23	FL	P	P	P	R1
52	FL-24	FL	P	P	P	R1
53	FL-25	FL	P	P	P	R1
54	FL-26	FL	P	P	P	R1
55	FL-27	FL	P	P	P	R1
56	FL-28	FL	P	P	N	R3
57	FL-29	FL	P	N	P	R2
58	FL-30	FL	P	P	P	R1
59	FL-31	FL	P	N	P	R2
60	FL-32	FL	P	P	P	R1
61	FL-33	FL	P	P	P	R1
62	FL-34	FL	P	P	P	R1
63	FL-35	FL	P	P	N	R3
64	FL-36	FL	P	P	P	R1
65	FL-37	FL	P	P	P	R1
66	FL-38	FL	P	P	P	R1
67	FL-39	FL	P	P	P	R1
68	FL-40	FL	P	P	N	R3
69	FL-41	FL	P	P	P	R1
70	FL-42	FL	P	P	N	R3
71	FL-43	FL	P	N	P	R2
72	FL-44	FL	P	P	P	R1
73	FL-45	FL	P	N	P	R2
74	FL-46	FL	P	N	P	R2
75	FL-47	FL	P	N	P	R2
76	FL-48	FL	P	P	P	R1
77	SC-1	SC	P	P	N	R3

78	SC-2	SC	P	P	P	R1
79	SC-3	SC	P	P	P	R1
80	SC-4	SC	P	P	P	R1
81	SC-5	SC	P	P	P	R1
82	SC-6	SC	P	P	P	R1
83	SC-7	SC	P	P	P	R1
84	SC-8	SC	P	P	P	R1
85	SC-9	SC	P	P	P	R1
86	SC-10	SC	P	N	P	R2
87	SC-11	SC	P	P	P	R1
88	SC-12	SC	P	N	P	R2
89	SC-13	SC	P	P	P	R1
90	SC-14	SC	P	N	P	R2
91	SC-15	SC	P	N	P	R2
92	SC-16	SC	P	N	P	R2
93	SC-17	SC	P	N	P	R2
94	SC-18	SC	P	N	P	R2
95	SC-19	SC	P	N	P	R2
96	SC-20	SC	P	P	P	R1
97	SC-21	SC	P	N	P	R2
98	SC-22	SC	P	N	P	R2
99	SC-23	SC	P	N	P	R2
100	SC-24	SC	P	P	N	R3
101	SC-25	SC	P	N	P	R2
102	SC-26	SC	P	N	P	R2
103	SC-27	SC	P	N	P	R2
104	SC-28	SC	P	N	P	R2
105	SC-29	SC	P	P	N	R3
106	SC-30	SC	P	P	N	R3

107	SC-31	SC	P	P	P	R1
108	SC-32	SC	P	P	P	R1
109	SC-33	SC	P	N	P	R2
110	SC-34	SC	P	P	P	R1
111	SC-35	SC	P	P	P	R1
112	SC-36	SC	P	N	P	R2
113	SC-37	SC	P	N	P	R2
114	SC-38	SC	P	N	P	R2
115	SC-39	SC	P	N	P	R2
116	SC-40	SC	P	P	P	R1
117	SC-41	SC	P	P	P	R1
118	SC-42	SC	P	P	P	R1
119	SC-43	SC	P	P	N	R3
120	SC-44	SC	P	P	N	R3
121	SC-45	SC	P	P	N	R3
122	SC-46	SC	P	P	P	R1
123	SC-47	SC	P	P	P	R1
124	SC-48	SC	P	P	N	R3
125	SC-49	SC	P	P	P	R1
126	SC-50	SC	P	P	N	R3
127	SC-51	SC	P	P	N	R3
128	SC-52	SC	P	P	N	R3
129	SC-53	SC	P	P	N	R3
130	SC-54	SC	P	P	P	R1
131	SC-55	SC	P	P	P	R1
132	SC-56	SC	P	P	N	R3
133	SC-57	SC	P	P	P	R1
134	SC-58	SC	P	P	N	R3
135	SC-59	SC	P	P	N	R3

136	SC-60	SC	P	P	P	R1
137	SC-61	SC	P	P	N	R3
138	SC-62	SC	P	P	N	R3
139	SC-63	SC	P	N	P	R2
140	SC-64	SC	P	P	P	R1
141	SC-65	SC	P	P	P	R1
142	SC-66	SC	P	P	P	R1
143	SC-67	SC	P	P	P	R1
144	SC-68	SC	P	P	P	R1
145	SC-69	SC	P	P	N	R3
146	SC-70	SC	P	P	P	R1
147	SC-71	SC	P	P	N	R3
148	SC-72	SC	P	P	P	R1
149	SC-73	SC	P	P	P	R1
150	SC-74	SC	P	P	N	R3
151	SC-75	SC	P	P	N	R3
152	SC-76	SC	P	P	N	R3
153	SC-77	SC	P	N	P	R2
154	SC-78	SC	P	P	P	R1
155	SC-79	SC	P	N	P	R2
156	SC-80	SC	P	N	P	R2
157	SC-81	SC	P	P	P	R1
158	SC-82	SC	P	P	N	R3
159	SC-83	SC	P	P	P	R1
160	SC-84	SC	P	P	P	R1
161	SC-85	SC	P	P	P	R1

Race determination was made according to the flowchart. P and N indicates positive and negative PCR amplification, respectively. R1 = Race 1, R2 = Race 2, R3 = Race 3.

Table A.2. Comparison of bioassay and PCR-based race differentiation results for all tested isolates.

Sl #	Isolate Name	Bioassay results	Molecular results
1	FL-1	R2	R2
2	FL-2	R3	R1
3	FL-4	R2	R2
4	FL-5	R3	R1
5	FL-6	R1	R1
6	FL-7	R3	R3
7	FL-8	R1	R1
8	FL-9	R3	R1
9	FL-10	R2	R2
10	FL-11	R3	R1
11	FL-12	R3	R3
12	FL-13	R2	R1
13	FL-14	R3	R1
14	FL-15	R3	R3
15	FL-17	R2	R2
16	FL-18	R3	R1
17	FL-21	R3	R1
18	FL-22	R2	R2
19	FL-23	R1	R1
20	FL-25	R3	R3
21	FL-27	R1	R1
22	FL-28	R3	R3
23	FL-29	R2	R2
24	FL-30	R2	R1
25	FL-31	R2	R2
26	FL-33	R1	R1
27	FL-34	R3	R1
28	FL-35	R2	R3
29	FL-36	R2	R1
30	FL-37	R1	R1
31	FL-38	R2	R1
32	FL-40	R3	R3
33	FL-42	R3	R3
34	FL-43	R2	R2
35	FL-46	R2	R2

36	SC-1	R1	R3
37	SC-2	R1	R1
38	SC-3	R1	R1
39	SC-4	R1	R1
40	SC-5	R1	R1
41	SC-6	R1	R1
42	SC-7	R1	R1
43	SC-8	R1	R1
44	SC-9	R1	R1
45	SC-10	R2	R2
46	SC-11	R1	R1
47	SC-12	R2	R2
48	SC-13	R1	R2
49	SC-14	R2	R2
50	SC-15	R1	R2
51	SC-16	R2	R2
52	SC-17	R2	R2
53	SC-18	R2	R2
54	SC-19	R2	R2
55	SC-20	R2	R1
56	SC-21	R2	R2
57	SC-22	R2	R2
58	SC-23	R2	R2
59	SC-25	R2	R2
60	SC-27	R2	R2
61	SC-31	R1	R1
62	SC-32	R1	R1
63	SC-33	R2	R2
64	SC-34	R1	R1
65	SC-35	R1	R1
66	SC-36	R2	R2
67	SC-37	R2	R2
68	SC-39	R2	R2
69	SC-40	R1	R1
70	SC-41	R1	R1
71	SC-42	R2	R1
72	SC-46	R1	R1
73	SC-57	R1	R1
74	SC-70	R1	R1
75	SC-77	R2	R2
76	SC-78	R2	R1

77	SC-79	R2	R2
78	SC-80	R2	R2
79	GA-1	R2	R2
80	GA-2	R2	R2
81	GA-3	R2	R2
82	GA-4	R3	R3
83	GA-5	R3	R2
84	GA-6	R3	R3
85	GA-7	R3	R3
86	GA-9	R3	R3
87	GA-13	R1	R1
88	GA-14	R1	R1
89	GA-20	R3	R3
90	GA-23	R3	R3
91	GA-24	R3	R3
92	GA-27	R2	R2
93	GA-28	R2	R2

APPENDIX B

APPENDIX TO CHAPTER 5

Table B.1. List of primers used in this study.

Primer name	Sequence	Assay	Conditions	Amplicon size (bp)	Source
FCYPA1upF	GCTTACGATCGGAGAAGAACA	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	336	This study
FCYPA1upR	AGGCCCATAGAGGGTAGTATAG				
FCYPA2upF	CCTTTGCTTCCTGCCTAGTT	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	347	This study
FCYPA2upR	GAGCTTCGAGTTGGGACAAT				
FCYPB1upF	GTGTTTGACCGTTGTGTTTGAG	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 57 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	364	This study
FCYPB1upR	CATGGACGGTTCCTGGAAATA				
FCYPB2upF	TATTTCCAGGAACCGTCCATG	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 60 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	393	This study
FCYPB2upR	CGTTCCGTTTCGAAGGATGA				
FCYPC1upF	CCCAGTACATAATAGCAGGAGTG	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	349	This study
FCYPC1upR	AAATTGGTCGCTCTGACTCAC				
FCYPC2upF	GTGAGTCAGAGCGACCAATTT	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 59 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	408	This study
FCYPC2upR	GGTGTCGGGATGAGGATTTG				
FCypA1F	TGAGGACGCGAATCCTTCTG	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 59 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	692	This study
FCypA1R	TGCTCCATGAGCTTCGAGTT				
FCypA2F	TCCCAACTCGAAGCTCATGG	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	759	This study
FCypA2R	CCAGGTGCTTGCATAGGTCT				
FCypA3F	CCTATGCAAGCACCTGGATCA	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	678	This study
FCypA3R	GTGGAATTGTGCAAATAGGGCA				
FCypA4F	TCCACCTCTACTGTTGCGAA	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	680	This study
FCypA4R	CGACCCCGCTTATACCAAGG				
FCypA5F	CCTTGGTATAAGCGGGGTCG	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	712	This study
FCypA5R	ACCCTACCCGCTTCTTGTTT				
FOCYP51Bpyes2-F	GGGTCTCCTCCAAGAACTT	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	692	Zheng et al. (2018) &
FCypB1R	CGAACCTCGGCGGAGATAAT				

					This study
FCypB2F	CGTGCCTATTATCTCCGCCG	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	672	This study
FCypB2R	ACAGGCATGGGAGACTTGAC				
FCypB3F	GTCAAGTCTCCCATGCCTGT	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	444	This study & Zheng et al. (2018)
FoCYP51Bpyes2-R	CTACTGCTGGCGTCTCTC				
FCypC1F	AGCACATTCGCAACCCTGTA	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	675	This study
FCypC1R	TTCATCACGCCGAAGCCATA				
FCypC2F	TTATGGCTTCGGCGTGATGA	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	691	This study
FCypC2R	CCGGCAGTCCAGGTATCTTTT				
FCypC3F	AAGATACCTGGACTGCCGGA	PCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	505	This study
FCypC3R	CACTGTTGGGACGCATCTA				
FCAq1F	TGGCTACCTTTGCCTCATAAC	qPCR	95 °C for 3 mins, 35 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 1 min, then 72 °C for 6 mins	120	This study
FCAq1R	GTCAGTTCCTTTCTCCAAGTCC				
Fn-1	TACCACTTGTTGCCTCGGC	qPCR	95°C for 2 mins, 40 cycles of 95°C for 10s and 60°C for 40s and a temperature ramp of 0.2°C/s	327	Zhang et al. (2006)
Fn-2	TTGAGGAACGCGAATTAAC				

Table B.2. Resistant phenotype and the relative *CYP51A* expression of the FON mutants

Resistant Group	EC_{50}	Resistance Factor	Relative Expression
Highly Resistant 1	47.679	9.562767	23.10286712836
Highly Resistant 2	172.228	34.5430113	38.0546276800871
Highly Resistant 3	184.78	37.0605106	46.6886510156855
HR mean	108.33	21.7272709	35.94871527
Intermediately Resistant 1	26.365	5.28791191	12.5099142900575
Intermediately Resistant 2	36.45	7.31061594	27.8576180254759
Intermediately Resistant 3	33.321	6.68304619	14.1232479406504
IR mean	31.77	6.37196895	18.16359342
Sensitive 1	4.217	0.84578511	8.75434961008591
Sensitive 2	6.969	1.39774163	11.79415374
Sensitive 3	4.079	0.81810706	4.642816
S mean	4.9859	1	8.39710634

Figure B.1. Molecular structure of prothioconazole

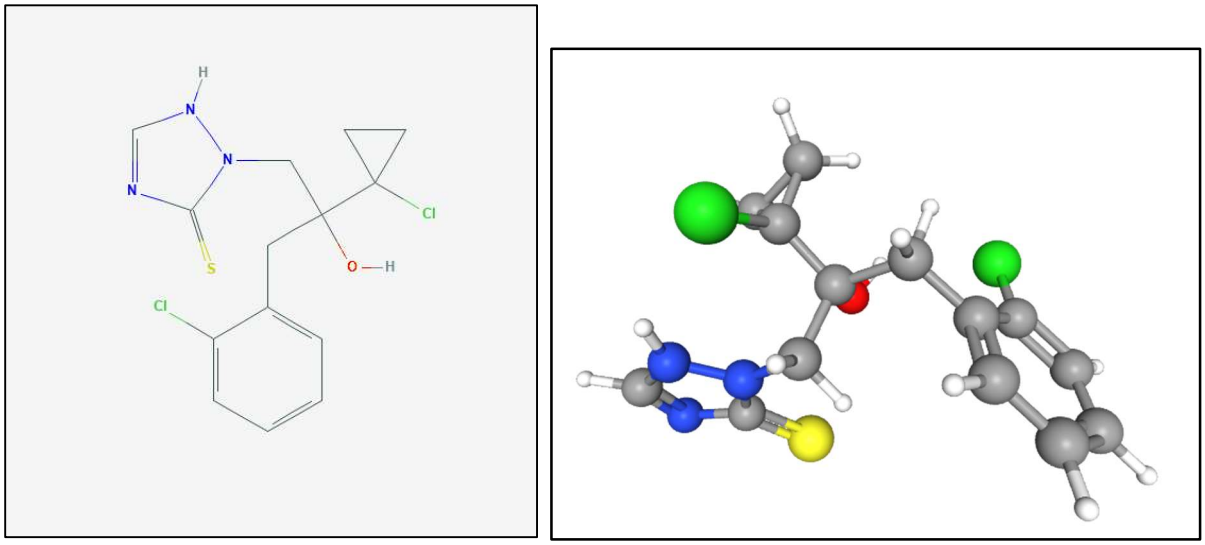

Figure B.2. Nucleotide sequence of *CYP51A* from sensitive and resistant isolates

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Sensitive1 ATGTTCTCACTCCTATACTACCCTCTATGGGCCTTTGCTTCCTGCCTAGT     50
           ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1 ATGTTCTCACTCCTATACTACCCTCTATGGGCCTTTGCTTCCTGCCTAGT     50

Sensitive51 TATCATCACTCTCAACGTCTTATACCAGAAGCTCCCTCGAAATGCCAACG    100
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant51 TATCATCACTCTCAACGTCTTATACCAGAAGCTCCCTCGAAATGCCAACG    100

Sensitive101 AACCTCCGTTAGTGTTCCACTGGCTTCCATTCGTTGGGAATGCTGTTGCT    150
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant101 AACCTCCGTTAGTGTTCCACTGGCTTCCATTCGTTGGGAATGCTGTTGCT    150

Sensitive151 TATGGACTCGACCCTTATGGTTTCTTTGTGAAGTGTCGAGAAAAGCACGG    200
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant151 TATGGACTCGACCCTTATGGTTTCTTTGTGAAGTGTCGAGAAAAGCACGG    200

Sensitive201 CGATGTCTTCACCTTTATCCTCTTCGGTCGAAAAATCGTTGCCTGTCTTG    250
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant201 CGATGTCTTCACCTTTATCCTCTTCGGTCGAAAAATCGTTGCCTGTCTTG    250

Sensitive251 GTGTTGACGGCAATGACTTTGTTCTCAACAGTCGAATTCAGGACGCCAAC    300
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant251 GTGTTGACGGCAATGACTTTGTTCTCAACAGTCGAATTCAGGACGCCAAC    300

Sensitive301 GCCGAAGAAATCTACAGTCCATTGACAACGCCTGTCTTTGGTAGTGATGT    350
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant301 GCCGAAGAAATCTACAGTCCATTGACAACGCCTGTCTTTGGTAGTGATGT    350

Sensitive351 CGTATACGATTGTCCCAACTCGAAGCTCATGGAGCAAAAGAAGTTTGTCA    400
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant351 CGTATACGATTGTCCCAACTCGAAGCTCATGGAGCAAAAGAAGTTTGTCA    400

Sensitive401 AGTTTGGCCTTACACAAAAGGCTCTCGAGTCCCATGTCCAGTTGATCGAG    450
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant401 AGTTTGGCCTTACACAAAAGGCTCTCGAGTCCCATGTCCAGTTGATCGAG    450

Sensitive451 CGAGAGGTTCTGGAGTACATCCAAGCTGTACCTTCATTCTCTGGAAAGTC    500
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant451 CGAGAGGTTCTGGAGTACATCCAAGCTGTACCTTCATTCTCTGGAAAGTC    500

Sensitive501 TGGCACAGTTGATGTATCCAAGGCAATGGCTGAGATAACCATCTTCACTG    550
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant501 TGGCACAGTTGATGTATCCAAGGCAATGGCTGAGATAACCATCTTCACTG    550

Sensitive551 CTGCTCGCTCTCTGCAGGGCGAAGAAGTTCGACGGAAGCTTACAGCTGAG    600
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant551 CTGCTCGCTCTCTGCAGGGCGAAGAAGTTCGACGGAAGCTTACAGCTGAG    600

Sensitive601 TTTGCAGCTCTGTATCATGACCTTGACCTAGGCTTCACTCCTGTAAACTT    650
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant601 TTTGCAGCTCTGTATCATGACCTTGACCTAGGCTTCACTCCTGTAAACTT    650

Sensitive651 CCTGTTCCCTTGGCTACCTTTGCCTCATAACCGACGTCGAGATGCTGCTC    700
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant651 CCTGTTCCCTTGGCTACCTTTGCCTCATAACCGACGTCGAGATGCTGCTC    700
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Sensitive701 ATGCAAAGATGAGAGAGATCTACATGGACATCATTAACGAACGAAGAAGA    750
             ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant701 ATGCAAAGATGAGAGAGATCTACATGGACATCATTAACGAACGAAGAAGA    750

Sensitive751 GGCGTAGGGGACTTGGAGAAAGGAACTGACATGATCGCCAACCTGATGAA    800
             ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant751 GGCGTAGGGGACTTGGAGAAAGGAACTGACATGATCGCCAACCTGATGAA    800

Sensitive801 TTGCGAGTACAAAAACGGGCAGCCGATTCCGGACAAAGAGATCGCGTACA    850
             |||||||||||||||||||||||||||||||||||||||||||| |||
Resistant801 TTGCGAGTACAAAAACGGGCAGCCGATTCCGGACAAAGAGATCGCGCACA    850

Sensitive851 TGATGATCACTCTTCTCATGGCTGGACAACACTCTTCGTCATCTGCTAGT    900
             ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant851 TGATGATCACTCTTCTCATGGCTGGACAACACTCTTCGTCATCTGCTAGT    900

Sensitive901 TCATGGATCATACTACATCTGGCTTCATCCACTGACATTGCTGAGGAACT    950
             ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant901 TCATGGATCATACTACATCTGGCTTCATCCACTGACATTGCTGAGGAACT    950

Sensitive951 CTACCAAGAGCAACTCATTAACTTGAGTGCCGATGGTGTTCTCCCTCCCC   1000
             ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant951 CTACCAAGAGCAACTCATTAACTTGAGTGCCGATGGTGTTCTCCCTCCCC   1000

Sensitive1001 TTCAGTACTCCGATCTCGACAAGCTTCCCCTTCTTCAGAATGTCGTCAAA   1050
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1001 TTCAGTACTCCGATCTCGACAAGCTTCCCCTTCTTCAGAATGTCGTCAAA   1050

Sensitive1051 GAAACACTCCGTGTTCATTCTTCCATTCACTCCATTCTGCGAAAGGTTAA   1100
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1051 GAAACACTCCGTGTTCATTCTTCCATTCACTCCATTCTGCGAAAGGTTAA   1100

Sensitive1101 AAGACCTATGCAAGCAACTGGATCACCTTACACCATCACCACAGACAAGG   1150
               ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1101 GAGACCTATGCAAGCAACTGGATCACCTTACACCATCACCACAGACAAGG   1150

Sensitive1151 TTCTCCTCGCTTCACCAACTGTTACAGCGTTGAGTGAAGAACACTTCACA   1200
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1151 TTCTCCTCGCTTCACCAACTGTTACAGCGTTGAGTGAAGAACACTTCACA   1200

Sensitive1201 GACGCCCAAAGATGGAATCCTCATCGGTGGGATAACAAACCCCAGGAGGA   1250
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1201 GACGCCCAAAGATGGAATCCTCATCGGTGGGATAACAAACCCCAGGAGGA   1250

Sensitive1251 GGCCGTGACGGACGATGTCATTGACTACGGCTACGGCGCTGTTTCTAAAG   1300
              |||||||||||||||||||||||||||||||||||||||| ||||||
Resistant1251 GGCCGTGACGGACGATGTCATTGACTACGGCTACGGCGCTGTTACTAAAG   1300

Sensitive1301 GAACGAAGAGCCCATACTTACCCTTTGGCGCTGGTCGGCATCGCTGCATC   1350
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1301 GAACGAAGAGCCCATACTTACCCTTTGGCGCTGGTCGGCATCGCTGCATC   1350

Sensitive1351 GGGGAGAAGTTTGCTTATGTCAACTTGGGCGTTATCGTCGCGACTTTGGT   1400
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1351 GGGGAGAAGTTTGCTTATGTCAACTTGGGCGTTATCGTCGCGACTTTGGT   1400
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Sensitive1401 GCGCAACTTCAGACTGTCGACTCTTGATGGCAAGCCTGGTGTTCCGGCAA   1450
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1401 GCGCAACTTCAGACTGTCGACTCTTGATGGCAAGCCTGGTGTTCCGGCAA   1450

Sensitive1451 CTGACTACACTTCTCTCTTCTCAAGGCCAGCCCAACCTGCATACATAAAC   1500
              ||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1451 CTGACTACACTTCTCTCTTCTCAAGGCCAGCCCAACCTGCATACATAAAC   1500

Sensitive1501 TGGGAGCGCAGGAGGGCTTAA   1521
              ||||||||||||||||||||
Resistant1501 TGGGAGCGCAGGAGGGCTTAA   1521
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Figure B.3. Amino acid sequence of *CYP51A* from sensitive and resistant isolates

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Sensitive1 MFSLLYYPLWAFASCLVIITLNVLYQKLPRNANEPPLVFHWLPFVGNAVA     50
           |||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant1 MFSLLYYPLWAFASCLVIITLNVLYQKLPRNANEPPLVFHWLPFVGNAVA     50

Sensitive51 YGLDPYGFFVKCREKHGDVFTFILFGRKIVACLGVDGNDFVLNSRIQDAN    100
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant51 YGLDPYGFFVKCREKHGDVFTFILFGRKIVACLGVDGNDFVLNSRIQDAN    100

Sensitive101 AEEIYSPLTTPVFGSDVVYDCPNSKLMEQKKFVKFGLTQKALESHVQLIE    150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant101 AEEIYSPLTTPVFGSDVVYDCPNSKLMEQKKFVKFGLTQKALESHVQLIE    150

Sensitive151 REVLEYIQAVPSFSGKSGTVDVSKAMAEITIFTAARSLQGEEVRRKLTAE    200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant151 REVLEYIQAVPSFSGKSGTVDVSKAMAEITIFTAARSLQGEEVRRKLTAE    200

Sensitive201 FAALYHDLDLGFTPVNFLFPWLPLPHNRRRDAAHAKMREIYMDIINERRR    250
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant201 FAALYHDLDLGFTPVNFLFPWLPLPHNRRRDAAHAKMREIYMDIINERRR    250

Sensitive251 GVGDLEKGTDMIANLMNCEYKNGQPIPDKEIAYMMITLLMAGQHSSSSAS    300
             |||||||||||||||||||||||||||||||| ||||||||||||||||||
Resistant251 GVGDLEKGTDMIANLMNCEYKNGQPIPDKEIAHMMITLLMAGQHSSSSAS    300

Sensitive301 SWIILHLASSTDIAEELYQEQLINLSADGVLPPLQYSDLDKLPLLQNVVK    350
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant301 SWIILHLASSTDIAEELYQEQLINLSADGVLPPLQYSDLDKLPLLQNVVK    350

Sensitive351 ETLRVHSSIHSILRKVKRPMQATGSPYTITTDKVLLASPTVTALSEEHFT    400
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant351 ETLRVHSSIHSILRKVKRPMQATGSPYTITTDKVLLASPTVTALSEEHFT    400

Sensitive401 DAQRWNPHRWDNKPQEEAVTDDVIDYGYGAVSKGTKSPYLPFGAGRHRCI    450
             |||||||||||||||||||||||||||||| ||||||||||||||||||
Resistant401 DAQRWNPHRWDNKPQEEAVTDDVIDYGYGAVTKGTKSPYLPFGAGRHRCI    450

Sensitive451 GEKFAYVNLGVIVATLVRNFRLSTLDGKPGVPATDYTSLFSRPAQPAYIN    500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Resistant451 GEKFAYVNLGVIVATLVRNFRLSTLDGKPGVPATDYTSLFSRPAQPAYIN    500

Sensitive501 WERRRA    506
             ||||||
Resistant501 WERRRA    506
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