The Pennsylvania State University

The Graduate School

Department of Plant Pathology and Environmental Microbiology

AN ECOLOGICAL LENS ON THE EMERGENCE OF PLANT PATHOGENICITY: COMPARATIVE STUDIES OF PATHOGENIC AND NON-PATHOGENIC *FUSARIUM OXYSPORUM*

A Thesis in

Plant Pathology

and International Agriculture and Development

by

Genna R. Tesdall

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

December 2018

The thesis of Genna R. Tesdall was reviewed and approved* by the following:

Maria del Mar Jiménez Gasco
Associate Professor of Plant Pathology
Thesis Advisor

David Geiser
Professor of Plant Pathology

Beth Gugino
Associate Professor of Plant Pathology

Mary Barbercheck
Professor of Entomology

Melanie Miller Foster
Assistant Professor of International Agriculture

Carolee Bull
Professor of Plant Pathology and Systematic Bacteriology
Head of the Department of Plant Pathology and Environmental Microbiology

*Signatures on file in the Graduate School

ABSTRACT

Banana is a staple food crop in the developing world, where 85% of production is traded and consumed locally and exports generate $5 billion USD annually. Fusarium wilt of banana, caused by Tropical Race 4 (TR4) of *Fusarium oxysporum (Fo)* f. sp. *cubense* is threatening Cavendish cultivars that dominate the export industry. To design sustainable solutions to manage Fusarium wilt of banana caused by TR4, for which no marketable resistant cultivars or chemical controls exist, scientists must understand the mechanisms of pathogenicity and the ecology of this organism, including the role of the pathogenicity-associated genes known as Secreted In Xylem (*SIX*) genes. This project examines the differences between pathogenic and non-pathogenic *Fo* isolates isolated from banana, tomato, and chickpea by examining the role of *SIX9*, evaluating phylogenetic relationships between these isolates, and detecting *SIX* genes in these isolates.

The role of *SIX*9 in pathogenic TR4 and Race 1 was examined by studying PEG-mediated knockout transformants *in vitro* and *in planta*. Mutants of TR4 displayed two *in planta* phenotypes: non-pathogenic and hypervirulent. Mutants of Race 1 isolates showed two *in-planta* phenotypes: non-pathogenic and pathogenic to Cavendish 'Gran Naine' and 'Gros Michel', or in other words, a TR4 *in planta* phenotype. The genetic underpinnings of these *in planta* phenotypes have yet to be determined. However, PCR and sequencing of insertion site amplicons indicated there may be more than one copy of *SIX9* in the genome of TR4 O-2052.

To understand evolutionary relationships between non-pathogenic isolates, phylogenetic relationships of *Fo* isolates from asymptomatic banana, tomato, and chickpea were inferred using *TEF-1α* and five novel genes, *04642, 06412, 10995, 15960,* and *15695*. This study designed primer pairs which showed high sequencing efficiency (>95%) and added 438 parsimony-informative characters to the 64 parsimony-informative characters from *TEF1-α*. Phylogenetic analysis indicated *Fo* isolates group together with isolates pathogenic and non-pathogenic to different hosts. No phylogenetic patterns were

identified based on the detection of *SIX* genes, mating-type loci, geographic origin, or origin within the plant (i.e.: root, crown, or stem).

This is the first study to report the presence of *SIX* genes in non-pathogenic isolates from tomato and chickpea. Twenty-nine endophytes of tomato, nineteen endophytes of chickpea, and eight *Fo* f. sp. *ciceris* were screened. There were eight PCR-based detections of *SIX8;* all eight detections were in *Fo* f. sp. *ciceris* isolates. *SIX9* was detected in five isolates; all were endophytes of chickpea. *SIX11* was detected in five isolates. Three of these detections were in three isolates characterized as *Fo* f. sp. *ciceris.* One detection was in an endophyte of tomato (2-1c1). One detection was in an endophyte of chickpea (cc44). *SI X14* was detected in 9 isolates. Eight of these detections were in isolates characterized as *Fo* f. sp. *ciceris* (100% of all *Fo* f. sp. *ciceris* isolates screened). One detection was in an endophyte of chickpea (cc44). *SIX1* was detected in one isolate, characterized as *Fo* f. sp. *ciceris* race 0 (*Foc*82108).

These studies contribute knowledge to the field of *Fo* biology and ecology by providing new affordable molecular tools, in the form of five core genome regions, for understanding *Fo* phylogenetic relationships with may more parsimony-informative characters than the current phylogenetic standard, *TEF1-α*. Furthermore, this research provides a starting framework to study relationships between *Fo* isolates non-pathogenic and pathogenic to their hosts. We find endophytes do not show clear phylogenetic relationships based on the core genome to predict pathogenicity or make other conclusions, like on host specificity. Furthermore, this study reports the first sequence-confirmed presence of *SIX* genes in non-pathogenic isolates from chickpea and tomato, prompting further questions about the ecological role of *SIX* genes. Finally, transformants of *Fo* f. sp. *cubense* TR4 revealed *in planta* phenotypes that eliminate pathogenicity, and in one mutant, induced hypervirulence. These results prompt further investigation into the molecular underpinnings of these phenotypes. We seek to answer the question: why are some *Fo* isolates plant pathogenic while others are not?

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

Literature Review

Fusarium oxysporum: a socioeconomically relevant organism

Fusarium oxysporum (Fo) is best known as a persistent soil-borne pathogen plaguing growers of over 120 different crops around the world (Ploetz and Evans 2015). *Fo* is the most economically important species of the *Fusarium* genus based on the number of hosts and losses associated with disease. Since *Fo* can persist in the soil, diseases caused by plant pathogenic *Fo* can result in dislocation of land use and markets (Backman 1987). *Fo* is found in most soils and has been observed from the Arctic (Kommedah et al. 1988) to the tropics (Sangalang et al. 1995), in the desert (Mandeel et al. 2005), grasslands (Opperman and Wehner, 1994), forests (Cabello and Aramarri, 2002), the sea floor (Palmero et al. 2010) across cultivated and wild landscapes (Laurence et al. 2012). *Fo* has also been isolated from marine algae (Granchinho et al. 2002). With reports on over 120 hosts, *Fo* is the most commonly reported *Fusarium* endophyte of wild and crop plants (Kuldau and Yates, 2000).

The most famous diseases caused by *Fo* feature high profile economic and food security impact. For example, the story of Fusarium wilt of banana, more commonly known as “Panama disease” or “Mal de Panama” in Latin American producing countries, is particularly captivating to the public imagination when publicized as the “banana apocalypse.” Since banana is used as a study system for objectives in this proposal, the case of Panama disease of banana is discussed in the literature review as a case study highlighting the importance of *Fo*.

Banana: a globally and domestically relevant system

Banana is the world’s most important fruit crop and the fourth most important food security crop (Ploetz 2015; Oritz and Vuylstek 1996). Over 100 million tons of bananas are produced annually around the world, and their annual worth is estimated at $5 billion USD (Panama Disease 2017). Only 15% of all produced bananas are exported; Latin America produces 80% of these export bananas (Vellema et al

2013). In 2009, banana was the most important export crop in Costa Rica, Ecuador, Panama, and Belize. It was the second and third most important export commodity in Colombia, Guatemala, and Honduras. In these countries, banana is the major source of foreign currency (Ploetz 2015; FAOSTAT 2015). As the top exporting country, Ecuador relies on banana production and trade to provide 380,000 jobs. Its share of the global export market has increased from 17% in the 1970s-1980s, to 30% in the 1990s, and continued growth today. Even more impressively, almost 90% of the 150,000 hectares registered for banana production in Ecuador, the world's top banana exporter, belongs to small and medium-size farms from 10-50 hectares (FAO 2003). Bananas grown in subtropical zones, in nations like South Africa, Brazil, India, and China, are produced for local consumption (Stover 1986, Daniells 2012). In Africa, banana is critical to the food security and income of some 70 million Africans (University of Stellenbosch, 2016).

History of Fusarium Wilt of Banana

Disease is the primary biotic constraint in banana produced both for export and local consumption (Ploetz and Evans 2015). Fusarium wilt is the largest threat to banana production in all parts of the globe and has long been recognized as one of the most destructive banana diseases (Ploetz and Evans 2015, Stover 1962, Stover and Simmonds 1987). Between the turn of the century and the 1950s, *Fo* f. sp. *cubense (Foc)* Race 1 wiped out all but isolated smallholder production of the main export cultivar of the era, 'Gros Michel'. The disease caused $2 billion USD in losses and destroyed over 40,000 hectares of banana production in Central and South America (Ghag et al. 2015).

The cultivar group Cavendish, resistant to *Foc* Race 1, became ubiquitous in export production as it replaced 'Gros Michel'. Cavendish bananas are currently accepted by growers and the market (Ghag et al 2015, Jeger et al. 1996; Ploetz et al. 2015; Vellema et al 2015). Cavendish makes up 95% of the global export production (D'Hont et al. 2012). It also produces 28% of locally consumed bananas around the world (Ploetz et al. 2015). TR4 has not been reported in Latin America and the Caribbean (LAC) to date.

The Cavendish cultivar group and the majority of cultivated bananas are susceptible to a new race of *Foc*, Tropical Race 4 (TR4) (Ploetz 2015). TR4 threatens a $5 billion USD annual industry and the

food security of millions in Latin America, Asia, and Africa. TR4 was first reported in South East Asia in 1992 (Pegg et al. 1992). Now, TR4 is present in Indonesia, Australia, and the Philippines; in 2013, it jumped continents to Mozambique, and in 2014 to Jordan, Lebanon, Oman, and Pakistan (Butler 2010; Garcia-Bastidas et al. 2014; Ordonez et al. 2015; Ploetz 2015; Ploetz et al. 2015).

The United States in the Banana Industry

The role of US enterprises in the banana trade is not common knowledge but can help stakeholders of The Pennsylvania State University understand the importance of US-based research in banana, which reaches beyond the connections between food security, peace, and push factors of immigration from LAC. Companies in the US have a long history of involvement in the LAC banana trade. The US-based United Fruit Company revolutionized the banana industry by consolidating a previously decentralized industry, especially through vertical integration of railroads, ports, and shipping facilities. Through the 20th century, the United Fruit Company also gained power in politics of the LAC and guided US international policy towards its interests in LAC (Striffler 2005). US companies Dole Food Corporation, Del Monte Fresh Fruit, and Chiquita Brands International (formerly United Fruit Company) are all US-based companies that gain revenue from banana production in LAC (Chiquita 2017, Dole 2017). Together, the US and Canada are the top importers of bananas, making up 38% of the global market (FAO 2003). People in the United States eat more bananas than apples and oranges combined (Panama Disease 2017).

Plant Disease Impacts to Livelihoods and Immigration

In recent years, the US experienced a spike in immigration from Central American nations. The push factors for immigration however were largely lost to the public eye. Kandel and colleagues (2015) identified coffee rust as one of the leading economic push factors leading to the spike of unaccompanied minor immigration to the US. The advent of coffee rust in 2013 in LAC caused many laborers and landowners to lose the coffee plantations their livelihoods depended upon by destroying 74% of the coffee crop in El Salvador, 70% in Guatemala, and 25% in Honduras. The loss of 200,000 jobs in these

three countries in 2013 and dropping employment and wages in the coffee industry throughout 2014 are identified as a major contributing factor to immigration. Impacts were compounded by high poverty rates, high violent crime rates, and slow economic growth (Kandel et al. 2015; Nevin 2016). Due to the importance of the banana industry in many LAC nations, the potential for devastation due to Fusarium wilt of banana in LAC banana production is not dissimilar to the threat of coffee rust, and thus should be considered integral to US domestic and foreign interests.

Fusarium Wilt Disease Management

Classical plant pathology outlines the ternary of conditions necessary to cause disease: appropriate environment, susceptible host, and virulent pathogen. The disease cycle illustrates this ternary in the field and can be used to identify potential intervention points (Figure 1.1). Fusarium wilt is extremely challenging to manage. Chlamydospores can survive in the soil for over 20 years, and even during vegetative growth, *Fo* has resistance to most fungicides.

Chemical soil treatments only work so long as toxic conditions are maintained; thus, infestation occurs even if the broad-spectrum fumigants can be used despite their environmental impacts and legal barriers, as the pathogen appears to survive at depths beyond the effective range of the treatment (Ploetz 2015; Backman 1987). Benomyl shows potential as a root dip treatment in a greenhouse setting to decrease disease severity (Nel et al. 2007), but benomyl has been banned in the US due to negative effects on human embryos (Colborn et al. 1994). Complicating matters further, resistant varieties are not accepted by the market and are not yet vetted in long-term field trials (Ghag et al. 2015; Ploetz 2015). Therefore, the best method of control is geographic isolation through quarantine. As a soil-borne pathogen, long-distance spread is thought to be primarily anthropogenic (Queensland Government 2015; Ploetz et al. 2015).

In the case of TR4, which is not yet globally distributed, exclusion is the most realistic current method of controlling a new outbreak of Fusarium wilt in banana. Realistic contingency plans to prevent and contain the spread of TR4 only exist in Australia, but the Australian extension program is not wholly

applicable to other regions and economic conditions. Governments and banana corporations are not investing in TR4 prevention and control for unclear reasons (Pocasangre and Magdama, personal communication). Therefore, the banana growers small and large must adopt rigorous phytosanitary practices to mitigate or prevent the worldwide devastation TR4 could have on the livelihoods of banana smallholders, laborers, and industry. Furthermore, preventative control measures for TR4 will also contribute to management of black sigatoka and other banana diseases that are notable barriers to production. This makes disease management relevant for today and the future of banana production in Latin America and beyond.

Phytosanitary containment practices include limiting the spread of spores on machinery, tools, shoes, and planting material. Machinery, tools and shoes can be disinfested using sodium hypochloride (Ghag et al. 2015). Planting material from tissue culture and pathogen-free seed can guarantee pathogen-free planting material, which also controls other diseases (Ploetz et al. 2015).

Since the host range of *Fo* includes over 120 crop species, it is unlikely that crop rotation is a viable means of control. *Fo* resides as an endophyte in many other plants and a saprophyte in the soil (Hennessy et al. 2005), also rendering crop rotation an illogical means of control. However, Hennessy's report of *Foc* were determined to be in weed hosts was determined via VCG analysis. These isolates were not tested for pathogenicity on banana; this details is crucial, as multiple races can be found within a VCG, and endophyte VCGs are largely unexplored (Dita et al. 2010; Ghag et al. 2015).

TR4 has also been detected on the exoskeletons of the banana weevil borer, *Cosmopolites sordidus* (Coleoptera: Curculionidae), which lives in the soil and feeds on the rooms and corm of the banana plant. Speculatively, banana weevil borer could carry *Foc* TR4 spores between banana plants in addition to pre-disposing plants by weakening them through room and corm feeding (Meldrum et al. 2013).

Resistant cultivars are also an option against specific races of *Fo*, and most resistant cultivars depend on monogenic resistance (Beckman 1987). The cultivar group Cavendish offers specific resistance against *Foc* Race 1 but is susceptible to TR4. In the tomato system (*Solanum lycopersicum*), race-specific resistance is available in commercial tomato germplasm. Lack of social and political acceptance of genetically modified organisms also pose barriers to resistance cultivars currently being developed.

Non-pathogenic *Fo* isolates have documented potential as biocontrol agents against plant pathogenic *Fo.* Suppressive soils, where disease incidence remains low despite presence of plant pathogenic *Fo* and a susceptible host, are most commonly associated with non-pathogenic *Fo* and fluorescent *Pseudomonas* spp. (Scher and Baker, 1980; Scher and Baker, 1982; Alabouvette, 1990; Postma and Rattink, 1992; Liu et al. 1995; Hoffland et al. 1996; Larkin et al. 1996; Van Loon et al. 1999). Fo47, a non-pathogenic *Fo* isolated from a soil suppressive to *Fo* f. sp. *lycopersici (Fol)* and *Fo* f. sp. *melonis*, was found to reduce Fusarium wilt disease incidence on flax (Duijff et al. 1999), carnation (Lemanceau et al. 1992, 1993), and tomato (Fuchs et al. 1997; Duijff et al. 1998). Non-pathogenic *Fo* and *F. solani* can also suppress Fusarium wilt of sweet potato under a variety of field conditions (Sneh 1998). A non-pathogenic *Fo* isolated from asymptomatic banana plants reduced disease incidence of Fusarium wilt in banana (Gerlach et al. 1999). Greenhouse tests of non-pathogenic *Fo* isolates reduced Fusarium wilt up to 87.4% and 75%, showing potential for biocontrol activity (Nel et al. 2006; Belgrove et al. 2011). These isolates have been genotyped by sequencing of the Intergenic Spacer Region of the rDNA (IGS) (Nel et al. 2006a), but their *SIX* genes, putative pathogenicity factors, have not been identified.

In vitro biocontrol assays between non-pathogenic and plant pathogenic *Fo* have been reported to show no inhibition, even when the non-pathogenic isolate reduces disease incidence in the greenhouse (Nel et al. 2006). This evidence supports the hypothesis plant-*Fo* interactions play an essential role for non-pathogenic isolates and their biocontrol activity.

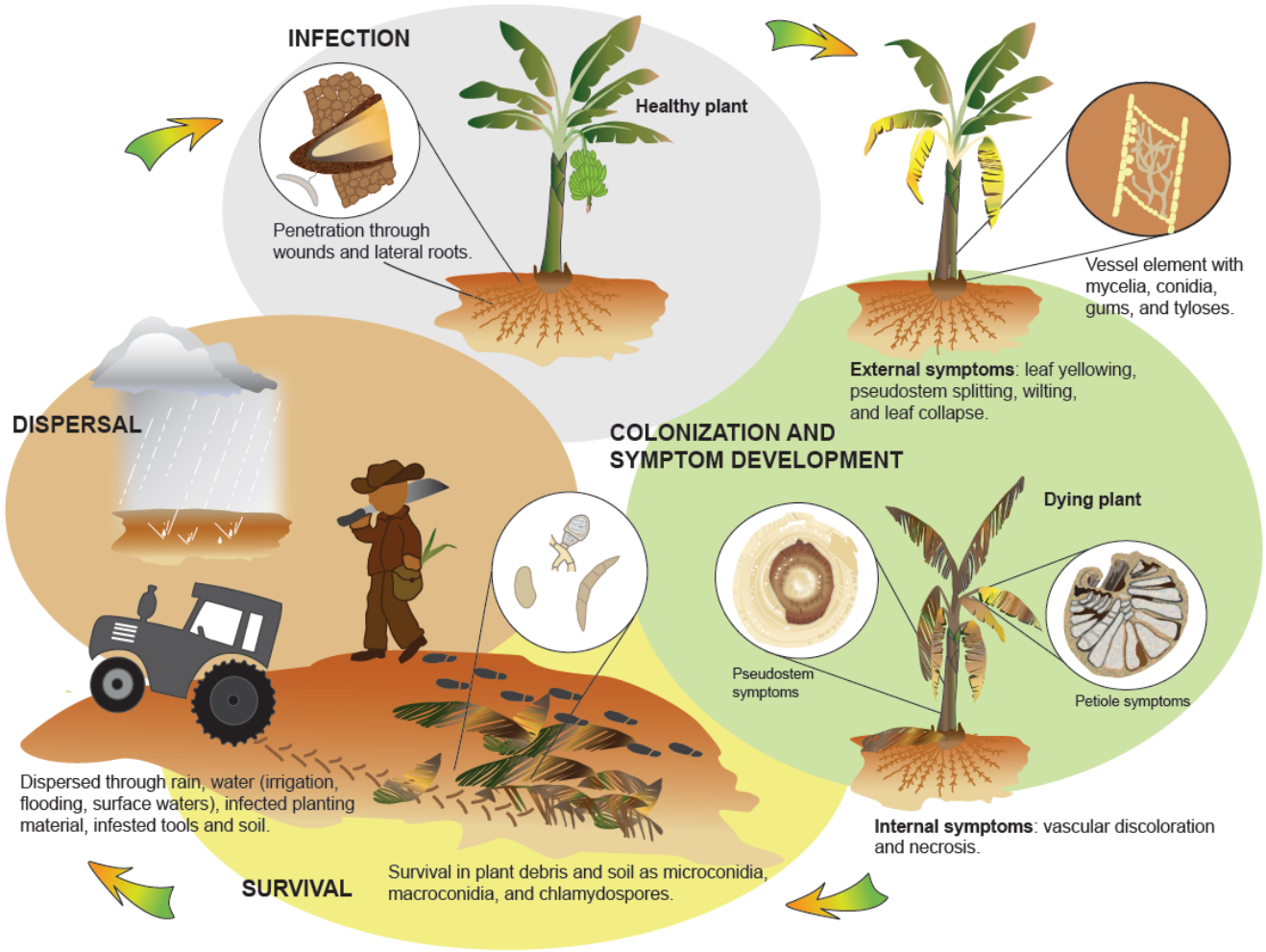
Isolates of non-pathogenic *Fo* have also been investigated as protective agents against nematodes which damage banana and tomato roots (El-Fatttah et al. 2007). Dual inoculation of *Fo* isolates V5w2 and Emb2.4o resulted in banana plant protection against the nematode *Radopholus similis* and the banana weevil *Cosmopolites sordidus* (Paparu et al. 2009). *Fo* has also been found to produce secondary metabolites toxic to plant parasitic nematodes (Hallmann and Sikora 1996).

Biology of *Fusarium oxysporum*

Morphologically, plant pathogenic and non-pathogenic isolates are indistinguishable and are often nearly indistinguishable from other species in the genus *Fusarium. Fo* produces three types of asexual spores. Microconidia are elliptical, are made of one cell, and are produced in the host rhizosphere, vasculature, and in liquid media. Macroconidia are elongated, pointed at the ends, and are made of three to five cells. Macroconidia are found on the surface of debris from infected plants and in sporodochia. Chlamydospores are oval, thick walled, and are produced in mature mycelia or macroconidia. Chlamydospores are survival structures which can remain viable in the soil for over 20 years. Conidiophores are both branched and unbranched monophialides (Nelson et al. 1983; Leslie et al. 2008). Figure 1.1 illustrates the disease cycle of Fusarium wilt of banana.

Fo has no observed sexual stage, neither *in situ* nor *in vitro*. However, molecular evidence suggests it would be hasty to rule out cryptic sexual reproduction (Taylor et al. 1999). In *Foc* and other plant-pathogenic *Fo*, both MAT1 and MAT-2 isolates have been observed (Fourie et al 2009; Yun et al. 2000; Magdama 2017).

Figure 1.1 Fusarium wilt disease cycle in the banana system. Designed by Magdama and Tesdall and illustrated by R Tesdall.



Host colonization

When pathogenic to a plant host, *Fo* causes vascular wilts of the host. It has also been shown to cause root rots, blub rots, and damping-off (Kang et al. 2014). Pathogenic isolates are found to colonize the host after infecting through the root apex or ruptures caused by the emergence of new lateral roots (Backman 1987; Booth 1971). Non-pathogenic isolates have also been found to colonize the root surface of tomato plants (Olivain et al. 2005) and have been isolated from asymptomatic tomato roots, crown, and stems (Demers et al. 2015). *Fo* enters the xylem mainly in the meristem of primary and lateral roots

(Czymmek et al. 2007). Study of the non-pathogenic isolate Fo47 revealed growth mainly in the root epidermal and outer cortical cells; comparatively, pathogenic strains were found to colonize the xylem (Olivain and Alabouvette 1999; Brader et al 2017). However, it remains unclear if there are differences in colonization patterns between other non-plant pathogenic *Fo* isolates, as the one isolate Fo47 is unlikely to represent all diversity in non-plant pathogenic *Fo*.

Taxonomy

In the past, *Fusarium* spp. were delimited using the morphological species concept based on cultural morphology (Wollenweber and Reinking 1946). However, due to differences in morphology caused by environmental variation, molecular techniques currently provide higher resolution of species limits within the genus (Geiser et al. 2004; 2013).

Fo is considered by some to be a species complex (*Fusarium oxysporum* Species Complex, FOSC) based on the application of genealogical concordance phylogenetic species recognition which proposed to separate *Fo* into two phylogenetic species (O'Donnel et al. 1998; Laurence et al. 2014). However, due to the limited diversity of isolates analyzed in these studies, isolates that had limited diversity in terms of geographic origin and were mainly plant-pathogenic, conclusions on the species complex remain the subject of further study. Increasing the number of phylogenetically-informative genes, especially those with many parsimony-informative characters, will create a more robust understanding of *Fo* phylogenetic relationships. Furthermore, including non-pathogenic *Fo* isolates, especially those from many geographic origins and host plants, will present a more complete picture of *Fo* as a species than what has been examined thus far in phylogenetic studies of pathogenic isolates originating mostly from temperate climates.

Molecular markers to study phylogenetic relationships and species boundaries within *Fusarium* have been based on conserved, housekeeping genes, such as Transcription Elongation Factor 1α (*TEF-1α*) (Geiser et al. 2013). *TEF-1α* is informative for elucidating evolutionary histories with *Fo* for several reasons. First, *TEF-1α* has been used to elucidate species in the genus *Fusarium;* differentiating species

within the genus can be extremely challenging and therefore elucidating species boundaries with genetic differences is extremely useful (Geiser et al. 2004). However, it is not possible to distinguish all Vegetative Compatibility Groups (VCGs) and formae speciales using *TEF-1α* sequences.

Host specificity

The designator forma specialis is an informal taxonomic grouping indicating host specificity within species of fungal pathogens. The race of an isolate is determined by inoculating a set of cultivars within a host species that show differential susceptibility. Many formae speciales of *Fo* have recognized races. However, despite having a broad host range, *Fo* plant pathogenic isolates tend to be highly host specific, causing disease on only a single plant species (Ma 2014). However, plant pathogenic *Fo* isolates can also colonize other hosts without causing disease (Hennessy et al. 2005). Therefore, the identifier "non-pathogenic" in this thesis is limited to the host species upon which the isolate has been tested. Given our current lack of knowledge on *Fo* host specificity, it is impractical to categorize an isolate as non-pathogenic to all potential hosts.

Fo f. sp. *lycopersici* (*Fol)* has three characterized races (1, 2, and 3) (Takken and Rep, 2010). Despite monophyly (Demers et al. 2014a; Jiménez-Gasco et al. 2002), *Fo* f. sp. *ciceris* is highly diverse. There are eight described pathogenic races: 0, 1A, 1B/C, 2, 3, 4, 5, and 6 (Haware and Nene, 1980; Jiménez-Díaz et al. 1993). The genetic basis for host resistance in chickpea has not been determined, but it is likely there is a gene-for-gene interaction between the pathogen and the host, as observed in *Fol*. Most resistance in chickpea cultivars is conferred by one or a few genes, offering further indication for this being a gene-for-gene system (Sharma and Muehbauer 2007).

Foc has four recognized races: 1, 2, subtropical race 4 (STR4), and TR4. Race 3 was previously thought to be pathogenic on *Musa* spp. but is now known to be pathogenic the closely related *Heliconia* spp. which includes ornamental plants. To understand race structure it is important to note domesticated bananas are often triploids originating from the diploid species *M. accuminata* and *M. balbasiana*.

Triploid banana hybrids indicate the origin of each set of chromosomes with an "A" for *M. accuminata* and "B" for *M. balbasiana.* Race 1 is pathogenic on 'Gros Michel' (AAA), 'Maqueno', 'Silk', 'Pome' (AAB), and 'Pisang Awak' (ABB). Race 2 is pathogenic on cooking bananas (ABB). In the presence of disease pre-disposing conditions, such as low temperatures, STR4 is pathogenic on the cultivar group Cavendish in addition to cultivars susceptible to Races 1 and 2 (Stover and Buddenhagen, 1986; Ploetz and Pegg, 2000).

Genetic diversity in races of *Fo* and other fungi was previously described using Vegetative Compatibility Groups (VCGs), although these techniques have now been surpassed by gene sequencing. VCG analysis relies on heterokaryon formation, which is genetically controlled by vegetative (*vic*) or heterokaryon (*het*) incompatibility loci. For a pair of individual fungi to be vegetative compatible, they must share the same allele at each *vic* locus. Thus, in an asexual fungus, it is expected for members of the same VCG to have extreme genetic similarity (Leslie 1993, Fourie 2011). However, characterization of diversity by VCG analysis also presents challenges, as it does not always illustrate the diversity of the organism. This is true in *Foc*, where 24 VGCs have been characterized (Fourie et al. 2011; Ordonez et al. 2015). In the past, VCGs have been associated different races, as VCG 01213 is mostly associated with TR4; however, multiple races can also be found within a VCG (Dita et al. 2010, Ghag et al. 2015). Overall, the observation of symptoms in a host determines the race, regardless of VCG.

However, in *Fo*, the forma specialis designation can be misleading when considering evolutionary relationships between isolates. Fourie and colleagues in 2009 found members of *Foc* separated into 2 groups and 8 lineages. Due to the polyphyletic nature of the forma specialis, researchers concluded that the ability to cause disease on banana evolved separately multiple times (O'Donnel et al. 1998). Conversely, evidence of monophyly has also been observed in the chickpea system, *Fo* f. sp. *ciceris* (Jimenez-Gasco et al. 2002; Demers et al. 2015).

Ecology: More Than a Pathogen

Investigating asymptomatic infections and non-pathogenic microbes is a promising new frontier for plant pathology, as it has been in the recent past in human clinical research (Bäckhed et al. 2012). Most plant pathology research to date focuses on plant pathogenic microorganisms. However, pathogenic microorganisms only represent a small fraction of microbial diversity, and even within a species, non-pathogenic isolates are thought to be more genetically diverse than plant pathogenic isolates (Demers et al. 2015). Thus, these studies included non-pathogenic isolates in comparison to plant pathogenic isolates in order to understand the mechanism of plant pathogenicity in *Fo.*

Fusarium oxysporum *as an endophyte*

In this thesis, an endophyte is an organism establishing an asymptomatic infection inside the plant (Kuldau and Yates 2000). This does not preclude the possibility that under different environmental conditions or on a different host, the organism could be a pathogen. Therefore, referring to endophytes as non-pathogens refers only to the host from which it was isolated and at the time it was isolated. Caution should be exercised when referring to an isolate as non-pathogenic, as it is well documented that plant pathogenic *Fo* can establish as asymptomatic infections in other hosts and live as a soil saprophyte (Gordon and Okamoto, 1990; Gordon and Martyn, 1997; Helbig and Carroll, 1984; Katan, 1971; MacDonald and Leach, 1976; Smith and Snyder, 1975; Suárez-Estrella et al. 2004).

Fo isolates are primarily non-pathogenic and have been reported to be one of the most commonly isolated root and soil associated fungus from asymptomatic crop plants (Laurence et al. 2011; Gordon et al. 1989). *Fo* has been found as an endophyte in many plant species, including tomato, chickpea, and banana. These endophytic isolates are excellent candidates to study differences between plant pathogenic and non-pathogenic isolates of *Fo* to understand the mechanism of plant pathogenicity.

Genetics and the Peculiar Case of *SIX*9

In order to understand the interaction between a plant pathogen and its host, plant pathologists often examine effectors, proteins secreted by the pathogen which affect the host plant. In general, it has

been observed that effector proteins in *Fo* are usually 300 amino acids in length, cysteine-rich, have non-orthologous proteins outside the genus or conserved functional domains, and have a signal peptide for secretion (Rep 2005). Researchers searched for protein products secreted in the xylem of the host plant, where plant pathogenic *Fo* colonizes the plant. The tomato-*Fol* pathosystem is a model system for understanding the effector genes and their protein products in *Fo.* In their first study, Houterman and colleagues (2007) isolated fungal proteins from the xylem sap of tomato plants infected by *Fol*. In this first isolation, the researchers named the corresponding genes Secreted In Xylem (*SIX*). To date, 14 *SIX* genes with functional protein products have been discovered in *Fol* (Rep et al. 2004; Lievens et al. 2009; Schmidt et al. 2013). Since their discovery in *Fol*, *SIX* genes have been found in many formae speciales. Table 1 summarizes the current status of *SIX* gene discovery in various *Fo* formae speciales, *Fo* endophytes, and selected related species.

Some *SIX* genes have been shown to confer race specificity in *Fol* (Houterman et al. 2008; Lievens et al. 2009). *SIX*1 is the avirulence protein recognized by tomato resistance gene I-3. It is involved in virulence when I-3 is not present in the tomato host (Rep et al. 2004, 2005). *SIX*3 is the avirulence gene recognized by tomato resistance gene I-2 (Houterman et al. 2009); *SIX*4 is the avirulence gene to genes I and I-1 in the tomato host as suppresses resistance derived from tomato genes I-2 and I-3 (Houterman et al. 2008, 2009). *SIX*6 in *Fol* has been found to have sequence homology in *Colletotrichum orbiculare* (57% protein sequence identity) and *C. higginsianum* (38% protein sequence identity) and *Fo* f. spp. *melonis, niveum, radicis-cucumerinum* (90% protein sequence identity)*, passiflorae* (93% protein sequence identity)*, vasinfectum* (89% protein sequence identity)*,* and *cubense* (60% protein sequence identity) (Gawehns et al. 2013). However, since most *SIX* genes have little to no sequence homology with other described genes, the role of *SIX* genes in host interactions remains to be discovered (Frasier-Smith 2014).

Most *SIX* genes have also been found to be located on chromosome 14 in *Fol* in addition to *in planta* secreted oxidoreductase (*ORX1*) (Houterman et al. 2007). Furthermore, chromosome 14 has been

observed to be mobile and conditionally dispensable, able to be transferred from *Fol* to non-pathogenic Fo47 and confer pathogenicity on tomato under experimental conditions. Chromosome 14 contains no housekeeping genes (Ma et al. 2010). *Fol* has been shown to lose chromosome 14 at an *in vitro* rate of 1 in 35,000 spores, and the loss of chromosome 14 results in a complete loss in pathogenicity on tomato (Vlaardingerbroek et al. 2016). Furthermore, the presence of *mimps*, miniature inverted-repeat transposable elements, are associated with the promotors of *SIX* genes. *Mimps* may be key when considering their potential functions and considering evolutionary relationships with non-pathogenic *Fo* (Schmidt et al., 2013). *Mimps* are class II transposable elements. Using *mimps in silico,* van Dam and Rep (2017) found additional *SIX* genes in the genomes of *F. fujikuroi* (*SIX2*), *F. proliferatum* (*SIX1* as a pseudogene and *SIX2*), *F. hostae* (*SIX2, SIX6, SIX7, SIX11*), and *F. agapanthi* (*SIX2* and *SIX11*).

Table 1.1: *SIX* gene detections in *Fusarium oxysporum* isolates from various hosts.

Host	f. sp.	*SIX1*	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*	*SIX8*	*SIX9*	*SIX10*	*SIX11*	*SIX12*	*SIX13*	*SIX14*	Source
Tomato	*lycopercisi*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	Gawehns et al. 2014
Arabidopsis					x					x						Thacher et al. 2012
Brassica										x						Taylor et al. 2016
Cotton	*vasinfectrum*						x									Chakrabarti et al. 2011
Cabbage	*betae*	x					x									Covey et al. 2014
Date Palm	*canariensis*	x						x			x					Laurence et al. 2015
Flax	*lini*	x						x			x		x	x		Laurence et al. 2015; Taylor et al. 2016
Banana	*cubense*	x	x		x*		x	x	x	x	x			x#		Meldrum et al. 2012, University of Queensland unpublished data; Taylor et al. 2016#
Onion	*cepae*			x		x		x		x			x		x	Sasaki et al. 2015; Taylor et al. 2016
Canola, cabbage	*conglutinans*	x			x											Kashiwa et al. 2013; Li et al. 2016
Watermelon	*niveum*						x									Niu et al. 2016
Pea	*pisi*							x			x	x	x	x	x	Taylor et al. 2016
Pinto bean	*phaseoli*						x		x			x				Taylor et al. 2016
Canary Island Date Palm	*canariensis*	x						x			x					Laurence et al. 2015
Carnation	*dianthi*							x		x	x		x			Taylor et al. 2016
Daffodil	*narcissi*							x		x	x		x			Taylor et al. 2016
Freesia	*freesia*							x			x		x	x	x	Taylor et al. 2016
Lilium	*lili*							x								Lievens et al. 2009
Melon	*melonis*						x									Lievens et al. 2009
Cotton	*vasinfectum*						x									Chakrabarti et al. 2010
Chickpea	*ciceris*						x		x			x		x	x	Williams et al. 2016
SIX Genes found in non-pathogenic isolates																
Onion										x					x	Taylor et al. 2016
Soil isolates		x					x		x	x		x			x	Rocha et al. 2016
Banana		x	x		x			x	x	x						Magdama 2017
SIX Gene homologs in other species																
F. proliferatum		x (PSUEDO)	x													van Dam and Rep 2017
F. hostae		x					x	x				x				van Dam and Rep 2017
F. agapanthi			x									x				van Dam and Rep 2017
Colletotrichum obiculare		x					x									van Dam and Rep 2017
C. higginsianum		x					x									van Dam and Rep 2017
F. verticilliodes			x													van Dam and Rep 2017

Horizontal gene transfer is currently accepted as an explanation for differences in inferred evolutionary relationships between *TEF-1α* and *SIX* genes. For example, horizontal gene transfer of small accessory chromosomes has been offered as an explanation for monophyly in *Fo* f. sp. *canariensis* for *SIX*1, *SIX*7, and *SIX*10 when *TEF-1α* does not support monophyly (Laurence et al. 2015). Similar differences in evolutionary relationships inferred based on *SIX* genes and *TEF-1α* are observed in *Foc*, *Fo* isolates from asymptomatic *Musa spp.* (Magdama 2017), and *Fo* soil isolates (Rocha et al. 2015). However, the presence of a highly similar *SIX*2 in many other *Fusarium* spp. (van Dam and Rep 2017) suggests, that *SIX*2 may have undergone vertical gene transfer.

Up until recent studies, small sets of non-pathogenic isolates had been screened for *SIX* genes; it was generally concluded that *SIX* genes were not present in non-pathogenic *Fo.* However, those that have searched extensively for *SIX* genes in endophytic and soil isolates found the presence of *SIX* genes. Researchers from Australia (Rocha et al. 2015) isolated 115 *Fo* from minimally disturbed non-agricultural soils in five out of six provinces in Australia. They found seven isolates (6%) with the presence of *SIX*9, two isolates each (2%) with the presence of *SIX*1 and *SIX*6, and one isolate each (0.87%) with the presence of *SIX*8, 11, and 14. Similarly, from a set of 103 *Fo* isolated from asymptomatic banana plants in Ecuador, two (1.9%) show evidence of *SIX*1, 11 (10.7%) for *SIX*2, (2.9%) for *SIX*4, *SIX*7, and *SIX*8 (a and b homologs), and 14 (13.6%) for *SIX*9. Magdama (2017) suggests that the presence of *SIX* genes in asymptomatic and soil isolates indicates that *SIX* genes have a role in *Fo*-host interactions other than pathogenicity, and plant pathogenic isolates result from a breakdown in the usual ecological niche of *Fo* as a mutualist with plant hosts.

*SIX*9 was first discovered as a putative effector protein by Ma and colleagues (2010) and described as a *SIX* gene by Schmidt and colleagues (2013). The protein was found to be an effector protein based on its presence *in silico*, the presence of a *mimp* in the promotor, and presence of its protein product in the xylem sap of infected tomatoes. mFot5, a Miniature Inverted-repeat Transposable Element (MITE) less than 500bp long, lies downstream of *SIX*9. mFot5s also lies downstream of *SIX*1/*SIX*2,

*SIX*3/5, and *SIX*10/12/7 clusters. mFot5 is part of the putative secondary metabolite cluster expressed during *Fol* infection in tomato (Schmidt et al. 2013).

Since *SIX*9 appears in both soil and endophytic banana isolates at the highest frequencies, *SIX*9 became an intriguing candidate for study in this proposal. Magdama (2017) also discovered the sequence of *SIX*9 in the putatively endophytic banana isolates studied was identical to the sequence of reference strains O-2052 (VCG01213), O-1966 (VCG0120) and O-1968 (VCG0123), characterized as TR4, STR4 and Race 1, respectively. When evolutionary relationships between these isolates are inferred using *SIX*9, three putative endophytes were included in a single cade with *Foc* isolates (Figure 3). Twelve putative endophytes form a distinct group, which is distinguished by 16 single nucleotide polymorphisms (SNPs) from *Foc* isolates. Sixteen SNPs in a 236bp region may indicate that *SIX*9 may not be functional in one or both of these groups, as genes that are no longer functional accumulate mutations at a higher rate than conserved genes. This possibility is kept in mind for a possible explanation for patterns in the data generated in this thesis.

Surprisingly, the *SIX*9 putative endophyte group is more closely related to ff. spp. *cepae, narcissi,* and *dianthi* than to *Foc* when inferring evolutionary relationships based on *SIX*9 (Magdama 2017). This finding drives us to aim to understand the relationships between these putatively endophytic isolates from banana and endophytic isolates from chickpea and tomato. *Fo* isolates from asymptomatic tomato and from soil in the same tomato fields did not show a pattern of genetic differentiation between soil and endophytic isolates as hypothesized. However, the analysis of genetic relationships was based on *TEF-1α*, which revealed 26 sequence types. These isolates had not been screened for *SIX* genes until the current study (Demers et al. 2015).

*SIX*9 knockout mutants of *Fo* f. sp. *radicis*-cucumerinum (*Forc)* have been studied by van Dam and colleagues (2017) in an effort to understand differences in pathogenicity between *Forc* and *Fo* f. sp. *cucumerinum* and *Fo* f. sp. *melonis (Fom)*, as *SIX*9 and *SIX*6 were not detected in *Fom* reference strains.

However, *SIX*9 gene knockout mutants did not exhibit reduced virulence on cucumber plants. Van Dam and colleagues (2017) concluded that *SIX*9 alone is not involved in pathogenicity for the cucumber pathosystem.

Phylogeographic patterns may be observed in phylogenetic groups based on *SIX* genes. For example, patterns emerge if we consider the host crop center of origin for the f. spp. closely related to the *SIX*9 putative endophyte group. By *SIX9*, the ff.spp. closely related to the *SIX*9 putative endophyte group are ff. spp. *cepae* (causing disease in onion)*, narcissi* (daffodil)*, dianthi* (carnation)*, pisi* (pea) and *niveum* (watermelon). Banana has its center of origin in south and south east Asia; onions in south Asia; daffodil in the Iberian peninsula; carnation in the Mediterranean region; peas in south Asia; watermelons and melons in southern Africa and south Asia, respectively (Khoury et al. 2016). Overlapping centers of origin of these host plants may be further evidence for the role of *SIX*9 in co-evolution and adaptation with the agricultural selection of the host plants.

The putative regulator *SIX Gene Expression 1* (*SGE1*) has been found to be essential for pathogenicity in *Fol* (Michielse et al. 2009). *SGE1* is found in the conserved genomic regions (Ma 2014). *SGE1* is a homolog of *Wor1* and *Ryp1*, the master regulators of morphological switching associated with the onset of pathogenicity observed in *Candida albicans* and *Histoplasma capsulatum,* respectively. *SGE1* is not essential for colonization or penetration of the root surface, but is needed for colonization of the xylem in tomato plants. *SGE1* knockout *Fol* mutants have decreased conidiation but exhibit similar to wild-type vegetative growth. *SGE1* expression is detected during saprophytic growth but is up-regulated 2 to 5 times during growth *in planta*. Expression of *SIX*1, *SIX*2, *SIX*3 and *SIX*5 has been found to be dependent on *SGE1* in *Fol*. Furthermore, *SGE1 Fol* knockout mutants were found to have biocontrol activity against *Fo* pathogenic on flax, reducing disease symptoms by 27 and 33% when the knockout inoculum load was 100 times the pathogenic inoculum load. A homolog of *SGE1* has also been found to regulate effector expression in *Verticilium dahliae* and *F. verticillioides* (Santhanam1 and Thomma 2013; Brown et al. 2014). However, *SGE1* is not exclusive to pathogenic *Fo.* In *Fo* f. sp. *betae* and in 11 non-

pathogenic isolates from sugar beet, all 11 were found to contain *SGE1* (Covey et al. 2014). Therefore, *SGE1* would be a logical candidate to include in *SIX* gene PCR screening to increase understanding of *SIX* gene expression patterns.

Avirulence genes are often candidates for molecular diagnostics, as they often determine host specificity (van der Does and Rep, 2007). Thus, *SIX* genes have become a candidate for molecular diagnostics. *Fol* race 1 contains *SIX*4, and thus can be distinguished from *Fol* race 2 and 3, which do not contain *SIX*4 (Lievens et al. 2009). In *Fo* f. sp. *vasinfectum (Fov)*, a PCR-based test using *SIX*6 was shown to distinguish *Fov* from 10 non-pathogenic isolates obtained from soil (Chakrabarti et al. 2011). A molecular diagnostic test to differentiate *Foc* TR4 from STR4 and races 1 and 2 has also been developed based on *SIX*8 (Fraser-Smith et al. 2014). However, a diagnostic test based on *SIX* genes has not be adopted by regulatory agencies like the Food and Agriculture Organization of the United Nations (FAO) and International Regional Organization for Agricultural Health (OIRSA) in quarantine plans for *Foc* in Latin America and the Caribbean (Dita et. al. 2010, 2011, 2013; Perez et al. 2014). Caution must be exercised in using molecular diagnostics from asymptomatic material, as diagnostic tests have been developed without screening against the vast majority of *Fo* diversity contained in non-pathogenic isolates. When considering non-pathogenic *Fo*, false positives are possible (Magdama 2017) and have already cost Australian banana growers in the fight against TR4 (The Daily Courier 2016). Similar issues with differentiating between pathogenic and non-pathogenic isolates of *Fo* from beets have also been noted in the literature (Covey et al. 2014).

Research Statement, hypotheses, and objectives

By 2050, global agricultural production will need to increase by 50% in order to feed our population, and plant pathology will be essential to the future of food security (OECD/FAO 2016). Annually, 10-16% of the global harvest, or $220 billion USD, is lost to plant diseases. Post-harvest losses claim another 6-12% of global food production. Even more concerning is that even though total crop production has exploded over the last 40 years, proportionally, crop losses to pests and pathogens have increased (Oerke 2006; Chakraborty and Newton 2011). Even with knowledge of current disease losses, most crop loss estimates due to climate change do not account for pest and disease losses, but focus on changes in temperature, precipitation, and carbon dioxide (Gregory et al. 2009, Chakraborty and Newton 2011). The discipline of plant pathology must innovate to change the trajectory of crop disease losses.

In order to spur innovation in plant pathology, we propose pathogens must be understood beyond their role as disease causing agents. The work in this thesis hinges on the assumption that understanding microbial symbionts and their interactions with their plant hosts will lead to better understanding of plant pathogenicity and its emergence.

However, classical plant pathology most often studies only plant pathogenic isolates of the microbial community. This is especially the case of the study organism, *Fusarium oxysporum (Fo)*. Several studies observed the majority of *Fo* diversity is hidden in little studied soil saprotrophs and non-pathogenic plant associates (Alves-Santos et al. 1999; Appel and Gordon 1994; Balmas et al. 2010; Edel et al. 2001; Elias et al. 1991; Gordon and Okamato 1992; Katan et al. 1994; Laurence et al. 2012; Lori et al. 2004). Understanding the diversity hidden in soil and non-pathogenic plant associates is crucial. Comparison between non-pathogenic and plant pathogenic isolates may reveal patterns that can predict the pathogenicity of an isolate, and elucidate the mechanisms by which pathogenicity to a host is acquired. The objectives of this thesis focus on this premise. In the future, these patterns could be used to improve the efficacy of disease detection, biocontrol selection, and agroecosystem design.

To highlight the importance of intra-species diversity, the banana (*Musa* spp.) system resurfaces as a compelling example. Early detection tests have been developed to detect *Fo* f. sp. *cubense (Foc)* Tropical Race 4 (TR4) *in planta*. These PCR-based detection methods are based on core genomic regions or genes on accessory chromosomes, like *SIX*8, proposed to be associated with plant pathogenicity, called Secreted In Xylem (*SIX*) genes (Dita et al. 2010; Schmidt et al. 2013). However, as Magdama (2017) discovered, the development of molecular detection methods excluded non-pathogenic *Fo* isolates, and the vast unconsidered diversity can lead to false positives (Magdama 2017). False positives already cost Australian banana growers millions (The Courier Mail, 2016). Thus, the objectives of this thesis seek to understand the mechanism of pathogenicity. What makes one *Fo* isolate pathogenic and another an endophyte?

Chapter 1: The role of *SIX*9 in *Fusarium oxysporum* f. sp. *cubense*- banana interactions

Based on the presence of *SIX9* in *Fo* endophytes from banana, soil isolates, and onion in addition to pathogenic *Fo* isolates, *SIX9* was a candidate gene for understanding functional gene-for-gene *Fo*-host interactions in the context of *Fo* diversity. Therefore, Chapter 1 tested the following hypothesis:

SIX9 *is essential to pathogenicity in Tropical race 4 and race 1 isolates of* Fusarium oxysporum *f.sp.* cubense *through its role in* Fo-*host interactions.*

The hypothesis was tested with the following objective:

To characterize the role of SIX9 in Foc-banana interactions by studying the phenotype of Foc SIX9 knockout mutants in vitro *and* in planta*.*

Chapter 2: Evolutionary relationships of non-pathogenic *Fusarium oxysporum* from banana, tomato, and chickpea

Understanding the phylogenetic relationships of *Fo* endophytes isolated from different host species will help identify if there is a phylogenetic basis for *Fo* lifestyle (endophyte, saprophyte), geographic distribution, or the presence of *SIX* genes. Therefore, chapter 2 tested the follow hypothesis:

Based on TEF-1α *and other phylogenetically informative genes, non-pathogenic isolates from different host species will not share phylogenetic relationships but display patterns based on geographic origin, area of isolation within the host plant, and detection of* SIX *genes. These phylogenetic relationships will differ when inferring relationships based on the accessory genome (SIX genes).*

The hypothesis was tested with the following objective:

To infer phylogenetic relationships between non-pathogenic and pathogenic Fo isolates from banana, chickpea, and tomato systems, and relate their evolutionary histories to formae speciales of Fo based on core and accessory genomes.

Chapter 2

The role of *SIX*9 in pathogenic *Fusarium oxysporum* f. sp. *cubense*-banana interactions

Introduction

The objective of chapter 2 is to characterize the role of *SIX*9 in *Foc*-banana interactions by testing *Foc SIX9* knockout mutants *in vitro* and effects on host phenotype *in planta.* Chapter 2 tests the hypothesis that *Foc SIX*9 knockout mutants will exhibit a reduction or absence of disease symptoms.

To test the hypothesis and provide evidence to understand the role of *SIX*9, mutants with a *SIX*9 knockout were compared with two isolates, one TR4 isolate, one Race 1 isolate in order to screen the knockout mutants for pathogenicity. The work in this master thesis generated knockout mutants for TR4 and Race 1 and characterized those using PCR testing, *in vitro* growth assays, and *in planta* phenotyping. However, any conclusions made from these studies do not rule out the possibility that the expression of *SIX*9, *SIX*9 knockout mutants, *in planta* colonization, and pathogenicity are subject to environmental factors such as temperature, humidity, and age of the host plant.

Materials and Methods

Fusarium oxysporum isolate maintenance and storage

Fo isolates collected from the root and corm of banana plants in Ecuador by Magdama (2017) and from the Fusarium Research Center (The Pennsylvania State University), were used in this study. Race 1 isolate EE44-M-GM2 was from Ecuador, Province Manabí, and Location Motalvo, and was obtained from banana 'Gros Michel.' Race 1 isolate EC28-M-GM1 was from Province Manabí, location Chone, and 'Gros Michel.' TR4 isolate TR4 0-2052 was obtained from the Fusarium Research Center. All isolates from the collection and mutants generated in this study are stored at -80°C as 25% glycerol 75% spore suspensions (Olivain and Alabouvette 1999). Working cultures were maintained on Carnation Leaf

Agar (CLA) prepared according the protocol available from the Fusarium Research Center at The Pennsylvania State University. These working cultures were maintained at 4°C to minimize contamination and desiccation.

Mutant generation

The *SIX*9 knockout mutants were created by replacing the *SIX*9 gene with the hygromycin phosphotransferase gene *(hph)* that confers resistance to the antibiotic hygromycin B via homologous recombination mediated by polyethylene glycol (PEG). All protocols were carried out according to Goswami (2012) and personal communication with Dr. Laura Ramos Sepulveda. Common challenges with PEG-mediated transformations of filamentous fungi include transient transformants and frequent multiple loci integrations (Liu and Friesen 2012). *Foc* Race 1 mutants were produced from isolates EC44-M-GM2 and EC28-M-GM1, isolated and characterized as *Foc* Race 1 by Magdama (2017). TR4 mutants were generated from the isolate number 0-2052 from the Fusarium Research Center (The Pennsylvania State University) using the primers described in Table 2.1.

To isolate the *hph* gene, *E. coli* bacteria containing a plasmid with the hygromycin gene were grown in Nutrient Agar (NA) amended with 1uL ampicillin for every 1mL media. The bacteria were grown for two days at 27°C. Then, the bacterial colonies were transferred to Luria Broth media amended with 1uL ampicillin for every 1mL media. The bacteria were grown between 12-14 hours at 37°C on a rotary shaker (250rpm). Then, the bacterial plasmid was extracted using a Sigma© GenElute™ HP Plasmid Miniprep Kit. The bacterial plasmid was a pGEM®-T easy Vector System from Promega. Storage copies of the *E. coli* were made using 150uL sterile, warm glycerol and 850uL of the LB overnight culture. The tubes were vortexed for thorough mixing and stored at -80°C.

After the plasmid extraction, to confirm presence of the *hph* gene in the plasmid, the *hph* gene was amplified using primers “Lhygromycin-F” and “Hygromycin-R” (Table 2.1) which are based on the left and right flanking regions of the *hph* gene (Figure 2.1D). A 1,678bp fragment was expected and observed in the PCR amplification (data not shown).

The entire hygromycin-B phosphotransferase gene and *SIX9* flanking regions were too large to be incorporated into the protoplast during transformation. Therefore, two overlapping cassettes of suitable size for incorporation into the protoplast were created (Figure 2.1B and C). The right-side construct was made using primers YG-F and RFRsix9 IN-R; the left-side construct was made using primers GY-F andRFRsix9 IN-R (Table 2.2B).

Figure 2.1 Illustration of homologous combination of the PEG-mediated transformation. This figure illustrates A) the original SIX9 gene with Left Flanking Region (LFR) and Right Flanking Region (RFR), B and C) transformation cassettes containing parts of the *hygromycin phosphotransferase* (*hph*) gene and the of the SIX9 gene, and D) the final expected *hph* gene insertion.

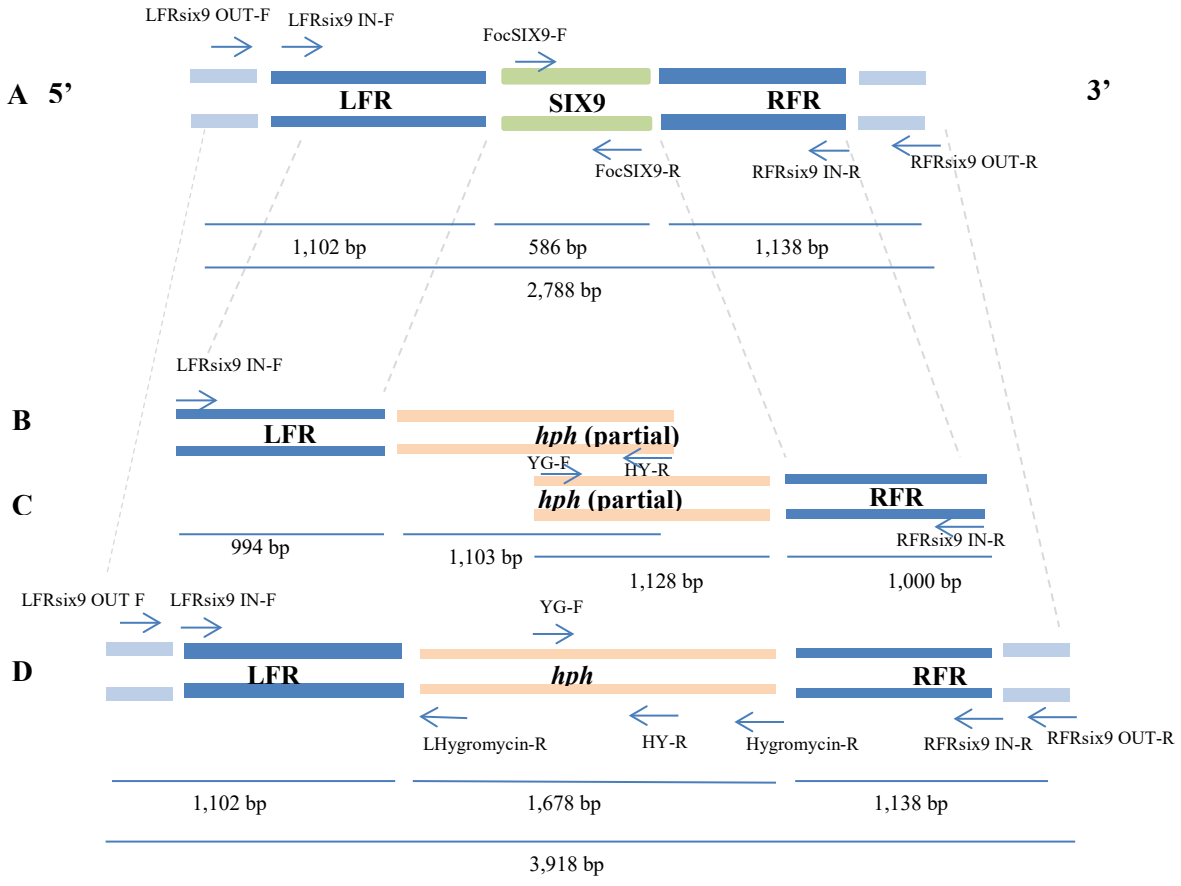


Table 2.1 Primers used for mutant generation and characterization. All primers were developed for this study using the software Geneious v7.1.4 with the exception of FocSIX9-F and FocSIX9-R (Magdama 2017).

Code	Primer sequence (5'-3')	Target	Amplicon Length (bp)
LFRsix9 OUT-F	CAGGCCCCTGGTAGCTTG	SIX9 region and	2,788
RFRsix9 OUT-R	GAGCTGCACGGGCTTCTC	Hygromycin resistance gene insertion region	3,672
Lhygromycin-R	CCACTAGCTCCAGCCAAGC	Hygromycin resistance gene	1,678
Hygromycin-F	CGACAGAAGATGATATTGAAGG		
LFRsix9 OUT-F	CCGTCAACCAAGCTCTGATA	*SIX9* Left Flanking Region	1,013
hygREV Set 1			
YG-F	CGTTATGTTTATCGGCACTTTGC	Internal region of hygromycin resistance gene	553
HY-R	GCTGCTCCATACAAGCCAACC		
YG-F	TGTTGTGCGCTTGTTAAGGA	Right flanking region construct	2,128
RFRsix9 IN-R			
LFRsix9 IN-F	TGGGGCCCTCCTTGAATTTT	Left flanking region construct	2,097
HY-R			
FocSIX9-F	GCAGTTGCGGCAATGGCT	SIX9	328
FocSIX9R	GCCCCATCTGGTATCCGACA		

To produce protoplasts, strains of *Foc* were inoculated in carboxyl methyl cellulose (CMC) media and grown for three days at 25°C on a shaker rotating at 150 rpm. Spores were harvested from CMC media and $1x10^6$ spores inoculated in 50mL Yeast Peptone Glucose media and grown at 25°C for 12-14 hours. Spores were then harvested and washed with 1M NH_4Cl. The spore cell walls were digested with the cell-wall degrading enzyme driselase for three hours, or until round protoplasts were observed microscopically. The protoplasts were harvested by centrifuge, washed with and suspended in Sorbitol Tris-Cl (STC), which served as an osmotic buffer to stabilize the protoplasts (Liu and Friesen 2012). The protoplasts were then transferred into chilled glass-tubes, where 30% PEG was co-incubated with 3-5ug DNA of the construct. After incubation at room temperature for 30 minutes, STC was added. The transformed protoplasts were gently pipetted into lukewarm regeneration media, and protoplasts from each tube were placed onto separate petri dishes. After 24 hours of incubation at 25°C, colonies containing the hygromycin phosphotransferase gene were selected by overlaying the regeneration media with complete media containing 300ppm hygromycin B. Colonies growing through the hygromycin B-amended complete media were plated onto half-strength PDA and maintained on CLA.

Mutant Characterization: *In-Vitro* Growth Assay

Mutants were tested for their growth and sporulation characteristics relative to their respective wild type isolates. Mutants were grown on PDA inoculated using PDA cores from fungal colonies grown for two weeks. Growth of the mutants was measured over 6 days at 28°C. Four radial measurements were taken with an electronic caliper for reach plate. Each mutant was replicated five times, and the entire growth assay was repeated three times. At the end of each experiment, serial dilutions of the plate were counted using a hemacytometer (Bright-Line Hemacytometer, Hausser Scientific, Horsham PA, USA) to determine sporulation after six days. The colony morphology of the mutants was also observed after one week of growth. The results of these experiments were statistically compared to the wild-types using t-tests in Microsoft Excel Version 14.0 and analyses for one entire repetition of the experiment are shown. The four measurements of the radii were averaged before statistical analysis.

Mutant Characterization: Insertion Detection

The insertion location of the *hph* gene was first detected by PCR amplification using primers LFRsix9OUT-L and RFRsix9OUT-R (Table 2.1). PCR reactions were carried out in 25 µL using Choice Taq Master mix (Denville), 0.5 µM of each primer, 10-20 ng of DNA and sterile deionized water. Genomic DNA used in the PCR reactions was extracted using a Qiagen Plant DNeasy Kit following the manufacturer's instructions. DNA was extracted from lyophilized mycelium grown in PDB for 72 hours. The resulting PCR products were visualized by gel electrophoresis and purified using ExoSAP-IT (USB Affymetrix Corporation, Cleveland, Ohio, USA) following the manufacturer's instructions. All PCR products were sent to the Genomic Core facility at The Pennsylvania State University for sequencing. These regions were sequenced using the same primers as used for PCR amplification. The reverse strands were sequenced if ambiguous nucleotides were noted in the forward strands. Sequences were edited and aligned using the software Geneious v7.1.4.

For preliminary screening of the mutants, primers LFRsix9OUT-F and HY-R (Table 2.1) were used to amplify an expected 1,013bp fragment, indicating the *hph* gene insertion occurred in the left

flaking region of *SIX9*. Using this technique, TR4 0-2052 Δ*SIX*9 #1, 2, 5, EC44-M-GM2 Δ*SIX*9: 2.2.5, 2.2.7, 2.2.8, 2.2.9, 3.2.4 and EC28-M-GM1 Δ*SIX*9: 1.1.2, 1.2.3, 3.1.2, 3.1.4, 3.1.6. were selected for further study (data not shown); the mutants ΔTR4 0-2052 #1, ΔTR4 0-2052 #5, ΔEC44-M-GM2 2.2.5, ΔEC44-M-GM2 2.2.7, ΔEC28-M-GM1 1.1.3, and ΔEC28-M-GM1 3.1.4 were studied *in planta* based on the aforementioned criteria.

To verify the insertion location, primer pairs LFRsix9OUT-F and hygREV and LFRsix9OUT-F and RFRsix9OUT-R (Table 2.1) were used to amplify the entire insertion region (Figure 2.2). Primer binding sites for LFRsix9OUT-F and RFRsix9OUT-R were outside the transformation construct. In Figure 2.1, *Foc*SIX9 primers (Table 2.2) were used to amplify *SIX9* to determine the presence or absence of *SIX9,* the gene knockout target, in the mutant genomes.

Pathogenicity Testing

Pathogenicity testing of the six mutants was conducted on hybrid bananas (*M. accuminata* triploids) 'Gros Michel' and Cavendish 'Gran Nain.' Tissue culture banana plants were supplied by Agristarts (Apopka, Florida, US). All banana plants were planted in 0.5L pots, in a steam-pasteurized soil mixture (40% Sungro Sunshine #4 potting mix and 60% Scots® Premium Top Soil), and grown at 28°C, 80% relative humidity and 14 hours light (Garcia-Bastidas et al. 2014) in Conviron Growth Chambers in the Department of Plant Pathology and Environmental Microbiology at The Pennsylvania State University following a complete randomized design and including eight replicates per treatment. The eight replicates per treatment were divided between two growth chambers (four replicates per growth chamber). The plants were acclimated for four weeks before inoculation in the same growth conditions above. The entire pathogenicity test was repeated two times.

Cornmeal sand inoculum and inoculation methods were performed as described by Demers (2012), Magdama (2017), Nene and Haware (1980), and Trapero Casas and Jiménez-Diaz (1985). Sand, cornmeal, and water were mixed by volume in the ratio 9:1:2, respectively. Sand and cornmeal were autoclaved twice with cooling to room temperature between autoclaving cycles. Then, double distilled

sterile water was added along with twelve 1cm^2 -plugs of PDA colonized by the *Fo* isolate to be tested. The *Fo* colonies were 7 days old at the time of inoculation. The inoculum grew for a minimum of two weeks at 24°C. Then, the cornmeal sand inoculum was homogenized by mixing and shaking with pasteurized soil mixture (40% Sungro Sunshine #4 potting mix, 60% Scots® Premium Top Soil) at a rate of 1:12 by weight. Banana plants were then repotted into the soil-inoculum mixture. At this time, samples of the soil inoculum were taken to determine inoculum concentration by plating by serial dilutions on NASH media prepared according to Leslie et al. (2008). The average number of colonies from four plates was used to estimate colony forming units (CFU)/mg soil.

Plants were watered every third day and fertilized every second week with the prescribed rate of Miracle Gro® Liquid Fertilizer. Two-spotted spider mites were managed using biocontrol mites *Neoseiulus californicus* and *Phytoseiulus persimilis* purchased from Beneficial Insectary, Inc. (Redding, California, US).

Positive controls were inoculated with wild type isolates (EC44-M-GM2, characterized as Race 1 from Ecuador, and TR4 isolate 0-2052, also known as *Foc*IL5). Negative controls were planted in non-inoculated steam-pasteurized soil mixture. Phenotypic differences in the host plant were considered as evidence for differences between the mutants and wild type *Fo*. Phenotypic differences were determined by Fusarium wilt disease severity based on internal and external symptoms (Figure 2.2).

To confirm the mutant caused the Fusarium wilt disease symptoms observed and to understand the extent of colonization of the host, re-isolations from various portions of the banana plant were done at the end of the experiment, six weeks post-inoculation. Fungal isolations were made on NASH media for the roots, corm, and pseudostem (4cm, 10cm and 25cm above the crown) after surface disinfestation. Samples were processed by rinsing materials with autoclaved water and surface disinfesting with 6.15% sodium hypochlorite for 1.5 minutes and 70% ethanol for 30 seconds in an aseptic environment. To confirm the identity of *Fo* recovered, plant material was plated on NASH media and recovered putative

mutants were plated on 150ppm hygromycin-amended Potato Dextrose Agar (PDA). Recovered isolates which grew on 150ppm hygromycin-amended PDA were concluded to be the inoculated isolates.

Statistical analyses

All numerical data was analyzed at the endpoints using a two tail t-test with the software Microsoft Excel Version 14.0. For disease severity data, ordinal data, non-parametric Krustal-Wallis test and Dunn's procedure were used to compare disease severities between mutant and wild-type inoculated banana plants with Microsoft Excel Version 14.0 with the software extension package XLSTAT Version 2018.6.

Figure 2.2: Fusarium wilt disease severity rating scale in banana. This scale is adapted from Li et al. 2014. The following scale ranges from 0-3, with 3 describing the most severe disease symptoms and 0 a lack of disease symptoms.

Results

Five ΔTR4 mutants were generated. Table 2.2 shows the name of the mutant isolate and the origin of each based on the tube number, plate number, and the number of days after the transformation event the isolate was discovered on the hygromycin B-amended complete media. Mutant isolates that

originated from different tubes and plates have no chance of being from the same transformation of a single protoplast, and thus are definitively different mutant isolates.

Sixteen race 1 mutants were generated from the race 1 isolate EC44-M-GM2. Nineteen race 1 mutants were generated from the isolate EC28-M-GM1. Race one isolate names include three numbers. The first number represents the tube number, the second number the plate number, and the third number the order in which it emerged from the plate.

Table 2.2 Origin of Tropical Race 4 transformants.

Isolate Name	Tube no.	Plate	Days after transformation
ΔTR4 0-2052 #1	2	2	2
ΔTR4 #2	2	1	3
ΔTR4 #3	2	1	3
ΔTR4 #4	3	2	4
ΔTR4 0-2052 #5	3	2	4

Mutant Characterization: *In-Vitro* Growth Assay

After growing for six days on PDA at 28°C, ΔTR4 0-2052 mutants displayed colony morphology, microscopic phenotype, and conidiation similar to the wild-type (Figure 2.3, Tables 2.3 and 2.4). ΔTR4 mutant colonies exhibited fluffy, aerial mycelium with pink to purple pigmentation, similar to the wild-type TR4. However, ΔTR4 0-2052 #1 grew significantly larger than the wild-type after five days of *in vitro* growth ($p=0.001$, Table 2.3). However, sporulation between ΔTR4 0-2052 #1 and the wild-type were not significantly different ($p=0.35$, Table 2.4). The mutant ΔTR4 0-2052 #5 had significantly more spores than the wild-type after five days of *in vitro* growth ($p=0.008$). However, ΔTR4 0-2052 #5 did not grow significantly larger than the wild-type in this same period of time ($p=0.39$, Table 2.3).

ΔEC44-M-GM2 and ΔEC28-M-GM1 mutants displayed significantly reduced growth and conidiation (Figure 2.3, Tables 2.5 and 2.6). The growth was measured after four weeks in order to better observe differences between the isolates. Conidiation was measured after five days of *in vitro* growth. Race 1 mutants exhibit a very different morphology than their corresponding wild-type race 1 isolates, for

both race 1 isolate transformations. Mutants showed highly reduced colony growth and formed white, compact mycelia that became radially ridged after three or more weeks of growth on PDA. When grown in full strength PDB on at shaker at 150 rpm at 28°C, race 1 mutants grew as many spherical colonies. Under the same conditions, wild type race 1 and ΔTR4 mutants grew as dispersed mycelia and became cloudy with suspended spores. Pink and purple pigments also developed when grown in PDB.

Figure 2.3 *In-vitro* mutant growth characterization of A) TR4 0-2052, B) ΔTR4 0-2052 #1, C) EC44-M-GM2, D) ΔEC44-M-GM2 2.2.7, E) EC28-M-GM1 and F) ΔEC28-M-GM1 1.1.3 after growing on PDA for six days at 28°C.

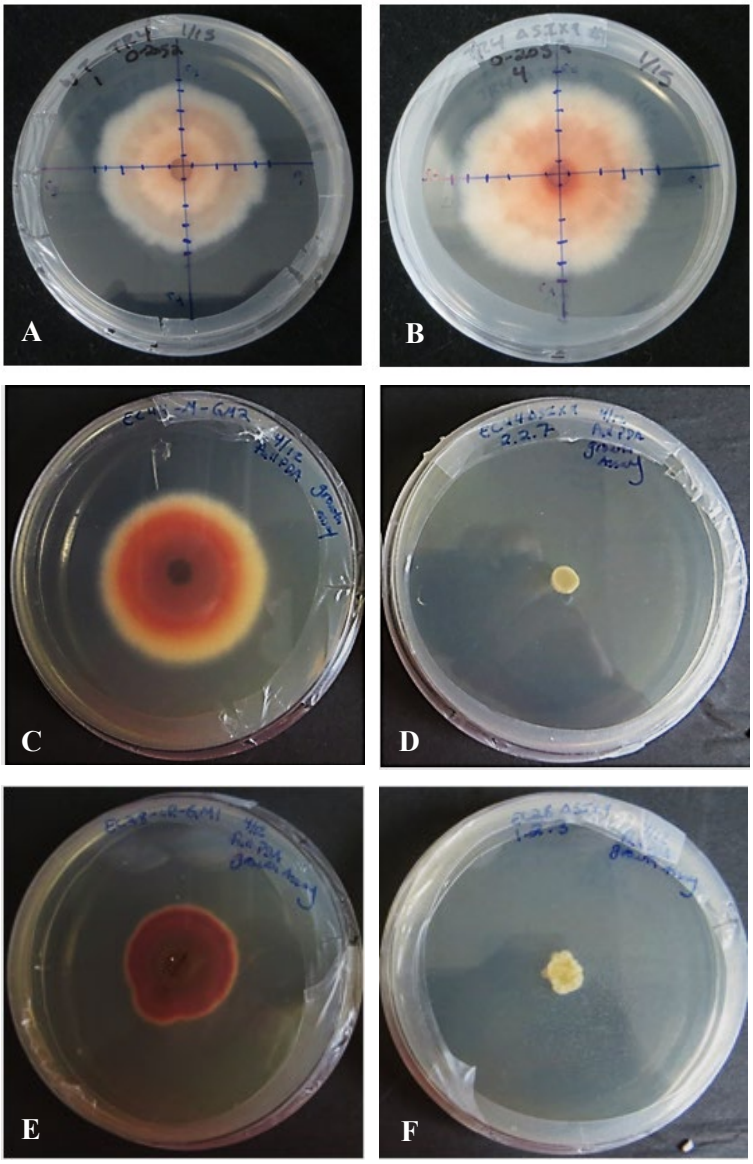


Table 2.3 Mean radius of Tropical race 4 mutant isolates after five days of growth on PDA. Cells highlighted in grey indicate the value is significantly different T tests (P<0.05) from the wild-type (TR4 0-2052). The mean radii of four replicates of each mutant isolate were considered.

Isolate	Average Radius (mm)	P-value	T-stat	Df
TR4 0-2052 WT	15.51			
ΔTR4 0-2052 #1	17.1	0.001	-3.82	19
ΔTR4 0-2052 #2	13.3	0.0003	4.45	19
ΔTR4 0-2052 #3	14.2	0.02	2.46	19
ΔTR4 0-2052 #4	11.1	<0.0001	10.20	19
ΔTR4 0-2052 #5	15.95	0.39	4.45	19

Table 2.4 Mean spore count of Tropical race 4 mutant isolates after five days of growth on PDA. Cells highlighted in grey indicate the value is significantly different (P<0.05) from the wild-type (TR4 0-2052). The mean four replicates of each mutant isolate were considered.

Isolate	Average Spore Count	P-value	T-stat	Df
TR4 0-2052 WT	4.4×10^3			
ΔTR4 0-2052 #1	3.7×10^3	0.35	0.95	19
ΔTR4 0-2052 #2	3.0×10^3	0.06	3.12	19
ΔTR4 0-2052 #3	9.0×10^2	<0.0001	7.97	19
ΔTR4 0-2052 #4	1.5×10^3	<0.0001	5.96	19
ΔTR4 0-2052 #5	1.4×10^4	0.008	-2.98	19

Table 2.5 Mean radius of race 1 mutant isolates after 4 weeks of growth on PDA. Cells highlighted in grey indicate the value is significantly different (P<0.05) from the wild-type (EC44-M-GM2). The mean radii of four replicates of each mutant isolate were considered.

Isolate	Average Radius (mm)	P-value	T-Stat	Df
EC44-M-GM2 WT	36.7			
Δ EC44-M-GM2 2.2.7	16.0	<0.0001	-13.1	11
ΔEC28-M-GM1 3.1.4	20.0	<0.0001	-10.4	11
ΔEC28-M-GM1 1.1.2	18.2	<0.0001	-18.2	11
ΔEC44-M-GM2 2.2.5	19.0	<0.0001	-12.6	11

Table 2.6 Mean spore count of race 1 mutant isolates after 5 days of growth on PDA. Cells highlighted in grey indicate the value is significantly different (P<0.05) from the wild-type (EC44-M-GM2). The mean of four replicates of each mutant isolate were considered.

Isolate	Average Spore Count	P-value	T-Stat	Df
EC44-M-GM2 WT	7.1×10^5			
Δ EC44-M-GM2 2.2.7	1.0×10^5	0.01	9.74	2
ΔEC28-M-GM1 3.1.4	2.0×10^5	0.02	6.83	2
ΔEC28-M-GM1 1.1.2	2.7×10^4	0.01	9.11	2
ΔEC44-M-GM2 2.2.5	3.3×10^4	0.01	9.26	2

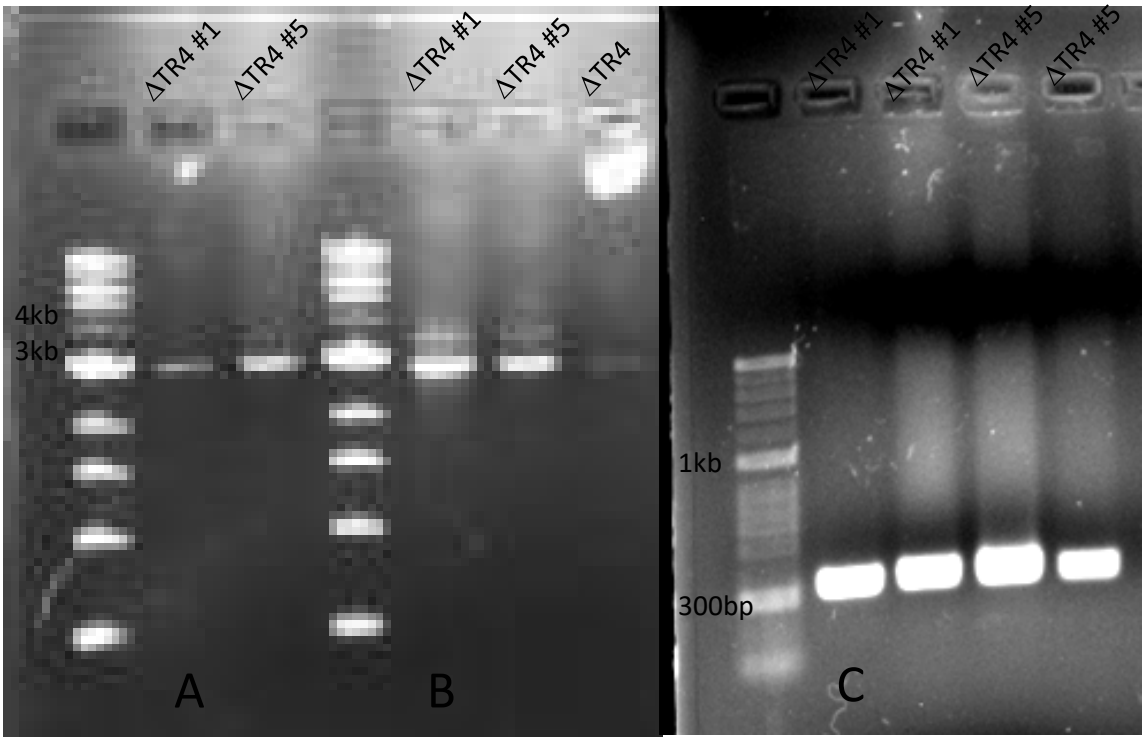
Mutant Characterization: Insertion Detection

For preliminary screening of the mutants, primers LFRsix9OUT-F and hygREV were used to amplify the expected 1,013bp fragment, indicating the *hph* gene insertion occurred in the left flaking region of *SIX9* (Figure 2.5A). Using this technique, ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5 were considered for further study.

To verify the insertion location, primers LFRsix9OUT-F and RFRsix9OUT-R were used to amplify the entire insertion region (Figure 2.2). Primer binding sites for LFRsix9OUT-F and RFRsix9OUT-R were outside the transformation construct. Two amplifications can be observed in the gel electrophoresis gel visualization using the aforementioned primer pair: one band around 3Kbp and one band just under 4Kbp (Figure 2.4B). The expected fragment size for the wild type region, which contains *SIX9,* is 2,788bp; the expected fragment size for the target transformants, which contains the *hph* gene, is 3,818bp. Both a 3kb and 4kb band can be observed in Figure 2.5B from amplification of genomic DNA from ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5, indicating that there is a region containing *SIX9* and containing the *hph* gene. Sanger sequencing of the PCR product of primers LFRsix9OUT-F and RFRsix9OUT-R, visualized in Figure 2.5B, resulted in sequencing results for the expected *hph* gene insertion; there was no sequencing result that showed *SIX9*. However, using the PCR products of LFRsix9OUT-F and RFRsix9OUT-R, expected target size PCR amplicons could be obtained from the primer pairs 1) FocSIX9-F and FocSIX9-R and 2) hygFWD and hygREV. Amplifications from FocSIX9-F and FocSIX9-R and hygFWD and hygREV resulted in sequence results which show an expected sequence for *SIX9* and for the *hph* gene, respectively.

FocSIX9 primers (Magdama 2017) were used to amplify *SIX9* from genomic DNA of the mutants ΔTR4 0-2052 #1 and ΔTR4 02052 #5. ΔTR4 0-2052 #1 and ΔTR4 02052 #5 displayed amplification for *SIX9* (Figure 2.4C). The identities of the fragments amplified by *FocSIX9* primers was also confirmed by Sanger sequencing.

Figure 2.4 Tropical race 4 mutant PCR amplification to identify the recombination insertion location of the hygromycin phosphotransferase gene. A) Primers LFRsix9out-F and hygREV amplify an expected 3Kbp target fragment from transformed genomic DNA. B) Primers LFRsix9OUT-F and RFRsix9OUT-R amplify an expected 2788kb target region from the wild type genomic DNA and an expected 3918bp target fragment from transformed genomic DNA. C) Detection of *SIX9* in ΔTR4 mutants using primers FocSIX9-F and FocSIX9-R to amplify a 328bp target region. Primers are from Magdama (2017.)



In planta mutant characterization

Banana plants were inoculated in two repeated experiments by planting them in soil mixtures infested with different mutants in fall 2017 and spring 2018. Table 2.7 shows the inoculum levels of the infested soil for each treatment and Table 2.8 shows the external disease symptoms of the banana plants three and four weeks post inoculation. Internal disease symptoms were congruent with external disease symptoms.

Table 2.7 *Fusarium oxysporum* mutant soil inoculum concentration for *in planta* testing.

Treatment	Soil inoculum concentration (cfu/g soil inoculum)	
	Fall 2017	**Spring 2018**
TR4 WT	1.1×10^5	2.5×10^5
EC44-M-GM2 WT	7.1×10^5	3.1×10^5
ΔTR4 0-2052 #1	3.3×10^4	1.3×10^5
ΔTR4 0-2052 #5	2.7×10^4	5.0×10^4
ΔEC28-M-GM1 1.1.2	2.7×10^4	3.7×10^5
ΔEC28- M-GM1 3.1.4	2.1×10^5	1.8×10^5
ΔEC44-M-GM2 2.2.5	3.3×10^4	4.5×10^5
ΔEC44-M-GM2 2.2.7	6.7×10^3	1.7×10^5

Overall, ΔTR4 0-2052 #1 showed statistically faster disease development than the wild-type TR4 O-2052 in both cultivars studied, Cavendish 'Gran Naine' ($p=0.013$, Figure 2.9) and 'Gros Michel' ($p=0.016$) in the Fall 2017 trial three weeks post innoculation (Figure 2.8). In both trials, disease symptoms were observed two weeks post-inoculation in ΔTR4 0-2052 #1, whereas there was no disease development in plants inoculated with the wild-type TR4 two weeks post-inoculation. The progression of disease was initially observed in the wild type TR4 0-2052 treatment in both cultivars and both repetitions three weeks post-inoculation. In both cultivars and in both repetitions, ΔTR4 0-2052 #1 showed no statistically significant differences from the wild-type four weeks post-inoculation, as the disease had progressed to plant death in both treatments. No disease developed in either cultivar inoculated with ΔTR4 0-2052 #5, and thus ΔTR4 0-2052 #5 disease severity was significantly different from the wild-type TR4 0-2052 in both cultivars and Fall 2017 and Spring 2018 (Table 2.9). Final values of *in vitro*

conidiation, final *in vitro* growth, and experimental inoculum load cannot explain these results, as there were no significant differences from the wild type in these morphological characteristics (Table 2.4). Moreover, these *in planta* differences cannot be explained with the molecular data obtained in this study (Figure 2.5).

Race 1 mutants ΔEC28-M-GM1 1.1.2, ΔEC28-M-GM1 3.1.4, and ΔEC44-M-GM2 2.2.7 all showed a complete loss of pathogenicity compared to the wild type (Table 2.5). In 'Gros Michel', these treatments showed significant differences from the wild-type at three ($p=0.012$, Table 2.10) and four weeks post-inoculation ($p=0.001$) by showing a complete loss of pathogenicity. In 'Gran Naine', these treatments showed no significant differences from the wild-type ($p=1$), as neither the wild-type race 1 nor the mutants caused disease on 'Gran Naine', a cultivar resistant to race 1. All banana plants inoculated with mutants that did not cause disease were grown for a total of six weeks post-inoculation to confirm the absence of delayed disease development.

Surprisingly, race 1 mutant ΔEC44-M-GM2 2.2.5 caused disease in both cultivars in both repetitions. Race 1 isolates should not cause disease on Cavendish cultivars, such as 'Gran Naine,' so the observation of disease in Cavendish caused by ΔEC44-M-GM2 2.2.5 indicated that that there was a change in this mutant that changed the phenotype from race 1 to TR4 ($p=0.0001$, Table 2.10). The severity of disease caused by ΔEC44-M-GM2 2.2.5 in 'Gros Michel' was significantly different than the wild-type EC44-M-GM2 three weeks post inoculation in both the Fall 2017 and Spring 2018 experiments ($p=0.004$, Table 2.10). However, both resulted in disease severity at four weeks post inoculation that was not statistically different ($p=0.423$ in Fall 2017, $p=0.284$ in Spring 2018). Therefore, the most conclusive observation that should be made about ΔEC44-M-GM2 2.2.5 is that it is able to cause disease on Cavendish 'Gran Naine'. Disease symptoms appeared at three weeks post-inoculation, similarly to the wild-type TR4 0-2052.

All mutants inoculated could be recovered from the plant material by plating on NASH media and hygromycin B-amended PDA. However, only mutants that caused disease, ΔTR4 0-2052 #1 and

ΔEC44-M-GM2 2.2.5 could be isolated from the corm and stem of the tested plants. Mutants that did not cause disease were only recovered from the roots of the tested plants.

Table 2.8 External Fusarium wilt disease symptom severity of Cavendish 'Gran Naine' and 'Gros Michel' banana plants inoculated with mutants. External symptoms were based on the disease rating scale in Figure 2.1. * indicates that the treatment has a statistically significantly different average mean ($p<0.05$) than the corresponding wild-type control (Tables 2.9, 2.10) across cultivars and repetitions.

External symptoms at 3 weeks post-inoculation																															
Treatment	**'Gros Michel'**																**Cavendish 'Gran Naine'**														
	Fall 2017								**Spring 2018**								**Fall 2017**								**Spring 2018**							
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TR4 WT	1	1	1	1	1	1	1	1	0	0	1	1	1	1	2	2	0	0	0	1	1	1	1	1	2	2	2	2	2	3	3	3
EC44-M-GM2 WT	1	1	1	1	1	2	2	3	0	0	1	1	1	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔTR4 #1*	3	3	3	3	3	3	3	3	0	1	2	3	3	3	3	3	1	1	2	2	2	2	3	3	2	3	3	3	3	3	3	3
ΔTR4 0-2052 #5*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔEC28-M-GM1 1.1.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔEC28-M-GM1 3.1.4	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔEC44-M-GM2 2.2.5	0	0	0	1	1	1	1	1	1	2	3	3	3	3	3	3	0	0	1	1	1	1	1	1	1	2	2	2	3	3	3	3
ΔEC44-M-GM2 2.2.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
External symptoms at 4 weeks post-inoculation																															
Treatment	**'Gros Michel'**																**Cavendish 'Gran Naine'**														
	Fall 2017								**Spring 2018**								**Fall 2017**								**Spring 2018**							
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TR4 WT	1	1	1	1	1	3	3	3	2	3	3	3	3	3	3	3	2	2	2	2	3	3	3	3	2	2	2	2	3	3	3	3
EC44-M-GM2 WT	2	2	2	3	3	3	3	3	1	1	1	1	2	2	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔTR4 0-2052 #1	2	3	3	3	3	3	3	3	1	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3
ΔTR4 0-2052 #5*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔEC28-M-GM1 1.1.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔEC28-M-GM1 3.1.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ΔEC44-M-GM2 2.2.5*	1	1	1	2	2	2	3	3	2	3	3	3	3	3	3	3	2	2	2	2	3	3	3	3	2	2	2	2	3	3	3	3
ΔEC44-M-GM2 2.2.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

.

Table 2.9 Krustal-Wallis test and Dunn's procedure for Fusarium wilt disease severity for banana plants inoculated with Tropical race 4 mutants. P-values shown are for pairwise comparisons using Dunn's procedure. Treatments highlighted in grey indicate treatments significantly different ($p<0.05$) from the wild-type. Each mutant is compared to the wild-type TR4 for that time point, where W3 indicates 3 weeks post-inoculation and W4 indicates 4 weeks post-inoculation. All analyses had 2 degrees of freedom.

	ΔTR4 0-2052 #1								**ΔTR4 0-2052 #5**							
	'Gros Michel'				**Cavendish 'Gran Naine'**				**'Gros Michel'**				**Cavendish 'Gran Naine'**			
	Fall 2017		Spring 2018		Fall 2017		Spring 2018		Fall 2017		Spring 2018		Fall 2017		Spring 2018	
	W3	W4	W3	W4	W3	W4	W3	W4	W3	W4	W3	W4	W3	W4	W3	W4
p-value	0.016	0.164	0.098	0.968	0.013	0.36	0.227	0.36	0.016	0.004	0.035	0.0001	0.089	0.001	0.003	0.001
Mean	3	2.9	0.9	2.9	2	2.9	2.9	2.9	0	0	0	0	0	0	0	0

Table 2.10 Krustal-Wallis test and Dunn's procedure for Fusarium wilt disease severity for banana plants inoculated with race 1 mutants. P-values shown are for pairwise comparisons using Dunn's procedure. Each mutant is compared to the wild-type race 1 (EC44-M-GM2) for that time point, where W3 indicates 3 weeks post-inoculation and W4 indicates 4 weeks post-inoculation. Treatments highlighted in grey indicate treatments significantly different ($p<0.05$) from the wild-type. All analyses had 4 degrees of freedom.

	ΔEC44-M-GM2 2.2.5								**ΔEC44-M-GM2 2.2.7, ΔEC28-M-GM1 1.1.2, ΔEC28-M-GM 3.1.4**				
	'Gros Michel'				**Cavendish 'Gran Naine'**				**'Gros Michel'**				**Cavendish 'Gran Naine'**
	Fall 2017		Spring 2018		Fall 2017		Spring 2018		Fall 2017		Spring 2018		Fall 2017& Spring 2018
	W3	W4	W3	W4	W3	W4	W3	W4	W3	W4	W3	W4	W3 & W4
P two tail	0.044	0.423	0.044	0.284	0.0001				0.012	0.001	0.012	0.001	1
Mean	0.6	1.9	2.6	1.9	0.75	2.5	2.4	2.5	0	0	0	0	0

Figure 2.5 Fusarium wilt disease external symptoms of banana plants four weeks post inoculation in fall 2017. A) Cavendish 'Gran Naine' non-inoculated control; B) 'Gros Michel' non-inoculated control; C) Cavendish 'Gran Naine' EC28-M-GM1 Δ 1.1.2; D) 'Gros Michel' EC28-M-GM1 Δ 1.1.2; E) Cavendish 'Gran Naine' EC28-M-GM1 Δ 3.1.4 F) 'Gros Michel' EC28-M-GM1 Δ 3.1.4; G) Cavendish 'Gran Naine' ΔTR4 0-2052 #5; H) 'Gros Michel' ΔTR4 0-2052 #5. I) Cavendish 'Gran Naine' TR4 0-2052; J) 'Gros Michel' TR4 0-2052; K) Cavendish 'Gran Naine' EC44-M-GM2; L) 'Gros Michel' EC44-M-GM2; M) Cavendish 'Gran Naine' ΔEC44-M-GM2 2.2.5; N) 'Gros Michel' ΔEC44-M-GM2 2.2.5; O) Cavendish 'Gran Naine' ΔTR4 0-2052 #1; P) 'Gros Michel' ΔTR4 0-2052 #1.

Discussion

The transformants resulting from replacing *SIX9* with the *hph* gene showed interesting and unexpected *in planta* phenotypes. Three transformation experiments were performed: one with TR4 0-2052, one with EC44-M-GM2, and another with EC28-M-GM1. The latter two isolates are characterized as *Foc* race 1. Transformation of TR4 0-2052 resulted in five transformants that grew in the presence of hygromycin B-amended media. Two of them (#1 and #5) were selected for further studies based on amplification of the targeted insertion regions. Those two transformants showed different *in planta* phenotypes. ΔTR4 0-2052 #1 showed faster disease development than the wild-type TR4 0-2052 in both cultivars studied, Cavendish 'Gran Naine' and 'Gros Michel.' Plants inoculated with ΔTR4 0-2052 #1 displayed disease symptoms at two weeks post inoculation, while plants inoculated with ΔTR4 0-2052 #5 remained asymptomatic in both cultivars studied for the entire experimental period, six weeks-post inoculation. Rates of *in vitro* conidiation, *in vitro* growth, and experimental inoculum load cannot explain the *in planta* differences between ΔTR4 0-2052 #1, ΔTR4 0-2052 #5, and the wild-type, as there were no significant differences from the wild type consistent between conidiation and *in vitro* growth. Moreover, these *in planta* differences cannot be explained with the molecular data obtained in this study. In the following section, several explanations are offered for these differences.

Mutant isolates which caused disease, ΔTR4 0-2052 #1 and ΔEC44-M-GM2 2.2.5 could be isolated from inoculated plant corms and stems. Isolates which did not cause disease were not able to be isolated from inoculated plant corms and stems. This observation offers two possibilities. The first is that the isolates which cause disease were able to act as a saprophyte, colonizing plant tissues after the senesced. The second possibility is that isolates which caused disease were able to colonize the plant more extensively than isolates that did not cause disease. The second possibility is intriguing, as it could indicate the role of *SIX9* as a host-colonization factor. Further research on the colonization of these mutants would be informative. Fluorescent labeling of test isolates could be considered in the future to observe colonization.

The PCR-detection of *SIX9* from ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5, including detection of both the wild-type insertion region (containing *SIX9)* and the mutant insertion region (containing *hph)*, do not explain *in planta* differences between these two mutants, as their molecular profiles thus far are identical. *SIX9* is detected by PCR and sequencing using the primers listed in Table 2.1. However, *SIX9* was not able to be sequenced from the PCR amplicon of the primer pair LFRsix9OUT-F and RFRsix9OUT-R from ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5. However, *SIX9* can be amplified and sequenced from the amplicon of primer pair LFRsix9OUT-F and RFRsix9OUT-R. These data also suggest the *hph* gene was integrated in the target region. The expected insertion region from LFRsix9OUT-F and RFRsix9OUT-R could be sequenced using both these primers. However, these data also suggest there is also a wild-type region containing *SIX9* in the genomes of ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5. These results could be explained by the presence of more than one copy of *SIX9* in the genome of TR4 0-2052. An additional copy (or additional copies) may have one or more polymorphisms at the primer site for *SIX9* detection, which could explain the failure in sequencing *SIX9* from the PCR amplicon LFRsix9OUT-F and RFRsix9OUT-R.

We consider the presence of multiple copies of *SIX9* to be possible despite the absence of previous reports in the literature, including literature which has accomplished whole genome sequencing of TR4 0-2052. Illumina-based sequencing is based on short reads, whose contigs must be assembled based on overlapping regions. This technique of genome assembly can overlook repetitive genomic regions. It is already understood that the accessory genome of *Fo* is rich in repetitive DNA and transposons. Additionally, *SIX9* has not been studied extensively in the literature. This limitation of Illumina-based sequencing should be considered in future studies.

There is also the possibility that the transformation resulted in additional insertions outside the targeted *SIX9* region. PEG-mediated transformation by homologous recombination is known to result in multiple loci integrations (Liu and Friesen 2012). Furthermore, *SIX* genes have been observed to have transposable elements (TEs) in their flanking regions. *SIX9* in *Foc* TR4 is known to have TEs MIMP1

(Genbank Accession Number AF076624.1) and MIMP2 (Genbank Accession Number AF076625.1) in the left flaking region of the *SIX9* gene. MIMP1 and MIMP2 were included in left flanking region of the construct. These TEs are class II TEs, which are DNA transposons (Schmidt et al. 2013). MIMPS belong to the Superfamily Tc1/mariner, the family MITES, and Order TIRs (Terminal Inverted Repeats). MITEs are non-autonomous TEs, meaning that they require an associated transposase for transposition. This transposase often has similar TIRs as the MITE. MITEs consist of TIRs flanking a short non-coding DNA region. TIRs consist of Terminal Inverted Repeats flanking a transposase ORF; in the *Fol* genome, they are the best represented order of class II TEs. Class II TEs appear to be preferentially inserted into or close to each other, and this can be observed in the left flanking region of *SIX9,* where MIMP1 and MIMP2 occur consecutively.

Overall, there were 55 MIMPs identified in the genome of *Fol;* MIMPs are miniature Impalas which are transposed by the Impala transposase. The Impala transposase was found to be active in *Fo* f. sp. *melonis* by transposon tagging. However, in *Fol4287,* all three Impala copies do not encode for the full length transposase, indicating that MIMPS present in *Fol4287* are not actively transposed. MIMP1 is also found in the promoter region of *SIX2, SIX7,* and the shared promoter of *SIX3* and *SIX5* in *Fol4287.* Overall in *Fol4287,* MIMP1 was found to be partially present 24 times and fully present 16 times; MIMP2 was partially present 7 times and fully present 6 times (Schmidt et al. 2013). Therefore, MIMP1 and MIMP2 are likely to occur in other locations of the *Foc* TR4 genome; thus it would be logical that an insertion of the construct could have occurred at one or multiple of these sites via homologous recombination. A full construct however would not be expected, as there is no known TE in the right flanking region of the construct. Therefore, it may be expected that an insertion at other locations of MIMP1 and/or MIMP2 would not include the entire construct with a functional *hph* gene. Instead, this insertion could mutate a region such as an expressed gene, like a *SIX* gene, or a promotor, thus changing gene expression, leading to the different phenotypes observed *in planta* in this study.

MIMPS have not been found to have an impact on expression of the *SIX* genes downstream. However, when a motif preceding MIMP1 (TCGGCA) was deleted in the promoter region of *SIX1* in *Fol4287*, there was no expression of *SIX1.* The authors of this study proposed this motif may be a transcription factor binding site for *SIX1.* Likewise, deletion of MIMP1 does not have an effect on expression of *SIX3* and *SIX5*, which share a promoter region. However, deletions in the number of TCGGCA motifs do affect *SIX3* and *SIX5* expression and consequent virulence in *Fol* (Schmidt et al. 2013). Sequencing of the insertion regions of ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5 does not reveal any deletion or insertion MIMPS relative to the wild-type. However, the *hph* gene contains one TCGGCA motif near the left-flanking region of the *hph* gene; thus, multiple integrations of the left-side construct could change the number of TCGGCA motifs present in the mutant, potentially leading to a source of phenotypic differences between of ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5. Furthermore, ectopic integrations and partial integrations of the construct may not have been detected by these molecular tests. Complicating matters further, if partial integrations occur commonly in PEG-mediated transformations, complementation studies, which are often considered essential for a complete gene-knockout study, may not reverse the mutation, or may add additional undetected mutations, making results of a complementation study inconclusive, even if the complementation has the same phenotype as the wild type. Therefore, gene-knockout studies and their conclusions should be approached with care, especially in a genome rich in TEs and repetitive elements, such as the *Fo* genome.

Nonetheless, the *in planta* phenotypes of ΔTR4 0-2052 #1 and ΔTR4 0-2052 #5 show promise in elucidating the differences between pathogenic and non-pathogenic isolates; ΔTR4 0-2052 #5 was non-pathogenic in Cavendish ‘Gran Naine’ and ‘Gros Michel.’ Thus, further examination of the genome may reveal differences between the mutants and the wild-type that are essential for pathogenicity.

Two different wild-type isolates of race 1, EC44-M-GM2 and EC28-M-GM1, were subjected to *SIX9* transformations. Two transformants of each EC44-M-GM3 (2.2.5 and 2.2.7) and EC28-M-GM1 (1.1.2 and 3.1.4) were selected for further study. The mutants of Race 1 isolates EC44-M-GM2 also show

interesting *in planta* phenotypes. ΔEC44-M-GM2 2.2.5 induced disease in both 'Gros Michel' and Cavendish 'Gran Naine'; that is, these isolates were changed from Race 1 to TR4 *in planta* phenotype. All the other race 1 mutants did not cause disease in either of the banana cultivars tested. However, it was not possible to obtain DNA of enough quality and quantity to yield consistent PCR results from the Race 1 mutant isolates. We conclude the altered *in vitro* morphology of the race 1 mutants, which showed a depressed growth rate, compact mycelium, and non-pigmented colonies, inhibited DNA extractions. The mutation(s) that resulted in these phenotypes may have resulted in higher production of polysaccharides or phenols that inhibited DNA extractions. However, the race 1 mutants were able to be isolated from the inoculated banana plants and colonized cornmeal-sand inoculum at rates similar to the wild-type.

For further study, PacBio whole genome sequencing, which features new long-read technology, could supplement current understanding of the *Fo* genome, including clarification on the amount of repetitive sequence in the genome and the number of copies of *SIX9*. Additionally, hybridization using southern blotting could be used to determine the copy number of *SIX9* in the genome of TR4 0-2052, ΔTR4 0-2052 #1, and ΔTR4 0-2052 #5. Southern blots would not capture other potential differences in the genome that may lead to differences in the *in planta* phenotypes. Therefore, if economically viable, PacBio sequencing should be considered to compare the genomes of TR4 0-2052, ΔTR4 0-2052 #1, and ΔTR4 0-2052 #5.

Revealing the genomic differences between the wild type and a mutant with no pathogenicity and between the wild-type and a mutant with hypervirulence may reveal clues to the mechanism of pathogenicity *Foc* TR4 evolved to cause disease on Cavendish. If scientists understand what mutations lead to virulence on a previously resistant cultivar, it can help breeders select traits that are less susceptible to having a pathogen overcome resistance, and it can help agriculturalists design agroecosystems that reduce selecting pressures for pathogen evolution of virulence. In the end, these are the types of sustainable, preventive solutions necessary in a global agricultural economy that increasingly reaches across borders, is threatened by climate change, and influenced by global and domestic politics.

Chapter 3

Evolutionary relationships of non-pathogenic *Fusarium oxysporum*

Introduction

The objective of this study was to infer phylogenetic relationships between non-pathogenic isolates from banana, chickpea, and tomato systems and relate their evolutionary history to formae speciales of *Fo* using phylogenetic models. This study is the first to analyze the phylogenetic relationships between non-pathogenic *Fo* isolates obtained from multiple plant host species and compare them to *Fo* pathogenic to various hosts. The goal of this study was to discover patterns in phylogenetic relationships between these isolates, providing evidence for the underpinnings of their host pathogenicity and host adaptation. To accomplish this goal, 95 isolates (Table 3.1) were sequenced at five novel phylogenetically informative loci, tested for the presence of *SIX* genes, and tested for mating type. The plant host, haplotype, origin of isolation within the plant, and geographic origin were also considered when examining patterns of phylogenetic relationships.

Thus, this study tests the hypothesis that based on *TEF-1α* and five phylogenetically- informative genes, non-pathogenic isolates from different host species will share phylogenetic relationships but display patterns based on geography, detection of *SIX* genes, and/or location of isolation within the plant (i.e.: root, crown, stem). As found in previous studies, these phylogenetic relationships differ when inferring relationships based on *SIX* genes, which are found in the accessory genome and thought to be horizontally transferred. Based on the idea that *SIX* genes can be horizontally transferred, we hypothesized that patterns in the detection of *SIX* genes could provide evidence towards their roles in *Fo*-host interactions, perhaps revealing *SIX* genes which act as colonization factors and pathogenicity factors.

Mating type has been detected in *Fo* isolates in the past and is considered indirect evidence for a history of sexual reproduction within the species (Fourie et al. 2009). As sexual reproduction is a

significant source of genetic variation within a species, this study also sought to detect the mating type of isolates in this study. As observed in *Fo* endophytes of banana (Magdama 2017) and pathogenic isolates of *Fo*, we suspected mating type in *Fo* endophytes from tomato and chickpea could be detected. We expected isolates of the same mating type and opposite mating type to group together; the detection of both mating types in a single isolate was not expected.

Materials and Methods

Phylogenetic relationships of non-pathogenic *Fo* isolates from chickpea, tomato, and banana

The *TEF1-α* region was used to infer phylogenetic relationships between selected *Fo* isolates from asymptomatic chickpea, tomato, and banana. Additionally, the sequences of five phylogenetically-informative genes from *Fo,* including genes *Fo*4287_04846, *Fo*4287_06412, *Fo*4287_10995, *Fo*4287_15395, and *Fo*4287_15690 were used to infer phylogenetic relationships of the 95 isolates (Table 3.1). These five genes were determined to be phylogenetically informative by previous studies by Geiser, are bioinformatically predicted to code for proteins, and are each located on separate chromosomes in the *Fo* core genome (D. M. Geiser, personal communication). Primers were generated using the software Geneious v7.1.4. based on *Fo* sequences generated by M.M. Jiménez-Gasco, D. M. Geiser, and M. Rep (unpublished data). The primer sequences and cycling conditions are described in Table 3.1.

DNA was generated from the selected isolates using the methods described in chapter two. PCR reactions were carried out in 25 µL using Choice Taq Master mix (Denville), 0.5 µM of each primer, 10-20 ng of DNA and sterile deionized water. The cycling conditions for amplification of gene targets were: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 45 s, primer annealing from 54°C- 59°C for 1 min, elongation at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The resulting PCR products were visualized by gel electrophoresis and purified using ExoSAP-IT (USB Affymetrix Corporation, Cleveland, Ohio, USA) following the manufacturer's instructions. All

PCR products were sent to the Genomic Core facility at The Pennsylvania State University for Sanger sequencing using the forward primer for the sequencing reaction.

Sequences were edited and aligned using the software Geneious v7.1.4. Distance matrices were used to determine isolates with 100% basepair identity for all six genes used in this study. Isolates with 100% identity are represented by a single isolate in downstream analysis (Table 3.2). The software MEGA7 (Molecular Evolution Genetics Analysis, Version 7.0.21) was used to generate phylogenetic trees from alignments generated by Magdama (2017), and Demers (2012) to construct phylogenies based on the Maximum Parsimony (MP) method. The MP methods were run with the Subtree-Pruning-Regrafting (SPR) algorithm and bootstrapping with 1,000 replicates. Isolates were selected to represent diversity in genetics, geography, host, *SIX* genes, and location of isolation from the host. Phylogenetic relationships between non-pathogenic isolates from tomato, chickpea, and banana were inferred and compared with reference pathogenic isolates from previous studies by Magdama (2017) and Demers (2012). The software PAUP*4 heuristic search was used to determine the number of parsimony informative characters provided by each gene alignment used in the MP analysis (Table 3.1).

SIX Gene Detection

PCR-based detection of *SIX* genes was used to detect the presence of *SIX*1- *SIX11, SIX13* and *SIX*14 in *Fo* isolates from asymptomatic tomato and chickpea. If *SIX* genes were detected by PCR and their amplicons visualized by gel electrophoresis, these *SIX* genes were sequenced and analyzed to infer phylogenetic relationships based on their *SIX* gene sequences.

DNA was generated following the same protocol as used in chapter two. Then, PCR primers and conditions used were as described by Rocha and colleagues (2015) for detection of *SIX* genes from soil isolates and by Magdama 2017 for detection in *Fo* isolated from asymptomatic banana (Table 3.2). For primers *SIX*1F/R through *SIX*8F/R PCR cycling conditions were: denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 45 s; annealing at 60°C for 45 s; and polymerization at

72°C for 1 min with a final extension at 72°C for 10 min (Medrum et al. 2012). *SIX*1, 2, 3, 5, 6, and 8 are all based on sequences for *SIX* genes in *Fo* f. sp. *cubense* and have the same cycling conditions (Meldrum et al. 2012). Primers for *SIX*7 are based on sequences from *Fol;* PCR conditions are: denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; and polymerization at 72°C for 1 min with a final extension at 72°C for 5 min (Chakrabarti et al. 2011). Cycling conditions for detection of *SIX4, SIX9, SIX*13, primers were developed by Magdama (2017) for *Foc,* will also be used at the annealing temperature 58°C. Detection of SIX10 through SIX11 and SIX14 were carried out according to Laurence et al 2015a and Rocha et al. 2015. PCR cycling conditions were: denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 45 s; annealing at 56°C for 45 s; and polymerization at 72°C for 1 min with a final extension at 72°C for 10 min.

Table 3.1 Primers used in this study for detection of *SIX* genes.

Code	Primer sequence (5'-3')	Target	Amplicon Length	Annealing Temp (C°)	Reference
*SIX*1F *SIX*1R	ATGGTACTCCTTGGCGCCCTC TGACAATGCGACCACGCCTCG	*SIX*1	260	60	Meldrum et al. 2012
*SIX*2F *SIX*2R	ACGACCTGGGCCATCTCGGT ACACCTTGACTGCGACGCAACG	*SIX*2	660	60	
*SIX*3F *SIX*3R	ACCGACCATCTTGCCTAAACATTTACC TTAACCACTCTGCCAAGGGGAACT	*SIX*3	555	60	
*FocSIX*4-F *FocSIX*4-R	TTGTGTACATGCTGGCGCT CCCGGAGTGAAGAAGAAGCT	*SIX*4	492	58	Magdama 2017
*SIX*5F *SIX*5R	TGCGCTTCGAGTACATCTCTGTTC CTGGTGAGATTTAGAGCAGTCAAAGCA	*SIX*5	326	60	Meldrum et al. 2012
*SIX*6F *SIX*6R	GGCTGCGTAGCTGGTCCCCT CATGTCATGAATGTACGCATGTCCCT	*SIX*6	611	60	
*SIX*7F *SIX*7R	CATCTTTTCGCCGACTTGGT CTTAGCACCCTTGAGTAACT	*SIX*7		55	Chakrabarti et al. 2011
*SIX*8F *SIX*8R	TCGCCTGCATAACAGGTGCCG TTGTGTAGAAACTGGACAGTCGATGC	*SIX*8	250	60	Meldrum et al. 2012
*FocSIX*9-F *FocSIX*9-R	GCAGTTGCGGCAATGGCT GCCCCATCTGGTATCCGACA	*SIX*9	328	58	Magdama 2017
*SIX*10F *SIX*10R	AAAAAGCAGGCTCCATGAAGCTCTTGTGGTTG AGAAAGCTGGGTCCTACTTAGACCTGGTAATTGTT	*SIX*10		56	Rocha et al. 2015; Laurence et al. 2015
*SIX*11F *SIX*11R	GATGTTCTCCAAAGCCATCC AGAATGCCACTCGGTGTGA	*SIX*11		56	
Foc*SIX*13-F Foc*SIX*13-R	ATTCCCTGGCGCTGACTTAG CTGTACTCCTAAGCGGTGGC	*SIX*13	399	58	Magdama 2017
*SIX*14F *SIX*14R	TTGCCACCTATGCATACCG TCCACATTCCTAAGCGAACC	*SIX*14		56	Rocha et al. 2015

Results

Primers for the genes *04642, 06412, 10995, 15960,* and *15695* were designed and showed high rates of amplification by PCR and efficacious Sanger sequencing (Table 3.1). 95 *Fo* isolates (87 endophytes and eight isolates pathogenic to chickpea) were included in this study (Table 3.3). For primer set *15960,* 82 isolates (86.3%) were successfully sequenced using the primers developed. Thirteen isolates (7.3%) were not successfully sequenced. However, five of these isolates had low sequencing success in at least one other locus. For locus *15695,* 91 isolates (95.8%) were successfully sequenced. Four isolates (4.3%) were not successfully sequenced, but three of these isolates had low sequencing success in at least one other locus. For locus *10995,* 83 isolates (87.4%) were successfully sequenced. Twelve isolates (12.6%) were not successfully sequenced, and six of these isolates had low sequencing success in at least one other locus. For locus *06412,* 86 isolates (90.5%) were successfully sequenced. Nine isolates (9.5%) were not successfully sequenced, and eight of these isolates had low sequencing success in at least one other locus. Finally, for locus *04642,* 81 isolates (85.3%) isolates were successfully sequenced. Forteen isolates (14.7%) were not successfully sequenced and seven of these isolates had low sequencing success in at least one other locus (Table 3.2).

The loci *04642, 06412, 10995, 15960,* and *15695* added many parsimony informative characters to phylogenetic analysis of evolutionary relationships between *Fo* isolates (Table 3.2). *TEF1-α* is standard when considering evolutionary relationships between *Fo* isolates. In this study, *TEF1-α* provided 64 parsimony informative characters. Only the locus *10995* provided fewer parsimony informative characters (45 characters) than *TEF1-α*. The locus *15965* provided the most with 145 parsimony informative characters. The locus *15690* provided 83 parsimony informative characters. The locus *04846* provided 97 parsimony informative characters. The locus *06412* provided 68 parsimony informative characters. By adding these five loci to the analysis of phylogenetic relationships between isolates, 438 parsimony informative characters were added to the 64 parsimony informative sites *TEF1-α* provided.

Table 3.2 Primers generated in this study for PCR amplification and sequencing of the loci *04642, 06412, 10995, 15960,* and *15695*. Sequencing was considered a success when the forward primer yielded high confidence base pair calls for at least 500bp. If there was a failure of sequencing at more than one locus, the result did not count towards the success rate of the primer, as the failure could reflect an issue with DNA quality rather than the primer. "N" represents no sequencing of the locus in this study. Parsimony informative characters were determined by parsimony heuristic search in PAUP4. * indicates these primers were designed by O'Donnell et al. 1998.

Code	**Primer Sequence (5'-3')**	**Target**	Amplicon	Annealing Temp (C°)	Sequencing success	Parsimony informative characters
15965FWD 15695REV	CATCCCAGACCCTTCTGATAAC TCTTCGGACTCTCCTCATCTC	*Fo*4287_15965	709bp	54	98.9%	145
15690-F 15690-R	CGTCGCGCAGAATACAACTG TGATACGCCCCTCAGAGTCA	*Fo*4287_15690	748bp	59	91.1%	83
04846-F 04846-R	GAGCCTGTGAAAAGTGCACG TCCCGCTCATCCTCAAGGTA	*Fo*4287_04846	738bp	54	92.0%	97
06412-F 06412-R	CTCATTCGTCGTCTCCCTCG TCTCAGCGAGAGTTGCGTTT	*Fo*4287_06412	710bp	54	98.9%	68
10995-F 10995-R	CAGCCACGATCAAGAATGCG GACTGTTGAGCTGGTGACGA	*Fo*4287_10995	523bp	54	93.3%	45
EF-1* EF-2*	ATGGGTAAGGARGACAAGAC GGARGTACCAGTSATCATGTT	*TEF1-α*	750bp	60	N	64

Table 3.3 Isolates used in this study, including host of origin, pathogenicity, *TEF1-α* haplotype based on previous studies, and regions analyzed in this research.

Isolate code	host plant	f. sp.[a]	haplotype[b]	TEF1-α	15690	15395	10995	6412	4846	Phylogenetics[d]	*SIX* genes tested[e]
1_1c1	tomato	NP	13*	+[c]	+	+	L	+	+	X	N
1_1r3	tomato	NP	4*	+	+	+	+	+	+	X	N
1_2c1	tomato	NP	12*	+	L[c]	L	+	+	+		+
1_2r2	tomato	NP	10*	+	+	+	+	+	+	X	+
1-4r3	tomato	NP	16*	+	+	+	+	+	+	X	+
1_4st1	tomato	NP	5*	+	+	+	+	L	L		+
2_1c1	tomato	NP	13*	-[c]	+	+	+	+	+	X	+
2_1r2	tomato	NP	5*	+	+	+	+	+	+		+
2_2st2/ Fo48	tomato	NP	8*	+	L	+	+	+	+	X	+
2_3c3/ Fo68	tomato	NP	21*	+	+	+	+	+	+		+
2_4r1	tomato	NP	2*	+	+	+	+	+	+		+
2_4st1	tomato	NP	13*	+	+	+	+	+	+	X	+
2s6	tomato	NP	7*	+	+	+	+	+	+	X	+
2s37	tomato	NP	11*	+	+	+	+	+	+	X	+
2s38	tomato	NP	23*	+	+	+	+	+	+		+
3_1r1	tomato	NP	13*	+	+	+	+	+	+		+
3_2c2/ Fo69	tomato	NP	20*	+	+	+	+	+	+	X	+
3_4r4	tomato	NP	18*	+	+	+	L	+	+		+
3_4st1	tomato	NP	2*	+	+	+	+	+	+	X	+
3_4st4	tomato	NP	14*	+	+	+	+	+	+	X	+

[a] NP indicates a non-pathogenic isolate obtained from an asymptomatic host.

[b] * indicates the haplotype determined by Demers et al. 2014. ** indicates the haplotype determined by Magdama 2017. Haplotypes with * and ** and the same corresponding number do not signify the same haplotype (ie: 7*≠7**).

[c] + indicates gene amplification by PCR and successful Sanger sequencing. L indicates a low-quality Sanger sequence read using both forward and reverse primers for the gene.

[d] X indicates the isolate was incorporated into phylogenetic analyses.

[e] N indicates the isolate was not tested for *SIX* genes. Isolates with a / in the isolate code indicate two isolate names have been used for the same isolate. The first isolate name is used in the Jimenez-Gasco Lab; the second isolate name is used in the Fusarium Research Center and is the name used in this thesis.

Table 3.3 (Continuation) Isolates used in this study, including host of origin, pathogenicity, *TEF1-α* haplotype based on previous studies, and regions analyzed in this research.

Isolate code	host plant	f. sp.[a]	haplotype[b]	TEF1-α	15690	15395	10995	6412	4846	Phylogenetics[d]	*SIX* genes tested[e]
3s1	tomato	NP	1*	+	+	+	+	+	+	x	+
3s36	tomato	NP	25*	+	+	+	+	+	+	x	+
4_2r2	tomato	NP	17*	+	+	+	+	+	+	x	+
4_4r1	tomato	NP	3*	+	+	+	+	+	+		+
4s38	tomato	NP	19*	+	+	+	+	+	+	x	+
7-96 Tunisia	chickpea	NP		+	+	+	+	+	+	x	+
CAV02_ELR	banana	NP	2**	+	+	+	L	+	+		Magdama 2017
CAV02_EO	banana	NP	21**	+	+	+	+	+	+	x	Magdama 2017
CAV04_EO	banana	NP	12**	+	L	L	L	L	+		Magdama 2017
CAV05_ELR	banana	NP	1**	+	+	L	+	+	+	x	Magdama 2017
CAV05_EO	banana	NP	19**	+	+	+	+	+	+	x	Magdama 2017
CAV06_EG	banana	NP	14**	+	+	+	L	+	+		Magdama 2017
CAV07_EO	banana	NP	5**	+	+	+	+	+	L	x	Magdama 2017
CAV09_EO	banana	NP	3**	+	L	+	+	+	+	x	Magdama 2017
CAV10_ELR	banana	NP	5**	+	L	+	+	+	+	x	Magdama 2017
CAV010_EO	banana	NP	15**	+	+	+	L	+	+		Magdama 2017
CAV013_EG	banana	NP	9**	+	+	+	L	L	L		Magdama 2017
CAV017_EO	banana	NP	22**	+	+	+	+	+	+	x	Magdama 2017
CAV022_ELR	banana	NP	18**	+	+	+	+	+	+	x	Magdama 2017
CAV026_EG	banana	NP	11**	+	L	+	L	L	L		Magdama 2017
CAV026_EO	banana	NP	4**	+	+	+	+	+	+	x	Magdama 2017
CAV027_EG	banana	NP	8**	+	+	+	+	+	+		Magdama 2017
CAV027_EO	banana	NP	16**	+	+	+	+	+	+	x	Magdama 2017
CAV029_ELR	banana	NP	6**	+	L	+	+	+	+	x	Magdama 2017
CAV030_ELR	banana	NP	6**	+	+	+	+	+	L	x	Magdama 2017
CAV032_ELR	banana	NP	6**	+	+	+	+	+	+	x	Magdama 2017
CAV034_EG	banana	NP	8**	+	L	L	+	+	+		Magdama 2017
CAV034_ELR	banana	NP	7**	+	+	+	+	+	+	x	Magdama 2017

Table 3.3 (Continuation) Isolates used in this study, including host of origin, pathogenicity, *TEF1-α* haplotype based on previous studies, and regions analyzed in this research.

Isolate code	host plant	f. sp.[a]	haplotype[b]	TEF1-α	15690	15395	10995	6412	4846	Phylogenetics[d]	*SIX* genes tested[e]
CAV036_EO	banana	NP	13**	+	+	+	+	+	L	x	Magdama 2017
CAV038_ELR	banana	NP	11**	+	+	+	+	+	L		Magdama 2017
CAV039_EO	banana	NP	12**	+	L	+	L	L	L		Magdama 2017
CAV042_EO	banana	NP	3**	+	L	+	+	+	+	x	Magdama 2017
CAV044_EO	banana	NP	20**	+	+	+	+	+	+	x	Magdama 2017
CAV048_EO	banana	NP	20**	+	L	+	+	+	+	x	Magdama 2017
CAV052_EO	banana	NP	11**	+	+	+	+	+	+		Magdama 2017
CAV053_EO	banana	NP	12**	+	+	+	+	+	+	x	Magdama 2017
cc42k	chickpea	NP		+	+	+	+	+	+	x	+
cc44J	chickpea	NP		+	+	+	+	+	+	x	+
cc61c	chickpea	NP		+	+	+	+	+	+	x	+
EC1-LR-CV1	banana	NP	17**	+	+	+	+	L	+	x	Magdama 2017
ET2A	chickpea	NP		+	+	+	+	L	L		+
ET4A	chickpea	NP		+	+	+	+	+	L	x	+
ET7A	chickpea	NP		-	+	+	+	+	+	x	+
ET9A	chickpea	NP		+	+	+	+	+	+	x	+
ET10A	chickpea	NP		+	+	+	+	+	+	x	+
ET11A	chickpea	NP		+	+	+	+	+	+	x	+
ET12A	chickpea	NP		+	+	+	L	+	+	x	+
ET13B	chickpea	NP		-	+	+	+	+	+	x	N
ET14A	chickpea	NP		+	+	+	+	+	+	x	N
ET15A	chickpea	NP		-	+	+	+	+	+	x	+
ET19A	chickpea	NP		+	+	+	L	L	L		+
ET23A	chickpea	NP		+	+	+	+	+	+	x	+
FL1c1/ Fo53	tomato	NP	5*	+	+	+	+	+	+	x	+
FL3r3	tomato	NP	24*	+	+	+	+	+	+	x	+
FL3st2	tomato	NP	4*	-	+	+	+	+	+		+
Fo448	chickpea	NP		+	+	+	+	+	+	x	+

Table 3.3 (Continuation) Isolates used in this study, including host of origin, pathogenicity, *TEF1-α* haplotype based on previous studies, and regions analyzed in this research.

Isolate code	host plant	f. sp.[a]	haplotype[b]	TEF1-α	15690	15395	10995	6412	4846	Phylogenetics[d]	*SIX* genes tested[e]
Fo802	chickpea	NP		+	+	+	+	+	L	x	+
Fo804	chickpea	NP		+	L	+	+	+	+	x	+
Fo816	chickpea	NP		+	+	+	+	+	+		+
Foc1992_R3N	chickpea	*ciceris*		+	+	+	+	+	+	x	+
Foc7989	chickpea	*ciceris*		+	+	+	+	+	+	x	+
Foc8012	chickpea	*ciceris*		+	+	+	+	+	+	x	+
Foc8605	chickpea	*ciceris*		+	+	+	+	+	+	x	+
Foc8607	chickpea	*ciceris*		+	+	+	+	+	+	x	+
Foc9093_PV1	chickpea	*ciceris*		+	+	+	+	+	+	x	+
Foc82108	chickpea	*ciceris*		+	+	+	+	+	+	x	+
FocUSA_3-1	chickpea	*ciceris*		+	+	+	+	+	+	x	+
GM1r2	tomato	NP	6*	+	+	+	+	+	+	x	+
GM3r3	tomato	NP	26*	+	+	+	+	+	+	x	+
GM4st4/ Fo74	tomato	NP	13*	+	+	+	+	+	L	x	N
GMs12	tomato	NP	9*	+	+	+	+	+	+	-	+
HZ4c1/ Fo49	tomato	NP	8*	+	L	+	+	+	+	x	N
MF2r1	tomato	NP	15*	+	+	+	+	+	+	x	N
MF4st2/ Fo52	tomato	NP	5*	+	+	+	+	+	+	x	N
MX4	chickpea	NP		+	+	+	L	L	L	x	+

Table 3.4 *Fusarium oxysporum* isolates in this study determined to be 100% identical at all nucleotide base pairs for the genes *TEF1-α, 04642, 06412, 10995, 15960,* and *15695.*

Representative Isolate	Isolates with no nucleotide differences in the six genes studied								
Fo57	Fo75	Fo58	3-1r1	Fo816	1-4st4	Fol051	Fonic001	Fol010	MF4st3
Fo74/ GM4st4	2-1r2								
Fo48/2-2st2	Fo68/2-3c3	Fo49/Hz4c1							
Fo59	Forl_CL57								
Fo69/3-2c3	Fonic012								
Fo5176	Focon_ PHW808								
FoBulbTu67	Folac								
Focubbpi_0901	Focubrrl36101	Focubst4_98	Focubnrrl 22615						
Folag001	Fomom001	Folag002	Folag 005						
Fom005	Fom006pilon	Fom013pilon	Fom004pilon	Fom013 pilon	Fom016 pilon				
Fon002	Fon037								
Fon019	Fon015blob2c								
Foq015	Foq037								
ET13B	ET23A								
Focub_115	Focub_B2								
Focubindia1	Focubindia17	Focubnrrl24409	Focubuva_ 8611						
Fol002	Fol004	Fol007	Fol014	Fol018	Fol026	Fol029	Fol038	Fol074	Fol4287 broad
Fon10	Fon013blob2c	Fon005blob3c	Fon020	Fon021blob3c					
Fonic001	Fonic003	Fonic010							
FophKOD886	FophKOD887	FophKOD888							
Foq011	Foq013								
Foq018b	Foq021	Foq030blob2c							
Forc024	Forc031	Forc016							

Sequences of *Fo* isolates used in this study were clone corrected; isolates with no polymorphisms at all six loci, *TEF1-α, 04642, 06412, 10995, 15960,* and *15695,* are listed in Table 3.4. Based on the six genes studied, *TEF1-α, 04642, 06412, 10995, 15960, and 15695*, endophytes from banana, chickpea, and tomato did not group by host plant species, geographic origin, nor detection of *SIX* genes. Endophytes from banana, chickpea, and tomato host plants group with *Fo* pathogenic on a variety of hosts, including *Fo* pathogenic to the host isolated from (ie: an isolate non-pathogenic to tomato groups with an isolate pathogenic to tomato and an isolate pathogenic to another host) (Figure 3.1). This is consistent with the

analysis of Magdama (2017) and Demers (2012), where pathogenic and non-pathogenic isolates from banana and chickpea, respectively, were found to group together.

Fo f. sp. *ciceris* formed its own group, labeled Clade 5C in Figure 3.1, which is exclusive to isolates pathogenic to chickpea except for one isolate: 7-96 Tunisia. The isolate 7-96 Tunisia also had a similar profile of *SIX* genes as isolates pathogenic to chickpea, as *SIX8, SIX11,* and *SIX14* were detected by PCR and Sanger sequencing (Tables 3.5, 3.6, and 3.7).

Interestingly, groups of endophytes from tomato and soils surrounding tomato plants grouped together throughout the phylogeny. For example, isolates 3s1, 2s37, and 3-4r4 formed their own group. These are labeled Clade 1 in Figure 3.1, all belong to MAT1-1, and did not have any detection for *SIX* genes. Isolates 3s1 and 2s37 were isolated from the soil around tomato plants and 3-4r4 was isolated from the roots of a tomato plant. Similarly, FL3st2, 1-1r3, Fo63, 4s38, and 3s36 group together, labeled Clade 2D, as do 2s38, 4-4r1, 2-4r1, 3-4st1, and Fo65, labeled Clade 4. These grouping do not appear to be based on the plant tissue from which the isolate was obtained (ie: root, crown, stem, soil). Groups of endophytes from banana also grouped together. For example, isolates CAV022-ELR, CAV010-ELR, CAV042-EO, CAV09-EO, CAV026-EO, CAV07-EO, CAV030-ELR, and CAV029-ELR grouped together and are labeled Clade 2B.

Tomato endophytes also grouped with *Fo* f. sp. *lycopersici.* For example, isolates Fo57, Fo75, Fo58, 3-1r1, 1-4st4, MF4st3, *Fol* 051, all clones at the six loci studied, and FL3r3 grouped with *Fol*001. Additionally, this group of isolates grouped with seven isolates of *Fo* f. sp. *nicotianae*. This group of isolates can be seen in Clade 3 of Figure 3.1. Endophytic isolates from tomato grouped together with *Fol* isolates regardless of the plant tissue from which the isolate was obtained (ie: root, crown, stem, soil).

Some banana endophytes also grouped with *Foc.* For example, isolates CAV038-ELR, EC1-LR-CV1, CAV044, CAV048, and CAV017-EO, seen in Clade 5B, grouped with pathogenic *Foc* isolates Focubnrrl22615, FocubB2, and FocubII5.

Figure 3.1 Phylogenetic relationships among *Fusarium oxysporum* pathogenic and non-pathogenic isolates from banana, chickpea, and tomato based on six loci: *TEF1-alpha, 04642, 06412, 10995, 15960,* and *15695.* The most parsimonious tree is displayed (CI:0.67; RI: 0.92). Bootstrap values are shown for 1000 replicates. There were a total of 2304 positions in the final dataset. All positions with less than 95% site coverage were eliminated. ● indicates MAT1-1 was detected in that isolate. ○ indicates MAT1-2 was detected in that isolate.

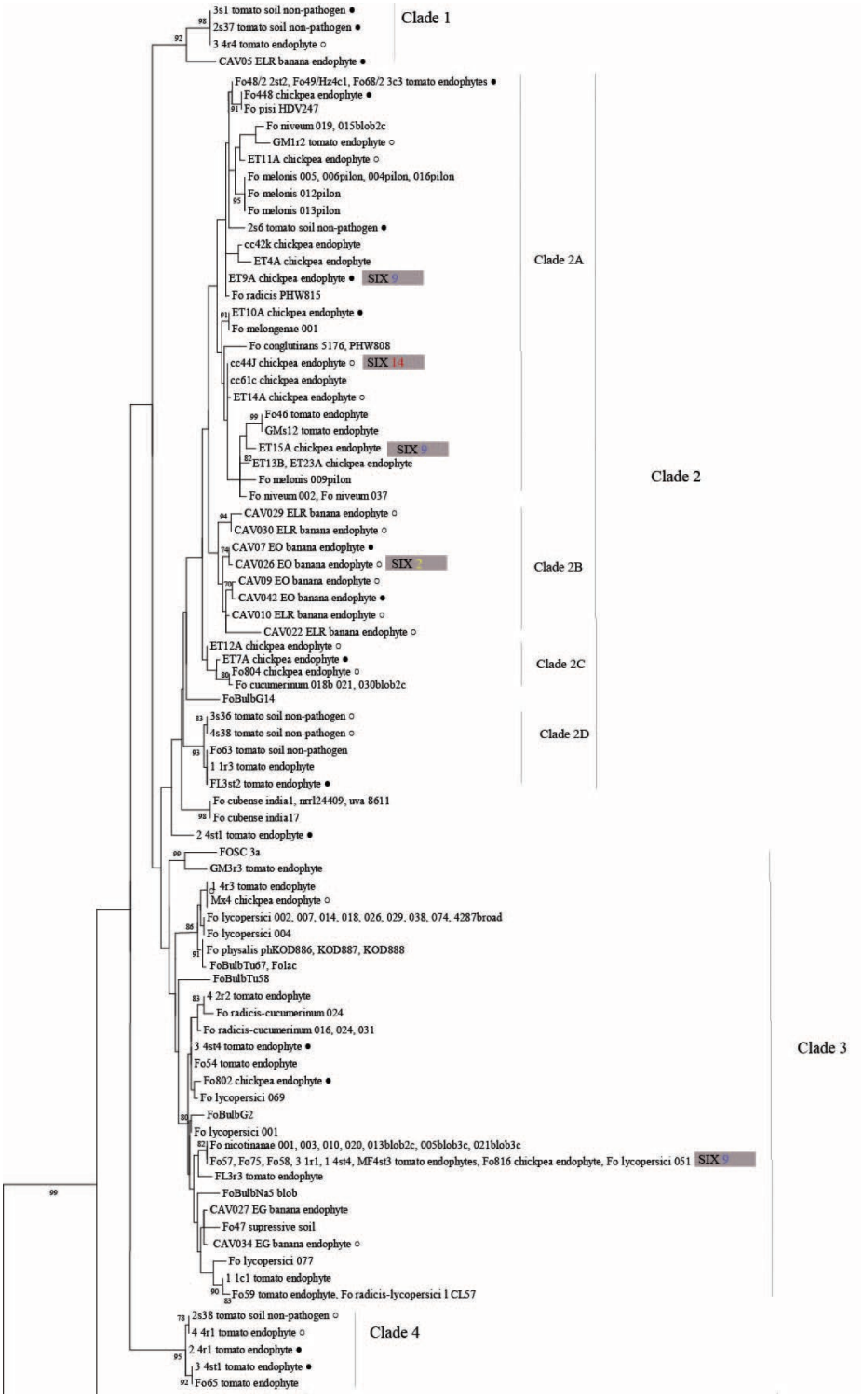
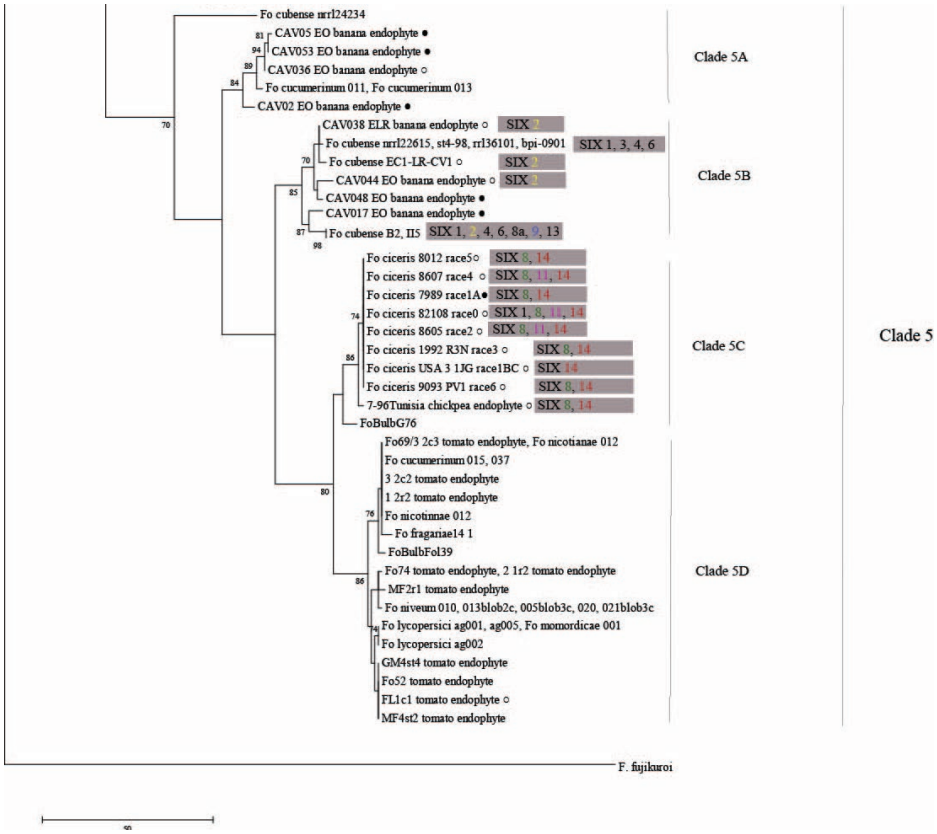


Figure 3.1 (continued) Phylogenetic relationships among *Fusarium oxysporum* pathogenic and non-pathogenic isolates from banana, chickpea, and tomato based on six loci: *TEF1-alpha, 04642, 06412, 10995, 15960,* and *15695*. The most parsimonious trees is displayed (CI:0.67; RI: 0.92). Bootstrap values are shown for 1000 replicates. There were a total of 2304 positions in the final dataset. All positions with less than 95% site coverage were eliminated. ● indicates MAT1-1 was detected in that isolate. ○ indicates MAT1-2 was detected in that isolate.



As in Magdama's (2017), in unpublished results from D. M. Geiser (personal communication), and Fourie et al. 2009, both mating types, MAT1-1 and MAT1-2, were detected within a single group of *Fo* isolates (Figure 3.1 and Table 3.5). No isolates were identified to be both MAT1-1 and MAT1-2 based on PCR results.

Table 3.5 Mating Type Detection by PCR. + indicates detection of the expected size PCR amplicon when visualized by gel electrophoresis. – indicates no amplicon.

Fo f. sp./ host plant	Isolate Code	MAT1-1 ●	MAT1-2 ○
ciceris	Foc8605 (R2)	-	+
ciceris	Foc8607 (R4)	-	+
ciceris	Foc82102 (R0)	-	+
ciceris	Foc8012 (R5)	-	+
ciceris	Foc 1992 R3N (R3)	-	+
ciceris	Foc 9093 PV1 (R6)	-	+
ciceris	Foc7989 (R1A)	+	-
ciceris	Foc USA 3-1 JG62 (R1B/C)	-	+
chickpea	7-96 Tunisia	-	+
chickpea	cc44J	-	+
chickpea	Fo448	+	-
chickpea	Fo802	+	-
chickpea	Fo804	-	+
chickpea	Fo816	+	-
chickpea	Mx6	+	-
chickpea	Mx4	-	+
chickpea	ET2A	+	-
chickpea	ET4A	-	+
chickpea	ET7A	+	-
chickpea	ET9A	+	-
chickpea	ET10A	+	-
chickpea	ET11A	-	+
chickpea	ET12A	-	+
chickpea	ET14A	-	+
chickpea	ET19A	+	-
tomato	1-2c1	+	-
tomato	1-2c2	-	+
tomato	1-4st4	+	-
tomato	2s6	+	-
tomato	2s37	+	-
tomato	2s38	-	+
tomato	2-1c1	+	-
tomato	2-1r1	+	-
tomato	2-2st1	+	-
tomato	2-3c3	+	-
tomato	2-4st1	+	-
tomato	2-4r1	+	-
tomato	3s1	+	-
tomato	3s36	-	+
tomato	3-1r1	+	-
tomato	3-4st1	+	-
tomato	3-4st4	+	-
tomato	4s38	-	+
tomato	4-2st1	+	-
tomato	4-4r1	-	+
tomato	FL1c1	-	+
tomato	FL3st2	+	-
tomato	GM1r2	-	+
banana	EC1-LR-CV1	-	+

SIX Gene Detection

Tables 3.5 and 3.6 summarize the detection of *SIX* genes in the *Fo* isolates screened in this study by PCR and by Sanger sequencing, respectively. All discussion in the results refers to *SIX* gene detection by Sanger sequencing, as not all PCR detections yielded sequencing results of high enough quality that could be identified as any known *SIX* gene. Non-target amplicon sizes and sequencing products were concluded to be artifacts of PCR.

Twenty-nine endophytes of tomato, 19 endophytes of chickpea, and eight *Fo* f. sp. *ciceris* were screened (53 isolates total). There were eight detections of *SIX8;* all eight detections were in *Fo* f. sp. *ciceris* isolates. The detection rate in the isolates screened was 14%. *SIX9* was detected in five isolates; all were endophytes of chickpea; the detection rate of *SIX9* in the isolates screened was 26.3%. *SIX11* was detected in five isolates. Three of these detections were in three isolates characterized as *Fo* f. sp. *ciceris* (37.5% of all *Fo* f. sp. *ciceris* isolates screened). One detection was in an endophyte of tomato (2-1c1) (3.4% of all *Fo* endophytes from tomato screened). One detection was in an endophyte of chickpea (cc44) (5.3% of all *Fo* endophytes from chickpea screened). *SIX14* was detected in nine isolates. Eight of these detections were in isolates characterized as *Fo* f. sp. *ciceris* (100% of all *Fo* f. sp. *ciceris* isolates screened). One detection was in an endophyte off chickpea (cc44) (5.3% of all *Fo* endophytes from chickpea screened). *SIX1* was detected in one isolate, characterized as *Fo* f. sp. *ciceris* (*Foc*82108).

In this study we screened SIX genes in representatives of all races of *Fo* f. sp. *ciceris*, with the first report of *SIX1* in *Fo* f. sp. *ciceris.* A previous study by Williams et al. (2016) described the presence of *SIX5, SIX8, SIX13,* and *SIX14* based on *in silica* analysis of the genome sequence of isolate *Fo* f. sp. *ciceris* 38-1 (of unknown race). In our study, *SIX14* was detected in all isolates of *Fo* f. sp. *ciceris* tested. *SIX8* was detected in all *Fo* f. sp. *ciceris* isolates tested except Race 1BC (ciceris USA 3-1 JG62). Race 0 (ciceris 82108) also had a homologue of *SIX1.*

In this study we also detected the presence of *SIX* genes in *Fo* isolates non-pathogenic to tomato and chickpea and confirmed their gene identity by Sanger sequencing. Homologues of *SIX8, SIX11,* and

SIX14 were detected in isolate cc44J, a non-pathogenic isolate from chickpea from Israel. The *SIX8* sequence from cc44J, however, was more similar to the sequence of *SIX8* from the banana race 1 isolate EC44-M-GM2 than it was to *SIX8* sequences from *Fo* f. sp. *ciceris* (Figure 3.2). Based on *TEF1-alpha, 04642, 06412, 10995, 15960,* and *15695*, cc44J also does not group with *Fo* f. sp. *ciceris.* This is important, as *Fo* f. sp. *ciceris* has been shown to be of monophyletic origin (Jiménez-Gasco et al. 2002; Demers et al. 2014). *Fo* f. sp. *ciceris* group closely with Fom009 and Fon002, pathogens of melon and watermelon, respectively.

Thirteen isolates showed PCR amplification for *SIX1.* When sequenced, these thirteen sequences aligned to each other but did not match any known sequence of *SIX1* in the GenBank database. This region showed an 80% sequence identity to an unnamed protein product from *F. venenatum* strain A3/5 (GenBank Accession number LN649231.1), although no publication is associated with this accession. This sequence was identified in isolates 1-4st1, 1-4r3, 2-1r2, 2s6, FL1c1, GM4st4/Fo74, MF2r1, MF4st2/Fo52, cc44, Fo802, Fo804, Mx9, and *Foc*8607. Eight of these isolates are endophytes of tomato, four are endophytes of chickpea, and one is characterized as *Fo* f. sp. *ciceris.*

Maximum Parsimony phylogenetic analysis revealed *SIX8* sequences from *Fo* f. sp. *ciceris* grouped together (Figure 3.2). As observed in the phylogenetic tree, there are two alleles of *SIX8,* named *SIX8a* and *SIX8b* (Fraiser-Smith et al. 2014). *SIX8* from *Fo* f. sp. *ciceris* appears to be homologous to *SIX8a.* The *SIX8a* from *Fo* f. sp. *ciceris* was most closely related to *SIX8a* from *Fo* f. sp. *passiflorae* and *Fo* f. sp. *cubense.* The isolate cc44J, an endophyte of chickpea, was most closely related to EC44-M-GM2, a Race 1 isolate of *Fo* f. sp. *cubense;* cc44J did not group with *Fo* f. sp. *ciceris* based on *SIX8* sequences nor the core genome.

SIX9 was only detected in endophytes of chickpea; it was the *SIX* gene most commonly encountered in the endophytes screened in this study, as it was in Magdama (2017) that studied endophytes from banana and Rocha et al. (2015) that focused on *Fo* isolates from soil. Isolates ET2A and ET23A were most related to *Fo* f. sp. *cubense* based on *SIX9* sequences (Figure 3.3). Fo816 was closely

related to *Fo* ff. ssp. *cepae, narcissi,* and *dianthi.* This group was closely related to a group of banana endophytes, the same group studied in Appendix A. Isolate ET12A did not group with other isolates. Isolates that group together based on *SIX9* did not group together based on their core genomes.

SIX11 was detected in *Fo* f. sp. *ciceris,* one endophyte of chickpea (cc44), and one endophyte of tomato (2-1c1). *Fo* f. sp. *ciceris* 8607 and *Fo* f. sp. *ciceris* 82108 group with cc44, 2-1c1, and *Fo* f. sp. *pisi*; this group is most closely related to *Fo* ff. ssp. *passiflorae* and *niveum* (Figure 3.4). *Fo* f. s. *ciceris* 8605 grouped separately from these groups. Isolates that group together based on *SIX11* did not group together based on their core genomes.

SIX14 was detected in *Fo* f. sp. *ciceris* and two endophytes of chickpea, cc44J and 7-96 Tunisia. Based on the core genome, isolate cc44J belongs to Clade 4 (Figure 3.1) and 7-96 Tunisia belongs to Clade 6B. *Fo* f. sp. *ciceris* 7989, 8605, 82108, 9093 PV1, 1992 R3N, and USA 3-1 JG62 were closely related based on *SIX14* sequences (Figure 3.6) and are closely related based on their core genome (Figure 3.1, Clade 6B). Isolate cc44J was most closely related to the group of *Fo* f. sp. *ciceris* based on the sequence of *SIX14;* next most closely related is *Fol1080.* Based on *SIX14,* isolate 7-96 Tunisia was related to *Fo* f. sp. *cepae* and *Fo* f. sp. *ciceris* 8012 and groups separately form the other *Fo* f. sp. *ciceris* isolates.

Table 3.6 *Fusarium oxysporum* isolates with PCR amplicons from *SIX* genes detection primer pairs. The size of the PCR amplicon, visualized by gel electrophoresis, is shown for each column. – indicates no known *SIX* gene was sequenced. Isolates tested with no detection are not shown. There were no detections for *SIX3, SIX4, SIX5, SIX6,* and *SIX10* and these genes are not listed in the table.

Fo f. sp./ host plant	Isolate code	*SIX1*	*SIX2*	*SIX7*	*IX8*	*SIX9*	*SIX11*	*SIX13*	*SIX14*
ciceris	USA 3-1JG62 (R1BC)	-	-	-	-	-	-	-	300bp
ciceris	8012 (R5)	500bp, 1kb	-	750bp	250bp	-	-	399bp	300bp, 1kb
ciceris	8605 (R2)	500bp, 1kb	-	750bp	250bp	-	300bp	399bp	300bp
ciceris	9093 PV1 (R6)	500bp, 1kb	-	-	250bp	-	-	-	300bp
ciceris	1992 R3N (R3)	500b, 1kb	1.7kb	-	250bp	-	-	-	300bp
ciceris	82108 (R0)	260bp	-	750bp	250bp	-	300bp	399bp	300bp
ciceris	8607 (R4)	500bp, 1kb	-	750bp	250bp	-	300bp	399bp	300bp
ciceris	7989 (R1A)	500bp, 1kb	-	-	250bp	-	300bp	399bp	-
chickpea	7-96 Tunisia	1kb	-	-	250bp	1kb	1kb	-	-
chickpea	cc44J Israel	1kb, 800bp	-	-	-	1kb	2kb	-	-
chickpea	ET10A	-	-	-	-	400bp	-	-	-
chickpea	ET11A	-	-	-	-	400bp	-	-	-
chickpea	ET12A	-	660bp	-	-	400bp	-	1kb	-
chickpea	ET14A	-	-	-	-	1kb	-	-	-
chickpea	ET15A	-	-	-	-	1kb	-	1kb	-
chickpea	ET16B	800bp	-	-	-	-	-	-	-
chickpea	ET23A	-	-	-	-	1kb	-	-	-
chickpea	ET2A	-	660bp	-	-	1kb	-	750bp	-
chickpea	ET4A	-	660bp, 1kb	-	-	-	-	-	-
chickpea	ET5A	800bp	-	-	-	-	-	-	-
chickpea	ET9A	-	-	-	-	-	-	1kb	-
chickpea	Fo802 Italy	800bp	-	-	-	1kb	-	-	-
chickpea	Fo804 Italy	800bp	-	-	-	1kb	-	-	-
chickpea	Fo816 Italy	-	-	-	-	1kb	-	-	-
chickpea	Fo448	1kb	-	-	-	-	-	-	-
chickpea	Mx446	1kb	-	-	-	-	-	1kb	-
chickpea	Mx6	1kb	-	-	-	-	-	-	-
chickpea	Mx9	800bp, 1kb	-	-	-	-	300bp	-	-
chickpea	Mx4	-	-	750bp	-	-	-	-	-
tomato	1-1r3	1kb, 800bp	-	-	-	-	-	-	-
tomato	1-2r2	1kb	-	-	-	-	-	-	-
tomato	1-4r3	800bp, 1kb	-	-	-	-	-	-	-
tomato	1-4st1	1kb	-	-	-	-	-	-	-
tomato	2-1c1	-	660bp	-	-	1000bp	2kb	-	-
tomato	2-1r2	1kb	2kb	-	-	-	-	-	-
tomato	2-2st2/Fo48	-	660bp	-	-	-	-	-	-
tomato	2-3c3/Fo68	-	660bp	-	-	-	-	-	-
tomato	2-4r1	-	660bp	-	-	-	-	-	-
tomato	2s38	-	2kb	-	-	-	-	-	-
tomato	3-2c2/ Fo69	1kb	660bp, 2kb	-	-	-	-	-	-
tomato	3-4st1	1kb	-	-	-	-	-	-	-
tomato	3s1	1kb	-	-	-	-	-	-	-
tomato	3s36	1.5kb, 1kb, 700bp	-	-	-	-	-	-	-
tomato	4-2r2	1kb	-	-	-	-	-	-	-
tomato	4-4r1	1kb	-	-	-	-	-	-	-
tomato	4s38	1kb, 700bp	-	-	-	-	-	-	-
tomato	FL1c1	1kb	-	-	-	-	-	-	-
tomato	GM1r2	-	660bp	-	-	-	-	-	-
tomato	GM4st4/Fo74	1kb	-	-	-	-	-	-	-
tomato	MF2r1	1kb	-	-	-	-	-	-	-

Table 3.7 *Fusarium oxysporum* isolates with detection of *SIX* genes verified by Sanger sequencing. + indicates the detection a *SIX* gene by PCR amplification and verification with Sanger sequencing. – indicates no known *SIX* gene was sequenced. * indicates the listed *SIX* genes were detected by Magdama (2017) and are listed for comparative purposes in this study. N indicates these isolates were not tested for the listed loci.

Fo f. sp./ host plant	Isolate code	*SIX1*	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*	*SIX8*	*SIX9*	*SIX10*	*SIX11*	*SIX13*	*SIX14*
cubense*	0-2052 (TR4)	+	+	-	-	-	+	-	+	+	N	N	+	-
cubense*	0-1966 (SubTR4)	+	+	-	+	-		+	+	+	N	N	-	-
cubense*	O-1968 (R1)	+	-	-	+	-	+	-	-	+	N	N	+	-
cubense*	FOC (R2)	+	-	-	+	-	+	-	-	+	N	N	+	-
cubense*	0-1080 (R2)	+	+	+	N	+	+	+	+	+	N	N	+	+
cubense*	EC44-M-GM2	+	+	-	+	-	-	+	+	+	N	N	-	-
cubense*	CAV027-EG	-	-	-	-	-	-	-	-	+	N	N	-	-
cubense*	CAV034-EG	-	-	-	-	-	-	-	-	-	N	N	-	-
cubense*	CAV030-ELR	N	N	N	N	N	N	N	N	N	N	N	N	N
cubense*	CAV029-ELR	N	N	N	N	N	N	N	N	N	N	N	N	N
cubense*	CAV044-EO	-	+	-	-	-	-	-	-	-	N	N	-	-
cubense*	CAV05-EO	-	-	-	-	-	-	-	-	-	N	N	-	-
cubense*	CAV017-EO	N	N	N	N	N	N	N	N	N	N	N	N	N
ciceris	1992 R3N (R3)	-	-	-	-	-	-	-	+	-	-	-	-	+
ciceris	7989 (R1A)	-	-	-	-	-	-	-	+	-	-	-	-	+
ciceris	9093 PV1 (R6)	-	-	-	-	-	-	-	+	-	-	-	-	+
ciceris	8012 (R5)	-	-	-	-	-	-	-	+	-	-	-	-	+
ciceris	8605 (R2)	-	-	-	-	-	-	-	+	-	-	+	-	+
ciceris	8607 (R4)	-	-	-	-	-	-	-	+	-	-	+	-	+
ciceris	82108 (R0)	+	-	-	-	-	-	-	+	-	-	+	-	+
ciceris	USA 3-1 JG (R1BC)	-	-	-	-	-	-	-	-	-	-	-	-	+
chickpea	7-96 Tunisia	-	-	-	-	-	-	-	+	-	-	-	-	+
chickpea	cc44	-	-	-	-	-	-	-	-	-	-	+	-	+
chickpea	Fo804	-	-	-	-	-	-	-	-	-	-	-	-	-
chickpea	Fo802	-	-	-	-	-	-	-	-	-	-	-	-	-
chickpea	Fo816	-	-	-	-	-	-	-	-	+	-	-	-	-
chickpea	Mx9	-	-	-	-	-	-	-	-	-	-	-	-	-
chickpea	ET2A	-	-	-	-	-	-	-	-	+	-	-	-	-
chickpea	ET19A	-	-	-	-	-	-	-	-	+	-	-	-	-
chickpea	ET23A	-	-	-	-	-	-	-	-	+	-	-	-	-
tomato	MF2r1	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	MF4st2/ Fo52	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	GM4st4/Fo74	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	FL1c1	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	1-4st1	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	1-4r3	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	2s6	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	2-1r2	-	-	-	-	-	-	-	-	-	-	-	-	-
tomato	2-1c1	-	-	-	-	-	-	-	-	-	-	+	-	-

Figure 3.2 Phylogenetic relationships among *SIX8* sequences of *Fusarium oxysporum* isolates. One of ten most parsimonious trees is displayed (CI: 0.86; RI: 0.97). Bootstrap values are shown for 1000 replicates. There were a total of 96 positions in the final dataset. All positions with less than 95% site coverage were eliminated. Yellow background represents isolation from banana; orange from chickpea.

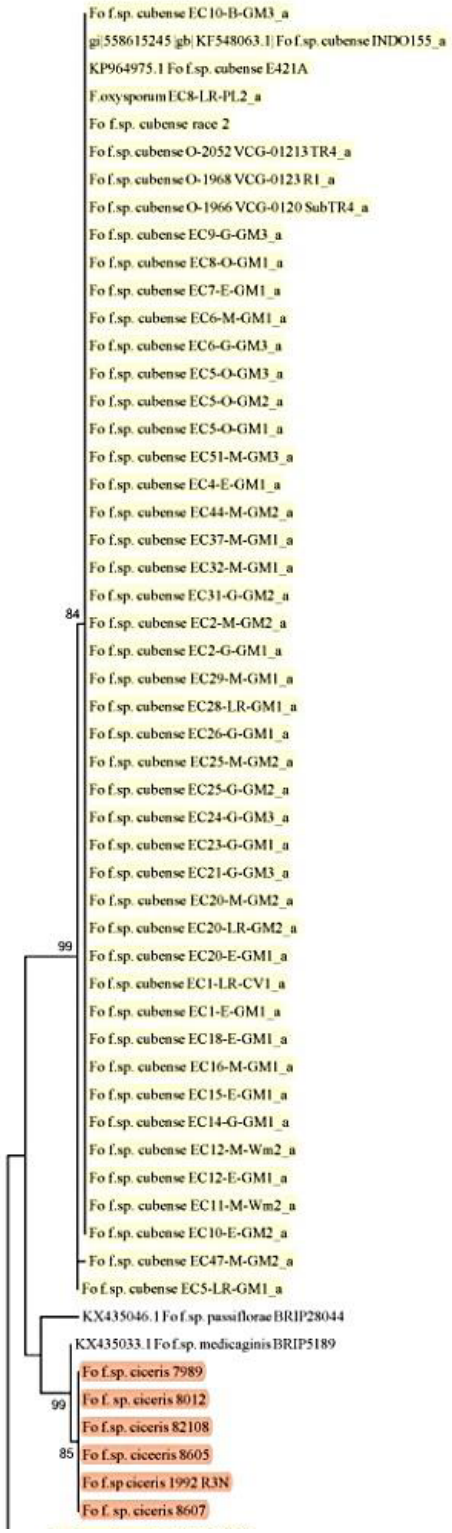


Figure 3.2 (continued) Phylogenetic relationships among *SIX8* sequences of *Fusarium oxysporum* isolates (continued). One of ten most parsimonious trees is displayed (CI: 0.86; RI: 0.97). Bootstrap values are shown for 1000 replicates. There were a total of 96 positions in the final dataset. All positions with less than 95% site coverage were eliminated. Yellow background represents isolation from banana; orange from chickpea.

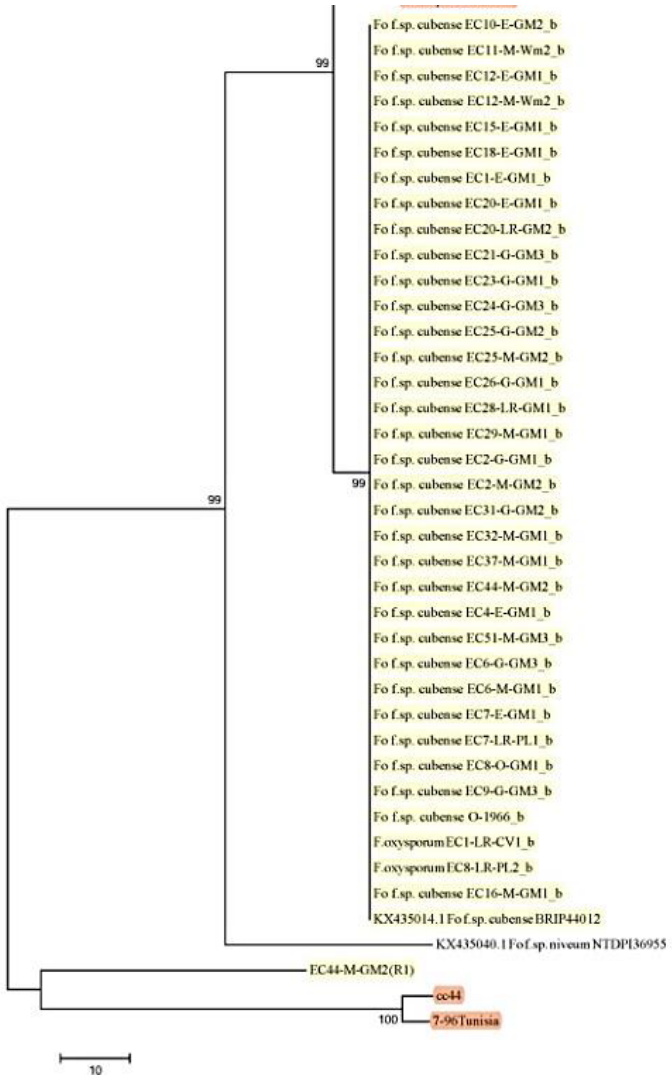


Figure 3.3 Phylogenetic relationships among *SIX9* sequences of *Fusarium oxysporum* isolates. One of four most parsimonious trees is displayed (CI: 0.75; RI: 0.97). Bootstrap values are shown for 1000 replicates. There were a total of 232 positions in the final dataset. All positions with less than 95% site coverage were eliminated. Green background represents isolation from tomato; yellow from banana; orange from chickpea.

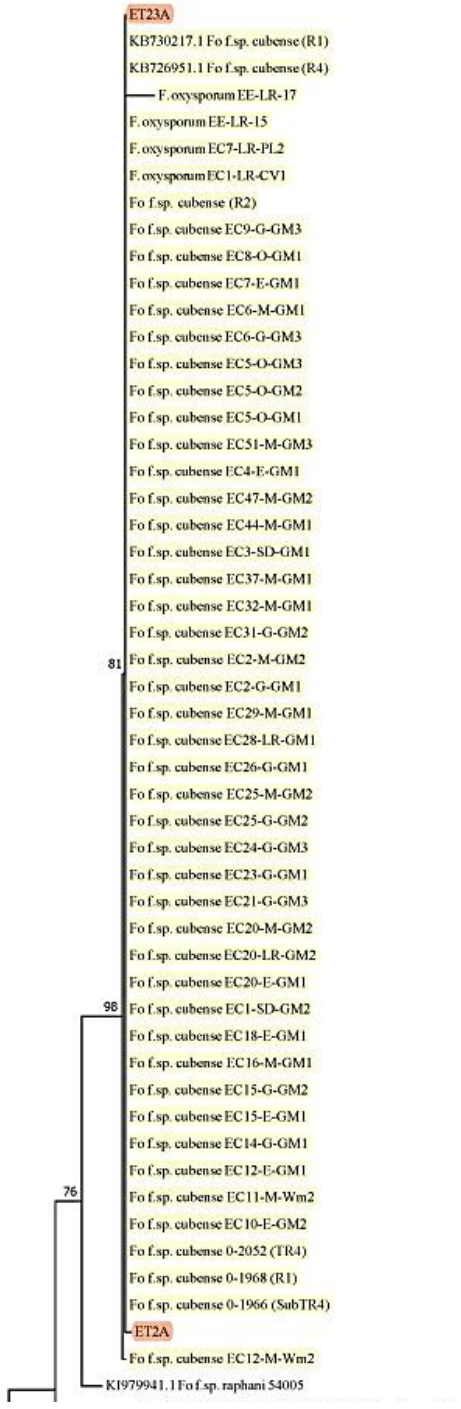


Figure 3.3 (continued) Phylogenetic relationships among *SIX9* sequences of *Fusarium oxysporum* isolates. One of four most parsimonious trees is displayed (CI: 0.75; RI: 0.97). Bootstrap values are shown for 1000 replicates. There were a total of 232 positions in the final dataset. All positions with less than 95% site coverage were eliminated. Green background represents isolation from tomato; yellow from banana; orange from chickpea.

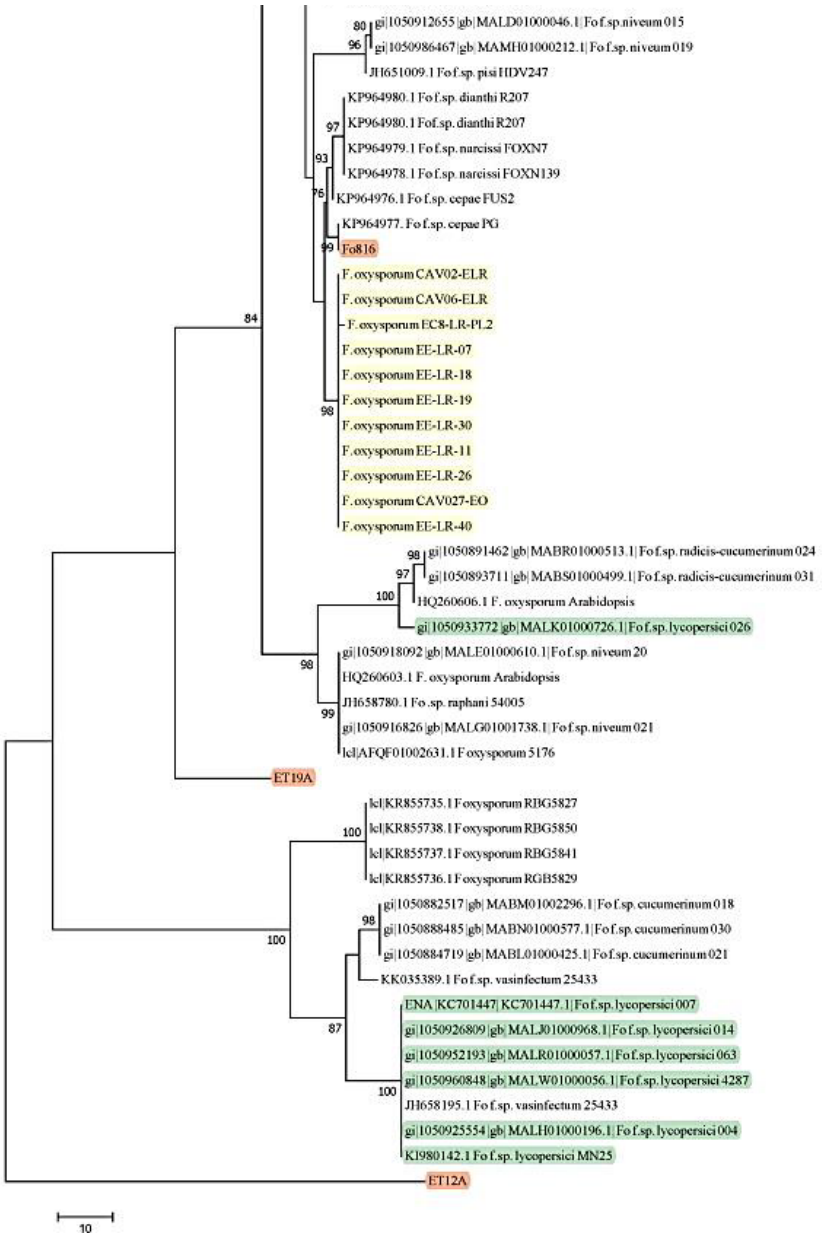


Figure 3.4 Phylogenetic relationships among *SIX11* sequences of *Fusarium oxysporum* isolates. One of ten most parsimonious trees is displayed (CI: 1.0; RI: 1.0). Bootstrap values are shown for 1000 replicates. There were a total of 216 positions in the final dataset. All positions with less than 95% site coverage were eliminated. Green background represents isolation from tomato; orange from chickpea.

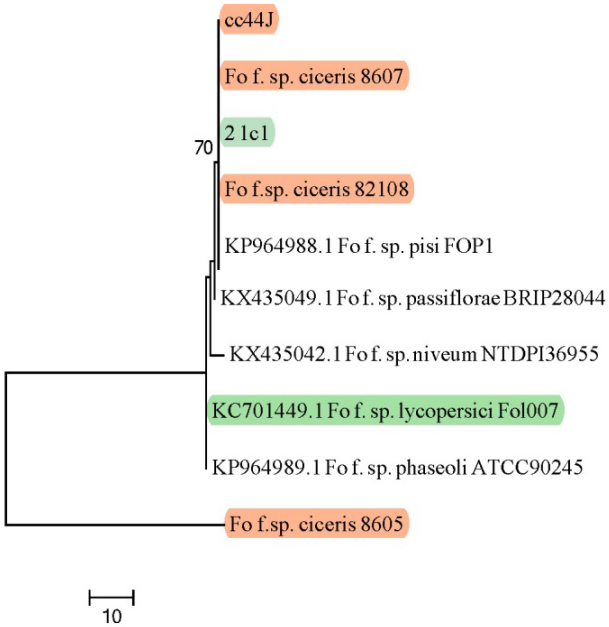
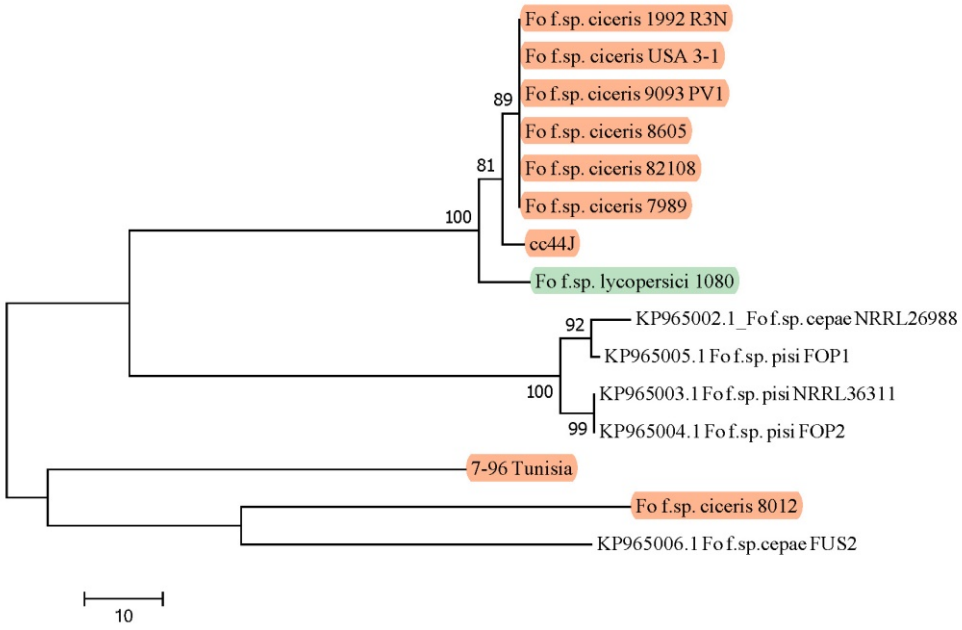


Figure 3.5 Phylogenetic relationships among SIX14 sequences of Fusarium *oxysporum* isolates. One of ten most parsimonious trees is displayed (CI: 0.81; RI: 0.88). Bootstrap values are shown for 1000 replicates. There were a total of 162 positions in the final dataset. All positions with less than 95% site coverage were eliminated. Green background represents isolation from tomato; orange from chickpea.



Discussion

The objective of this research was to examine relationships between endophytic and pathogenic isolates from banana, chickpea, and tomato hosts with the goal of determining patterns based on host and geographic origins and plant pathogenicity. Phylogenetic analysis with five novel loci, *SIX* gene detection and phylogenetic analysis, and mating type determination were used to examine patterns in the *Fo* isolates studied. This research developed new phylogenetic markers that add 438 parsimony-informative characters to the standard locus *TEF1-α,* which provided 64 parsimony-informative characters. The MP tree generated using all six sequence regions was congruent with MP trees based on 41 core genome loci, and provides higher resolution than the current standard *TEF1-α* and resolution congruent to 41 core genome loci. This research also studied phylogenetic relationships between *Fo* endophytes from banana, chickpea, and tomato, and of those and isolates pathogenic to banana, chickpea, tomato, as well as other hosts. Results indicate that in most cases endophytes from banana, chickpea, and tomato are not phylogenetically related and do not share common ancestors based on host of origin, although some exceptions were identified. For example, some of the non-pathogenic isolates studied were closely related with pathogenic isolates. The presence of *SIX* genes was detected in endophytes from tomato and chickpea, and their PCR-based detection was confirmed by amplicon sequencing. *SIX* genes were also studied in all races of *Fo* f. sp. *ciceris*, the causal agent of Fusarium wilt of chickpeas. Interestingly, phylogenetic relationships of *SIX* gene sequences did not necessarily show patterns based on pathogenicity or host of origin. Finally, a selection of the isolates studied were typed as MAT1-1 and MAT1-2 based on PCR analysis. As expected, isolates of the same and opposite mating types grouped closely together (Arie et al. 2000; Lievens et al. 2009; Yun et al. 2000), perhaps providing indication for potential cryptic sexual reproduction in the species (Taylor et al. 2009). In the search for sexual reproduction in *Fo,* isolates from this study, especially those closely related in clades containing both MAT1-1 and MAT1-2 isolates, could be considered for further investigation.

Five novel core genome loci and *TEF1-α* improve phylogenetic analysis of *Fo*

The five core-genomic markers developed in this research for phylogenetic studies (*04642, 06412, 10995, 15960,* and *15695)*, in addition to *TEF1-α*, increase the confidence in conclusions made based on phylogenetic analyses. Patterns observed in previous phylogenetic studies using *TEF1-α* are consistent with the results yielded by adding these five new genes. The primers generated in this research targeting such genes for sequencing had high rates of success (>90%) in PCR amplification and Sanger sequencing. Using the same reference isolates, the MP phylogeny was congruent to a MP phylogeny generated using 41 core genome sequences based on whole genome sequences of the analyzed isolates (Geiser, Jiménez-Gasco, and Rep, unpublished data). Groups of *Fo* from chickpea and *Fo* f. sp. *ciceris* were congruent to previous analysis based on *TEF1-α* (Demers 2012). The phylogenetic structure of *Fo* isolates from banana and *Fo* f. sp. cubense was also congruent with the phylogenetic analysis based on *TEF1-α* (Magdama 2017), but provided resolution with bootstrap support of several previously unresolved groups. Thus, these regions, amplified and sequenced with the primers developed in this research, can provide a quick, affordable, and reliable alternative to whole genome sequencing for determining phylogenetic relationships between members of *Fo* while improving the phylogenetic resolution and increasing the number of phylogenetically-informative characters considered in single-gene based phylogenetic analysis.

Affordable means of phylogenetic analysis are especially important as research grows to include endophytic *Fo* isolates, which represent a majority of the diversity of the species, but have been little studied up to this point (Alves-Santos et al. 1999; Appel and Gordon 1994; Balmas et al. 2010; Deltour et al. 2018; Demers et al. 2015; Edel et al. 2001; Elias et al. 1991; Gordon and Okamato 1992; Katan et al. 1994; Laurence et al. 2012; Lori et al. 2004). Many non-pathogenic isolates from many host plants and geographic origins must be included in future analyses in order to understand the full diversity of *Fo* as a species, and to delineate species boundaries within this group, and whole genome sequencing of many isolates will not be economically feasible for many research groups in the near future. Sanger sequencing of six loci is an affordable means of sequencing and inferring phylogenetic relationships which will

improve the accessibility of phylogenetic studies for research groups around the globe, thus laying the foundation for increased knowledge generation on *Fo.* By using the primers developed in this study, increased confidence in phylogenetic analysis will also be comparable across research groups, which can aid international collaboration.

Phylogenetic analysis based on five novel core genome loci and *TEF1-α* suggests diverse evolutionary origins of endophytes and *SIX* genes

The MP tree produced in this study yielded groups that combined isolates from many different hosts, pathogenic isolates, and non-pathogenic isolates. When particular groups are examined individually, isolates representing all the pathogenic diversity of *Fo* f. sp. *ciceris* group together, some endophytes of tomato group together, and some endophytes of banana group together in distinct clades. Non-pathogenic and pathogenic isolates also group together.

Groups of isolates mostly from tomato roots and tomato soils, such as the group of isolates in Clades 1 and 3C (Figure 3.1) might be explained by considering the ecological niche of these isolates to be the soil and superficial interactions with plant roots. In contrast, groups of isolates which include pathogenic and non-pathogenic isolates from the same plant, such as Clades 6A, 6B, 6D, might be explained by considering the ecological niche of these isolates to be adapted to interaction with their plant hosts. Differences between the pathogenic and non-pathogenic isolates which group together based on their core genomes have yet to be discovered. However, we speculate that agricultural ecosystems select for host-adapted *Fo,* as host adaptation could ensure reproductive success and dissemination of *Fo.* In non-agricultural ecosystems, adapting to the host plant may not be advantageous, as it may be difficult to locate a new suitable host-plant to colonize. Likewise, causing aggressive disease which quickly kills the host-plant can lead to an evolutionary dead end for a microorganism, as it may be difficult to locate a new suitable host-plant to colonize. However, under agricultural conditions, where most *Fo* isolates studied originate from, *Fo* isolates which cause disease may not be at an evolutionary disadvantage as they would be in wild settings, where the next suitable host-plant is close by. Thus, we speculate that agroecosystems

may select for host-adapted *Fo* and allow for disease-causing isolates to develop. This hypothesis requires further studies with a diversity of *Fo* isolates from wild settings.

Fo f. sp. *ciceris* shows a distinct monophyletic clade, consistent with previous results (Jiménez-Gasco et al. 2002; Demers et al., 2014). Unlike *Fo* f. sp. *ciceris,* endophytes isolated from chickpea were not closely related to each other and grouped with isolates from other hosts, non-pathogenic as well as plant pathogenic. The exception is 7-96 Tunisia, which groups with isolates of *Fo* f. sp. *ciceris*. Isolate 7-96 Tunisia was tested for pathogenicity to the chickpea universal susceptible cultivar 'P2254' and confirmed to be non-pathogenic to chickpea (Demers 2012). In addition, 7-96 Tunisia tested positive for *SIX8* and *SIX14*, genes which were also detected in most isolates of *Fo* f. sp. *ciceris* screened. However, based on *SIX14,* 7-96 Tunisia groups apart from *Fo* f. sp. *ciceris* isolates and non-pathogenic isolate cc44J. These different *SIX14* alleles may represent a sequence changes in the *SIX14* protein product which could alter host-plant recognition, facilitating pathogenicity on the host.

Furthermore, based on *SIX8* sequences, isolate 7-96 Tunisia and cc44J group separately from *Fo* f. sp. *ciceris* isolates. As proposed for *SIX14,* the differences in *SIX8* sequence may represent polymorphisms in *SIX8* which result in a change (loss or gain) of host-plant recognition. Due to its profile of *SIX* genes and grouping with *Fo* f. sp. *ciceris,* chickpea endophyte 7-96 Tunisia may represent the link between the endophytic and pathogenic lifestyles in the chickpea host. Thus, future comparative studies between isolates of *Fo* f. sp. *ciceris* and 7-96 Tunisia have the potential to be informative in understanding the differences between *Fo* isolates pathogenic and non-pathogenic to chickpea, and uncover potential mechanisms of pathogenicity.

Based on the presence of *SIX8, SIX11,* and *SIX14* in *Fo* f. sp. *ciceris* isolates, we speculate that these genes may be related to host adaptation to chickpea and different cultivars of chickpea. Due to their presence in 7-96 Tunisia and cc44J, which are non-pathogenic to chickpea as confirmed by testing on the universal susceptible cultivar 'P2245' (Demers 2012), we speculate that these genes may be more specifically involved in *Fo*-host interactions rather than explicit pathogenicity. The data in this chapter cause speculation that some, but not all, *SIX* genes are involved in host recognition of a colonizing

microbe, and differences in host recognition can lead to differing pathogenicity phenotypes. Further study of *in planta* expression of the *SIX* genes detected in this study, and protein conformation predictions could test this hypothesis.

Furthermore, the detection of both MAT1-1 and MAT1-2 in the *Fo* f. sp. *ciceris* clade may explain the high diversity of races pathogenic to chickpea present in this monophyletic group. If cryptic sexual reproduction exists in *Fo,* perhaps *Fo* f. sp. *ciceris* has generated some of its phenotypic traits from recombination. Both mating type loci should be sequenced from these isolates of *Fo* f. sp. *ciceris* to confirm their identities and functionalities.

Interestingly, there are also groups of endophytes and soil-isolates from tomato and banana throughout the phylogeny, all without detection of any *SIX* genes and which have detections of MAT1-1 or MAT1-2 in each isolate. There are no correlations between the groups and the niche from which the isolate originated (root, crown, stem, or surrounding soil). There is also one group of banana endophytes isolated from Cavendish banana plants which all also includes isolates of both MAT1-1 or MAT1-2. CAV026-EG has a homologue of *SIX2,* whose sequence groups with *SIX2* sequences from *Foc* isolates (Magdama 2017). No other banana endophyte from this clade has detection of any other *SIX* gene. Based on a previous study demonstrating *in vitro* horizontal gene transfer in *Fol* (Ma et al. 2010), it is possible that horizontal gene transfer occurred between CAV026-EG and a *Foc* isolate.

Further evidence for horizontal gene transfer can be observed by examining the distribution of isolates with a *SIX* gene sequence in the phylogeny. Tomato endophyte 2-1c1 does not group near other isolates with *SIX11* sequences. The same is true for ET19A and ET23A in the case of *SIX9.* Chickpea endophyte Fo816 is even determined to be a clone at the six loci studied to endophytes of tomato, but contains *SIX9* sequence that groups with endophytes of banana. One group of *Fo* isolates from banana includes both pathogenic and non-pathogenic isolates. Interestingly, EC1-LR-CV1 (race 1), and endophytes CAV044-EO and CAV038-ELR and the *Foc* isolates in this group all contain identical

sequences of *SIX2* (Magdama 2017). The sequence of *SIX2* is also identical to that of CAV026-EO, belonging to the banana endophyte group.

Sequence comparison of *SIX* gene sequences showed isolates of a given forma specialis do not always share identical alleles. Previous studies showed isolates pathogenic to the same host share nearly identical *SIX* alleles. *SIX* genes and their surrounding regions have been proposed as a detection method for formae speciales (Frasier-Smith et al. 2014; Hogg et al. 2007; Magdama 2017; van Dam et al. 2016; van Dam et al. 2017). In this study, *Fo* f. sp. *ciceris* isolates harbored different *SIX14* and *SIX11* alleles, despite this forma specialis being monophyletic. The sequence of *SIX11* from tomato endophyte 2-1c1 was more similar to *SIX11* sequences from *Fo* f. sp. *ciceris* 8607, 82108, and chickpea endophyte cc44J than to *Fo* f. sp. *lycopersici* isolate Fol007 (GenBank accession KC701449). Chickpea endophytes ET2A and ET23A had *SIX9* alleles similar to *Foc*, and *SIX9* alleles of chickpea endophyte Fo816 grouped with banana endophytes. Chickpea endophyte cc44J and *Foc* race 1 isolate EC44-M-GM2 shared closely related *SIX8* alleles.

Taken together, the diversity found in *SIX* gene sequences of isolates pathogenic to and colonizing the same host offers an explanation for false positives described by van Dam et al. (2017) in their molecular detection assay for *Fo* strains targeting cucurbit plant species. Some assays are based on pathogenicity-associated regions, such as *SIX* genes and regions flanking them, which thus far have been shown to be specific to a forma specialis (Fraser-Smith 2014; van Dam 2017) and IGS regions (Dita et al. 2010). The revelation that *SIX* genes and pathogenicity-associated regions may not be host-specific is crucial to developing molecular tests for plant-pathogenic formae speciales of *Fo,* which are in high demand for early diagnosis, plant quarantine, and assessment of potential biocontrol strains of *Fo* (Wang et al 2013). Conclusions made about the specificity of a *SIX* gene sequence to pathogenicity must be limited to a single pathosystem, and primers for detection must be tested against a large diversity of endophytes.

Previous studies, such as that of Jelinski et al. (2017) used PCR to detect the presence of *SIX* genes in *Fo* isolates obtained from soil. However, in their study, PCR amplicons were not sequenced. As can be observed from comparing PCR detections of *SIX* genes and confirmation by Sanger sequencing, many PCR amplicons were determined to be non-target amplifications. Thus, sequencing is crucial in the detection of *SIX* genes and is strongly recommended as a standard for future studies which aim to detect *SIX* genes. Since detected *SIX* genes were sequenced it was also possible to analyze the relationships the *SIX* genes using phylogenetic methods.

Conclusions

This study is unique in combining endophytes from multiple hosts to compare their phylogenetic relationships. This study showed that as in banana and tomato *Fo* endophytes and their respective formae speciales, endophytes and pathogens from different hosts are phylogenetically closely related. This is the first study to report Sanger sequenced *SIX* genes in *Fo* endophytes from tomato and chickpea. There are no clear patterns based on the isolates' origins from within the host plant, geography, mating type, or the detection of *SIX* genes, indicating that non-pathogenicity and colonization of a particular host species may have emerged multiple times throughout the evolutionary history of *Fo*. It also appears that host specificity is not predicted by the *SIX* genes screened in this study nor the genes analyzed as phylogenetically informative from the core genome. This forces us to consider that the mechanism of pathogenicity is hidden elsewhere in the biology of *Fo.* There is also the possibility that *SIX* genes may be present in isolates for which there was no PCR-based detection, maybe due to polymorphisms at the primer binding sites that would allow *SIX* genes to evade detection. Complicating matters further, pathogenicity may also be determined by the expression of *SIX* genes in different environments. In this study and that of Magdama (2017), *SIX* genes were detected by PCR and confirmed by Sanger sequencing, but were not tested for *in vitro* nor *in planta* expression. It is possible that although *SIX* genes were detected in endophytes, they are not expressed. Studying *SIX* gene expressions in endophytes is an important line of future research, especially for the case of *SIX9*, which shows high rates of detection in

non-pathogenic isolates. Further study of *SIX* genes and their recognition in different plant hosts is also essential to understanding potential shortcomings of molecular diagnostic tools, which often rely on pathogenicity-associated regions for diagnosis. This study showed that pathogenicity-associated regions are not always host specific and may explain false positives of molecular diagnostic tests. It is thus imperative that further research includes non-pathogenic isolates of *Fo*, as understanding the species diversity and control of pathogenic isolates depends on it.

Literature Cited

About Dole. 2017. Dole. http://www.dole.com/AboutDole

Alves-Santos, F. M. Benito, E. P. Eslava, A. P. Díaz-Mínguez, J. M. 1999. Genetic diversity of *Fusarium oxysporum* strains from common bean fields in Spain. Appl. Environ. Microbiol. 65:3335-3340.

Appel, D. J. Gordon, T. R. 1994. Local and regional variation in populations of *Fusarium oxysporum* from agricultural field soils. Phytopathol. 84:786-791.

Balmas V, Migheli Q, Scherm B, Garau P, O 'Donnell K, Ceccherelli G, et al. 2010. Multilocus phylogenetics show high levels of endemic fusaria inhabiting Sardinian soils (Tyrrhenian Islands). Mycologia 102:803–812.

Bäckhed F, Fraser C, Ringel Y, Sanders M, Sartor R, Sherman P, Versalovic J, Young V, Finlay B. 2012. Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications. Cell Host & Microbe 12:611-622.

Beckman C. 1987. The nature of wilt diseases of plants. St. Paul, Minn. APS Press.

Belgrove A, Steinberg C, Viljoen A. 2011. Evaluation of Nonpathogenic *Fusarium oxysporum* and *Pseudomonas fluorescens* for Panama Disease Control. Plant Disease 95:951-959.

Booth C. 1971. The genus *Fusarium*. Farnham Royal, Bucks. England: Commonwealth Agricultural Bureaux.

Brown D, Busman M, Proctor R. 2014. *Fusarium verticillioides* SGE1 Is Required for Full Virulence and Regulates Expression of Protein Effector and Secondary Metabolite Biosynthetic Genes. Molecular Plant-Microbe Interactions 27:809-823.

Cabello, M. Arambarri, A. 2002. Diversity in soil fungi from undisturbed and disturbed *Celtis tala* and *Scutia buxifolia* forests in the eastern Buenos Aires province, Argentina. Microbiol. Res. 157:115-125.

Chakraborty S, Newton A. 2011. Climate change, plant diseases and food security: an overview. Plant Pathology 60:2-14.

Chiquita. 2017. Chiquita. http://www.chiquita.com/

Colborn T, vom Saal F, Soto A. 1994. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Impact Assessment Review 14:469-489.

Covey P, Kuwitzky B, Hanson M, Webb K. 2014. Multilocus Analysis Using Putative Fungal Effectors to Describe a Population of *Fusarium oxysporum* from Sugar Beet. Phytopathol. 104:886-896.

Czymmek K, Fogg M, Powell D, Sweigar J, Park S, Kang S. 2007. *In vivo* time-lapse documentation using confocal and multi-photon microscopy reveals the mechanisms of invasion into the Arabidopsis root vascular system by *Fusarium oxysporum*. Fungal Genetics and Biol. 44: 1011-1023.

Dita MA, Waalwijk C, Buddenhagen IW, Souza JT, Kema GHJ. 2010. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. Plant Pathol. 59:348–357.

Dong X, Wang M, Ling N, Shen Q, Guo S. 2016. Potential role of photosynthesis-related factors in banana metabolism and defense against *Fusarium oxysporum* f. sp. *cubense*. Environ. and Experimental Botany 129:4-12.

Demers J. 2012. Evolutionary ecology of *Fusarium oxysporum*-plant interactions. Doctoral dissertation. The Pennsylvania State University.

Demers JE, Garzon CD, and Jiménez-Gasco MM. 2014. Striking genetic similarity between races of *Fusarium oxysporum* f. sp. *ciceris* confirms a monophyletic origin and clonal evolution of the chickpea vascular wilt pathogen. European Journal of Plant Pathology 139: 303-318.

Demers J, Gugino B, and Jiménez-Gasco M. 2015. Highly Diverse Endophytic and Soil *Fusarium oxysporum* Populations Associated with Field-Grown Tomato Plants. Applied and Environ. Microbiol. 81: 81-90.

Diener A. 2012. Visualizing and Quantifying *Fusarium oxysporum* in the Plant Host. Molecular Plant-Microbe Interactions 25: 1531-1541.

El-Fattah A, Dababat A, Sikora RA. 2007. Influence of the mutualistic endophyte *Fusarium oxysporum* 162 on *Meloidogyne incognita* attraction and invasion. Nematology 9:771-776.

Elias KS, Schneider RW, Lear MM. 1991. Analysis of vegetative compatibility groups in nonpathogenic populations of *Fusarium oxysporum* isolated from symptomless tomato roots. Can. J. Bot. 69:2089-2094.

FAO. 2003. The World Banana Economy, 1985-2002. Food and Agriculture Organization of the United Nations. http://www.fao.org/docrep/007/y5102e/y5102e04.htm

Fourie G, Steenkamp E, Gordon T, Viljoen A. 2009. Evolutionary Relationships among the *Fusarium oxysporum* f. sp. *cubense* Vegetative Compatibility Groups. Applied and Environmental Microbiology 75:4770-4781.

Fourie G, Steenkamp E, Ploetz R, Gordon T, Viljoen A. 2011. Current status of the taxonomic position of *Fusarium oxysporum* formae speciales *cubense* within the *Fusarium oxysporum* complex. Infection, Genetics and Evolution. 11:533-542.

Fraser-Smith S, Czislowski E, Meldrum R, Zander M, O'Neill W, Balali G, Aitken E. 2014. Sequence variation in the putative effector gene *SIX*8 facilitates molecular differentiation of *Fusarium oxysporum* f. sp. *cubense*. Plant Pathology 63:1044-1052.

García-Bastidas F, Ordóñez N, Konkol J, Al-Qasim M, Naser Z, Abdelwali M, Salem N, Waalwijk C, Ploetz R, Kema G. 2014. First Report of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 Associated with Panama Disease of Banana outside Southeast Asia. Plant Disease 98:694-694.

Geiser D, Jiménez-Gasco MM, Kang S, Makalowska I, Veeraraghavan N, Ward T, Zhang N, Kuldau G, O'Donnell K. 2004. FUSARIUM-ID v. 1.0: A DNA Sequence Database for Identifying Fusarium. European Journal of Plant Pathology 110:473-479.

Gerlach KS, Bentley S, Moore NY, Aitken EAB, Pegg KG. 1999. Investigation of non-pathogenic strains of *Fusarium oxysporum* for suppression of Fusarium wilt of banana in Australia. In: Alabouvette C, ed. Second International Fusarium Workshop. Dijon, France: INRA-CMSE, 945–53.

Ghag SB, Shekhawat UKS, and Ganapathi T. R. 2015. Fusarium wilt of banana: biology, epidemiology and management. Int. J. Pest Manag. 61:250–263.

Gordon T, Martyn R. 1997. The Evolutionary Biology of Fusarium. Annual Review of Phytopathol. 35:111-128.

Gordon TR, Okamoto D. 1992. Variation within and between populations of *Fusarium oxysporum* based on vegetative compatibility and mitochondrial DNA. Can. J. Bot. 70:1211-1217.

Gordon T, Okamoto D, Jacobson D. 1989. Colonization of Muskmelon and Non-susceptible Crops by *Fusarium oxysporum* f. sp. *melonis* and Other Species of *Fusarium*. Phytopathol. 79:1095.

Granchinho SCR, Franz CM, Polishchuck E, Cullen WR, and Reimer KJ. 2002. Transformation of arsenic (V) by the fungus *Melnis* isolated from the alga *Fucus gardneri.* Applied Organometallic Chemistry 16:721-726.

Gregory P, Johnson S, Newton A, Ingram J. 2009. Integrating pests and pathogens into the climate change/food security debate. Journal of Experimental Botany 60:2827-2838.

Gaur PM, Tripathi S, Gowda CLL, Ranga Rao GV, Sharma HC, Pande S, Sharma M. 2010. Chickpea Seed Production Manual. Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. 28 pp.

Haegi A, Catalano V, Luongo L, Vitale S, Scotton M, Ficcadenti N, Belisario A. 2013. A newly developed real-time PCR assay for detection and quantification of *Fusarium oxysporum* and its use in compatible and incompatible interactions with grafted melon genotypes. Phytopathology 103:802-810.

Hall B. 2011. Phylogenetic trees made easy. Sunderland, Massachusetts: Sinauer.

Hallmann J, Sikora RA. 1996. Toxicity of fungal endophyte secondary metabolites to plant parasitic nematodes and soil-borne plant pathogenic fungi. European Journal of Plant Pathol. 102:155-162.

Hennessy, C. Walduck, G. Daly, A. and Padovan, A. 2005. Weed hosts of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in northern Australia. Australasian Plant Pathology. 34: 115.

Hogg AC, Hohnston RH, Dyer AT. 2007. Applying real-time quantitative PCR to fusarium crown rot of wheat. Plant Dis 91:1420-1426.

Houterman, P. Cornelissen, B. and Rep, M. 2008. Suppression of Plant Resistance Gene-Based Immunity by a Fungal Effector. PLoS Pathogens.

Houterman P, Speijer D, Dekker H, De Koster C, Cornelissen B, Rep M. 2007. The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. Molecular Plant Pathol. 8 215-221.

Ito, S. Nagata, A. Kai, T. Takahara, H. and Tanaka, S. 2005. Symptomless infection of tomato plants by tomatinase producing *Fusarium oxysporum* formae speciales nonpathogenic on tomato plants. Physiological and Molecular Plant Pathol. 66 183-191.

Jiménez-Gasco MM, Milgroom MG, Jiménez-Díaz RM. 2002. Gene genealogies support *Fusarium oxysporum* f. sp *ciceris* as a monophyletic group. Plant Pathol. 51: 72-77.

Kandel W, Bruno A, Seelke C, Taft-Morales M. 2015. Unaccompanied Alien Children: Potential Factors Contributing to Recent Immigration. Current Politics and Economics of the United States, Canada and Mexico 17:453-483.

Kang S, Demers J, Jiménez-Gasco M, Rep M. 2014. *Fusarium oxysporum*. In: Genomics of Plant-Associated Fungi and Oomycetes: Dicot Pathogens. Springer-Verlag Berlin Heidelberg. 99-119.

Katan J. 1971. Symptomless carriers of the tomato Fusarium wilt pathogen. Phytopathol. 61: 1213-1217.

Katan T, Berliner R, Katan J. 1994. Vegetative compatibility in populations of *Fusarium oxysporum* from wild carnation. Mycol. Res. 98: 1415-1418.

Khoury CK, Achicanoy HA, Bjorkman AD, Navarro-Racines C, Guarino L, Flores-Palacios X, Engels JMM, Wiersema JH, Dempewolf H, Sotelo S, Ramírez-Villegas J, Castañeda-Álvarez NP, Fowler C, Jarvis A, Rieseberg LH, and Struik PC. 2016. Origins of food crops connect countries worldwide. Proc. R. Soc. B 283: 20160792.

Kommedahl T, Abbas HK, Burnes PM, Mirocha CJ. 1988. Prevalence and toxigenicity of *Fusarium* species from soils of Norway near the Arctic Circle. Mycologia. 80: 790-794.

Kuldau GA, Yates IE. 2000. Evidence for *Fusarium* endophytes in cultivated and wild plants. In: C. W. Bacon, and White, J. F. Jr., Microbial Endophytes. Marcel Dekker, Inc. New York. 85-117.

Laurence MH, Burgess LW, Summerell BA, Liew ECY. 2012. High levels of diversity in *Fusarium oxysporum* from non-cultivated ecosystems in Australia. Fungal Biol. 116: 289-297.

Laurence M, Summerell B, Liew E. 2015. *Fusarium oxysporum* f. sp. *canariensis*: evidence for horizontal gene transfer of putative pathogenicity genes. Plant Pathol. 64:1068-1075.

Leslie J, Summerell B, Bullock S. 2008. The *Fusarium* Laboratory Manual. 1st ed. Hoboken: John Wiley & Sons.

Li C, Chen S, Zuo C, Sun Q, Ye Q, Yi G, Huang B. 2011. The use of GFP-transformed isolates to study infection of banana with *Fusarium oxysporum* f. sp. *cubense* race 4. European Journal of Plant Pathol. 131:327-340.

Li E, Wang G, Xiao J, Ling J, Yang Y, Xie B. 2016. A *SIX*1 Homolog in *Fusarium oxysporum* f. sp. *conglutinans* is Required for Full Virulence on Cabbage. PLOS ONE. 11.

Lievens B, Houterman P, Rep M. 2009. Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* and discrimination from other formae speciales. FEMS Microbiology Letters. 300: 201-215.

Lin YH, Chen KS, Chang JY, Wan YL, Hsu CC, Huang JW, Chang PFL. 2010. Development of the molecular methods for rapid detection and differentiation of *Fusarium oxysporum* and *F. oxysporum* f. sp. *niveum* in Taiwan. N Biotechnol 27:409-418.

Liu Z, Friesen T. 2012. Polyethylene Glycol (PEG)-Mediated Transformation in Filamentous Fungal Pathogens. In: Bolton M, Thomma B, ed. by. Plant Fungal Pathogens: Methods and Protocols, Methods in Molecular Biology. Springer Science+Business Media, LLC. p. 365-375.

Lori G, Edel-Hermann V, Gautheron N, Alabouvette C. 2004. Genetic diversity of pathogenic and nonpathogenic populations of *Fusarium oxysporum* isolated from carnation fields in Argentina. Phytopathol. 94:661-668.

Ma L. 2014. Horizontal chromosome transfer and rational strategies to manage Fusarium vascular wilt diseases. Molecular Plant Pathology 15:763-766.

Ma L, van der Does H, Borkovich K, Coleman J, Daboussi M, Di Pietro A, Dufresne M, Freitag M, Grabherr M, Henrissat B, Houterman P, Kang S, Shim W, Woloshuk C, Xie X, Xu J, Antoniw J, Baker S, Bluhm B, Breakspear A, Brown D, Butchko R, Chapman S, Coulson R, Coutinho P, Danchin E, Diener A, Gale L, Gardiner D, Goff S, Hammond-Kosack K, Hilburn K, Hua-Van A, Jonkers W, Kazan K, Kodira C, Koehrsen M, Kumar L, Lee Y, Li L, Manners J, Miranda-Saavedra D, Mukherjee M, Park G, Park J, Park S, Proctor R, Regev A, Ruiz-Roldan M, Sain D, Sakthikumar S, Sykes S, Schwartz D, Turgeon B, Wapinski I, Yoder O, Young S, Zeng Q, Zhou S, Galagan J, Cuomo C, Kistler H, Rep M. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature, 464: 367-373.

Magdama F. 2017. Population Biology of *Fusarium oxysporum* associated with banana in Ecuador. Doctoral dissertation. The Pennsylvania State University.

Mes J, Haring M, Cornelissen B. 2000. Foxy: an active family of short interspersed nuclear elements from Fusarium oxysporum. MGG - Molecular and General Genetics. 263:271-280.

Meldrum R, Daly A, Tran-Nguyen L, Aitken E. 2013. Are banana weevil borers a vector in spreading *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in banana plantations? Australasian Plant Pathol. 42:543-549.

Meldrum R, Fraser-Smith S, Tran-Nguyen L, Daly A, Aitken, E. 2012. Presence of putative pathogenicity genes in isolates of *Fusarium oxsyporum* f. sp. *cubense* from Australia. Australasian Plant Pathol. 41: 551-557.

Nel B, Steinberg C, Labuschagne N, Viljoen A. 2007. Evaluation of fungicides and sterilants for potential application in the management of Fusarium wilt of banana. Crop Protection 26:697-705.

Nelson P, Toussoun T, Marasas W. 1983. *Fusarium* Species: An Illustrated Manual for Identification. University Park and London: The Pennsylvania State University Press.

Nene YL, Haware MP. 1980. Screening chick-pea for resistance to wilt. Plant Dis. 64: 379-380.

O'Donnell K, Kistler H, Cigelnik E, Ploetz R. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences 95:2044-2049.

OECD/FAO 2016. OECD-FAO Agricultural Outlook 2016-2025. OECD Publishing, Paris.

Oerke E. 2005. Crop losses to pests. J. Agric. Sci. 144:31.

Olivain C, Alabouvette C. 1999. Process of tomato root colonization by a pathogenic strain of *Fusarium oxysporum* f. sp. *lycopersici* in comparison with a non-pathogenic strain. New Phytologist 141:497 510.

Olivain C, Humbert C, Nahalkova J,. Fatehi J, L'Haridon F, Alabouvette C. 2006. Colonization of Tomato Root by Pathogenic and Nonpathogenic *Fusarium oxysporum* Strains Inoculated Together and Separately into the Soil. Applied and Environmental Microbiol. 72: 1523-1531.

Opperman L, Wehner FC. 1994. Survey of fungi associated with grass-roots in virgin soils on the Springbok Flats. S. African J. Bot. 60: 67-72.

Ortiz R, Vuylsteke D. 1996. Recent advances in Musa genetics, breeding, and biotechnology. Plant Breeding Abstracts 66:1355-1363.

Palmero D, De Cara M, Moreno MM, Iglesias C, Tello JC. 2010. Stimulation of mycelial growth of pathogenic and seabed isolates of *Fusarium oxysporum* in presence of salts. African J. Microbio. Res. 4: 1859-1861.

Panama Disease. 2017. Panamadisease.org. http://panamadisease.org/en/infofacts

Paparu P, Dubois T, Coyne D, Viljoen A. 2009. Dual inoculation of *Fusarium oxysporum* endophytes in banana: effect on plant colonization, growth and control of the root burrowing nematode and the banana weevil. Biocontrol Sci Technol 19:639-655.

Paparu P, Macleod A, Dubois T, Coyne D, Viljoen, A. 2009. Efficacy of chemical and fluorescent protein markers in studying plant colonization by endophytic non-pathogenic *Fusarium oxysporum* isolates. *BioControl*, 54: 709-722.

Pegg KG, Moore NY, Sorensen S. 1992. Fusarium wilt in the Asian Pacific region. In: Valmayor RV, Ploetz RC, Lee SW, Roa VN. 1993. Pingtun, Taiwan (Los Banos, Laguna, Philippines: TBRI, ASPNET, INIBAP).

Ploetz 2005. Panama Disease, an old nemesis rears its ugly head: Part 1, the beginnings of the banana export trades. Plant Health Progress.

Ploetz R. 2015. Management of Fusarium wilt of banana: A review with special reference to tropical race 4. Crop Protection 73:7-15.

Ploetz RC, Pegg K. 2000. Fusarium wilt. In: Diseases of Banana, Abaca and Enset. Jones, D.R. (Ed). CABI Publishing, Wallingford, UK. 143- 159.

Ploetz R, Freeman S, Konkol J, Al-Abed A, Naser Z, Shalan K, Barakat R, Israeli Y. 2015. Tropical race 4 of Panama disease in the Middle East. Phytoparasitica 43:283-293.

Stover RH. 1986. Disease management strategies and the survival of the banana industry. Annu. Rev. Phytopathol. 24:83-91

Rep M. 2005. Small proteins of plant-pathogenic fungi secreted during host colonization. FEMS Microbiol. Letters 253: 19-27.

Rep M, Van Der Does H, Meijer M, Van Wijk R, Houterman P, Dekker H, De Koster C, Cornelissen B. 2004. A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. Molecular Microbiology 53:1373-1383.

Rocha L, Laurence M, Ludowici V, Puno V, Lim C, Tesoriero L, Summerell B, Liew E. 2015. Putative effector genes detected in Fusarium oxysporum from natural ecosystems of Australia. Plant Pathol. 65:914-929.

Santhanam P, Thomma B. 2013. *Verticillium dahliae Sge1* Differentially Regulates Expression of Candidate Effector Genes. Molecular Plant-Microbe Interactions 26:249-256.

Sangalang AE, Burgess LW, Backhouse D, Duff J, Wurst M. 1995. Mycogeography of *Fusarium* species in soils from tropical, arid and Mediterranean regions of Australia. Mycol. Res. 99: 523-528.

Sharma KD, Muehlbauer FJ. 2007. Fusarium wilt of chickpea: Physiological specialization, genetics of resistance and resistance gene tagging. Euphytica 157: 1-14.

Sneh B. 1998. Use of non-pathogenic or hypovirulent fungal strains to protect plants against closely related fungal pathogens. Biotechnol. Adv.16:1-32

Stover RH. 1986. Disease management strategies and the survival of the banana industry. Annu. Rev. Phytopathol. 24:83-91

Striffler S. 2005. Banana wars. 1st ed. Durham, NC: Duke Univ. Press.

Taylor J, Jacobson J, Fisher M. 1999. The Evolution of Asexual Fungi: Reproduction, Speciation and Classification. Ann. Rev. Phytopathol. 37:197-246.

Taylor A, Vagany V, Jackson A, Harrison R, Rainoni A, Clarkson J. 2016. Identification of pathogenicity-related genes in *Fusarium oxysporum* f. sp. *cepae*. Molecular Plant Pathol. 17:1032–1047.

The Courier Mail. 2016. Panama-infested Tully banana farm sells in Australian Banana Growers' Council $4.5m buyout.

Trapero-Casas A, Jiménez-Díaz RM. 1985. Fungal wilt and root rot diseases of chickpea in southern Spain. Phytopathol. 75:1146-1151.

University of Stellenbosch. 2016. Banana Production in Africa. Banana Fusarium Wilt in Africa.

van Dam P, Fokkens L, Ayukawa Y, van der Gragt M, ter Horst A, Brankovics B, Houterman P, Arie T, Rep M. 2017. A mobile pathogenicity chromosome in *Fusarium oxysporum* for infection of multiple cucurbit species. Scientific Reports 7.

van Dam P, Fokkens L, Schmidt SM, Linmans JHJ, Kistler HC, Ma LJ, Rep M. 2016. Effector profiles distinguish *formae speciales* of *Fusarium oxysporum.* Environ Microbiol. 18:4087-4102.

van Dam P, Sain M, ter Horst A, van der Gragt M, Rep M. 2017. Use of Comparative Genomics-Based Markers for Discrimination of Host Specificity in *Fusarium oxysporum.* Appl. and Environ. Microbiol. 84:01868-17.

Visser M, Gordon T, Wingfield B, Wingfield M, Viljoen A. 2004. Transformation of *Fusarium oxysporum* f. sp. *cubense*, causal agent of Fusarium wilt of banana, with the *green fluorescent protein (GFP)* gene. Australasian Plant Pathol. 33:69.

Vellema S, Stoorvogel JJ, Jansen K, Kema GHJ, Cruz J, Erima R, Garcia Bastidas F, Montiflor M, Ordonez Roman N, Salacinas M, Segura Mena R. 2013. The threat of Panama disease to the role of banana and plantain in realizing food security: linking local realities to a global problem. Poster presentation in First International Conference on Global Food Security, Noordwijkerhout, The Netherlands. September 29- October 2.

Wang C, Lin Y, Lin Y, Chung W. 2013. Modified primers for the identification of nonpathogenic *Fusarium oxysporum* isolates that have biological control potential against Fusarium wilt of cucumber in Taiwan. PLoS One 8:e65093.

Williams A, Sharma M, Thatcher L, Azam S, Hane J, Sperschneider J, Kidd B, Anderson J, Ghosh R, Garg G et al. 2016. Comparative genomics and prediction of conditionally dispensable sequences in legume–infecting *Fusarium oxysporum* formae speciales facilitates identification of candidate effectors. BMC Genomics 17.

Appendix: The role of *SIX*9 in *Fusarium oxysporum*-banana interactions

Introduction

The objective of this research was to characterize the role of *SIX*9 in *Fusarium oxysporum (Fo)* isolated from banana by evaluating the effects of *Fo* isolates containing and not containing the gene *SIX*9 on the banana plant phenotype.

This research is guided by the hypothesis that *Fo* isolates containing *SIX*9 cause a different banana plant host phenotype. The hypothesis of this research is based on unmeasured observations from previous research that banana plants inoculated with *Fo* endophytes appeared larger in diameter, height, and overall were more vigorous (personal communication, F. Magdama and M. M. Jiménez-Gasco). Previous studies evaluating *Fo* endophytes in the chickpea system also reported unmeasured observations that chickpea plants inoculated with *Fo* endophytes were more resilient when challenged with drought stress (personal communication, M. M. Jiménez-Gasco).

Magdama (2017) identified *SIX9* as the *SIX* gene most frequently detected among *Fo* endophytes from banana. The *SIX9* gene sequence obtained from the eleven *Fo* banana endophytes was only sixteen nucleotides different than the wild-type TR4 0-2052 and appeared to be conserved among this group of endophytes (Magdama 2017). However, the role of *SIX9* is currently unclear. In a study where *SIX9* was knocked out in *Fo* f. sp. *radicis-cucumerinum,* mutants lacking *SIX9* did not show a change in disease phenotype compared to the wild-type (van Dam et al. 2017). These data, along with the discovery of *SIX9* in non-pathogenic *Fo* isolates from banana, lead us to hypothesize that *SIX9* may play a role in *Fo*-plant interactions, perhaps allowing *Fo* to colonize the plant host. The conservation of *SIX9* among the *Fo*-banana endophytes, along with previous reports of *Fo* endophytes conferring drought resilience in the chickpea system, lead to the hypothesis that *Fo* endophytes containing *SIX9* may confer a benefit to the plant host. Therefore, in this study, we measured horticultural characteristics of banana plants inoculated with *Fo* endophytes isolated from banana.

Materials and Methods

In the first set of experiments, putatively non-pathogenic *Fo* isolates from banana collected by Magdama (2017) were compared based on the presence or absence of *SIX*9 genes in these isolates and their phylogenetic relationships. A group of eleven isolates (listed below), isolated from asymptomatic banana plants, was found to contain *SIX*9 and form a single group when phylogeny was inferred based on maximum likelihood using a 236nb region of the *SIX*9 gene (Madgama 2017). The colonization and phenotype of these 11 isolates was compared to three isolates, also collected from asymptomatic banana plants in Ecuador, which did not contain any described *SIX* gene. These three putative endophytes lacking *SIX* genes group with the *SIX*9 putative endophytes based on *TEF-1α* and IGS regions. Based on these criteria, the selected three putative endophytes lacking *SIX* genes are: EE-LR-02, EE-LR-49, and EE-LR-50. The three *SIX*9-carrying putative endophytes were the following: EC8-LR-PL2, EE-LR-07, EE-LR-11, EE-LR-18, EE-LR-19, EE-LR-26, EE-LR-30, EE-LR-40, CAV02-ELR, CAV06-ELR, and CAV027-EO.

These tests also served as a pathogenicity test for the eleven *SIX*9-carrying putative endophytic isolates collected from asymptomatic material. In a second experiment, the number of *Fo*-isolates containing *SIX9* was decreased to three for comparison to the three *Fo* isolates that do not contain *SIX9.* These three *SIX9*-carrying endophytes were: EC8-LR-PL2, EE-LR-07 and CAV06-ELR.

For both experiments, the Cavendish ‘Gran Naine’ and ‘Gros Michel’ were used. Plants were inoculated after 8 weeks in the growth chamber at the same growth conditions described in chapter two. For Cavendish ‘Gran Naine’, each treatment was replicated in four plants; for ‘Gros Michel’ each treatment was replicated in three plants. For the second set of experiments, only Cavendish ‘Gran Naine’ was used. Each treatment was replicated in four plants. To measure the effect of endophytes on the plant host, height, diameter, and disease symptoms were measured for each plant after six weeks post-inoculation. Cornmeal-sand inoculum was prepared as described in Chapter 2. At the end of the

experiment, fungal re-isolations were made on NASH media for the roots, corm, and pseudostem (4cm, 10cm and 25cm above the crown) after surface disinfestation. Samples were processed as described in Chapter 2.

Results

For the first set of experiments, banana plants were inoculated after 16 weeks of acclimation to the growth chamber. Due to difficulties with two-spotted spider mites, growth chamber light intensity, and producing inoculum, the banana plants had undergone a great deal of stress. Banana plant size also varied. The variation in banana size was distributed among the treatments. For the second set of experiments, banana plants were inoculated after 12 weeks. In both experiments, treatment plants were inoculated with soil-inoculum mixture using the methods described in Chapter 2. Soil inoculum was quantified using methods described in Chapter 2; inoculum concentration is listed in Table A.1. In both experiments, treatment plants were measured for height and diameter differences eight weeks post-inoculation (Table A.2).

Inoculum load for the infested soil for the first round of experiments can be seen in Table A.1. Soil was also dilution-plated on one plate of PDA. PDA plates showed similar colony numbers to NASH.

Table A.1 Soil inoculum concentration for *in planta* testing. N indicates this isolate was not tested.

Isolate	CFU/mg soil	
	Experiment 1	Experiment 2
EC8-LR-PL2	6.5×10^4	5×10^5
EE-LR-07	2.4×10^5	2×10^3
EE-LR-11	2.0×10^5	N
EE-LR-18	2.3×10^6	N
EE-LR-19	3×10^4	N
EE-LR-26	3×10^4	N
EE-LR-30	2.6×10^5	N
EE-LR-40	1.0×10^6	N
CAV02-ELR	6.3×10^4	N
CAV06-ELR	2.0×10^5	$3x\ 10^5$
CAV027-EO	3.1×10^5	N
EE-LR-02	3.3×10^4	$1.1x\ 10^6$
EE-LR-49	5×10^4	4.9×10^6
EE-LR-50	2.2×10^5	1×10^5
EC44-M-GM2	9.5×10^5	4×10^3
TR4 0-2052	5.9×10^5	9×10^5

Putatively non-pathogenic isolates from asymptomatic banana plants had a *Fo* disease severity rating of 0 in 'Gros Michel' and Cavendish 'Gran Naine.' Positive controls, TR4 0-2052 on Cavendish 'Gran Naine' and TR4 0-2052 and EC44-M-GM2 on 'Gros Michel,' showed disease severity ratings of 3 (dead plants) at the end of the experiment.

In both repetitions of the experiment, non-pathogenic isolates were be isolated consistently from the roots, corm, stem, and petioles of inoculated banana plants.

Differences between the treatments were evaluated using t-tests. For the spring 2018 trial, where only Cavendish 'Gran Naine' was tested, there were no significant differences between treatments (Table A.3).

For the Cavendish 'Gran Naine' plants in the summer 2017 trial, plants inoculated with EE-LR-50 were significantly taller than the control. The pseudostem of plants inoculated with EE-LR-50 were also significantly wider than the non-inoculated control. Plants inoculated with EE-LR-07 were also significantly larger than the non-inoculated control based on both height and pseudostem diameter. Overall, the *SIX9*-endophytes were significantly taller than the control. *SIX9*-endophytes, non-*SIX9* endophytes, and the control all had differences between groups based on diameter.

For 'Gros Michel' plants in the summer 2017 trial, plants inoculated with EE-LR-49 were significantly wider than those of the non-inoculated control. Psuedostems of plants inoculated with either non*SIX9*-carrying endophytes and *SIX9*-carrying endophytes were wider than the non-inoculated controls.

Table A.2 Average change in banana plant height (cm) and diameter (mm) 8 weeks post-inoculation. Change in height (cm) is considered to be ΔH= $H_f - H_0$, where H_f = final height and H_0 = initial height, and change in diameter (mm) is considered to be ΔD= $D_f - D_0$, where D_f = final height and D_0 = initial height.

		Spring 2018	Summer 2017	Summer 2017
		Cavendish 'Gran Naine'	Cavendish 'Gran Naine'	'Gros Michel'
Control	ΔH	10.75	4	10.3
	ΔD	5.9	2.4	5.9
EE-LR-02	ΔH	14	10.4	15.3
	ΔD	6.38	17.1	19.4
EE-LR-49	ΔH	9.75	5	12.7
	ΔD	5.25	7.1	12.7
EE-LR-50	ΔH	14	17	23.9
	ΔD	6.15	10.8	2.7
EE-LR-07	ΔH	12	15.25	9.7
	ΔD	5.68	12.8	
EC8-LR-PL2	ΔH	12.25	9.25	4.4
	ΔD	6.98	9.9	17
CAV06-ELR	ΔH	10.75	7.75	6.1
	ΔD	6.85	9.1	14.3

Table A.3 T-statistic and t critical two-tail for spring 2018 Cavendish 'Gran Naine.' Significant differences (P<0.05) are highlighted in grey, where change in height (cm) (ΔH= $H_f - H_0$, where H_f = final height and H_0 = initial height), and change in diameter (mm) (ΔD= $D_f - D_0$, where D_f = final height and D_0 = initial height) are considered. Differences based on A) t-test values, where the control mean of change in height (H) was 10.75cm and the control mean of the change in diameter (D) was 5.9mm. B) T-test comparisons of treatments grouped by isolates containing *SIX9*, non*SIX9*-isolates, and non-inoculated control based on change in height and C) change in diameter.

A	**EE-LR-02**		**EE-LR-49**		**EE-LR-50**		**EE-LR-07**		**EC8-LR-PL2**		**CAV06-ELR**	
	non*SIX9*-endophytes						*SIX9*-carryingendophytes					
	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD
t-statistic	-1.0	-0.8	0.3	0.44	-0.9	-0.2	-0.3	0.19	-0.5	-0.67	0	-0.4
P two tail	0.4	0.48	0.8	0.7	0.4	0.9	0.8	0.9	0.6	0.54	1	0.7
Df	3	3	3	3	3	3	3	3	3	3	3	3
Mean	14	6.38	9.75	5.25	14	6.15	12	5.68	12.25	6.98	10.75	6.85

B		Control (mean=5.9mm)	
	t-stat	P two tail	Df
SIX9-carrying endophytes (mean=6.5mm)	-0.54	0.6	11
non*SIX9*-endophytes (mean=5.9mm)	-0.99	0.34	11
		SIX9-carrying endophytes	
non*SIX9*-endophytes	0.3	0.8	11

C		Control (mean=5.9mm)	
	t-stat	P two tail	Df
SIX9-carrying endophytes (mean=6.5mm)	-0.6	0.57	11
non*SIX9*-endophytes (mean=5.9mm)	-3.5	1	11
		SIX9-carrying endophytes	
non*SIX9*-endophytes	-0.6	0.54	11

Table A.4 T-statistic and t critical two-tail for summer 2017 Cavendish 'Gran Naine.' Significant differences (P<0.05) are highlighted in grey, where change in height (cm) (ΔH= $H_f - H_0$, where $H_f =$ final height and $H_0 =$ initial height), and change in diameter (mm) (ΔD= $D_f - D_0$, where $D_f =$ final height and $D_0 =$ initial height) are considered. Differences based on A) t-test values, where the control mean of change in height (H) was 4cm and the control mean of the change in diameter (D) was 2.4mm. B) T-test comparisons of treatments grouped by isolates containing *SIX9*, non*SIX9*-isolates, and non-inoculated control based on change in height and C) change in diameter.

A	**EE-LR-02**		**EE-LR-49**		**EE-LR-50**		**EE-LR-07**		**EC8-LR-PL2**		**CAV06-ELR**	
	non*SIX9*-endophytes						*SIX9*-carrying endophytes					
	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD
t-statistic	-1.7	-2.8	-0.1	-0.7	-3.2	-3.8	-7.3	-4.2	-2.1	-3.1	-1.1	-1.4
P two tail	0.17	0.07	0.9	0.56	0.048	0.03	0.005	0.02	0.12	0.051	0.34	0.3
Df	3	3	3	3	3	3	3	3	3	3	3	3
Mean	10.4	17.1	5	7.1	17	10.8	15.25	12.8	9.25	9.9	7.75	9.1

B	Control (mean=4cm)		
	t-stat	P two tail	Df
SIX9-carrying endophytes (mean=10.8cm)	-4.1	0.001	11
non*SIX9*-endophytes (mean=10.8cm)	-2.1	0.06	11
	SIX9-carrying endophytes		
non*SIX9*-endophytes	0.01	0.99	11

C	Control (mean=2.35mm)		
	t-stat	P two tail	Df
SIX9-carrying endophytes (mean=10.6mm)	-4.4	0.001	11
non*SIX9*-endophytes (mean=11.8mm)	-3.1	0.01	11
	SIX9-carrying endophytes		
non*SIX9*-endophytes	-4.4	0.001	11

Table A.5 T-statistic and t critical two-tail for summer 2017 'Gros Michel.' Significant differences (P<0.05) are highlighted in grey, where change in height (cm) (ΔH= $H_f - H_0$, where H_f = final height and H_0 = initial height), and change in diameter (mm) (ΔD= $D_f - D_0$, where D_f = final height and D_0 = initial height) are considered. Differences based on A) t-test values, where the control mean of change in height (H) was 10.3cm and the control mean of the change in diameter (D) was 5.9mm. B) T-test comparisons of treatments grouped by isolates containing *SIX9*, non*SIX9*-isolates, and non-inoculated control based on change in height and C) change in diameter.

A	**EE-LR-02**		**EE-LR-49**		**EE-LR-50**		**EE-LR-07**		**EC8-LR-PL2**		**CAV06-ELR**	
	non*SIX9*-endophytes						*SIX9*-carrying endophytes					
	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD	ΔH	ΔD
t-statistic	-0.7	-3.2	-1	-14.2	0.7	-0.8		1.19	-1.3	-0.1	-1.4	-1.2
P two tail	0.6	0.08	0.4	0.004	0.6	0.47		0.35	0.3	0.9	0.3	0.4
Df	2	2	2	2	2	2		2	2	2	2	2
Mean	15.3	19.4	12.7	23.9	2.7	9.7		4.4	17	6.1	14.3	7.4

B	Control (mean=9cm)		
	t-stat	P two tail	Df
SIX9-carrying endophytes (mean=10cm)	0.4	0.7	29
non*SIX9*-endophytes (mean=11.1cm)	0.4	0.7	8
	SIX9-endophytes		
non*SIX9*-endophytes	0.6	0.57	8

C	Control (mean=5.8mm)		
	t-stat	P two tail	Df
SIX9-carrying endophytes (mean=5.9mm)	-0.1	0.9	8
non*SIX9*-endophytes (mean=17.7mm)	-4.2	0.002	8
	SIX9-carrying endophytes		
non*SIX9*-endophytes	3.7	0.006	8

Discussion

The pathogenicity test of *SIX9*-endophytes confirmed that these isolates, although they contain *SIX9*, do not cause disease on Cavendish 'Gran Naine' and 'Gros Michel.' Therefore, these isolates do not belong to race 1 or TR4 of *Foc*. However, it is important to note that pathogenicity on other hosts and in differing environmental conditions were not be tested, and thus, these isolates will remain putatively nonpathogenic, even after a pathogenicity test on 'Gros Michel' and Cavendish 'Gran Nain.' These isolates may have undescribed genetic or epigenetic differences besides the presence of *SIX*9 that have not yet been described. Therefore the conclusions drawn are discussed within the context of these isolates and our knowledge of *Fo*.

When considering the effect of the *Fo* isolates on host plant effects, overall, results between the spring 2018 and summer 2017 experiments were inconsistent. Inconsistent results are not surprising considering the stress banana plants underwent before the summer 2017 experiment. Therefore, in a best case, the results of this experiment may be interpreted as experiments with two different environmental conditions. The summer 2017 experiments should be considered as an experiment where plant stress is a variable.

In summer 2017, banana plants inoculated with isolates EE-LR-49, EE-LR-50, and EE-LR-07 showed at least one significantly improved measurement of growth at least one cultivar. Psueodstems of plants inoculated with EE-LR-49 were significantly wider than the controls in 'Gros Michel.' In Cavendish 'Gran Naine', plants inoculated with the isolate EE-LR-07 were taller and had wider pseudostems than the controls in the summer 2017 experiment. It may also be possible that growth promotion of the plant host only occurs at lower inoculum loads. Therefore, these isolates should also be considered for further investigation.

The only *SIX9*-endophyte that improved plant growth was EE-LR-07. This isolate only conferred an advantage in summer 2017. However, the inoculum load for EE-LR-07 for the spring 2018 experiment (2×10^3cfu/mg soil) was lower than the inoculum load for the other isolates, which was on average on the order of 10^5cfu/mg soil. This difference may explain the lack of differences in the spring 2018 experiment. Therefore, EE-LR-07 should also be considered for further study.

Inconsistency between the Spring 2018 and Summer 2017 trials could be due to the stresses the test plants were exposed to before the trial in Summer 2017. These stresses were not administered before the Spring 2018 trial. Therefore, it could also be useful to expose test plants to different stress factors before inoculating to determine if these isolates confer a host advantage under particular stress conditions. Water stress, temperature, insect pressure, and crowding should be considered for stress factors to test in future experiments.

Ideally, *Fo* endophytes could be used to promote plant growth in the field, where banana production takes place. However, effects on plant growth are known to vary between growth chamber, greenhouse, and field experiments. Therefore, if these isolates continue to promote banana plant growth under growth chamber conditions, these isolates should be considered for field investigation. Even if results are not confirmed under field conditions, these *Fo* isolates could be beneficial for the establishment of banana transplants in nurseries or the conservation of banana germplasm. Further studies could also investigate these applications.

This study begins to provide applied evidence that *Fo* affects the plant beyond pathogenicity. There is nuance to the effects *Fo* endophytes have on their hosts; we are only beginning to understand these effects. However, the effects of *Fo* endophytes on their hosts may reveal the main ecological niche of *Fo.* Based on the genetic diversity of *Fo* within endophytes of the species, and now on the results of this experiment, we suspect that *Fo* may have a symbiotic relationship with the plant host, analogous to the symbiosis observed between plants and root mycorrhizae. Gathering data on the ecological niche of *Fo* is ultimately crucial to understanding the differences between pathogenic and non-pathogenic isolates of *Fo;* in the end, understanding the main ecological role of *Fo*, and the factors that change this role, are key to sustain able solutions to the diseases *Fo* causes.