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Verticillium Dahliae Causes the Fungal Wilting Disease of Cotton Plants Grown on the Mississippi State North Farm

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By

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The emergence and spread of *Verticillium* wilt were observed in cotton plants at the R.R. Foil Plant Science Research Center at Mississippi State during the late summer in 2013 and 2014. Several fungi with different morphology and growth characteristics were isolated from diseased cotton plants. Genomic DNA was extracted from the isolated fungal species and used in molecular typing via PCR amplification and DNA sequencing analysis of the ribosomal internal transcribed spacer (ITS) region. A total of five fungal genera were identified, and *Verticillium* sp. was the most frequently isolated genus. The isolated *Verticillium* strains could be *Verticillium dahliae*, *Verticillium longisporum* or even *Verticillium albo-atrum*. A PCR-based genotyping method using *VTA2* (*Verticillium* transcription activator) gene specific primers confirmed that the isolated *Verticillium* strain was *Verticillium dahliae*, and it caused *Verticillium* wilt in Mississippi cotton plants. Pathogenicity tests (Koch's postulates) confirmed the earlier qualitative identifications of *Verticillium dahliae* in the greenhouse.

DEDICATION

I would like to dedicate this work to Allah who helped and guided me throughout my graduate study. I would also like to dedicate this work to my family who encourage and support me throughout this journey.

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LIST OF ABBREVIATIONS

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide BLAST
bp	Base pair (s)
BSA	Bovine serum albumin
C	Cytosine
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
E. coli	*Escherichia coli*
eLRR-RLPs	extracellular leucine-rich repeat class of receptor-like proteins
EDTA	ethylenediaminetetraacetate
g	Gram (s)
G	Guanine
G.	*Gossypium*
gDNA	Genomic deoxyribonucleic acid

hr	Hour (s)
HGT	Horizontal Gene Transfer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
ITS1-F	Internal transcribed spacer 1 forward primer
L	Liter (s)
LB	Luria-Bertani
kb	Kilobase (s)
LRR	leucine-rich repeat
M	Molar
mg	milligrams
min	Minute (s)
miRNAs	MicroRNAs
mL	milliliters
mM	millimolar
nt	Nucleotide
OD	Optical density
PDA	Potato dextrose agar
PVP	Polyvinylpyrrolidone
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid

rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
s	Second (s)
sp.	species
spp.	species
sVTA2-F	specific *Verticillium* transcription activator 2 forward primer
sVTA2-R	specific *Verticillium* transcription activator 2 reverse primer
T	Thymine
TAE	Tris-base Acetate EDTA
Tet	Tetracycline
TRR	Terminator ready reaction
U	Unit (s)
UV	UltraViolet
V	Volt (s)
V.	*Verticillium*
Va	*Verticillium albo-atrum*
Vd	*Verticillium dahliae*
VdVTA2	*Verticillium dahliae Verticillium* transcription activator 2
Vd2	*V. dahliae* race 2
Vl	*Verticillium longisporum*
VTA2	*Verticillium* transcription activator 2
VW	*Verticillium* wilt

w/v	Mass/volume
X g	Number of times the gravitational force (G-Force)
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
°C	Degree Celsius
µL	microliter
µM	micromolar

CHAPTER I

INTRODUCTION AND BACKGROUND

Cotton Plant

Cotton has been cultivated in the Old World for at least 7,000 years (5th millennium BC). Evidence of planting cotton has been observed at the Mehrgarh site in Pakistan, where early cotton threads have been saved in copper beads (Moulherat et al. 2002). The current English name of cotton came initially from the Arabic languish 'quotn' نِطُق which began to be employed circa 1400 AD (Metcalf, 1999). Commonly, cotton refers to species that create spinnable fibers (lint) on their seed coats (Regulator, 2013). Since then, all modern-day literature has used this nomenclature for cotton.

Cotton plants are a shrub native to tropical and subtropical areas around the world, covering the Americas, Africa, and India (Pickering, 2008). The greatest varieties of wild cotton species are found in Mexico, and then followed by Australia and Africa. Cotton was domesticated independently in both the old and new worlds (Hasta luego, 2012).

During the years between statehood and the Civil War, Mississippi's economy grew rapidly. Mississippi became the largest and most dynamic cotton-producing state in the United States of America between 1817 and 1860. The cotton crop was so valuable and it was referred as white gold (Casil, 2010).

By the 1830s, Mississippi was the state to have the highest cotton yields in the United States of America, which resulted in high demand for enslaved labor. The population and cotton production statistics tell a simple, but significant story (Eugene, 2006). The growth of Mississippi's population before its admission to statehood and afterward is clearly related to the rise of cotton stock. Cotton production in Mississippi expanded from nothing in 1800 to 535.1 million pounds in 1859 (Phalen, 2014).

Taxonomy of the Cotton

Cotton belongs to the genus *Gossypium*, which was named by Linnaeus in the middle of the eighteenth century. The genus has been classified under both the Malvaceae (mallow family) and Bombacaceae plant family and in both of the Hibsceae and *Gossypieae* tribes (Box, 2000). Nowadays, the genus is firmly arranged in the *Malvales* order, the *Malvaceae* family and tribe *Gossypieae*, due to the uniqueness of the lysigenous glands found completely in species within the genus. These glands contain a number of sesquiterpenes, conjointly described as gossypol. Only those species of Gossypium yielding seed hairs can be called cotton correctly (Regulator, 2013).

Gossypium hirsutum is also known as American cotton, Bourbon cotton, Cotton Belt cotton, Upland cotton, and West Indian cotton (Small et al., 2009).

The taxonomy of *Gossypium* is still a counterfactual history topic. According to Smith, the genus *Gossypium* includes 43 species (Smith, 1995), which consist of 37 diploid species (2n = 2x = 26) and six tetraploid (2n = 4x = 52) species. Fifty species of the cotton genus *Gossypium L.* (*Malvaceae*) are classified into eight genome groups, designated A through G and K (Cronn et al., 2002), on the basis of chromosome pairing affinities (Endrizzi et al., 1984). However, other authors list 49 diploid (2n = 26) species

in total but include only five allotetraploids (2n = 52) (Tabbasam et al. 2014), which are a monophyletic assemblage derived from an individual allopolyploidization event that happened 1.5 million years ago (An et al., 2008). The genomes of the five tetraploid species are designated $(AD)_1$ through $(AD)_5$ for their genome constitutions (Singh, 2010; Zhang et al., 2008).

Cultivation of Cotton

It is believed that cotton has been planted and cultured in the United States since 1621 (Blake, 1869). Four *Gossypium* species are cultivated, including the diploids *G. arboreum* L. and *G. herbaceum* L. and the tetraploids *G. hirsutum* L. and *G. barbadense* L. The principal cultivated cotton species are *G. hirsutum* and *G. barbadense*. 'Upland' cotton (*G. hirsutum* L.) comprises nearly 95 percent of the world's cotton fiber yields (Chen et al., 2007; Guo et al., 2008), and approximately 5% of the global fiber production is from *G. barbadense* (d'Eeckenbrugge et al., 2014; Percy et al., 2006) which originally grew in Egypt, Peru, Sudan, the USA, and parts of the former Soviet Union. *G. arboreum* is grown largely in India, and *G. herbaceum* is grown in the dry regions of Africa and Asia (Stewart et al., 2010). The genetic diversity among modern varieties of upland cotton is quite narrow, as reported by isozyme analysis (Wendel et al., 1992).

Usage of Cotton

Cotton is the world's largest natural fiber crop, and it is also an essential source of oil and protein (Zhang et al., 2011). Cottonseed is used to provide vegetable oil for cooking (Company et al., 1942); cottonseed meal, which is the highest quality nutrition protein supplement for poultry and livestock (Briggs, 2014); cottonseed hulls, a fodder to

feed cattle and other livestock (Sarwatt et al., 2004); and linters, a cellulosic feedstock for multiple consumer and industrial products. Cottonseed meal is an excellent source of protein and it can be used in various ways, alone or with other protein sources (Rojas et al., 1969). Furthermore, the hard outer covering of the cottonseed, which is typically known as the hull, is removed from the cotton seeds before extracting the oil. Cottonseed hulls serve as an excellent source of feed for the cattle and other livestock as they contain approximately 6% to 8% linter cotton (Gregg et al., 2010; Zaborsky, 1981) and approximately 95% cellulose (Francis, 1914). Moreover, cottonseed oil can be utilized as cooking oil (Small, 2009) and salad dressing (Station et al., 1934). Cottonseed oil was the first vegetable oil used in the United States (Small, 2009). In addition, Cottonseed oil was also used for medical purposes (Thurman, 1940).

Vascular Wilt

Vascular wilt diseases are caused by pathogenic fungi (Dimond, 1971). The fungi enter into the xylem vessel of a host plant. They multiply inside the vessels and cause a blockage of water transport (Saravanan et al., 2004). The disease symptoms caused by the fungi involve wilting and death of the leaves, which is frequently followed by the death or serious deterioration of the entire host plant (Publishing, 2010; Schall, 2008). Vascular wilts occur in group because symptoms related to the appearance of the pathogen in the vascular system of the plant host are usually in the xylem tissue (Fradin et al., 2009). Several fungal genera, including *Ceratocystis, Fusarium, Ophiostoma,* and *Verticillium* cause vascular wilt diseases (Chaube and Spundhir, 2005) although the *Verticillium* and *Fusarium* species are the most common ones (Fradin, 2009). Each of them causes wilt disease in many major crops. *Ceratocystis fagacearum* causes vascular

wilt in oak trees. *Fusarium* causes vascular wilt in crops. *Ophiostoma ulmi* causes Dutch elm disease. *Verticillium* causes vascular wilt diseases in field crops, ornamentals, fruit, and forest trees. Two *Verticillium* species, *V. albo-atrum* and *V. dahliae*, attack hundreds of plant types. Many host plants are attacked by particular types or species of the fungi (Chaube and Spundhir, 2005; Fradin et al., 2009; Pieterse et al., 2014; Schall, 2008).

Verticillium wilt (VW) or tracheomycosis is a vascular wilt disease (Caroselli, 1957). Like other wilt diseases, the VW disease is highly destructive and causes significant damage to many major cultivated and wild-type crop species of commercial plants (Rosenthal et al., 2012). VW causes huge economic loss to many crops and ornamental plants in multiple regions of the world (Zhao et al., 2014). In 1950, VW was first reported in peanuts (*Arachis hypogaea* L.) in New Mexico (Woodward et al., 2011). VW is widespread in the standard commercial cultivars of the cotton plants and has been observed across the US Cotton Belt, from South Carolina to California (Pérez-Artés, 2000).

Verticillium species have several special features. They have many hosts (Schneiter et al., 1997), and their propagules can survive in infected soil for many years as microsclerotia (Figure 1.A), or as mycelium or conidia (Figure 1.B) in the vascular system of perennial plants (Zhang et al., 2014). Figure 1.C shows the conidiophore, which is the key for identification of the Verticillium fungi (Bolda et al., 2013).

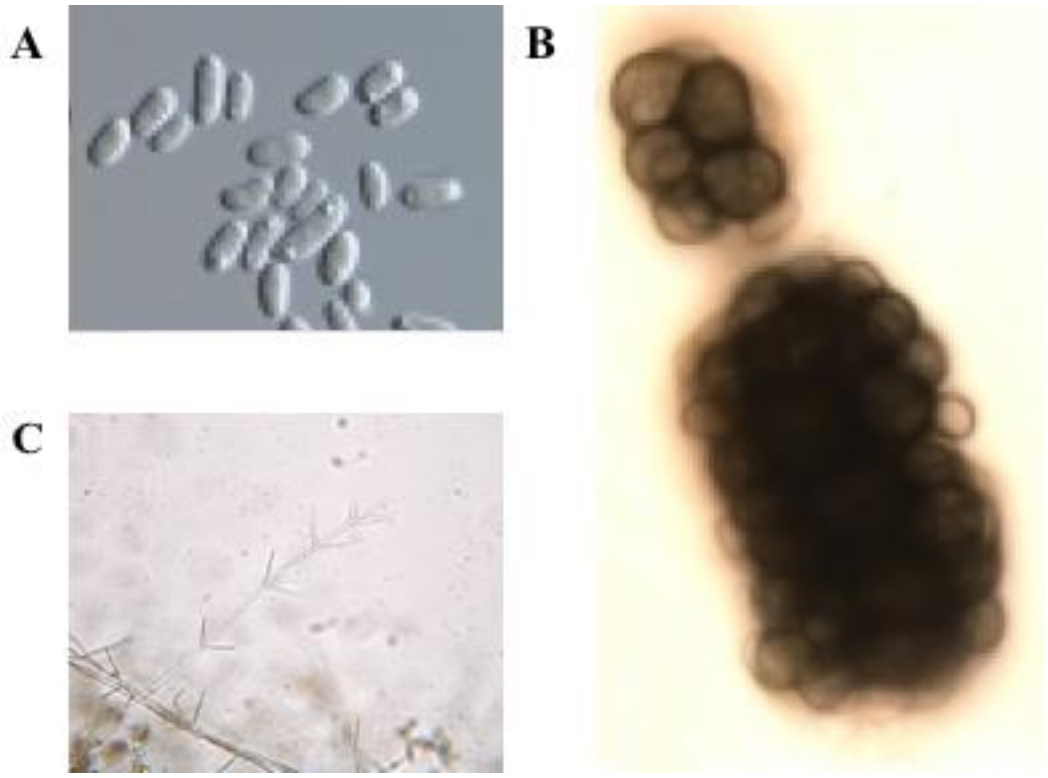

Figure 1 Morphological features of *Verticillium dahliae* strain PD322

A, Conidia. B, Microsclerotia (Subbarao, 2011). C, Conidiophore (Bolda, 2013).

Under optimal growing conditions, *Verticillium* spp. produce dark microsclerotia (Isaac, 1967). Microsclerotia may survive under field conditions for up to 14 years in the absence of the host (Wilhelm, 1955). *Verticillium* spp. also have an unknown sexual state and reproduces asexually, which suggests that somatic mutation is a dominant force in generating genetic diversity (Amyotte et al., 2012).

The way that *Verticillium* spp. infect host plants is also distinctive from multiple pathogens since they do not produce appressoria (a bulbous formation) (Kempken, 2013; Tran et al., 2014). *Verticillium* spp. penetrate young roots, settles into the xylem and phloem cells of the vascular tissue, and then flows, along with water and nutrients, to and within the roots, stems, and leaves (Horsfall, 2012; Kailis et al., 2007; Vossen, 2007;

Welbaum, 2015). The reduced water and nutrient transport in chlorotic and necrotic parts decreases lint yield, yield components, and substantially affects fiber quality such as fiber length (Mejda et al., 2007).

Verticillium spp. have a wide host range. When they infect host plants, symbiotic relationships can be developed into three different types: susceptibility, tolerance and resistance (Robb, 2007). With high levels of symptoms and fungal amount, a host plant is said to be susceptible (Lucas et al., 2012). When there are low levels of symptoms and high levels of fungal quantity, a host plant is characterized as a tolerant responder. A host plant is described as a resistant responder, when there are both low levels of symptoms and fungal amount. The way in which plants resist *Verticillium* spp. are another exciting feature of the disease. In most plants, such as potatoes, resistance is polygenic; nevertheless, the dominant single-gene resistance is observed in cotton and potato (Jansky et al., 2004; Kawchuk et al., 2001; Lynch et al., 1997). The mechanism of single-gene resistance to *Verticillium* spp. however is unknown (Gururani et al., 2012; Klosterman et al., 2009).

Verticillium Wilt

Nees von Essenbeck built the genus *Verticillium* in 1816. The genus *Verticillium* Nees is one of the world's major plant pathogens causing wilt diseases (Brown et al., 1979). The genus *Verticillium* was classified based on its unique morphological characteristics (Station, 1952), "verticillate conidiophores." More than 50 *Verticillium* species have been reported that are categorized taxonomically based on their characteristics such as size and type of asexual reproductive spores and structures to survive, and most *Verticillium* species are not even plant pathogens. There has been

much controversial effort over the classification and nomenclature of *Verticillium* spp. (Isaac, 1967). After revision of the original genus, six plant pathogenic *Verticillium* spp. have been established. Karapapa and coworkers (1997) have suggested a potential seventh species of *Verticillium*. The seven plant pathogenic *Verticillium* spp. are *V. albo-atrum*, *V. dahliae Klebahn*, *V. nigrescens*, *V. nubilum*, *V. tricorpus*, *V. theobromae* (Turconi), and *V. longisporum comb.* nov (Karapapa et al., 1997).

V. albo-atrum and *V. dahliae* cause major crop losses around the world (Paredes-Lopez, 1999). The most significant characteristics that distinguish these two species biologically and geographically are the temperature difference in the growth, the survival of microsclerotia, and the types of dark resting mycelium (Pegg et al., 2002). The best growth temperature for *V. albo-atrum* is 20 to 25ºC, whereas *V. dahliae* favors slightly higher temperatures, about 25 to 28ºC (Agrios, 2005; Calderón et al., 2014). At the temperatures higher than 28ºC, all pathogenic *Verticillium* species have a drastic decrease in growth rate, sporulation efficiency, spore viability, and haploid growth rate (Trigiano, 2013). Unlike the *V. dahliae*, *V. albo-atrum* does not form microsclerotia. However, *V. albo-atrum*, however, produces melanized mycelia (Puhalla, 1973). In addition, *Verticillium* spp. can also be differentiated at the species level using PCR-based assays that utilize small differences in the internal transcribed spacer (ITS) region base sequence of ribosomal genes (Inderbitzin et al., 2013). The identification and genetic variations of *Verticillium* spp. and other fungi have been conducted via ITS DNA sequencing (Ibáñez-Escribano et al., 2014; Porras-Alfaro et al., 2014). The ITS regions are part of the ribosomal RNA operon, and they are often used for fungal systematics and classification (Porras-Alfaro, 2014). Two ITS regions, ITS1 and ITS2, are present in the fungal rRNA

operon. ITS1 is located between the 18S rRNA and the 5.8S rRNA genes, while ITS2 is present between the 5.8S rRNA and the 28S rRNA genes. The entire rRNA operon is transcribed as a primary transcript with the order of 18S rRNA - ITS1 - 5.8S rRNA - ITS2 - 28S rRNA (Ibáñez-Escribano, 2014; Mayta et al., 2000; Nimri et al., 2002; Porras-Alfaro, 2014). The two ITS sequences are removed from the primary transcript during RNA processing. Because of their non-functional role, the two ITS regions have higher degrees of mutation/variation (Nimri, 2002) than the 5.8S rRNA, 18S rRNA, and 28S rRNA genes (Ibáñez-Escribano, 2014), and they are used in fungal species identification.. Two primers, ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) are often used in PCR amplification of the ITS1 and ITS2 regions for identification of fungal species (Figure 2.A) (Liu et al., 2011; Quirós et al., 2008; Zhu et al., 2013). However, for purposes of routine identification, an important, new study has found that the use of ITS2 alone is usually sufficient for species-level identification (Endotoxin et al., 2013; Padmavati et al., 2013; Waddington, 2009).

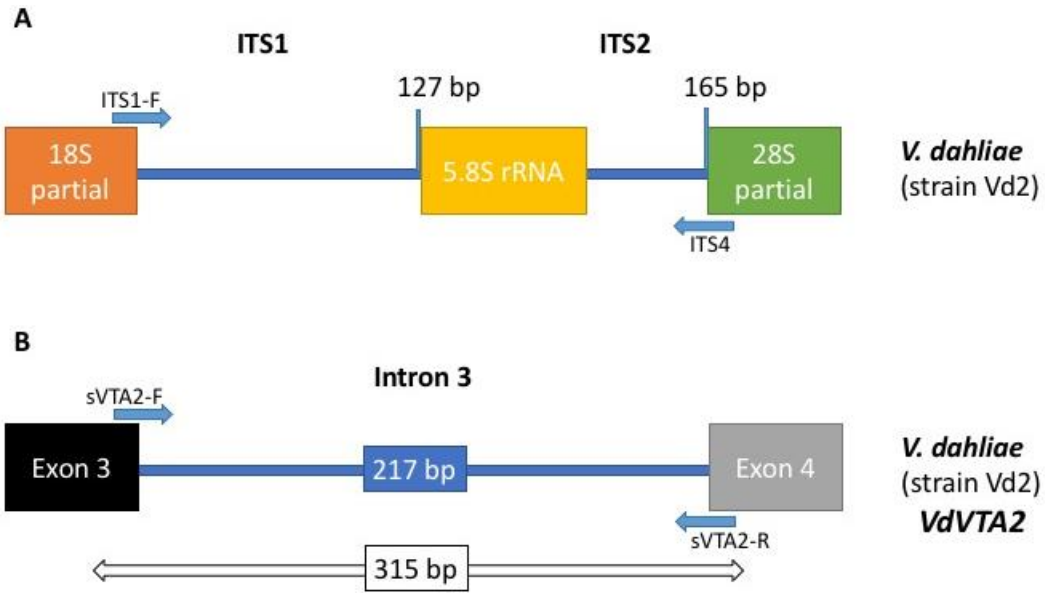

Figure 2 Schematic representation of ribosomal genes bridged by ITS regions and intron 3 (I3) of *VTA2* gene of *V. dahliae* (*VdVTA2*)

A, Schematic representation of ribosomal genes bridged by ITS regions, which are targeted by PCR amplification using the primer pair (ITS1-F/ITS4). **B**, Schematic representation of intron 3 (I3) of *VTA2* gene of *V. dahliae* (*VdVTA2*). The VTA2 gene is a spcific barcode for identification of *V. dahliae*. The specific primer pair (sVTA2-F/sVTA2-R) amplifying this 315 bp of intron and exons is indicated by blue arrows. The third intron (I3) (217 bp) of *V. dahliae* was indicated in blue box.

Further, it has been reported that *V. dahliae* and *V. albo-atrum* could be differentiated by restriction fragment length polymorphism (RFLP) (Society et al., 1995; Tjamos, 2000). Moreover, the ITS region can be used to discriminate between *V. dahliae* and *V. albo-atrum*, but for *V. longisporum*, this method is limited. Tran et al. (2013) have reported that the *VTA2* gene can serve as a barcode marker to distinguish between hybrid *V. longisporum* isolates and haploid *V. dahliae* or *V. albo-atrum* strains by a simple PCR reaction. In order to distinguish among *V. dahliae, V. albo-atrum,* and *V. longisporum*, two PCR primers, *s*VTA2-F and *s*VTA2-R have been used to amplify the third intron region (I3) of the *VTA2* gene (Tran, 2014; Tran et al., 2013) (Figure 2.B).

The Disease Cycle of Verticillium Wilt

The life cycle of pathogenic *Verticillium* spp. can be divided into three stages: dormant, parasitic, and saprophytic (Fradin et al., 2006). The stage of dormancy contains inhibition and germination of resting structures in the soil. The stage of parasites includes root penetration, root-cortex colonization and endodermis, and movement to the xylem, xylem colonization of the stems and leaves, expression of symptoms and killing or consuming the host plant. The stage of saprophytes is the production of resting structures in the dead host (Schnathorst, 1981).

The formation of microsclerotia as known resting structures plays a significant role in the survival, distribution and epidemiology of the disease in most plants (Fradin, 2006; Schnathorst, 1981). Microsclerotia present in infested soil is inhibited from germination due to mycostasis in the stage of dormancy. In the state of dormancy, the microsclerotia can survive for many years in the soil (Tjamos et al., 1995). In the field conditions, *V. dahliae* can survive up to 14 years (Haverkort et al., 1995) as conidia and microsclerotia (Soesanto et al., 2001). *Verticillium* sp. develops the parasitic stage after it enters the host through wounds or at the root cap. Inside the root, the cortex is colonized (Narisawa et al., 2002). The penetration is usually intracellular or intercellular (Lopez-Llorca et al., 2002). Hyphae enter the cortex directly, with no need to form the appressoria (Pegg, 2002). Hyphae enter the endodermis and attack the xylem vessels (Isaac and Lloyd, 1959) where the conidia are developed in the cortex (Hake et al., 1996). When they are inside the stem, conidia move quickly through the xylem. Conidia may become trapped in vessel end walls, germinate, enter adjacent vessel elements, and continue colonization and increase infection (Schall, 2008; Schnathorst, 1981; Welbaum,

2015). Many researchers have found that the rate of conidia formation in the host plant correlates with the attack of the strain, with strongly conidiated *Verticillium* strains being more virulent (Fradin, 2006; Schnathorst, 1963). At this stage, the host plant begins to show symptoms. Upon the appearance of symptoms, the host is defined as susceptible or resistant. Unlike other fungi casing vascular wilt diseases such as *Ceratocystis* spp., *Fusarium* spp. and *Ophiostoma* spp., *Verticillium* spp. lives in the xylem vessels until the host is dying (Figure 3). At this point, the fungus enters into a saprophytic stage and forms microsclerotia (Nyvall, 2013) that return to the ground when the host dies and decays (Fradin, 2006).

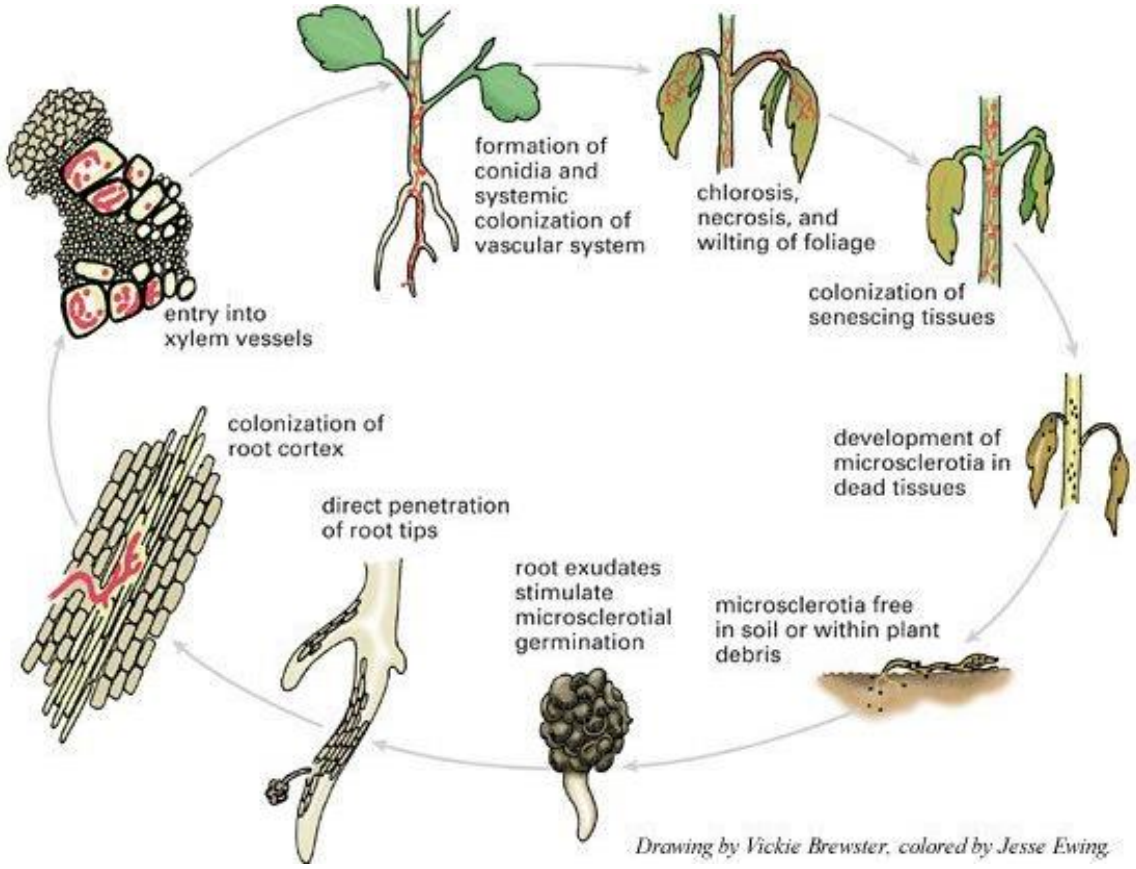

Figure 3 The life cycle of *V. dahliae* on potato

In the soil, the cycle begins with resting structures (microsclerotia). Plant root exudates stimulate germination of microsclerotia. Consequently, the germinated *V. dahliae* infects roots and penetrates the plants within the root tips. The fungus quickly occupies the xylem vessels, colonizes vascular systems and forms asexual conidia. The symptoms including chlorosis, necrosis and wilt of leaves appear early. When the host is dead, *V. dahliae* forms microsclerotia for the following infection cycle(Berlanger et al., 2000).

Symptomatology and Epidemiology

The symptoms of VW are similar to those of Fusarium wilt and other fungal vascular wilt diseases (Agrios, 2005). The symptoms usually can be classified as external which are visible to the naked eye or internal or microscopic symptoms located inside the xylem vessels of the host (Mace, 2012). The visible external symptoms include stunting, leaf epinasty, abscission, and the development of distinctive foliar

symptoms (Kramer, 2012; Mace, 2012). The symptoms are flaccidity (wilt) and chlorosis, followed by necrosis of successive branches, and then abrupt collapse and death of the entire plant (Research, 1971). Internal symptoms may involve vascular browning which represents the deposition of melanin-like compounds on the walls of xylem vessels and neighboring parenchyma cells in plants (Yadeta and Thomma, 2013). It also leads to premature foliage senescence, transpiration and increased leaf temperature ending in yield loss (Bowden et al., 1990).

Many *Verticillium* spp. that have been isolated can generate a wide variety of symptoms in some host plants, while others require specialized hosting (Fernandez et al., 2006; Kranz et al., 1977; Pohronezny, 2003; Production and Eucarpia, 1993; Region, 1973; Rimmer et al., 2007). As a result of the very different symptoms on various host plants, there are no particular symptoms that are related to the plants infected with *Verticillium* spp. (Fradin, 2006).

The initial emergence of VW in a field are normally mild and local (Agrios, 2012). The attacks on plants become successively more severe and widespread in subsequent years as the inoculum builds up (Khan, 2001). VW occurs after widespread of the pathogenic fungus, and more virulent strains of the fungus appear until the crop is overwhelmed by the pathogen (Agrios, 2012; Khan, 2001).

There are other factors that affect the epidemiology of diseases caused by *Verticillium* spp., which include the strains of the pathogen, interactions with other pathogens, inoculum density, soil type, suppressive and conducive soils, nutritional status of the soil, temperature of the soil and air, water, movement of propagules by wind

machines and animals, prevalence of weed hosts, extent of rainfall and irrigation, crop cultivars, and plant densities (Mace et al., 1981; Mace, 2012).

Economic Importance and Disease Management

VW is one of the most damaging plant diseases that occur in herbaceous perennial and biennial plants. In fact, nearly all major agronomic plant species, except herbs, are prone to infection, causing several billions of dollars in yield losses each year (Klosterman et al., 2011). Average yield losses of 3.1 percent were reported in the ten-year period in the United States of America (EROĞAN, 2013; Zhang et al., 2011), between 3.5 and 5 percent in the state of New Mexico (Zhang, 2011), and current annual estimated losses caused by the disease are about 1.5 million bales worldwide (Kirkpatrick et al., 2001). VW has been recorded as the most significant disease of the major potato varieties grown in North America (Hachey et al., 2002).

VW is difficult to control due to several combined factors of the long-term survival of microsclerotia of the pathogen (Pegg, 2002) and its growth within the vascular host plant tissues. All of these factors making chemical and cultural attempts to protect plants against VW become inefficient or impractical (Shittu, 2014).

Among *Verticillium* spp., *V. dahliae* appears to be the most challenging pathogen to combat (Jiménez-Gasco et al., 2013). It can exist for many years in different internal tissues of many hosts and in the soil which makes it harder to monitor (Wildon et al., 1935). The dark resting structures of *Verticillium* spp. provide the initial inoculant (Hu et al., 2014); consequently, they serve as excellent targets for the management of *Verticillium* spp.

The control methods that reduce the level of inoculum involve physical methods – heat treatments and soil solarization, chemical alternatives for soil fumigation, and crop rotation. Crop rotation is less effective due to a wide host range of more than 200 dicotyledonous plants (Woodward et al., 2011). One of the greatest practical ways to combat the disease is the removal of plants that have been diagnosed with VW. Currently, the most efficient way to prevent Verticillium disease is the breeding of cultivated crops with high resistance to VW disease, which is the most economical and efficient means of decreasing loss from VW (Zhang et al., 2014).

Verticillium spp. and Host Plant Interactions

When *Verticillium* spp. infect cotton plants, three kinds of host pathogenic relationships - susceptibility, resistance, or tolerance may occur (Sherf et al., 1986). In the susceptible relationship, symptom level and numbers of *Verticillium* sp. are significant. In the resistance status, both symptom level and numbers of *Verticillium* sp. remain low (Ben et al., 2013). In the tolerant relationship the levels of symptoms are low but with high amounts of *Verticillium* sp. (Genetics et al., 2003). The type of relationship that would occur is determined by the cellular interaction between the host plant and the pathogen that appeares inside the xylem vessels of the host (Genetics, 2003).

Cotton planted in the North farm at the R.R. Foil Plant Science Research Center at Mississippi State have been reported to be infected by pathogenic fungi (Baird et al., 2004; Scott et al., 2013; Smith et al., 2013). However, there is limited information regarding the regulation of cotton in response to fungal infections. There are relatively few historical studies on cotton wilt caused by *Verticillium albo-atrum*. Wyllie et al. (1970) isolated *Verticillium albo-atrum* and *Verticillium nigrescens* from diseased cotton

plants in California that consistently yielded a pathotype causing darkening of the vascular tissues (Ashworth Jr et al., 1972; Wyllie et al., 1970). Studies conducted by Paplomatas et al. (1992) have shown that VW of cotton caused by *Verticillium dahliae* Kleb. posed huge economic losses in California (Paplomatas et al., 1992). This current study was to determine whether cotton wilt was caused by *Verticillium albo-atrum* or *Verticillium dahliae* Kleb. (at the R.R. Foil Plant Science Research Center at Mississippi State). *V. dahliae* is one of the most aggressive soil-borne plant pathogens that mainly infects cotton plants and can be particularly destructive for its vascular propagation and wilting development.

The overall goal of this study is to identify fungal pathogens that cause wilt diseases on cotton plants found in the North Farm, Mississippi State University. Also, this current research was undertaken with a view towards seeking a better knowledge of the cellular, and molecular basis of *Verticillium* wilt in the cotton plants. It has been believed that the findings obtained from this study could also improve knowledge of interactions between the host and other vascular wilt pathogens. Therefore, the present study was performed in four successive stages. The first stage was an exploratory step where significant biological questions about *Verticillium* wilt were asked, particularly with the respect to the endophytic properties of many soil-borne fungi with different morphology and growth characteristics from diseased cotton plants at the R.R. Foil Plant Science Research Center at Mississippi State during the late summer in 2013 and 2014. Stage two was centered on extracting genomic DNA from the isolated fungal species and used in molecular typing via PCR amplification and DNA sequencing analysis of the ribosomal internal transcribed spacer (ITS) region. The third stage was to answer the

question that whether the isolated *Verticillium dahliae* (MSCT67) can cause the vascular wilt disease in cotton plants. The isolated *Verticillium* spp. (MSCT67) was thus used in pathogenicity test using Koch's postulates in the greenhouse (Zhang et al., 2013; Yuan et al., 2011). In the last stage of this study, distinguish between the *Verticillium* spp. were done by using the PCR-based genotyping method with species-specific primers. The isolated *Verticillium* sp. was confirmed to be *Verticillium dahliae* and was it could cause vascular wilt disease in cotton plants.

CHAPTER II

MATERIALS AND METHODS

Isolation of Multiple Fungal Pathogens from Diseased Mississippi Cotton Plants

Cotton plants with the symptom of *Verticillium* wilt (VW) were observed at the R.R. Foil Plant Science Research Center of Mississippi State University. The stem tissues of the diseased cotton plants were collected, and *Verticillium* spp. and other fungi were isolated from the cotton tissues by using a standard aseptic technique. The stem tissues were sliced with a sterile scalpel into thin sections, dipped into 70% ethanol for 30 S, rinsed in double distilled water, and then placed on Potato Dextrose Agar (PDA, Disco, Detroit, MI) (compositions of 39 mg/mL PDA, 15 µg/mL of tetracycline) plates. The PDA plates with upside down were incubated at 28 to 30°C for 1 to 2 weeks, and fungi grown on the plates were further transferred to fresh PDA plates for the purification of fungal cultures. Multiple purified fungal cultures were found to have phenotypes and morphological characteristics that are typical or similar to *Verticillium* spp. (later identified and confirmed to be *Verticillium* by molecular typing).

Pathogenicity Test Based on Koch's Postulates

Based on Koch's postulates the isolated *Verticillium* sp. and other fungal pathogens were used in a pathogenicity test to confirm whether it could cause the disease in cotton plants with VW symptoms (Zhang et al., 2013; Yuan et al., 2011) . For the test, cotton seeds were germinated and grown in pots containing sterilized topsoil in a

greenhouse at 28-23°C day/night temperatures and in 60% to70% relative humidity (Bhat et al., 2003). The bottom part of the young plants was then lightly cut with a razor blade, and an agar block of *Verticillium* culture was inoculated into the wound location. After two months, the plants were checked to determine if VW symptoms were developed in cotton plants, and plant stem tissues were then collected for the re-isolation of the *Verticillium* spp.

Extraction of *Verticillium* spp. Genomic DNA

Fungal genomic DNA samples were isolated using a modified method as previously described (Krohn, 2010; Liu, 2011). In combination with heat treatment, fungal mycelia were quickly dismantled, and the cellular DNA was liberated into the extraction buffer. A 0.6 ml microcentrifuge tube was filled with 100-µL of modified extraction buffer containing 0.05 M carbonate buffer, pH 9.6, 0.05% of Tween 20, 0.2% of BSA, and 2% of PVP 40. Mycelia of fungal cultures on PDA plates were picked using a sterile stainless steel micro-spoon and suspended into the buffer. The suspension was heated at 95°C in a dry bath for 15 min. After heating, the sample was kept on ice for 1 min, vortexed for 10 s, and then briefly centrifuged at 13,000 rpm16,000 x g for 1 min with Eppendorf Centrifuge 5415D. One µL of the suspension was used as DNA template for polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) regions of the fungal ribosomal DNA (Liu, 2011).

PCR Amplification of Ribosomal ITS Regions

The ITS region covering ITS1, 5.8S rRNA gene, and ITS2 was amplified by PCR using two fungi-specific primers, the forward primer ITS1-F (5′-CTTGGTCATTTAGAG

GAAGTAA-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Liu, 2011; Quirós, 2008; Zhu, 2013). PCR amplifications were performed in a Labnet MultiGene II Thermal Cycler. A PCR reaction mixture was prepared by pipetting one µL of Prime *Taq*™ DNA polymerase (5 unit/µL, GenScript), one µL of the extracted genomic DNA, one µL of forward primer ITS1-F (0.5 µM), one µL of reverse primer ITS4 (0.5 µM), five µL of 10 x *Taq* Buffer (500 mM KCl, 100 mM Tris HCl (pH 9.0 at 25°C), 15 mM $MgCl_2$, and 1% Triton X-100; GenScript), one µL of 10 mM dNTPs and 40 µL of ddH_2O into a 0.2 ml thin wall tube. The sample was mixed by vortexing and then subjected to PCR amplification by an initial denaturation at 94°C for 1 min, following by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 20 s, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were analyzed immediately by agarose gel electrophoresis.

Analysis of Ribosomal ITS PCR Products

The amplified ITS PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel. To prepare the 1% (w/v) agarose gel, 0.25 g of agarose was weighed and transferred to a 125 mL flask containing 25 ml of 1X buffer (20 mM Tris-HCl, pH 7.5, 7.5 mM sodium acetate, 0.5 mM EDTA, made from 50X stock solution). The agarose suspension was microwaved with occasional swirling until the agarose was completely dissolved. Five µL of 5-mg/mL ethidium bromide were then added to the agarose solution, and the solution was poured into a gel box and left for 30 to 60 min to allow hardening. After hardening, 1X TAE agarose gel buffer was added to submerge the gel, and the samples of the PCR products mixing with 1 µL loading dye along with GeneRuler 1 kb DNA ladder were loaded into the wells of agarose gel. The

electrophoresis was run for approximately 70 min at constant 70V until the fast moving bromophenol blue (BP) dye reaches the middle of the gel. The gel was photographed under the UV light with a BioDoc-it Imaging System (UVP).

Purification of Ribosomal ITS PCR product

The PCR product was purified with a QIAquick™ PCR Purification Kit (Qiagen, Hilden, Germany). The ITS PCR product and five volumes (500 µL) of buffer PB [5 M guanidine hydrochloride (Gu-HCl), 30% isopropanol] were mixed in a 1.5 mL microfuge tube by vortexing. The PCR mixture was applied on a QIAquick spin column (maximum binding capacity 10 µg) that was placed into a 2-mL collection tube and centrifuged for 1 min at the maximum speed 16,000-x g 13,000 rpm. The flow-through buffer was then discarded. The QIAquick column was placed back into the same tube. A 0.75 mL (750 µL) buffer PE (10 mM Tris-HCl, pH 7.5, 70% ethanol) was added to the QIAquick column for washing and centrifuged again for 1 min at 16,000 x g 13,000 rpm. The flow-through buffer was then discarded, and the QIAquick column was centrifuged for an additional 1 min at the maximum speed 16,000-x g 13,000 rpm to remove residual ethanol. The QIAquick column was placed in a clean 1.5 mL microfuge tube, and 50 µL of Buffer EB (10 mM Tris-HCl, pH 8.5) were added to the center of the QIAquick membrane to elute the DNA. The column was left for 1 min and then centrifuged for 1 min. The purified PCR product (total 100 µL) was stored at -20°C.

Ligation of purified Ribosomal ITS PCR product to pGEM®-T Easy Vector

The purified PCR product was ligated with the pGEM®-T Easy Vector by using T4 DNA ligate. The ligation reaction mixtures was prepared by pipetting five µL of 2X

Rapid Ligation Buffer [60mM Tris-HCl (pH 7.8), 20mM $MgCl_2$, 20mM DTT, 2mM ATP and 10% polyethylene glycol (MW8000, ACS Grade)], one µL of pGEM®-T Easy vector (50 ng), five µL of purified PCR product, one µL of T4 DNA ligase (3 Weiss units/µL) and eight µL of sterile distilled water into a 1.5 ml microfuge tube for the reaction. The sample mixture was gently mixed, and the ligation reaction was conducted overnight at 16°C or 1 hr at room temperature. The ligation mixture was transformed into *E. coli* XL1-Blue competent cells.

Preparation of *E. coli* XL1-Blue competent cells

A single colony of *E.coli* XL1-Blue cell stock was inoculated into 20 ml of sterile Luria-Bertani (LB) medium [10 g/L Bacto- tryptone (Difco), 5 g/L Bacto yeast extract (Difco), 10 g/L NaCl, pH 7.0] and the culture was grown overnight at 37°C with constant shaking. One ml of overnight grown culture of *E.coli* XL1-Blue was then inoculated into a 250 ml flask containing 20 ml of sterile LB media and cultured for 1 hr at 37°C with continuous shaking (250 rpm) until the OD_{600} reached 0.5. The culture was then transferred into a 50 ml centrifuge tube, and cells were collected by centrifugation at 8,000 X g for 10 min at 4°C. After decanting and discarding the liquid layer, the cells were suspended in 1/2 volume (10 mL) of ice-cold 0.1 M $CaCl_2$ and kept on ice for 15 min. The cells were collected by centrifugation at 8,000 g for 10 min at 4°C, resuspended in 2 ml of 0.1 M $CaCl_2$ and kept on ice for use immediately (Mandel et al., 1970).

Transformation of *E. coli* XLl-Blue Competent Cells with pGEM®-T Easy/ Ribosomal ITS ligation mixture

Ten µL of the pGEM-T Easy/ ITS ligation mixture were added to a 1.5 mL tube containing 100 µL of competent *E. coli* XL1-Blue cells. The sample was gently mixed and incubated on ice for 30 min. The tube was then placed in a 42°C water bath for 90 s and then in ice for 2 min. Five hundred µL of LB medium were added to the transformed cells, and the cells were incubated in a 37°C shaker at 250 rpm for 60 min. Aliquots (50 and 100 µL) of transformed cells along with 10 µL of 0.1 M IPTG (galactosidase inducer) and 16 µL of 20 mg/ml of X-gal (galactosidase substrate) were added onto LB/amp (50 mg/mL) plates. The mixture was spread until the solution was completely absorbed onto LB. The plates were placed upside down and incubated at 37°C overnight. Several (3–5) colorless (positive) clones were selected and grown in a LB (Luria-Bertani) liquid medium containing 50 µg/mL of ampicillin for the isolation of recombinant pGEM-T easy plasmids.

Isolation of Recombinant pGEM®-T Easy Plasmids

The white clones were inoculated into 1.5 mL LB/amp media and grown in a 37°C shaker for 12 hr. The cells were then collected by centrifugation at 16,000-x g 13,000 rpm and used for the isolation of recombinant plasmids using the Qiagen Spin Mini prep kit.

The 1.5 mL bacterial culture was poured into a 1.5 mL microfuge tube and centrifuged for 5 min at 16,000-x g 13,000 rpm. The supernatant was discarded and the cell pellet was resuspended in 250 µL of buffer P1 by vortexing until no cell clumps were visible. Buffer P2 (250 µL) were added to the cell suspension to lyse the cells by gently

inverting the tube 4 to 6 times with no vortexing. The lysis reaction was allowed to proceed for not more than 5 min. Buffer N3 (350 µL) were then added to the lysate and followed by gently inverting the tube immediately 4 to 6 times. The sample was centrifuged for 10 min at 16,000-x g 13,000 rpm and the supernatant was applied to the QIAprep spin column by decanting or pipetting and followed by centrifugation for 1 min at 16,000-x g 13,000 rpm. The flow-through was discarded, and the spin column was washed with 0.75 mL of PE buffer and then centrifuged for 1 min at 16,000-x g 13,000 rpm. The column was centrifuged for an additional 1 min at 16,000-x g 13,000 rpm to remove residual wash buffer and then placed in a clean 1.5 mL microfuge tube. To elute the plasmid, 50 µL of Buffer EB were added to the center of the spin column, left for 1 min, and centrifuged for 1 min at 16,000 x g 13,000 rpm. The tube containing the recombinant plasmid was labeled and stored at -20°C.

Analysis of Recombinant pGEM®-T Easy Plasmids

The recombinant pGEM-T easy plasmids were digested by the restriction enzymes *Eco*Rl and analyzed by 1% agarose gel electrophoresis. Five µL of the recombinant plasmid DNA, 2 µL of sterile water, 1 µL of 10X restriction buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% Triton X-100, pH 7.5), and 1 µL (20 U/µL) of *Eco*RI, 0.1 mM EDTA, 10 mM DTT, 0.15% Triton X-100, 200 µg/ml BSA and 50% glycerol) were pipetted into a 0.6 mL centrifuge tube. The sample was gently mixed by vortexing and incubated at 37°C for 1 hr. The digested DNA samples along with one µL of GeneRuler 1 kb DNA Ladder were loaded into the wells of a 1% agarose gel. The electrophoresis was carried out at 70 volts until the BP dye reaches the middle of the gel. The gel was photographed under the UV light with a BioDoc-it Imaging System.

Sequencing of Ribosomal ITS Regions

The recombinant pGEM-T easy plasmids containing the ITS insert were sequenced using the dideoxynucleotide chain termination method (Sanger method) with the BigDye terminator V1.1 cycle sequencing kit from Applied Biosystems. One µL (10 uM) of M13 forward universal primer (17mer): 5′-(GTTTTCCCAGTCACGAC)-3′, 2 µL of recombinant pGEM-T easy/ITS plasmid, 2 µL of terminator ready reaction (TRR) mix, 3 µL of 5X sequencing buffer, and 12 µL of sterile double distilled water were transferred into a 0.2 ml microfuge tube. The solution was gently mixed and subjected to PCR amplification via an initial denaturation at 96°C for 2 min and following by 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min in a thermal cycler (Labnet Multi Gene II). The cycle sequencing sample was transferred into a 1.5 ml tube containing 2 µL of 3M-sodium acetate, pH 4.7 and 50 µL 96% ethanol.

The tube was vortexed and then centrifuged for 10 min at 16,000-x g 13,000 rpm. The supernatant was carefully and completely removed with a pipette and discarded. The pellet was rinsed with 250 µL of 70% ethanol, centrifuged for 5 min at 16,000-x g 13,000 rpm, and vacuum dried for 15 min in a Savant Speed Vac Concentrator using the Labconco Freeze Dry System. The pellet was resuspended in 20 µL of Hi-Di formamide, heated at 95°C for 2 min to denature DNA, and then loaded onto the ABI Prism 310 Genetic Analyzer.

Identification of Fugal Species from Diseased Mississippi Cotton Plants

Three recombinant pGEM-T easy plasmids containing the ITS insert were sequenced and all the three clones generated identical sequence. The ITS sequence data

was analyzed and then used to identify the fungal species isolated from diseased cotton plants with the BLASTn program. The multiple sequence alignment of ITS from different *Verticillium* spp. were done with the ClustalW program (Li, 2003).

Distinguish Between the *Verticillium* spp. Based on PCR Amplification

The third intron region (I3) of the *Verticillium* transcription activator *VTA2* gene was amplified by PCR and used to distinguish between *V. dahliae*, *V. albo-atrum*, and *V. longisporum* (Tran, 2013). The reaction mixture for PCR amplification of the *VTA2* intron was prepared by pipetting 1 µL of the extracted gDNA, 1 µL (10 µM) of each *s*VTA2-F (5′-GCACGTCACCATGCAGTC-3′) and 1 µL (10 µM) *s*VTA2-R (5′-CAGCTTCTTCCTCCTTCTTGC-3′), 1 µL of Prime *Taq*™ DNA polymerase (5 unit/µL, GenScript), 5 µL of 10 x *Taq* Buffer (500 mM KCl, 100 mM Tris HCl (pH 9.0 at 25°C), 15 mM $MgCl_2$, and 1% Triton X-100; GenScript), 1 µL of 10 mM, and 40 µL of ddH2O into a 0.2 ml thin wall PCR tube. The sample was mixed by vortexing and subjected to PCR amplification using a Labnet MultiGene II Thermal Cycler. The amplification was performed by an initial denaturation at 94°C for 1 min and followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 20 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Amplified PCR products were analyzed by electrophoresis on a 1 % agarose gel as previously described.

CHAPTER III

RESULTS

Isolation of *Verticillium* spp. and other fungi from diseased Mississippi cotton plants

The symptoms of *Verticillium* wilt (VW) were observed on cotton plants at The R.R. Foil Plant Science Research Center of Mississippi State University during the late summer of 2013 and 2014. The visible external symptoms including stunting, leaf epinasty, abscission, and the development of distinctive foliar symptoms were noted on the cotton plants (Figure 4). Internal symptoms including vascular browning that represented the deposition of melanin-like compounds on the walls of xylem vessels and neighboring parenchyma cells were also observed in diseased cotton plants (Yadeta, 2013).

Figure 4 Symptoms of *Verticillium* wilt of cotton plants caused by *Verticillium* species at the R.R. Foil Plant Science Research Center during the late summer in 2013 and 2014

Diseased cotton plants in the field showed yellowing, leaf necrosis, twig and branch dieback (A and C) and vascular tissue discoloration (B and D). Plants with both mild (A and B) and severe (C and D) symptoms were observed.

The stem tissues of the diseased cotton plants were collected, and fungi that infected the plants were isolated from the cotton tissues by using a standard aseptic technique (Ashraf et al., 2012; Goud et al., 2003; Kim et al., 2001; Smith, 1965). The stem tissues were sliced with a sterile scalpel into thin sections, and then transferred onto PDA/tetracycline plates (Figure 5). The antibiotic tetracycline served to control bacterial contamination. The PDA plates were incubated at 28 to 30°C (Hassan et al., 2014) for 1 to 2 weeks, and fungi grown on the plates were transferred to fresh PDA plates for further purification of fungal cultures. Two ungal cultures, MSCT67 and MSCT70, were

isolated and purified from the 2013 and 2014 plant samples, respectively. MSCT67 and MSCT70 were found to have phenotypes and morphological characteristics that are typical or similar to *Verticillium* spp. (Figures 6 and 7). MSCT67 produced conidia spores (Figure 8) and the dark resting structures of microsclerotia (Figure 9B) as cultures age. The production of microsclerotia is one of the characteristic features of *V. dahliae*. The mycelium morphology (Figure 9A) and microsclerotia production (Figure 9B) strongly suggested that MSCT67 and MSCT70 were *Verticillium* sp., and it was later identified and confirmed to be *Verticillium dahliae* by molecular typing. Several fungi were also isolated from the 2014 cotton plants with vascular wilt diseases using the PDA plates. Molecular typing later identified the species of these fungi (see section 2).

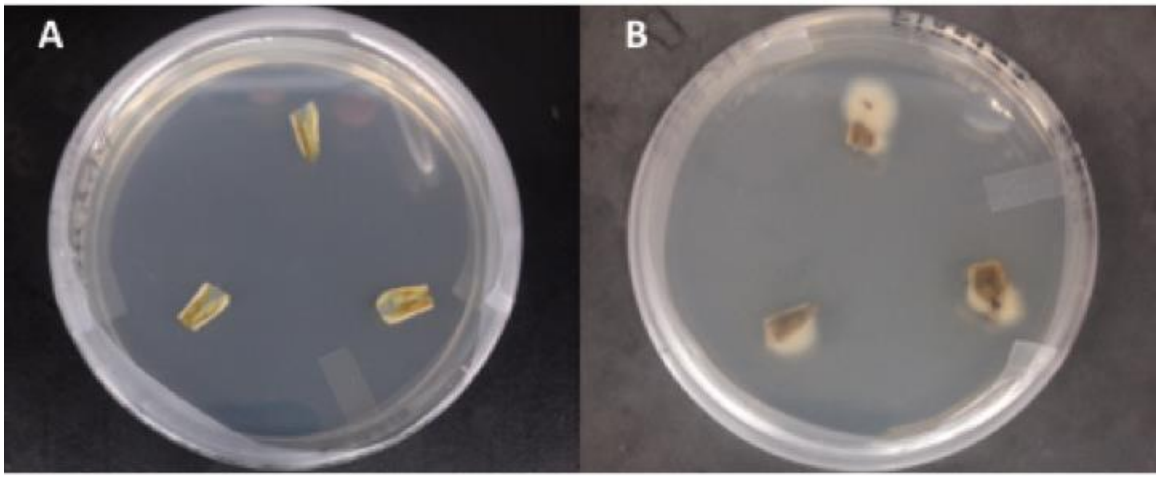

Figure 5 Isolation of *Verticillium* species and other fungi from diseased cotton plants

A, Stem tissues of diseased cotton plants on PDA/tetracycline plates. **B**, Growth of fungi from vascular tissues after incubation at 28 to 30°C for 3 days

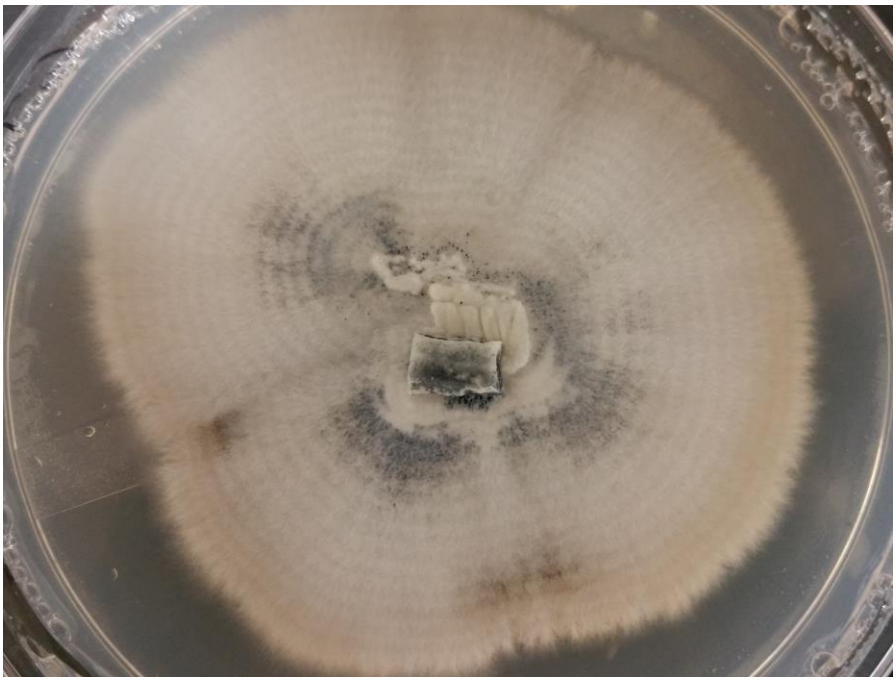

Figure 6 Growth of *Verticillium dahliae* on PDA at 28 to 30°C for 20 days

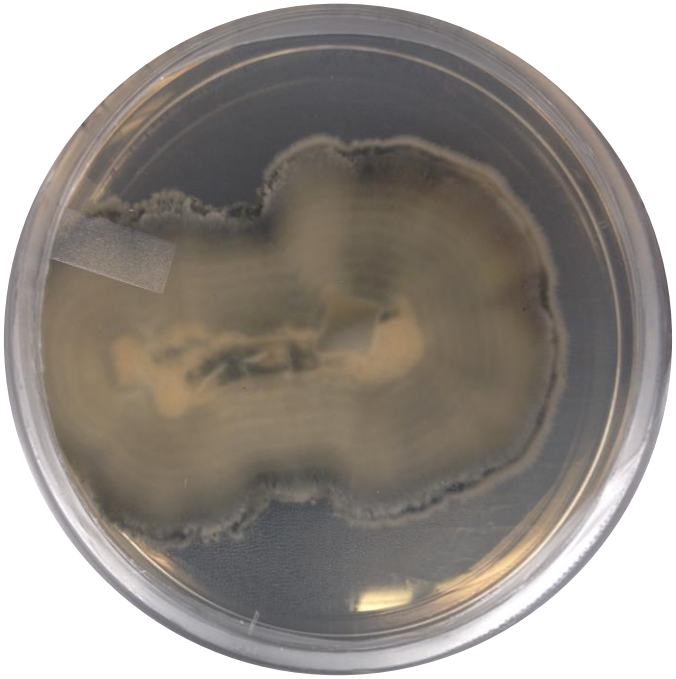

Figure 7 Production of dark microsclerotia by *Verticillium dahliae* on PDA culture plates

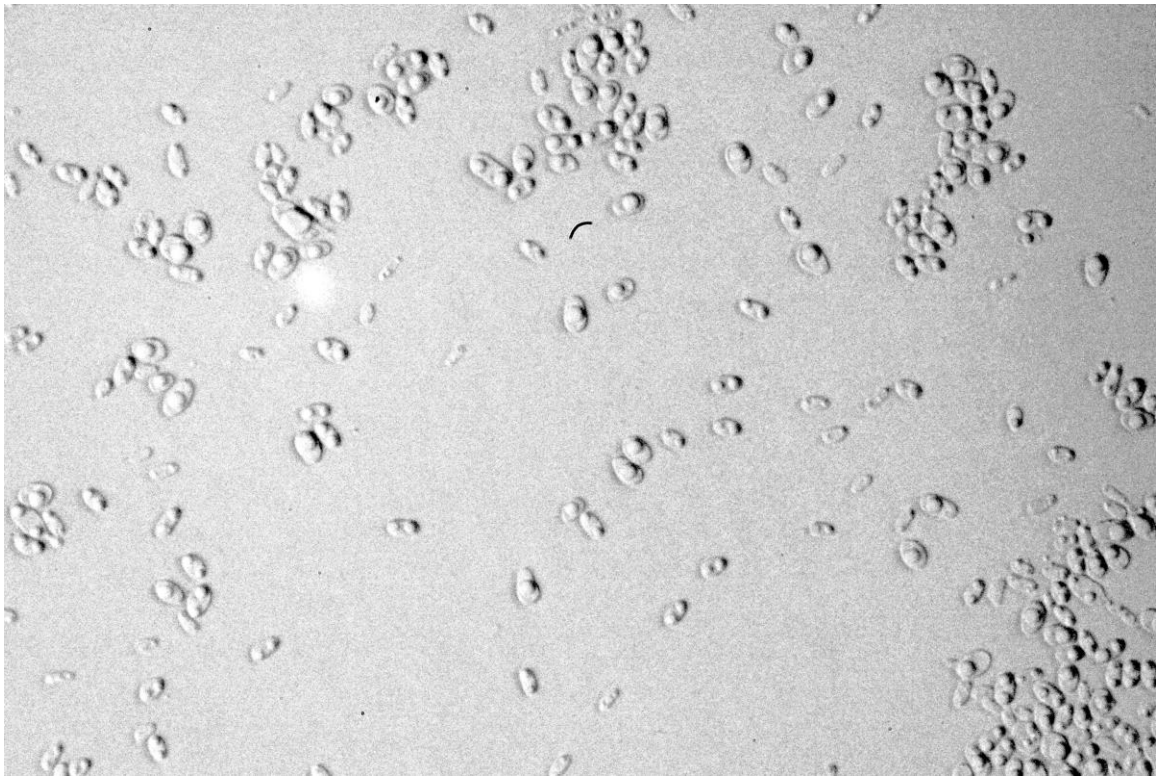

Figure 8 Conidia from *Verticillium dahliae* isolate MSCT67 grown on PDA/tet plates

The picture was taken at 40X magnification using an Olympus BH-2 microscope

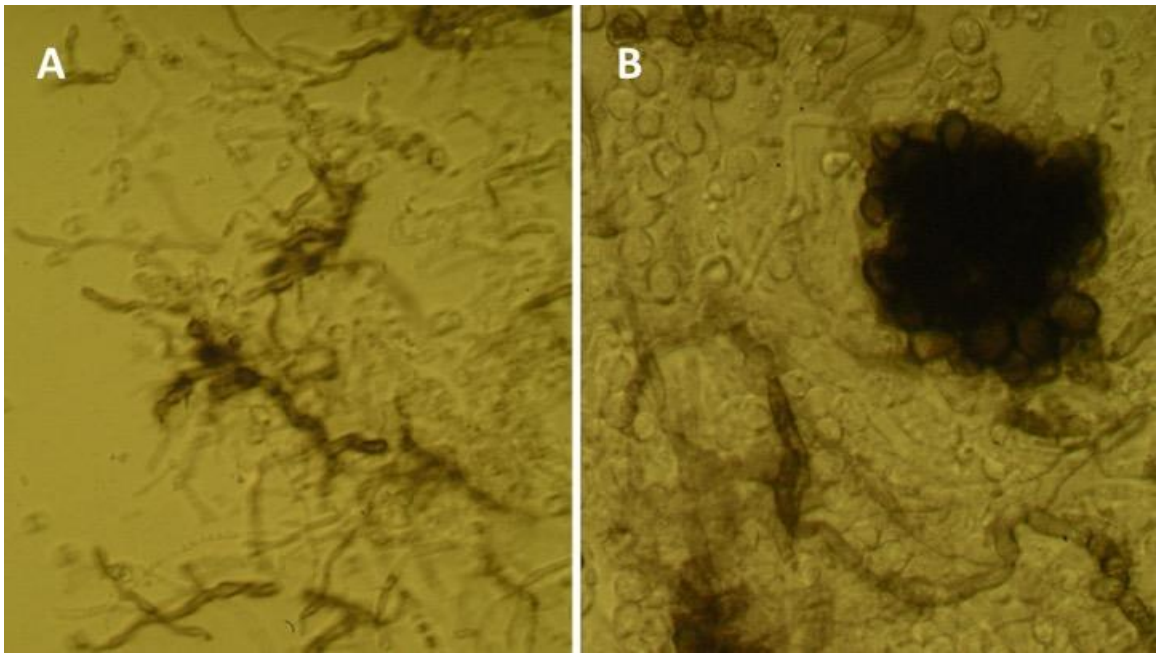

Figure 9 The mycelia and resting structures of *Verticillium dahliae* isolate MSCT67

A, the melanized mycelia of *V. dahliae*. **B**, the dark microsclerotia of *V. dahliae* from PDA/tet plates. The picture was taken at 40X magnification using an Olympus BH-2 microscope

Identification and genetic variations of *Verticillium* spp. and other fungi isolated from diseased Mississippi cotton plants

Identification of the two fungal isolates MSCT67 and MSCT68 were conducted by PCR amplification and DNA sequencing of internal transcribed spacer (ITS) regions of ribosomal genes. Genomic DNA samples from MSCT67 and MSCT68 were amplified by PCR using the ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers designed on the basis of 18S rRNA and 28S rRNA gene sequences, respectively (Gardes et al., 1993; Nouri et al., 2012). Analysis of the PCR products containing ITS1, 5.8S rRNA gene and ITS2 on a 1% agarose gel indicated that they had sizes ranging from 589 to 600 bp (Figure 10).

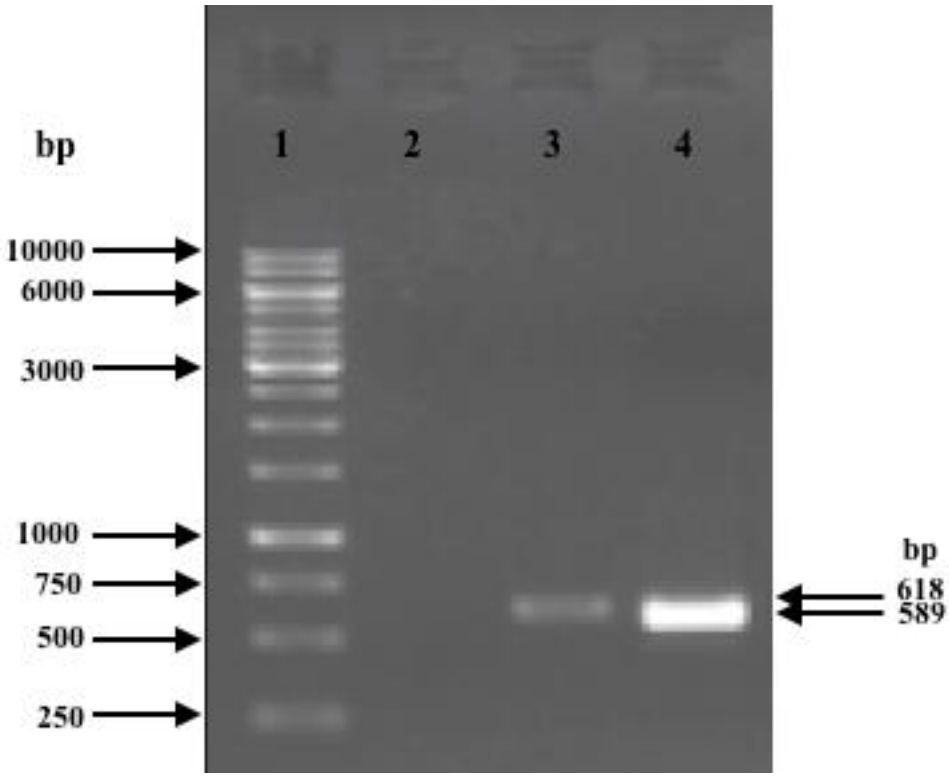

Figure 10 Analysis of the PCR-amplified fragments containing ITS regions of *Verticillium* isolates on 1% agarose gel

Lane 1 contains GeneRuler 1 kb DNA ladder, Lane 2 contains a PCR reaction without DNA template (negative control), lane 3 contains a unique DNA band of 618 bp amplified from genomic DNA of MSCT68, and lane 4 contains the unique DNA bands of 589 bp amplified from genomic DNA of the isolate MSCT67.

The PCR products were cloned into the pGEM-T Easy vector, and recombinant pGEM-T Easy plasmids were isolated, digested with the restriction enzymes *Eco*RI and analyzed by 1 % agarose gel electrophoresis. The restriction digestion generated a large size (3 kb) of linear pGEM-T Easy plasmid and two small DNA fragments with sizes around ~295 bp (Figure 11). Most of fungal 5.8S RNA genes contain a conserved *Eco*RI site within the center of the genes (Figures 16, 17, 18, 19, and 20) (Jobes et al., 1997; White et al., 1990), and *Eco*RI digestion cleaved the 589 bp ITS (ITS1-5.8S RNA gene-ITS2) (Figure 10) into two 295 bp DNA fragments (Figure 12). The results of restriction

digestion indicated that the ITS amplified from the genomic DNA of MSCT67 was successfully cloned into the pGEM-T Easy plasmid.

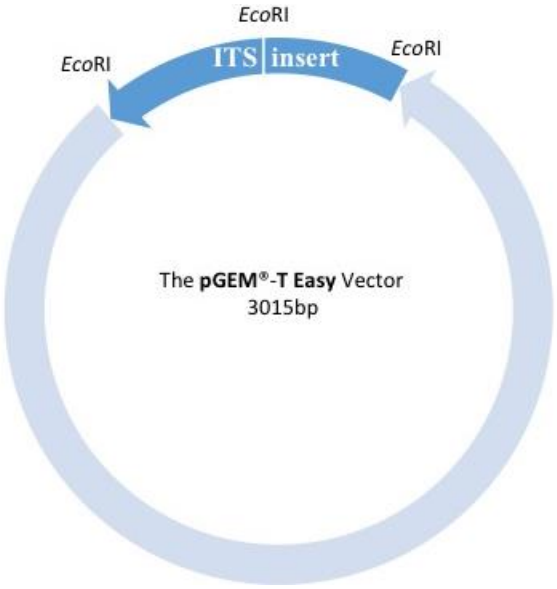

Figure 11 Schematic representation of *Eco*RI sited (E) in the recombinant pGEM-T Easy plasmid containing the ITS insert.

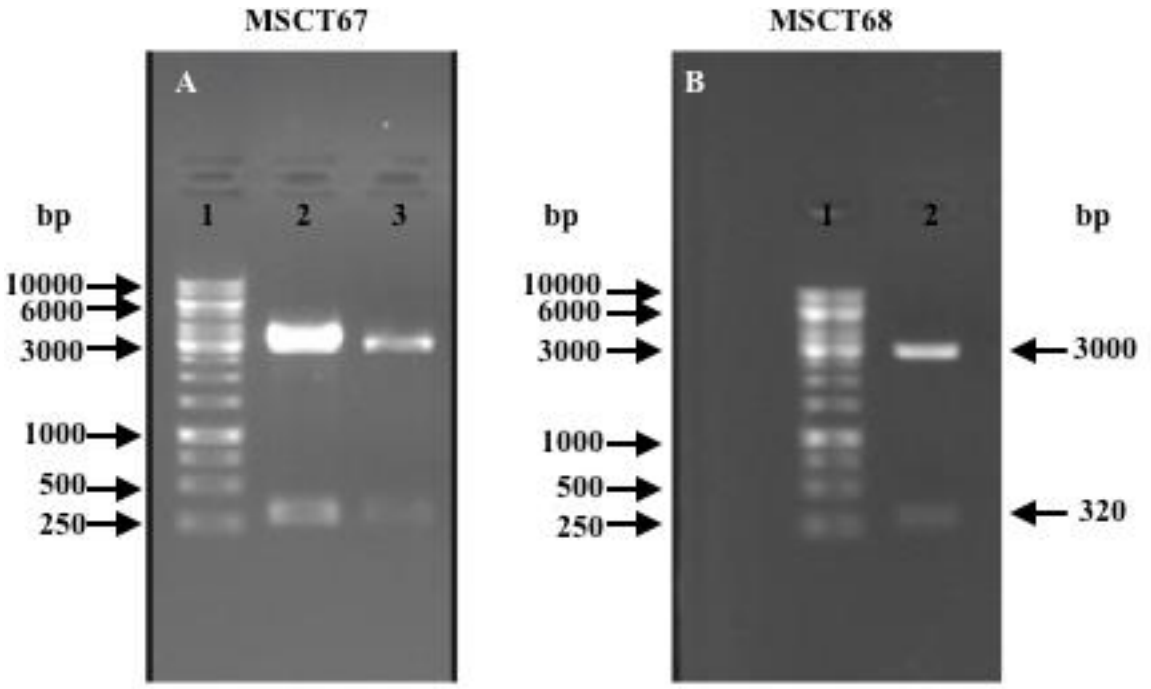

Figure 12 Analysis of the recombinant pGEM-T Easy plasmids containing ITS inserts

A, Two clones of MSCT67 recombinant pGEM-T Easy plasmids were digested with *Eco*RI, and the digested plasmids were analyzed on a 1% agarose gel (lanes 2 and 3). Lane 1 contains GeneRuler 1 kb DNA ladder. **B**, A clone of MSCT68 recombinant pGEM-T Easy plasmids was digested with *Eco*RI (lane 2). A GeneRuler 1 kb DNA ladder (lane 1) was also included for electrophoresis for size comparison.

These recombinant plasmids containing the ITS insert were sequenced with the universal primer using an ABI310 Prism automated DNA analyzer. The ITS sequence (ITS1, 5.8S ribosomal RNA, and ITS2) of MSCT68 491 bp is shown in Figure 16. The MSCT68 sequence was then used to search DataBank with the BLASTn program (Altschul et al., 1990) , and it was found that it had 100% homology with the ITS sequence of two *Diaporthe* species and three *Phomopsis* species, as shown in Figure 24. The ITS BLAST results therefore couldn't be used to identify the fungal species of MSCT68. Analysis of ITS sequences of MSCT67 (Figures 13 and 14) via BLAST search found that the sequences had 100% identity with *V. dahliae* complete sequence from

GenBank (strain Vd2; GenBank accession: HE972023.1; E-value = 0.0; 100% Query cover). There was also an exact match in ITS sequences between MSCT67 isolate and *Verticillium longisporum* (strain Vl19; GenBank accession: HE1972013.1; e-value = 0.0; 100% Query cover) and other strains (Figure 25). A more comprehensive comparison of ITS regions of ribosomal DNA in four *Verticillium* spp. are shown in Figures 25, 26, and 27.

Three *Verticillium* species, *V. dahliae*, *V. longisporum* and *V. albo-atrum* have been identified to be plant pathogens, and they can be distinguished based on their ITS1 and ITS2 sequences (Figures 13 and 14). *V. dahliae* and *V. albo-atrum* differ not only in ITS1 but also in ITS2. There are four pyrimidine nucleotide exchanges in ITS1 between *V. dahliae* and *V. albo-atrum* (strains Va1, Va2, Va3, Va4) at positions 73, 85, 87, and 90 and two pyrimidine exchanges in ITS2 at positions 97 and 126. The ITS sequences of all strains (Va1 to 4) of *V. albo-atrum* sequenced share 99% identity to each other (Tran, 2013).

V. dahliae is also distinct to *V. longisporum* Vl18, which has two pyrimidine exchanges in ITS2 at positions 97 and 126 and one pyrimidine exchange in ITS1 at positions 125 of *V. longisporum* Vl18 and one pyrimidine exchange in ITS2 at the position 90 of *V. albo-atrum* Va1. The ITS of *V. longisporum* Vl18 is homologous to the ITS of *V. albo-atrum* except for two pyrimidine exchanges in the ITS1 at positions 90 and 125 (Figures 13, 14). *V. longisporum* Vl18 has been described to be closely related to *V. albo-atrum* Va1 than to *V. dahliae*, and *V. longisporum* Vl19 is more closely related to *V. dahliae* than to *V. albo-atrum*, which is supported by the phylogenetic tree analysis of the inferred evolutionary relationships between *V. dahliae*, *V. albo-atrum* Va1, *V.*

longisporum Vl18, and *V. longisporum* Vl19 (Figure 15). The ITS sequence of ribosomal DNA from *Verticillium* isolate MSCT67 was used to search DataBank using BLASTN 2.2.32+ tools (Altschul, 1990). The ITS1 and ITS2 sequences of MSCT67 from BLAST results are 100% identical to those of *V. dahliae* and *V. longisporum*. Blast searches and analysis of the ITS region (ITS1 and ITS2) of the fungal isolate MSCT67 suggested that it could be *V. dahliae* or *V. longsporum* (Figure 25). Figures 26 also shows the results of BLAST searches of the ITS1 region of the fungal isolate MSCT67, and Figures 27 presents the BLAST analysis of the ITS2 region. Five fungal cultures were isolated from the 2014 diseased cotton plants as well, and they are identified as *Diaporthe* sp. (Figure 16), *Verticillium* sp. (MSCT70) (Figure 17), *Setosphaeria* sp. (Figure 18), *Fusarium* sp. (Figure 19), and *Phoma* sp. (Figure 20) by sequencing their ITS regions of ribosomal DNA (Table 1). Additional considerations are presented when we attempt to tie in this particular species to the wilt disease state. At this point, peer review literature data was utilized in order to generate the assumption, which later became the hypothesis, that a species of *Verticillium* sp. was responsible for infecting the cotton plant.

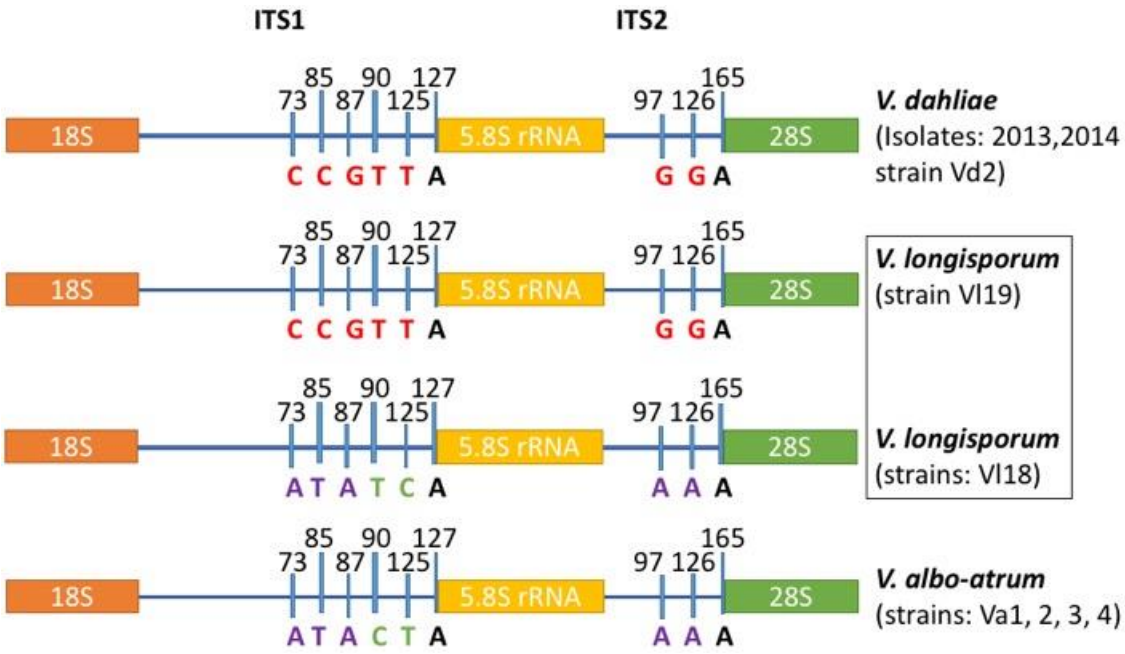

Figure 13 Analysis of ITS region of ribosomal DNA in *Verticillium* plant pathogens

The ITS region of *Verticillium* spp. comprises 129 nt of ITS1 and 165 nt of ITS2. The ITS of *V. longisporum* Vl19 is identical to that of *V. dahliae* isolate MSCT67. The four pyrimidine exchanges between *V. dahliae* and *V. albo-atrum* in ITS1 at positions 73, 85, 87, and 90 and two pyrimidine exchanges in ITS2 at positions 97 and 126 are indicated. The collected sequences were aligned by clustalW2 (www.ebi.ac.uk/Tools/clustalW2/index.html)

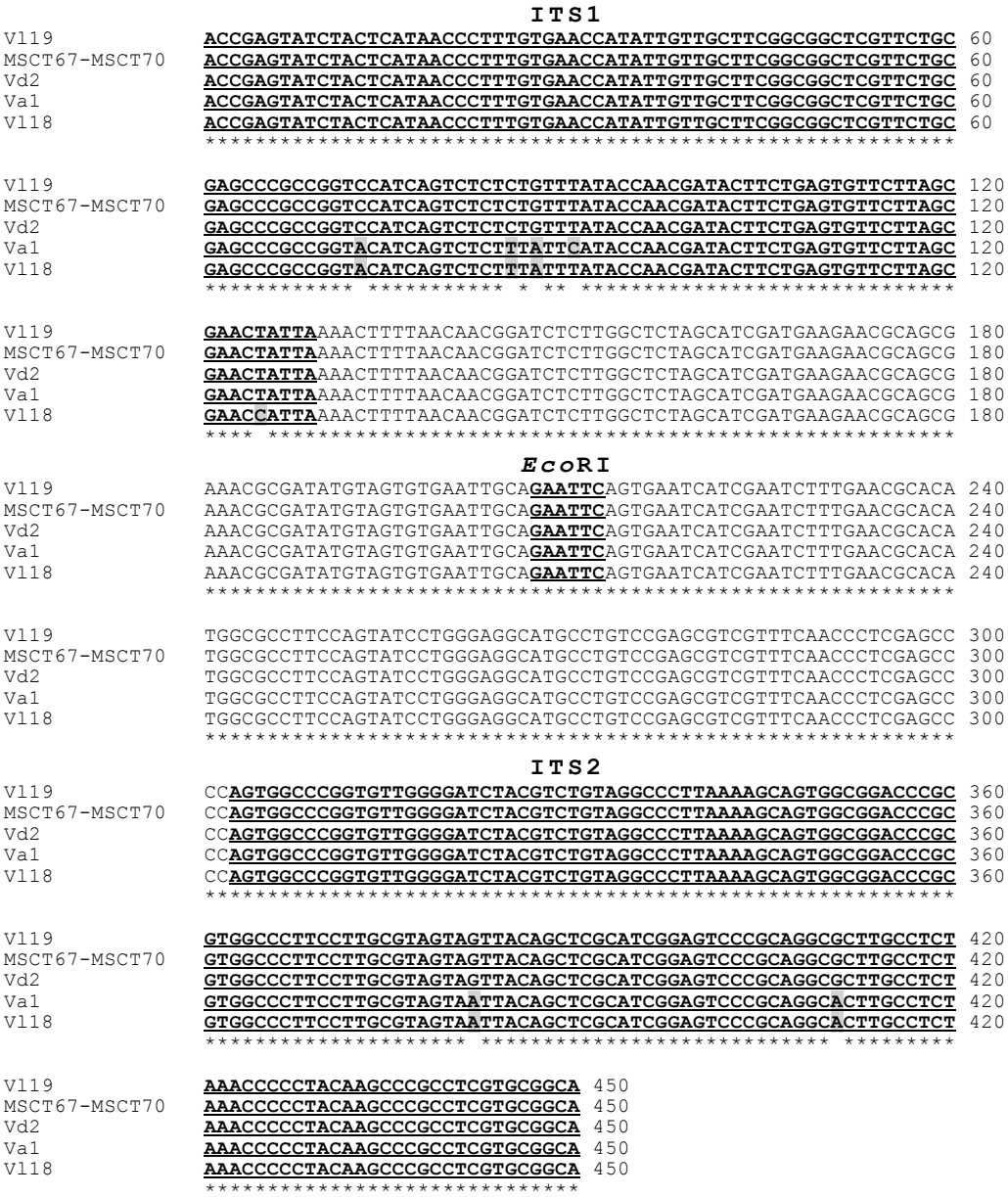

Figure 14 Multiple sequence alignment of ITS region of ribosomal DNA in *Verticillium* plant pathogens

Identical nucleotides are indicated by stars (*). The ITS region in bold type comprises 129 nt of ITS1 and 165 nt of ITS2. The 5.8S rRNA gene is located between the ITS1 and ITS2 regions. The *Eco*RI sites in the 5.8S rRNA gene are underlined. The ITS of *V. longisporum* Vl19 is identical to that of *V. dahliae* isolate. The four pyrimidine exchanges between *V. dahliae* and *V. albo-atrum* in ITS1 at positions 73, 85, 87, and 90 and two pyrimidine exchanges in ITS2 at positions 97 and 126 are indicated. The sequences were aligned with the ClustalW2 program (www.ebi.ac.uk/Tools/clustalW2/index.html)

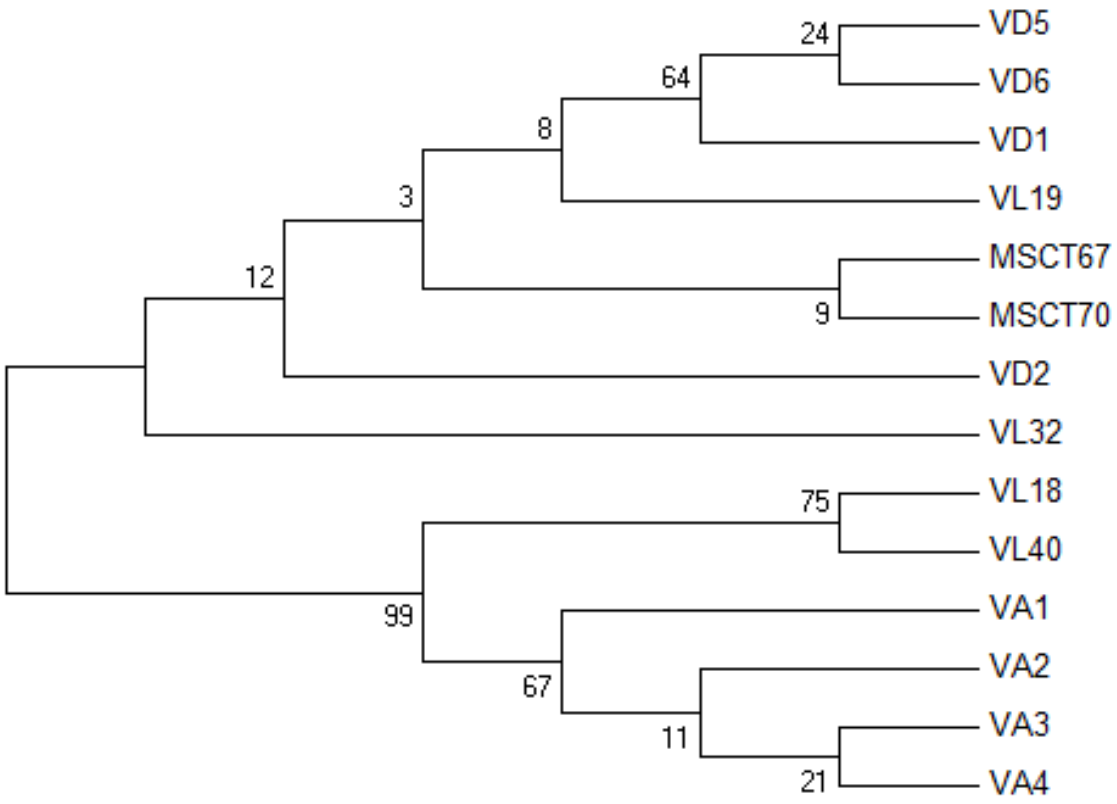

Figure 15 Construction of a phylogenetic tree of *Verticillium* spp.

Including *V. longisporum* (Vl19, Vl32), *V. dahliae* (Vd1,Vd2,Vd5,Vd6), *V. dahliae* isolate (MSCT67 and MSCT70), *V. longisporum* (Vl18,Vl40), and *V. albo-atrum* (Va1-4) using nucleotide sequences of internal transcribed spacer ITS1, 5.8S ribosomal RNA gene, and internal transcribed spacer ITS2 and a web service of phylogeny.fr. The ITS of *V. longisporum* (Vl18,Vl40) are more closely related to *V. albo-atrum* (Va1-4). The branches are represented in a cladogram, which ignore the branch length.

Table 1 Fungi identified in infected cotton plants

Time	Cultivar		Genera[a]	Genera[b]	Genera[c]
Summer 2013	Unknown	MSCT67	*Verticillium* sp.	*Verticillium* sp.	*Verticillium* sp.
		MSCT68	*Diaporthe* sp.	*Diaporthe* sp.	*Diaporthe* sp.
Summer 2014	Unknown	MSCT70	*Verticillium* sp.	NA	NA
	TM1		*Diaporthe* sp.	NA	NA
			Setosphaeria sp.	NA	NA
			Fusarium sp.	NA	NA
			Phoma sp.	NA	NA

(a) Identified from the infected cotton plants collected from the field.
(b) Fungi identified in (a) were used to infect cotton plants grown in a greenhouse as Koch's postulates and pathogenicity test. Infected plants were sectioned, incubated on PDA plates and fungi re-identified isolated and used the same method as in (a).
(c) Fungi identified in (b) were used to infect cotton plants grown in a greenhouse as the second cycle.
NA denotes not applicable.

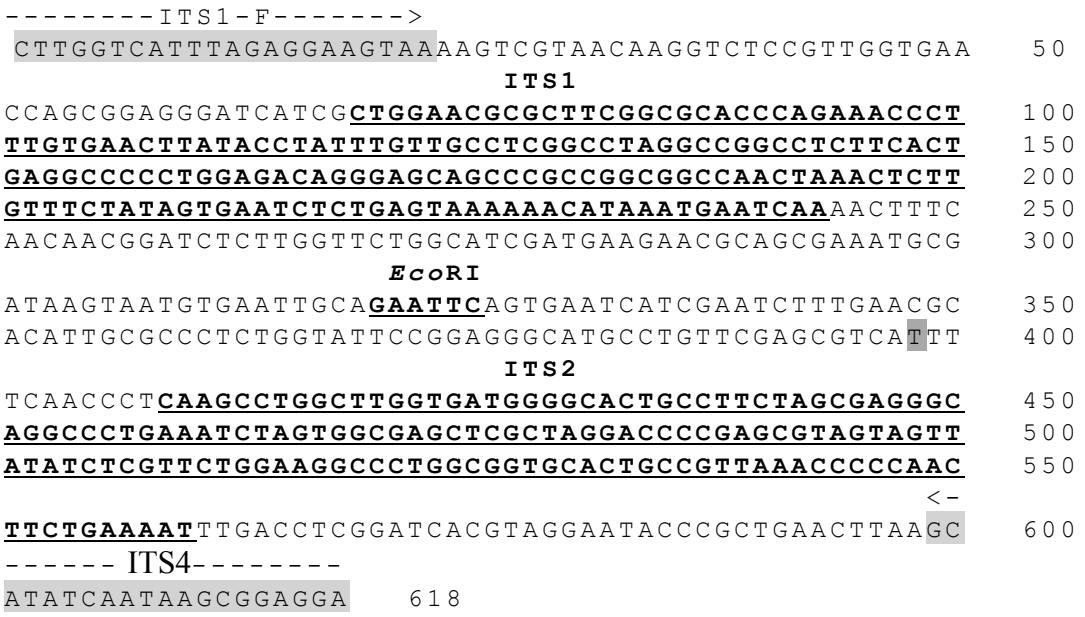

Figure 16 Nucleotide sequence of ITS and 5.8S rRNA gene of *Diaporthe* sp. isolate

The ITS region in bold type comprises 228 nt of ITS1 and 218 nt of ITS2. The one additional nucleotide between *Diaporthe* sp. isolate and other strains of *Diaporthe phaseolorum* is located at the position 155 of 5.8S rRNA gene. The 5.8S rRNA gene is located between the ITS1 and ITS2 regions. The *Eco*RI site in the 5.8S rRNA gene is underlined. ITS1-F and ITS4 are two PCR primers used to amplify the ITS region. The collected sequences were aligned by clustalW2 (www.ebi.ac.uk/Tools/clustalW2/index.html)

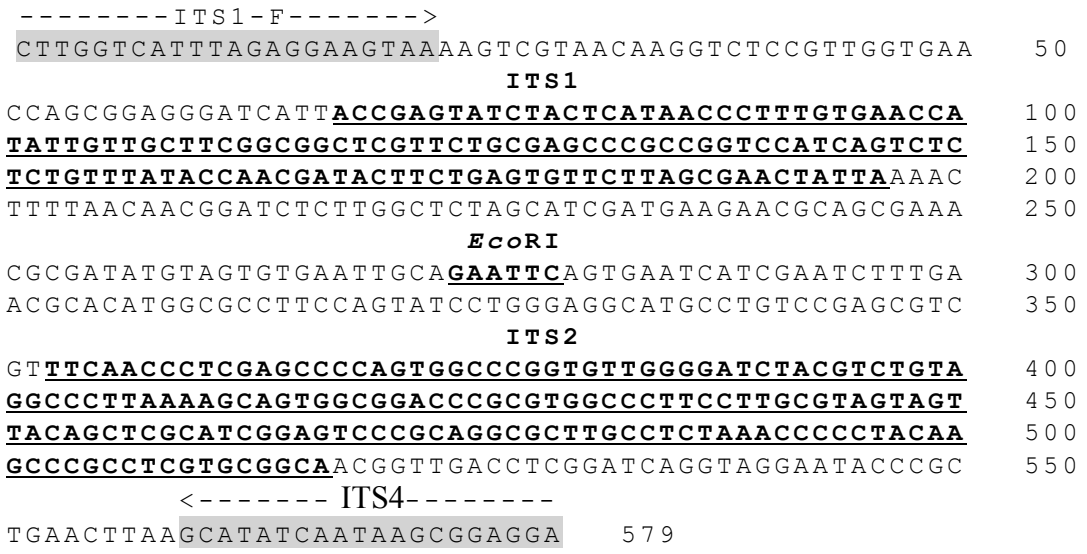

Figure 17 Nucleotide sequence of ITS and 5.8S rRNA gene of *Verticillium dahliae* isolate (MSCT67 and MSCT70 samples)

The ITS region in bold type comprises 129 nt of ITS1 and 165 nt of ITS2. The 5.8S rRNA gene is located between the ITS1 and ITS2 regions. The *Eco*RI site in the 5.8S rRNA gene is underlined. ITS1-F and ITS4 are two PCR primers used to amplify the ITS region.

Figure 18 Nucleotide sequence of ITS and 5.8S rRNA gene of *Fusarium* spp. Isolate

The ITS region in bold type comprises 148 nt of ITS1 and 152 nt of ITS2. The 5.8S rRNA gene is located between the ITS1 and ITS2 regions. The *Eco*RI site in the 5.8S rRNA gene is underlined. ITS1-F and ITS4 are two PCR primers used to amplify the ITS.

```
--------ITS1-F-------->
CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAA    50
                          ITS1
CCTGCGGAAGGATCATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTT    100
ACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGAT    150
TGGACACATTTAAACCCTTTGTAGTTGCAATCAGCGTCTGAAAAACTTTA    200
ATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA    250
                                    EcoRI
ACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCAT    300
CGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCT    350
                          ITS2
GTTCGAGCGTCATTTGTACCTTCAAGCTTTGCTTGGTGTTGGGTGTTTGT    400
CTCGCCTCTGCGCGCAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATT    450
GATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTC    500
CAAAAAGTACATTTTTTACACTCTTGACCTCGGATCAGGTAGGGATAC      548
```

Figure 19 Nucleotide sequence of ITS and 5.8S rRNA gene of *Phoma* spp. Isolate

The ITS region in bold type comprises 139 nt of ITS1 and 158 nt of ITS2. The 5.8S rRNA gene is located between the ITS1 and ITS2 regions. The *Eco*RI site in the 5.8S rRNA gene is underlined. ITS1-F and ITS4 are two PCR primers used to amplify the ITS

```
                          ITS1
GGGTGTGGTTTGCTGGCTACAGCGTCCGCCCCAAGTATTTTTCACCCATG    50
TCTTTTGCGCACTTTTTGTTTCCTGGGCGAGTTCGCTCGCCACCAGGACC    100
CAACCATAAACCTTTTTTTATGCAGTTGCAATCAGCGTCAGTATAATAAT    150
TCAATTTATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG    200
                                    EcoRI
AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAA    250
 TCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCGT    300
                          ITS1
GCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGT    350
CTTTTTGTCTCTCCCCTTGTTGGGGGAGACTCGCCTTAAAACGATTGGCA    400
GCCGACCTACTGGTTTTCGGAGCGCAGCACAAATTTGCGCCTTCCAATCC    450
ACGGGGCGGCATCCAGCAAGCCTTTGTTTTCTATAACAAATCCACATTTT    500
                                          <---- ITS4-
GACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGC    550
------
GGAGGA    556
```

Figure 20 Nucleotide sequence of ITS and 5.8S rRNA gene of *Setosphaeria* spp. Isolate

The ITS region in bold type comprises 139 nt of ITS1 and 180 nt of ITS2. The 5.8S rRNA gene is located between the ITS1 and ITS2 regions. The *Eco*RI site in the 5.8S rRNA gene is underlined. ITS1-F and ITS4 are two PCR primers used to amplify the ITS

Pathogenicity Test Based on Koch's Postulates

One question that needs to be asked, however, is whether the isolated *Verticillium dahliae* (MSCT67) and can cause the vascular wilt disease in cotton plants. The isolated *Verticillium* spp. (MSCT67) was thus used in a pathogenicity test using Koch's postulates. For the test, cotton seeds were germinated and grown in pots containing sterilized topsoil in a greenhouse at 28/23°C day/night temperatures and 60% to70% relative humidity (Bhat, 2003; Breeding, 2005; EROĞAN, 2013; Hanson, 2000; Pérez-Artés, 2000; Zhu, 2013). An agar block of *Verticillium* culture was inoculated into the wound location at the bottom of the cotton plants lightly cut with a razor blade (Figure 21). After growth for two months, no vascular wilt symptoms were observed in the plants (Figure 21). However, upon cutting a cross-section of the stem from the plants, the vascular tissue appeared discolored, from brown to black. The cross sections of plant stem tissues were subsequently transferred to PDA/tet plates, and fungal cultures were purified and re-isolated. Genomic DNAs were isolated from the two fungal cultures and used for amplification of ITS regions. DNA sequencing analysis of the ITS regions confirmed that the re-isolated fungi was indeed *V. dahliae*. The isolated fungi were again used to infect cotton plants as the second cycle, and identical fungal species were isolated and identified in the infected cotton plants (Table 1).

Figure 21 Pathogenicity test of *Verticillium*-infected cotton plants in the greenhouse.

Distinguish between the *Verticillium* spp. Based on Intron 3 of *VTA2* Gene

The ITS region is useful to discriminate between *V. dahliae* and *V. albo-atrum*, but it is not suitable for distinguish between *V. dahliae* and *V. longisporum*. *V. longisporum* is an allodiploid hybrid derived from the two haploid species of *V. dahliae* and *V. albo-atrum*. *V. longisporum* contains nearly double amount of nuclear DNA when compared to other *Verticillium* species such as *V. dahliae* and *V. albo-atrum*. The *Verticillium* transcription activator *VTA2* gene has been recently shown to be a barcode marker for *V. longisporum*. In order to distinguish among *V. dahliae, V. albo-atrum,* and *V. longisporum*, two PCR primers, *s*VTA2-F (5′- GCACGTCACCATGCAGTC -3′), and *s*VTA2-R (5′- CAGCTTCTTCCTCCTTCTTGC -3′) have been used to amplify the third

intron region (I3) of the *VTA2* gene (Tran, 2014, 2013). Two I3 PCR products with sizes of 315 and 380 bp would be amplified from the genomic DNA of *V. longisporum*, and a single DNA band of 315 or 380 bp amplified from the genomic DNA of *V. dahliae* or *V. albo-atrum*, respectively.

From the ITS sequences, MSCT67 is identified and classified as *Verticillium* sp. However, MSCT67 could be *V. dahliae*, *V. longisporum* or even *V. albo-atrum*. Therefore, there is a need to identify which species it is. The genomic DNA of MSCT67 was isolated and used for PCR amplification of the I3 intron of VTA2 with primers *s*VTA2-F and *s*VTA2-R. Analysis of PCR products by agarose gel electrophoresis indicated that only a single DNA band of 315 bp was amplified (Figure 22), suggesting that the isolated *Verticillium* sp. is not *V. albo-atrum* or *V. longisporum*, but is *V. dahliae*. The 315 bp DNA fragment was sequenced, and the BLAST search data (Altschul, 1990) indicated that the 315 bp sequence has 100% homology with the VTA2 sequence of *V. dahliae* and *V. longisporum* (Figure 23). The sequence of *VTA2* gene of *V. dahliae* is 100% identical to *V. dahliae* VdLs.17 chromosome 4, *V. dahliae* VdLs.17 hypothetical, *V. dahliae* JR2, and *V. longisporum* Vl19 Table 2 than other *Verticillium* species based on BLASTn searches.

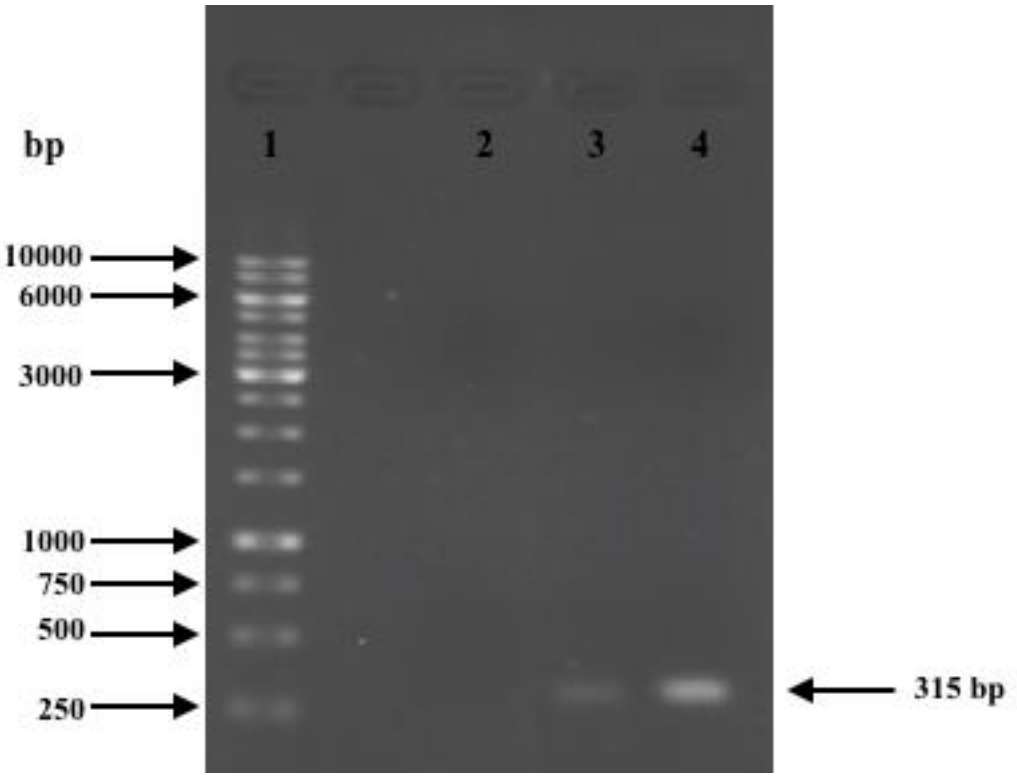

Figure 22 Analysis of intron 3 (I3) of *VTA2* gene of *Verticillium* isolates by 1% agarose gel electrophoresis

The 315 bp I3 was amplified by PCR with primers *s*VTA2-F and *s*VTA2-R using the MSCT67 genomic DNA as templates isolated from different dates (lanes 3 and 4). Lane 1 contained GeneRuler 1 kb DNA ladder, and Lane 2 contains a PCR reaction no DNA template (negative control).

------- *s*VTA2-F------>
CAGCTTCTTCCTCCTTCTTGCGCTGCTTCCATTCCTTACGAATCTCTTTG 50

Intron 3 (I3) of *VTA2* gene

AATTCT**GCAGTCACGCATTAGCTTCTGGCCTTTTCATCACCCAAGTCCCC** 100
GTAGCTCGGGTCGCCCATAGAAGCTAGTCTCAATTTTACGCCTGGACACA 150
CTACTCGGCGCGCTCTTCAACAGGGGCCCAACAGGAAAGCCCCCACATTG 200
CGCGTCCCGCATAGAACAGCGACAGGGATGGTGTGAAGAACAGGGCAAAG 250
<--
ATGGAGGTTGTAGGTGCATACCTTCAGGAGTTCGCTTCGTGCCGTGCGAC 300
---- *s*VTA2-R----
TGCATGGTGACGTGC 315

Figure 23 A nucleotide sequence of intron 3 (I3) of *VTA2* gene of *V. dahliae* isolate

Two primers, *s*VTA2-F and *s*VTA2-R, were used to amplify the intron. The third intron (I3) (217 bp) of *V. dahliae* was indicated in bold type and underlined. A single DNA band of 315 bp was amplified by PCR with the genomic DNA from *V. dahliae* isolates (2013 and 2014 samples).

Table 2 *Verticillium* spp. matched 100% with the *VTA2* sequence of *V. dahlia* in BLASTn program

Verticillium spp.	Strain	E-value	Identity	GenBank accession
V. dahliae	VdLs.17 chromosome 4	1e-162	100%	CP010983.1
V. dahliae	JR2	1e-162	100%	CP009078.1
V. dahliae	VdLs.17 hypothetical	1e-162	100%	XM_009655082.1
V. longisporum	Vl19	1e-162	100%	HE972130.1

CHAPTER IV

DISCUSSION

The emergence and spread of *Verticillium* wilt (VW) recently found in cotton plants at the R.R. Foil Plant Science Research Center at Mississippi State could pose a considerable threat to Mississippi cotton growers because some breeding lines appeared to be extremely susceptible to the disease. VW is a vascular wilt disease and widespread in the standard commercial cultivars of the Upland cotton plants, and it has been observed across the US Cotton Belt, from South Carolina to California (Pérez-Artés, 2000). Infected cotton plants had a decrease in lint yields and yield components, and often with low fiber and seed quality. The disease is caused by one of the three pathogenic *Verticillium* species: *V. dahliae*, *V. longisporum* and *V. albo-atrum*, and there is a need to identify which *Verticillium* species causes VW in diseased Mississippi cotton plants. The identification of *Verticillium* species will be useful for Integrated Pest Management and for the breeders to develop cotton lines that are tolerant to VW. *V. dahliae* is morphologically distinct to *V. albo-atrum*. *V. albo-atrum* has melanized resting mycelium (Puhalla, 1973) whereas *V. dahlia* has no dark mycelium. *V. dahliae*, however, produces black microsclerotia (Isaac, 1967), the resting structures. *V. longisporum*, an interspecies of the hybrid of *V. dahlia* and *V. albo-atrum*, containing nearly twice amounts of nuclear DNA compared to *V. dahliae* and *V. albo-atrum*, has asexual spores twice longer than *V. dahlia*. These morphological differences among the

three *Verticillium* spp., however, can be reliably used to identify and differentiate them among each other. Microsclerotia production (Figure 9B) confirmed that MSCT67 and MSCT70 were *Verticillium dahliae* and caused *Verticillium* wilt in cotton plants.

Molecular tools have been recently developed for diagnosis and identification of fungal species. For examples, restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP), have been used for diagnosis of *V. longisporum* and have demonstrated that it is an interspecies hybrid between *V. dahlia* and *V. albo-atrum*. Internal transcribed spacer (ITS) regions, ITS1 and ITS2, of ribosomal genes have been lately used for identification of fungal species. The ITS1 region is located between the 18S rRNA and 5.8S rRNA genes, and ITS2 is present between the 5.8S rRNA and 28S rRNA genes. Both the ITS1 and ITS2 sequences of *V. dahliae* are different from those in *V. albo-atrum* and *V. longisporum* V118 (a virulent strain) (Fig. 1.6). The ITS sequences of *V. dahliae*, however, are identical to those of *V. longisprorum* 119 (a non-virulent strain). *V. longisporum* has double amounts of nuclear DNA and contains two alleles of the *VTA2* genes. Amplification of the *VTA2* intron 3 in *V. longisporum* resulted in the generation of two PCR products with sizes of 315 and 385 bp, and a single DNA band of 315 bp was amplified from *V. dahlia*. Using the ITS sequences and amplification of the *VTA2* genes, MSCT67 and another *Verticillium* isolate were accurately identified to be *V. dahliae*. In most years, only trace amounts of VW are reported for Mississippi. However, under conditions favorable for VW (cold temperatures), significant losses can and do occur (Blasingame, 1997 - 2012). The loss of an estimated 13,714 bales of cotton in 2011 to bacterial blight (*Xanthomonas*

axonopodis pv. Malvacearum) in Mississippi demonstrated the potential loss associated with diseases when conditions favor a pathogen (Blasingame et al., 2012).

The VW symptoms including stunting, yellowing, necrosis, and interveinal chlorosis were not observed in infected cotton plants in the pathogenicity test conducted in the greenhouse. The tan and light brown color of vascular ring, however, representing the presence of *Verticillium* was seen in the dissected stem tissues from the plants and *V. dahliae* was able to be re-isolated from the stem tissues and identified via amplification and sequencing of the ribosomal ITS regions. One of the possible reasons for no observed VW symptoms is that VW generally develops in cold weather (late summer), particularly in the low night temperature below 65°C. The pathogenicity test in this research was conducted in the greenhouse from March to May, and infected cotton plants were grown at higher temperatures. The VW symptoms could be observed if the pathogenicity test were carried out in the field during the late summer or the inoculated plants were grown for a longer time.

V. dahliae has no sexual stage, which suggests that somatic mutation is a major force in contributing to the genetic diversity of this pathogen. A recent study indicated that integration of transposable elements into the fungal genome through horizontal gene transfer (HGT) is a driving force in *Verticillium* genome evolution and inter- and intra-specific genome variation (Amyotte, 2012). *V. dahliae* is very difficult to eradicate because the fungus can survive in the soil for many years as microsclerotia, persistent resting structures present in diseased plant tissues. Control of the fungus via soil fumigation is impractical, expensive and harmful to the environment. Crop rotation is also ineffective due to a wide host range of more than 200 dicotyledonous plants. There

are no effective fungicides for treating infected plants, and comprehensive prevention and control of VW can only rely on the development and planting resistant varieties. *Ve1* was the first gene identified as conferring resistance to *V. dahliae* in tomato (Fradin, 2009). Vel-mediated resistance signaling is activated by an effector Ave1, which was identified by a powerful tool of population genetics through genome and RNA sequencing (de Jonge et al., 2012). *Ve1* provides resistance in tomato against race 1 strain of *V. dahliae* but not to race 2. Transfer of the tomato *Ve1* gene to Arabidopsis also mediates resistance to VW (Fradin, 2009), suggesting that resistance signaling is conserved between tomato and Arabidopsis. Several genes (*Gbve* and *Gbve1*) for resistance to VW have also been isolated from *Gossypium barbadense* (Zhang et al., 2011; Zhang et al., 2012a). The three resistance genes *Ve1*, *Gbve* and *Gbve1* all encode extracellular leucine-rich repeat class of receptor-like proteins (eLRR-RLPs). These proteins have similar domains, containing leucine rich repeats (LRR), a signal peptide, and a transmembrane domain. The LRR has been hypothesized to participate in the particular recognition of the pathogen effector protein Ave1. Upland cotton (*G. hirsutum*) has low to moderate levels of resistance to VW (Kheiri and Fatahi, 2010). It has been recently reported that a number of the Acala type Upland cotton cultivars, such as Acala Nem-X, have fairly high levels of resistance to VW (Zhang et al., 2012b). Although variability for resistance to VW in Upland cotton has been demonstrated, most cultivars and accessions evaluated are considered susceptible. Resistance to VW, if any, is largely unknown for most commercial cultivars grown in Mississippi. It is not only prudent to characterize levels of resistance to *V. dahliae* in Mississippi grown cultivars, buy also to identify/isolate the gene(s) conferring resistance to VW in an effort to minimize the

genetic vulnerability in Mississippi' cotton crop. To understand the regulation of cotton in response to *Verticillium* infection, Zhang et al. (2015) had constructed small RNA libraries from *Verticillium*-infected *G. hirsutum* L. and *G. barbadense* L. and identified differentially expressed miRNAs and their targets via RNA deep sequencing and degradome analysis. The targets of the miRNAs with altered expression included many transcription factors (e.g. auxin response factors ARF, MYB, NAC, HD-ZIP and AP2-like factor), leucine-rich repeat (LRR) containing protein, LRR and NB-ARC domains-containing disease resistance –like protein proteins and a diverse novel proteins such as ubiquitin carboxyl-terminal hydrolase family protein, purine permease 3, TT12-2 MATE transporter, glutathione S-transferase, lipoxygenase, and intracellular protein transport protein USO1. These experimental results suggested that miRNAs could regulate global gene networks in cotton development upon infection by *V. dahliae* (Zhang, 2015).

CHAPTER V

CONCLUSIONS

The main objective of the current research was to identify fungal pathogens that cause wilt diseases on Mississippi cotton plants. Consequently, four basic phases were selected in the study of *Verticillium* wilt on the cotton crop at the R.R. Foil Plant Science Research Center at Mississippi State during the late summer in 2013 and 2014. The first phase was to collect stem tissues of the diseased cotton plants and isolating several soil-borne fungi with different morphology and growth characteristics via culturing on PDA plates. Phase two was to extract genomic DNAs from the isolated fungal species and used them in molecular typing via PCR amplification and DNA sequencing analysis of the ribosomal internal transcribed spacer (ITS) region. Using the ITS1 and ITS2 regions of the rRNA genes within the pathogen allowed for the species to be identified at the molecular level as *V. dahliae*. The third phase was to answer the question whether the isolated *Verticillium dahliae* (MSCT67) can cause the vascular wilt disease in cotton plants. The isolated *Verticillium* spp. (MSCT67) was thus used in pathogenicity test using Koch's postulates in the greenhouse (Zhang, 2013; Yuan, 2011). In the last stage of this study, distinguish between the *Verticillium* spp. was done by using the PCR-based genotyping method with species-specific primers. Two PCR primers, *s*VTA2-F and *s*VTA2-R, designed on the basis of the *VTA2* gene were used for PCR amplification to

confirm that the isolated *Verticillium* sp. was *Verticillium dahliae* and could cause *Verticillium* wilt in cotton plants.

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APPENDIX A

THE OUTPUT OF BLAST DATABASE SEARCH

Sequences producing significant alignments:		Score (Bits)	E Value	Ident
gb|KJ590725.1|	Diaporthe ueckerae isolate FAU658 internal tra...	907	0.0	100%
gb|FJ785448.1|	Phomopsis sp. STAM 60 18S ribosomal RNA gene, ...	907	0.0	100%
gb|EU301113.1|	Diaporthe phaseolorum strain MUCC219 18S ribos...	907	0.0	100%
gb|AY745020.1|	Phomopsis longicolla strain STAM-33 18S riboso...	907	0.0	100%
gb|AY050627.1|	Phomopsis sojae internal transcribed spacer 1,...	907	0.0	100%

Figure 24 The output of BLAST Database search with the ITS sequences of ribosomal DNA of *Diaporthe* sp. isolates (2013 and 2014 samples).

Sequences producing significant alignments:	Score (Bits)	E Value	Ident
gb|CP010980.1| Verticillium dahliae VdLs.17 chromosome 1, com...	239	2e-59	100%
gb|CP009075.1| Verticillium dahliae JR2 chromosome 1, complet...	239	2e-59	100%
dbj|AB551181.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551183.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551184.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551185.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551186.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551192.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551195.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551196.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551206.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551214.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
dbj|AB551215.1| Verticillium dahliae genes for ITS1, 5.8S rRN...	239	2e-59	100%
gb|HQ692909.1| Verticillium dahliae isolate VSP 08-33 C4 18S ...	239	2e-59	100%
gb|HQ692910.1| Verticillium dahliae isolate SL1-1 18S ribosom...	239	2e-59	100%
ref|NR_126124.1| Verticillium dahliae ITS region; from TYPE m...	239	2e-59	100%
gb|HQ206756.1| Verticillium dahliae strain PD403 18S ribosoma...	239	2e-59	100%
gb|HQ206815.1| Verticillium dahliae strain PD504 18S ribosoma...	239	2e-59	100%
gb|HQ441164.1| Verticillium dahliae strain CF1 18S ribosomal ...	239	2e-59	100%
gb|JN595826.1| Verticillium dahliae isolate NZFS3558 18S ribo...	239	2e-59	100%
gb|JQ070078.1| Verticillium dahliae strain ATCC 44571 18S rib...	239	2e-59	100%
gb|JQ902034.1| Verticillium dahliae isolate V01-A1 18S riboso...	239	2e-59	100%
gb|JQ902035.1| Verticillium dahliae isolate V02-A1 18S riboso...	239	2e-59	100%
gb|JQ647437.1| Verticillium dahliae culture-collection CABI:I...	239	2e-59	100%
gb|JQ647438.1| Verticillium dahliae culture-collection CABI:I...	239	2e-59	100%
gb|JQ647447.1| Volutella ciliata culture-collection CABI:IMI6...	239	2e-59	100%
gb|JX308315.1| Verticillium dahliae strain DB110712 internal ...	239	2e-59	100%
gb|JX276654.1| Verticillium dahliae isolate VeDh-Rhi-1 18S ri...	239	2e-59	100%
emb|HE972013.1| Verticillium longisporum 18S rRNA gene (parti...	239	2e-59	100%
emb|HE972023.1| Verticillium dahliae 18S rRNA gene (partial),...	239	2e-59	100%
gb|JX997431.1| Verticillium dahliae strain 12-141 18S ribosom...	239	2e-59	100%
gb|HQ703411.1| Verticillium dahliae isolate C9 18S ribosomal ...	239	2e-59	100%
gb|KJ018789.1| Verticillium longisporum strain BC13091625 int...	239	2e-59	100%
gb|KJ696553.1| Verticillium dahliae strain QZ12100902 18S rib...	239	2e-59	100%
gb|KJ744368.1| Verticillium dahliae strain PM17 18S ribosomal...	239	2e-59	100%
gb|KM106204.1| Verticillium dahliae isolate VD21 internal tra...	239	2e-59	100%
gb|KM106208.1| Verticillium dahliae isolate VD10 internal tra...	239	2e-59	100%
gb|KM106209.1| Verticillium dahliae isolate VD32 internal tra...	239	2e-59	100%
gb|KM000048.1| Verticillium dahliae isolate DB13AGO056 intern...	239	2e-59	100%
gb|KF878394.1| Verticillium dahliae isolate VEGY1 18S ribosom...	239	2e-59	100%
gb|KF878396.1| Verticillium dahliae isolate VEGY3 18S ribosom...	239	2e-59	100%
gb|JQ629939.1| Verticillium dahliae isolate VD1 18S ribosomal...	239	2e-59	100%
gb|JQ629941.1| Verticillium dahliae isolate VD5 18S ribosomal...	239	2e-59	100%
gb|JQ629942.1| Verticillium dahliae isolate VD6 18S ribosomal...	239	2e-59	100%
gb|JQ629944.1| Verticillium dahliae isolate VD11 18S ribosoma...	239	2e-59	100%
gb|JQ629945.1| Verticillium dahliae isolate VD12 18S ribosoma...	239	2e-59	100%
gb|JQ629949.1| Verticillium dahliae isolate VD32 18S ribosoma...	239	2e-59	100%
gb|HQ206719.1| Verticillium dahliae strain PD323 18S ribosoma...	239	2e-59	100%
ref|NR_126126.1| Verticillium longisporum ITS region; from TY...	239	2e-59	100%
gb|JF704205.1| Verticillium dahliae isolate DB14311 18S ribos...	239	2e-59	100%
emb|HE972025.1| Verticillium dahliae 18S rRNA gene (partial),...	239	2e-59	100%
gb|KM106203.1| Verticillium dahliae isolate VD1 internal tran...	239	2e-59	100%
gb|KM503139.1| Ilyonectria radicicola strain CYLD internal tr...	239	2e-59	100%
gb|JQ629947.1| Verticillium dahliae isolate VD30 18S ribosoma...	239	2e-59	100%
gb|JQ629948.1| Verticillium dahliae isolate VD31 18S ribosoma...	239	2e-59	100%
gb|JQ629958.1| Verticillium longisporum isolate VDL44 18S rib...	239	2e-59	100%

Figure 25 The output of BLAST Database search with the ITS1 and ITS2 sequences of ribosomal DNA of *V. dahliae* isolates (MSCT67 and MSCT70 samples).

Sequences producing significant alignments:		Score (Bits)	E Value	Ident
gb|KP822065.1|	Verticillium dahliae isolate NV-M1-1 internal ...	305	1e-79	100%
gb|CP010980.1|	Verticillium dahliae VdLs.17 chromosome 1, com...	305	1e-79	100%
gb|JQ629949.1|	Verticillium dahliae isolate VD32 18S ribosoma...	305	1e-79	100%
gb|JQ629944.1|	Verticillium dahliae isolate VD11 18S ribosoma...	305	1e-79	100%
gb|JQ629942.1|	Verticillium dahliae isolate VD6 18S ribosomal...	305	1e-79	100%
gb|JQ629941.1|	Verticillium dahliae isolate VD5 18S ribosomal...	305	1e-79	100%
gb|JQ629940.1|	Verticillium dahliae isolate VD2 18S ribosomal...	305	1e-79	100%
gb|JQ629939.1|	Verticillium dahliae isolate VD1 18S ribosomal...	305	1e-79	100%
gb|KM461712.1|	Uncultured fungus isolate DGGE gel band 15 18S...	305	1e-79	100%
gb|KF878396.1|	Verticillium dahliae isolate VEGY3 18S ribosom...	305	1e-79	100%
gb|KF878394.1|	Verticillium dahliae isolate VEGY1 18S ribosom...	305	1e-79	100%
gb|KM013462.1|	Verticillium dahliae strain SWUKJ5.1710 intern...	305	1e-79	100%
gb|KM013461.1|	Verticillium dahliae strain SWUKJ5.1330 intern...	305	1e-79	100%
gb|KM013460.1|	Verticillium dahliae strain SWUKG5.1640 intern...	305	1e-79	100%
gb|KM013459.1|	Verticillium dahliae strain SWUKG5.1610 intern...	305	1e-79	100%
gb|KM013458.1|	Verticillium dahliae strain SWUKG5.1520 intern...	305	1e-79	100%
gb|KM013457.1|	Verticillium dahliae strain SWUKG2.0720 intern...	305	1e-79	100%
gb|KM013456.1|	Verticillium dahliae strain SWUKD5.1820 intern...	305	1e-79	100%
gb|KM013455.1|	Verticillium dahliae strain SWUKD4.0211 intern...	305	1e-79	100%
gb|KM013454.1|	Verticillium dahliae strain SWUKD3.0620 intern...	305	1e-79	100%
gb|KM013453.1|	Verticillium dahliae strain SWUKD3.0610 intern...	305	1e-79	100%
gb|KM013452.1|	Verticillium dahliae strain SWUKD3.0110 intern...	305	1e-79	100%
gb|KM000048.1|	Verticillium dahliae isolate DB13AGO056 intern...	305	1e-79	100%
gb|KM106209.1|	Verticillium dahliae isolate VD32 internal tra...	305	1e-79	100%
gb|KM106208.1|	Verticillium dahliae isolate VD10 internal tra...	305	1e-79	100%
gb|KM106204.1|	Verticillium dahliae isolate VD21 internal tra...	305	1e-79	100%
gb|CP009075.1|	Verticillium dahliae JR2 chromosome 1, complet...	305	1e-79	100%
gb|KJ744368.1|	Verticillium dahliae strain PM17 18S ribosomal...	305	1e-79	100%
gb|KF493995.1|	Uncultured Verticillium clone TVD_ITS1F-ITS4_9...	305	1e-79	100%
gb|KJ696553.1|	Verticillium dahliae strain QZ12100902 18S rib...	305	1e-79	100%
gb|KJ018789.1|	Verticillium longisporum strain BC13091625 int...	305	1e-79	100%
gb|HQ703411.1|	Verticillium dahliae isolate C9 18S ribosomal ...	305	1e-79	100%
gb|KC834724.1|	Verticillium dahliae isolate CAV1003 internal ...	305	1e-79	100%
gb|KC156640.1|	Verticillium dahliae isolate Vd170 18S ribosom...	305	1e-79	100%
gb|KC156639.1|	Verticillium dahliae isolate Vd138 18S ribosom...	305	1e-79	100%
gb|KC156638.1|	Verticillium dahliae isolate Vd166 18S ribosom...	305	1e-79	100%
gb|KC156637.1|	Verticillium dahliae isolate Vd162 18S ribosom...	305	1e-79	100%
gb|KC156636.1|	Verticillium dahliae isolate Vd168 18S ribosom...	305	1e-79	100%
gb|KC156635.1|	Verticillium dahliae isolate Vd158 18S ribosom...	305	1e-79	100%
gb|KC156634.1|	Verticillium dahliae isolate Vd136 18S ribosom...	305	1e-79	100%
gb|JX997431.1|	Verticillium dahliae strain 12-141 18S ribosom...	305	1e-79	100%
emb|HE972023.1|	Verticillium dahliae 18S rRNA gene (partial),...	305	1e-79	100%
emb|HE972013.1|	Verticillium longisporum 18S rRNA gene (parti...	305	1e-79	100%
gb|JX276654.1|	Verticillium dahliae isolate VeDh-Rhi-1 18S ri...	305	1e-79	100%
gb|JX308315.1|	Verticillium dahliae strain DB110712 internal ...	305	1e-79	100%
gb|JQ647447.1|	Volutella ciliata culture-collection CABI:IMI6...	305	1e-79	100%
gb|JQ647437.1|	Verticillium dahliae culture-collection CABI:I...	305	1e-79	100%
dbj|AB735536.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
gb|JQ902035.1|	Verticillium dahliae isolate V02-Al 18S riboso...	305	1e-79	100%
gb|JQ902034.1|	Verticillium dahliae isolate V01-Al 18S riboso...	305	1e-79	100%
gb|JQ070078.1|	Verticillium dahliae strain ATCC 44571 18S rib...	305	1e-79	100%
gb|JN595826.1|	Verticillium dahliae isolate NZFS3558 18S ribo...	305	1e-79	100%
gb|HQ441164.1|	Verticillium dahliae strain CF1 18S ribosomal ...	305	1e-79	100%
gb|GU799602.1|	Verticillium dahliae isolate 566 internal tran...	305	1e-79	100%
gb|JF810409.1|	Verticillium dahliae strain R-B internal trans...	305	1e-79	100%
dbj|AB628076.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB628075.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
gb|HQ206756.1|	Verticillium dahliae strain PD403 18S ribosoma...	305	1e-79	100%
dbj|AB551214.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%

Figure 26 The output of BLAST Database search with the ITS2 sequences of ribosomal DNA of *V. dahliae* isolates (MSCT67 and MSCT70 samples).

ref|NR_126124.1|	Verticillium dahliae ITS region; from TYPE m...	305	1e-79	100%
gb|HQ692910.1|	Verticillium dahliae isolate SL1-1 18S ribosom...	305	1e-79	100%
gb|HQ692909.1|	Verticillium dahliae isolate VSP 08-33 C4 18S ...	305	1e-79	100%
gb|HQ692908.1|	Verticillium dahliae isolate VSP 08-33 A3 inte...	305	1e-79	100%
dbj|AB551215.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551211.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551209.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551207.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551206.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551197.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551196.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551195.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551192.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551191.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551190.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551189.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551188.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551187.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551186.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551185.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551184.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551183.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551182.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551181.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551180.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551179.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551178.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551177.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551176.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551174.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551173.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551172.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
dbj|AB551171.1|	Verticillium dahliae genes for ITS1, 5.8S rRN...	305	1e-79	100%
gb|GU461636.1|	Verticillium dahliae isolate Ca1352 18S riboso...	305	1e-79	100%
gb|GU461628.1|	Verticillium dahliae isolate Le1343 18S riboso...	305	1e-79	100%
gb|GU461623.1|	Verticillium dahliae isolate Le1340 18S riboso...	305	1e-79	100%
gb|GU461622.1|	Verticillium dahliae isolate Le1338 18S riboso...	305	1e-79	100%
gb|GU461620.1|	Verticillium dahliae isolate Le1335 18S riboso...	305	1e-79	100%
gb|GU461618.1|	Verticillium dahliae isolate Le1333 18S riboso...	305	1e-79	100%
gb|GU461615.1|	Verticillium dahliae isolate Oe1329 18S riboso...	305	1e-79	100%

Figure 26 (continued)