EDUARDO MONDLANE UNIVERSITY

FACULTY OF AGRICULTURE AND FORESTRY ENGINEERING

MASTERS OF SCIENCE IN CROP PROTECTION

Diversity of *Fusarium oxysporum* f. sp. *cubense* in Mozambique and Associated In Vitro Response to Fungicides, Biocontrol-Agents and Phenolic Compounds

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MAPUTO, OCTOBER 2016

Diversity of *Fusarium oxysporum* f. sp. *cubense* in Mozambique and associated in vitro response to Fungicides, Biocontrol-agents and Phenolic compounds

BY

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Submitted in Partial Fulfillment of the Requirements
for a Master of Science Degree in Crop Protection
Faculty of Agriculture and Forestry Engineering
Eduardo Mondlane University

Maputo, October 2016

DECLARATION

I, Amugoli Otuba Moses, a Master's student in Crop Protection, declare that the work contained in this thesis is my own, resulting from my own investigation and that it has not previously, in its entirety or part, been submitted for a degree to any other University.

Signature ..... Date ..11.. / .....11...../ 2016

This Thesis entitled "Diversity of *Fusarium oxysporum* f. sp. *cubense* in Mozambique and associated in vitro response to Fungicides, Biocontrol agents and Phenolic compounds" was submitted as a research project for the Master's degree in Crop Protection, Faculty of Agronomy and Forestry Engineering of the University Eduardo Mondlane.

Maputo, ...11../ .....11...../ 2016

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

MINAG	Ministério da Agricultura-Moçambique (Ministry of Agriculture in Mozambique)
PDA	Potato Dextrose Agar
NPK	Nitrogen, Phosphorus, and Potassium
MBC	Microbial Biological Control
MBCA	Microbial Biological Control Agents
MM	Minimal Media
CLM	Chlorate Media
CAV	Cultural Collection, Department of Pathology, Stellenbosch University
ISR	Induced Systemic Resistance
Foc	*Fusarium oxysporum* f. sp. *cubense*

ACKNOWLEDGEMENT

Most importantly, I would like to thank the Almighty God for saving me and leading me through numerous of journeys.

My sincere thanks go to the following people and institutions. You are deeply appreciated.

My Supervisors Prof. Ana Maria Mondjana, Prof. Altus Viljoen and Dr. Glaudina Mostert. It was honourable being under your guidance. Thank you for the unlimited encouragement and commitment.

Prof. Domingos Cugala, Prof. Luisa Santos, Dr. Amélia Jorge Sidumo, Dr. Helder Zavale, Dr. Joao Bila, dr. Jadwiga Massinga, Eng. Amandio Muthambe, Prof. Tomás Chiconela among other teaching and administrative staff for the academic and moral guidance I received throughout my study period.

Stellenbosch University especially the department of Plant Pathology for allowing me accomplish my study in your facility. Special thanks to the staff of the Fusarium Laboratory for the extra hand you gave me in accomplishing my investigations.

The Ministry of Agriculture of Mozambique, especially Dr. Serafina Mangana and Eng[a] Antonia Vaz for the guidance and financial support I received during the surveys.

Colleagues in the Crop Protection class; Benedict, Emma, Elias, Joseph, Ronald, Andrea etc. for the social support we shared all throughout the study period in Mozambique. You will dearly never be forgotten.

My family for the smiles and love you have always given me. My guardians thank you for your undivided love, encouragement and prayers. My sisters and brothers thank you for the moments we share.

SUMMARY

Panama disease or Fusarium wilt of banana is the most devastating disease of banana in the world. *Fusarium oxysporum* f. sp. *cubense* (*Foc*), the causative organism, has the potential to survive in soil under unfavourable conditions for over 3 decades while awaiting a potential host. This has further complicated management of Panama disease. Resistant cultivars (currently not existing for Tropical race 4 or TR4), are the only strategy that will effectively overcome the devastating impact of Panama disease. This study sought to investigate the extent of Panama disease and to identify potential management strategies for further field evaluation against *Foc* in Mozambique.

The first epidemic of Panama disease was noticed in the mid-20th century. Thereafter, the impacts of Panama disease have been felt where ever it occurs. **Chapter 1** provides the general introduction to Panama disease and the scope of the study. It describes the existing problem, purpose and the details of the expected achievements in the study.

Fusarium oxysporum f. sp. *cubense* and banana (the host crop) are said to have co-evolved in Asia. **Chapter 2** is a review on banana and Panama disease. It also describes management measures that have been studied and applied over the years in attempt to prevent, control and eradicate Panama disease both globally and within Mozambique.

Recently in 2013, the most devastating strain, Tropical race 4 (TR4) was reported in Mozambique. This was the first time TR4 has been reported in Africa ever since its first report in Asia in 1990. However, Panama disease (probable race 1 and 2) was already existent in Mozambique. **Chapter 3** describes the distribution of Panama disease in Mozambique. It provides the vegetative compatibility groups (VCGs) that have been identified in the country.

Host resistance is the only means through which Panama disease has been successfully managed since its catastrophic appearance in the 1950s. Various measures have been tested but have given inconsistent, short lived or poor results in the control of Panama disease. **Chapter 4** looks at the effects of some biological control agents, available fungicides and phenolic compounds on *Foc* in vitro. It suggests the using of an integrated approach that includes *Foc* suppressive phenolic compounds in the management of Panama disease. The most inhibitory compounds are identified for the above regard. **Chapter 5** provides the study conclusions and recommendations for further

studies and management of Panama disease. This study is aimed at providing a background for more studies and practices that can generate efficient measures for the management Panama disease in Mozambique.

CHAPTER ONE: GENERAL OVERVIEW

1.1 Introduction

1.1.1 Background

Banana (Musa species) is the fourth most important food commodity (after rice, wheat and maize) grown in less developed countries with small scale farmers contributing about 87% of the total production chiefly for consumption and sale in local markets (Machovina, 2015; Ayala—Silva *et al*., 2009; Janick and Paull, 2008; Frison *et al*., 2004). The industry has enormously contributed to increased export earnings and employment of thousands in developing countries (Evans and Ballen, 2015). In Mozambique, banana production is recovering despite virtually being destroyed during the civil war (GDS report, 2005; TechnoServe, 2002). The crop is mainly grown in the Provinces of Manica, Maputo and Nampula respectively (Calima, 2014; MINAG, 2014). Currently, Mozambique contributes about 0.5% to world banana exports and has got a very high potential to expand future production given the country's enormous chunk of well drained and unused fertile land (Calima, 2014; FAO, 2014; TechnoServe, 2002).

Banana is mainly consumed as dessert but some subgroups such as plantain can be cooked as food or used as medicine (Kumar *et al*., 2012; Uazire *et al*., 2008). Besides sale of banana bunches and suckers, several products including woven fibre handcrafts and alcohol have been used domestically and sold for income, not withholding the use of banana leaves and suckers in social functions and traditional rituals (BARNESA Report, 2008).

Banana crop holds great value as a source of income, food and in maintenance and improvement of soil fertility and structure, effectively minimizing soil erosion (BARNESA Report, 2008). However, banana production is exposed to various challenges which include among others, pests (Asian fruit flies, banana weevils and nematodes) and diseases (Black sigatoka, anthracnose, and most recently Panama disease - Tropical race 4) (Pérez-Vicente *et al*., 2014; Calima, 2014; Uazire *et al*., 2008).

Tropical race 4 (TR4), one of the races of *Fusarium oxysporum* f. sp. *cubense* (*Foc*), was confirmed in 2013 as new disease threatening banana production in Mozambique (MINAG, 2014; Pérez-

Vicente *et al*., 2014). Having first been reported in 1876 by J. Bancroft in Australia, Panama disease has since been re-occurring in different races wiping out major export cultivars in major banana exporting countries (Ploetz, 2005). TR4 overcame the resistance in Cavendish cultivars, the current export subgroup (today accounts for about 99% of world banana exports) which replaced the Gros Michal subgroup that was wiped out by race 1 of *Foc* in the mid-20th century (Pérez-Vicente *et al*., 2014; Ploetz, 2005). Cavendish cultivars (Williams, ASDIA, dwarf Cavendish, Grand Naine and Chinese Cavendish) also form much of banana cultivars grown in Mozambique both for local consumption and export (MINAG, 2014). *Foc,* a soil borne pathogen, can persist in the soil and dead tissue for over 3 decades as chlamydospores which are induced to germinate by secondary and primary root exudates from proximal banana plants (Stover, 1972). Susceptible cultivars and characteristic persistence of *Foc* have further increased the likelihood of spread of TR4 in Mozambique hence calling for implementation of extensive counter measures to check the spread of the disease in the country.

1.1.2 Statement of the Problem

Panama disease (Fusarium wilt of banana), caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is among the most destructive plant diseases in the history of export agriculture (Visser *et al.*, 2010; Ploetz, 2000; Correll, 1991; Stover, 1962). The disease, proposed to have originated from Asia, is associated with three pathogenic races (Race 1, 2, and 4) characterized based on virulence to individual or subgroups of banana cultivars (Ploetz and Pegg, 1997; Moore *et al.*, 1993; Correll, 1991; Stover, 1962; Stover and Waite, 1960).

As described by Correll (1991), *Foc* races have further been characterized into vegetative compatibility groups (VCGs) from work initiated by Puhalla (1985). Vegetative compatibility occurs when alleles at the corresponding vic loci of paired nitrate non-utilizing (nit) mutants are identical. Twenty-four vegetative compatibility groups have been associated with *Foc* worldwide (Visser *et al.*, 2010; Ploetz, 2000; Ploetz and Pegg, 2000; Katan, and Di Primo, 1999).

In 1990, TR4, a strain that is now threatening the global banana industry was reported in Malaysia, spread to Indonesia, Australia and South East Asia in less than a decade and is currently reported in Africa and the Middle East (Ploetz, 2015; Pérez-Vicente *et al*., 2014; Masdek *et al*., 2003;

Nasdir, 2003). Infected suckers or rhizome pieces used as planting material are the principal means through which Panama disease is spread (Ploetz and Pegg, 2000). However, locally it can also be spread through contaminated soil attached to planting material, farm machinery and footwear, water running through infested soil and use of contaminated farm management tools (Pérez-Vicente *et al*., 2014; Ploetz and Pegg, 2000).

Noronha (1970) and Plumb-Dhindsa and Mondjane (1984), reported Panama disease to have had a significant impact on banana production in Mozambique although the races were not specified. In 2013 however, TR4 was confirmed to be present in two private farms in Mozambique (MINAG, 2014; Pérez-Vicente *et al*., 2014). The two plantations, Matanuska and Jacaranda are in Nampula Province. Coincidently, new investments in commercial banana production were being made in Nampula and Maputo Provinces with the aim of boosting banana production in the country (Calima, 2014).

The discovery of TR4 in Mozambique has posed a very big threat to the high potential banana industry. TR4 is carrying with it a 'scare of devastation' which earlier strains caused to major banana exporting countries worldwide and this disaster could also be replicated in Mozambique. This is worsened by the challenge faced in eradicating the soil persistent *Foc* pathogens. Ineffectiveness on *Foc* (Moore *et al.,* 2001) and the negative impacts on consumers and agro-ecosystems, have raised public concern regarding the increasing use of synthetic pesticides in agriculture (Xue, *et al.,* 2015**:** Woo *et al.,* 2014: Ploetz, 1994). Conventional breeding for resistance in banana has also been limited by triploidy and consumer taste preferences (Xue, *et al.,* 2015**:** Viljoen, 2002). Hence, the introduction of other disease control alternatives such as novel chemical compounds, antagonistic microorganisms and secondary plant metabolites (phenolic compounds) can offer optimistic control results against Panama disease. In addition to stimulating the production of antagonistic phenolic compounds within the banana plants, biocontrol agents provide the most consumer and environmentally friendly alternative as these agents are a part of the existing agro-ecosystem (Xue, *et al.,* 2015; Thangavelu *et al.,* 2003).

Despite earlier and present reports giving evidence of Panama disease in Mozambique, the geographical distribution and genetic diversity (VCGs) of the specific strains of *Foc* have not been investigated. Generating VCGs of *Foc* in Mozambique will give a clear picture of the genetic

constitution of *Foc* in the country in relation to all other VCGs across the world. With the aid of GIS mapping, the distribution of various *Foc* strains in Mozambique can also be determined. Mapping is important in the implementation of regulatory measures such as quarantine to check the further spread of Panama disease to healthy areas. Integrated use of good cultural practices, commercial biocontrol products, phenolic compounds and effective fungicides can potentially provide a long-term Panama disease management strategy in the absence of a resistant banana cultivar given the decades' long persistence of *Foc* in infested banana fields.

1.1.3 Objectives

The major objective of the study is to establish the genetic diversity of *Foc* in Mozambique and the proficiency of commercially available fungicides, phenolic compounds and biological control products in the management of Panama disease.

Specifically, to;

1. Establish the distribution of *Fusarium oxysporum* f. sp. *cubense* in Mozambique.
2. Identity the different vegetative compatibility groups (VCGs) of *Fusarium oxysporum* f. sp. *cubense* present in Mozambique.
3. Determine the potential of phenolic compounds, fungicides and biological control agents in the control of Panama disease.

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CHAPTER TWO: LITERATURE REVIEW

2.1 Banana

2.1.1 Description

Banana is classified under the order Zingiberales, family Musacea*e* and genus *Musa* and includes a worldwide collection of over 1,000 landraces (Simmonds, 1962; Heslop-Harrison and Schwarzacher, 2007). The landraces encompass both sweet or dessert bananas (43% of world production) and the cooking bananas (57% of world production) which includes the plantain subgroup. The arborescent monocotyledonous perennial herb is composed of a corm, which is an underground true stem and a pseudostem which bears a meristem from which leaves and flowers are initiated (UNCST, 2007) (Figure 1). The pseudostem which can grow to a height of 2 to 8 meters in farmed varieties and 10 to 15 meters in wild types is made up of tightly rolled overlapping leaf bases (Karamura and Karamura, 1995; UNCST, 2007). The corm, about 30 cm, from which roots ranging from 50 to 100cm initiate, forms buds which develop into eyes and later become suckers which are commonly used as vegetative reproductive organs (Karamura and Karamura, 1995; UNCST, 2007).

When the eleventh-last leaf has been produced, the shoot meristem transforms into an inflorescence without a photoperiodic requirement (Australian Government, 2008; Purseglove, 1972). Once transformed, the inflorescence begins to elongate upwards at an average of 8 cm per day until it emerges through the centre of the pseudostem after about a month (Simmonds, 1959a). Once emerged, it is classed as a compound spike that later bends under its own weight bearing immature inflorescence encased inside a purple bract often referred to as a bell (Simmonds, 1959a). The bracts contain 5- 15 double whorls of floral parts comprising of female flowers at the proxirnal end (closest to the base of the peduncle), male flowers at the distal end (closest to the tip of the peduncle), and neuter or hermaphrodite flowers occasionally present in between (Australian Government, 2008). Each of the nodes is covered by a purple bract that open in sequence (1 per day) from base to tip, becoming reflexed before being shed. As the hands of fruits begin to develop from the female flowers, the male flowers are usually shed leaving the peduncle bare except for the very tip, which consists of a ' male bud' (also referred to as the bell) containing the last-formed

of the male bracts and flowers (Australian Government, 2008). Once flowering has stopped, the pseudostem dies giving way to the younger generation of suckers that formed on its corm (Jones, 2000).

Figure 1: Description of banana plant growth stages. A – Fully grown banana plant. B – A banana plant with two of its suckers. C – A newly emerged unrolling banana leaf. D – A developing banana inflorescence that has bent due to its own weight.

Traditionally, conventional vegetative means have been used by various farming communities to propagate banana since most cultivars of *Musa* have less than 1% germination ability in soil and are completely or nearly female-sterile (Pillay *et al.*, 2002; Simmonds, 1959; Ngomuo and Ndakidemi, 2014). These include the use of suckers (out growths from lateral buds originating from corms) and rarely, complete or divided corms with one or several buds to produce new plants. However, conventional propagation of banana using suckers and corms is very slow and has significantly contributed to spread of diseases, decreasing yields and loss of superior plant genetic traits (Hussein, 2012). As an option, use of tissue culture in banana propagation is widely gaining acceptance especially in areas of disease management (Ali, *et al.,* 2011). According to Ngomuo and Ndakidemi (2014), mass propagation and clean planting material can be attained with the use of tissue culture. Use of tissue culture in banana production has also provided superiority over conventional propagation through optimal yield, uniformity, disease-free planting material, true to type plants, mass multiplication over a shorter time, cheaper and safer transportation of tissue culture plantlets, conservation of germplasm and production of more vigorous, higher yielding and better quality banana fruits (Hwan *et al*., 1976; Ngomuo and Ndakidemi, 2014).

2.1.2 History of Banana

Musa acuminata Colla (A genome) and hybrids between *Musa acuminata* Colla and *Musa balbisiana* Colla (B genome) are the wild Southeast Asian progenitor species from which most known edible bananas having chromosome numbers 22, 33 and 44 were domesticated in a complex process that involved intra and interspecies hybridization, polyploidization, seed sterility, parthenocarpy, and somatic mutations (Simmonds and Shepherd, 1955; Mbida *et al*., 2006; Mohapatra *et al*., 2010: Australian Government Report, 2008). Malesia (Malay Peninsula, Indonesia, Philippines, New Guinea and later India) is the origin and the centre of initial domestication and diversification of banana before its spread to the tropics of Asia, Africa and the Americas (Mbida *et al*., 2006; Simmonds, 1962: Australian Government Report, 2008: UNCST, 2007). According to Mohapatra *et al.* (2010), polyploidy and hybridization of the A and B genome gave rise to diploid (AA, BB, AB), triploid (AAA, AAB, ABB, BBB) and tetraploid (AAAA, AAAB, ABBB, AABB) bananas. Four categories of banana (recent introductions - RI, the Indian Ocean Complex - IOC, the Eastern African AAA highland group - EA-AAA, and the African plantain group - AP-AAB) are found today in Africa (De Langhe *et al.*, 1996; Mbida *et al*., 2006).

Mostly grown near towns and townships, RI are composed of about 10 high quality dessert fruit cultivars (Mbida *et al*., 2006). The IOCs include edible-AA, AB, AAA, AAB, ABB, an almost complete spectrum of banana genomes often cultivated along the coastal areas of the Indian Ocean (Simonds, 1959; De Langhe *et al.*, 1996; Mbida *et al*., 2006). The EA-AAA consists of 60 cultivars grown in the areas stretching from Uganda southwards to Mozambique and southern Africa and not in anywhere else in the world (Karamura, 1999).

Despite suggesting three scenarios, the introduction of banana into Africa is not well understood (Neumann and Hildebrand, 2009). First, it is believed that starting more than 2000-3000 years ago, banana came into Africa through several waves via the Indian Ocean (Neumann and Hildebrand, 2009; De Langhe *et al*., 1994; De Langhe and De Maret, 1999). The second theory supposes that banana entered eastern Africa via Madagascar by people of Malaysian-Indonesian origin in the first millennium A.D. (Neumann and Hildebrand, 2009; Rossel 1998; Simmonds 1966; Smartt and Simmonds, 1995; Vansina 1984:90). According to Neumann and Hildebrand (2009), the contact between Africa and Asia fueled an exchange of such crops as banana despite having an unclear timing of introductions. However, data outside archaeology upholds for the earlier scenario of banana introduction into Africa (Neumann and Hildebrand, 2009). The third scenario as proposed by Blench (2009), suggests that plantains entered western Africa as part of an Indo-Pacific crop package that included taro (*Colocasia esculenta* (L.) *Schott*) and water yam (*Dioscorea alata* L.) (Neumann and Hildebrand, 2009).

2.1.3 Importance of Banana

Musa cavendishii, M. paradisiaca and *M. sapientum* are the three commonly grown banana species in the world (Mohapatra *et al*., 2010). The sweeter and less starchy *M. cavendishii* is a pure triploid (AAA group) acuminate also known as dessert banana while both the starchier *M. paradisiaca* and *M. sapientum* belong to the AAB group. *M. sapientum* also known as the true banana can be eaten raw when fully mature.

Food, pharmaceuticals, feed and packaging are among the important uses of whole banana plant (Mohapatra *et al.*, 2010: Kumar *et al.*, 2012). The banana fruit is not only rich in carbohydrates and antioxidants but also minerals especially potassium and iron, making it an ideal food for infants

and mothers (Mohapatra *et al.*, 2010). The nutrient rich peel can also be used as animal feed, biogas production resource, base material for alcohol production and for pectin extraction. Banana fibre can also be used to produce biodegradable ropes and the leaves used as animal feed, wrapping of various products and thatching.

In Mozambique, banana takes apart in the basket of staple foods grown with a contribution of more than half from small scale farmers particularly in the central region (Calima *et al.*, 2014; FAO, 2011). Commercially, Mozambique exports over 85,000 metric tons of bananas (0.5% global market share) to East Europe, Middle East, South Africa (with small re-exports on to Botswana and Swaziland), Zimbabwe, and Zambia (Calima *et al.*, 2014). According to Calima *et al.* (2014), fifteen medium to large commercial plantations are in operation in Mozambique. Matanuska (in Nampula) and Bananalandia (in Maputo) are the two largest plantations while a range of companies operate in the South, among which are Beluzi Bananas, Lda (an organic banana operation), Rio Verde, and AAA Enterprises.

According to Calima *et al.* (2014), Matanuska and Jacaranda export from Northern Mozambique to the Middle East and East Europe, while plantations from Namaacha and Boane in Maputo export to South Africa. Banana production has been increasing in Mozambique and exports have reached an annual average of USD 70.9 million in 2011-2014 (Cruz and Mafambissa, 2016).

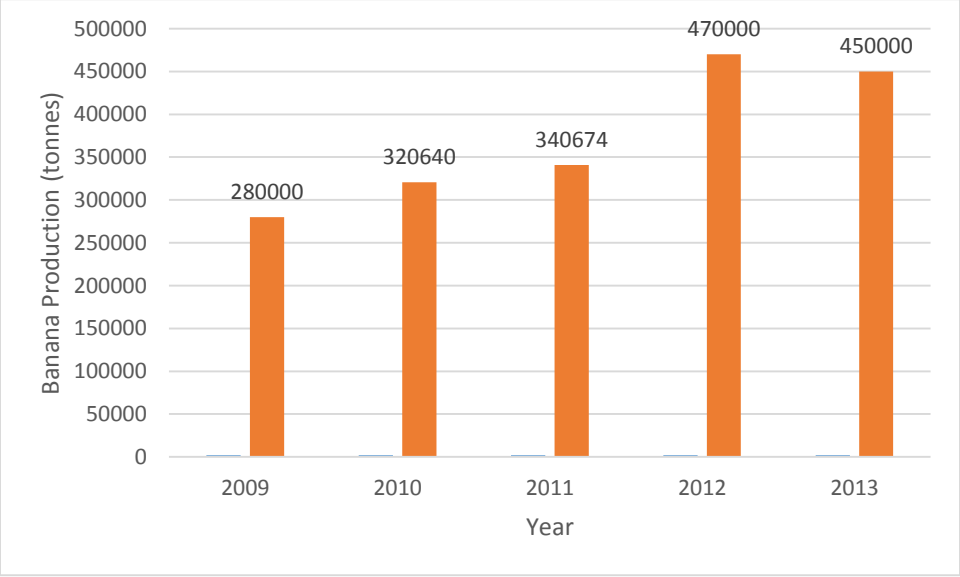

Figure 2: Banana Production from Mozambique, 2009-2013. Source: FAOSTAT (2016).

However, just like other agricultural sectors in the country, the banana industry has continued to be plugged by various problems. These challenges include; limited government support, harsh climatic conditions especially in the south of Mozambique, poor production technologies and extension services, pests such as nematodes and weevils and diseases like Banana bunchy top virus, Sigatoka and Panama disease (Uazire *et al*., 2008). Most recently (2013), a devastating strain of Panama disease (TR4) was discovered to be present in Mozambique and it is the only country in Africa in which the disease has been confirmed (MINAG, 2014).

2.2 Panama disease (Fusarium wilt of banana)

Fusarium oxysporum f. sp. *cubense* (*Foc*) is the causative agent of Panama disease or Fusarium wilt of banana and is among the most catastrophic fungal diseases in agriculture. The species *Fusarium oxysporum* is among 10% of the species in kingdom fungi that have been described scientifically and is the most widely spread species in the genus Fusarium (Toome and Aime, n.d.; Leslie and Summerell, 2006). *Fusarium oxysporum* are saprophytes characterized by the ability to survive for long periods of time in soil habitats where they exist as a usual constituent of organic matter and rhizosphere of many plant species world over (Gordon and Martyn, 1997; Garrett, 1970). The species are morphologically indistinguishable and composed of both non-pathogenic

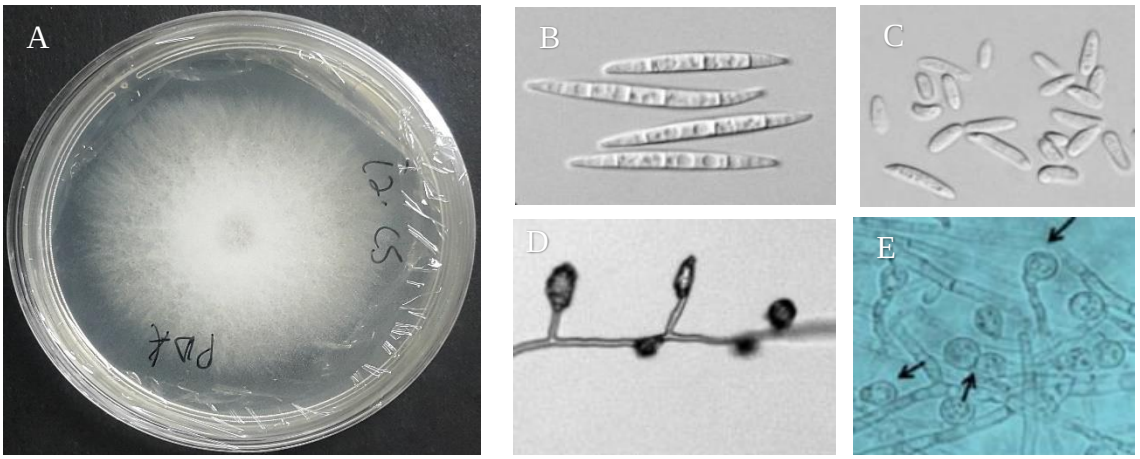

Figure 3: Morphological traits of Foc. A – Foc race 4 culture on PDA media. B – Macroconidia. C – Microconidia. D – Microconidia in situ. E – Chlamydospores. B, C and D – Adopted from Leslie and Summerell (2006) and Modified. E – Adopted from Pérez-Vicente and Dita (2014).

and pathogenic root-rotting or vascular wilt causing organism responsible for Fusarium wilts of several plants (Baayen *et al.,* 2000; Gordon and Okamoto, 1992). Distinctively, *Fusarium oxysporum* produce; microconidia in false heads on short phialides formed on the hyphae, four to eight celled sickle-shaped macroconidia formed in sporodochia on branched conidiophores from monophialides and the smooth or rough walled chlamydospores in pairs or singly in hyphae or conidia (Leslie and Summerell, 2006; Nelson *et al.*, 1983) (Figure 3). Chlamydospores have aided the survival (over 3 decades) and dispersal of *Fusarium oxysporum* through space (various soil habitats) and time as they are resistant to desiccation and unfavourable environmental conditions.

Due to a high level of host specificity, *Fusarium oxysporum* has been divided into *formae speciales*. More than 150 formae speciales have been described capable of causing wilts of several agricultural crops including Panama disease in banana (Armstrong, 1981; Fourie *et al.,* 2009; Fourie *et al.,* 2011). Division into *formae speciales* is based on pathogenicity of species of *Fusarium oxysporum* on a host or group of hosts (O'Donnell *et al.*, 2009; Correll, 1991). Further, *formae speciales* are divided into races based on virulence to a set of differential host cultivars that vary in disease resistance.

The disease is caused by three strains of *Foc* which include: Race 1, Race 2 and Race 4 (Pérez-Vicente *et al.*, 2014). Race 3 was excepted from the *Foc* strains since it is virulent to only *Heliconia spp* and not *Musa spp* (Ploetz, 2005). According to CABI (2007), Race 1 attacks: *Musa textilis* (abacá), 'Gros Michel' (AAA genome), 'Maqueño' (AAB genome), 'Silk' (AAB genome), 'Pome' (AAB genome), Pisang Awak (ABB genome), 'I.C.2' (AAAA genome- bred hybrid), Race 2 attacks: 'Bluggoe' (ABB genome), bred AAAA hybrids and Race 4 attacks: Cultivars in the Cavendish subgroup (AAA genome), such as 'Dwarf Cavendish', 'Grand Nain' and 'Williams' plus cultivars susceptible to race 1 and race 2.

Formae speciales of *Fusarium oxysporum* have been grouped further based on vegetative compatibility (Puhalla, 1985). Vegetative compatibility has provided for characterization of sub-specific groups of fungi based on genetics as opposed to host-pathogen interactions (Correll, 1991). Vegetative compatible fungi, applied to study relationships within *Foc*; are those that can form functionally diploid cells (heterokaryons) from haploid cells (Visser, 2010; Leslie, 1990; 1993; Puhalla, 1985). The prevailing technique of vegetative compatibility groups (VCG) was

initially developed by Cove (1976) using *Aspergillus nidulans* and later improved by Puhalla and Correll *et al.* in 1985 and 1987 respectively with *Fusarium oxysporum* (Pérez-Vicente *et al.* 2014). According to Pérez-Vicente *et al.* (2014), "in the *Foc-Musa* relationship where genetic analysis of pathogenicity is practically impossible, VCGs are strong indicators of pathogenic behaviour and are important tools in the study of biology and genetics". This is possible because vegetative compatibility in *Fusarium* species requires alleles of at least each of the 10-corresponding vegetative incompatible (*vic*) loci from paired fungal strains to be identical since VCGs constitute closely related clonally derived sub-populations (Puhalla and Spieth, 1985; Pérez-Vicente *et al.*, 2014; Correll, 1991). At least 24 vegetative compatibility groups have been identified within *Foc.* (Fourie *et al.*, 2011).

2.2.1 History of *Foc*

Fusarium. oxysporum f. sp. *cubense* is supposedly said to have co-evolved with banana in Southeast Asia (Ploetz and Pegg, 1997; Pegg *et al.*, 1993). Therefore, spread of the pathogen to new areas in Asia could have been through the first lot of vegetative planting material in suckers/rhizomes during the prehistoric times and later to the rest of the world except countries around the Mediterranean Sea, South-Pacific Islands, parts of Melanesian and Somalia (Bentley *et al.*, 1995; Stover, 1962; Stover and Simmonds, 1987). Further, failure by early farmers to associate symptoms to the subsequent decline in banana yields despite being visible on planting material could have facilitated the inevitable spread of Panama disease during the prehistoric times (Jones, 2002). It is likely that the disease was afflicting susceptible banana cultivars in many locations around the world by the time it was first described on the highly susceptible cultivar 'Silk' (AAB genome) in Australia in the 19th century (Stover, 1962; Bancroft, 1876). Therefore, details of the initial spread of the disease, which seems to have occurred before intensive research on plant diseases and their associated effects on agriculture, are not known.

It was only after the commercial production of the cultivar 'Gros Michel' (AAA genome) started in the Latin American/Caribbean region late in the 19th century that the disease became important (Stover, 1962). Here it acquired the name Panama disease after the country where it first caused extensive damage. Although the pathogen was almost certainly disseminated with planting material of 'Gros Michel', it is highly likely that it already existed in many locations in small

plantings of cultivars like 'Silk', which were introduced to the Americas in colonial times. Gros Michel was a cultivar with massive bunches of large and flavourful fruit that was resistant to damage and hence enabling its intact shipment (Ploetz, 2005). However, being susceptible to *Foc* Race 1, Gros Michel production had been severely affected by the mid-1900s in major banana exporting countries (Ploetz, 2005). This massive flavourful cultivar was replaced by the Cavendish cultivars that were more resistant to the *Foc* Race 1 (Pérez-Vicente and Dita, 2014). It was the discovery of 'Valery', a commercially acceptable cultivar in the Cavendish subgroup (AAA genome) that saved the banana industry due to its resistance to populations of *Foc* present in the Americas (Jones, 2002). Hence, Valery replaced 'Gros Michel' in the 1960s to be the main cultivar of the trades. Since 'Valery', other Cavendish cultivars, such as 'Robusta', 'Williams' and 'Grand Nain', have also gained prominence. These Cavendish cultivars however were smaller, less flavourful and more susceptible to damage than the Gros Michel.

The resistance in the Cavendish cultivars was however, overcome by a new Race of *Foc*, Race 4 (Pérez-Vicente and Dita, 2014). In Asia, the destructive pinch of Panama disease was first felt in Taiwan in the late 1960s on the newly adapted Cavendish plantations lowering the production to 10% of the previous output and subsequently increasing production cost which severely affected banana export (Pérez-Vicente and Dita, 2014; Masdek *et al*., 2003; Nasdir, 2003). Later, in the 1990s, TR4 is reported to have devastated banana plantations in Malaysia and Indonesia before spreading to Australia and South East Asia in less than a decade. Recently in 2013, TR4 was reported in Africa (Mozambique), Oman and Jordan (Pérez-Vicente and Dita, 2014).

Information on the history of Panama disease is scarce for Mozambique. Noronha (1970) noted the presence of the disease in Mozambique though he did not specify the present races. Later, Plumb-Dhindsa and Mondjane (1984), highlighted the importance of the disease as of great importance in one or more areas in Mozambique. TR4, the new strain of *Foc* was however confirmed to be present in Mozambique in 2013 (MINAG, 2014) after an outbreak in one of the banana commercial farms (Matanuska) in Nampula Province, Northern Mozambique.

2.2.2 Signs and symptoms of Panama disease

Panama disease on banana commences when the *Foc* spores encounter the xylem through wounds or injuries on the plant (Sequeira *et al.*, 1958). In case of *Foc* soil inoculum, the spores germinate when in proximity to banana roots penetrating the root tips of small lateral or feeder roots before progressing to the rhizome (Ploetz, 2000; Beckman and Ploetz, 1990; Sequeira *et al.,* 1958). In the xylem, *Foc* produce microconidia that accumulate upstream affecting water movement in the plant. Beneath sieve cells, blocked spores germinate, spread and block the entire plant water conducting system resulting in typical wilt symptoms (Stover *et al.,* 1961). Internally, the *Foc* invasion is manifested as a distinguishing discoloration of yellow or brownish spots and streaks within the vascular strands of the rhizome (most severe between stele and cortex) and pseudostem which in advanced stages of infection become brick red to brown in colour (Ploetz, 2006; Ploetz, 2000; Stover, 1962; Wardlaw, 1961).

Externally, faint brown streaks and/or spots appear on older leaf sheaths and there can be longitudinal splitting of the outer leaf sheaths in the pseudostem (Gang *et al.,* 2013; Ploetz, 2000). Wilting in infected plants will then become evident first with yellowing of older leaves along the leaf margins, continuing into the midrib till the leaves turn completely brown and die (Figure 4). These symptoms will then progress to younger leaves of the banana plant. External symptoms may not appear until the banana plant is exposed to stressful conditions or during bunching stage (Brandes, 1919). Bending at the petiole close to the midrib, the leaves then gradually collapse hanging down to form a "skirt" of dead leaves around the pseudostem (Ploetz, 2000; Pérez-Vicente et al., 2014). Being the last to show symptoms, younger leaves often remain erect showing bristle-like appearance. (Pérez-Vicente *et al*., 2014). In younger infested plants, growth continues and the emerging leaves have a reduced, shrivelled and distorted lamina.

Fusarium wilt of banana disease may manifest on leaves as characteristic "yellow or green leaf syndrome" (Pérez-Vicente *et al*., 2014). Yellow leaf syndrome, the most noticeable and typical symptom of Fusarium wilt on banana is characterized by a yellow border on older leaves progressing to the younger leaves. As opposed to the yellow leaf syndrome, leaves in the green leaf syndrome remain predominantly green until the petioles bend and collapse. *Fusarium*

oxysporum f. sp. *cubense* symptoms cut across for all races hence specific strains cannot be distinguished by observable symptoms in disease banana plants (Stover, 1962; Ploetz and Pegg,

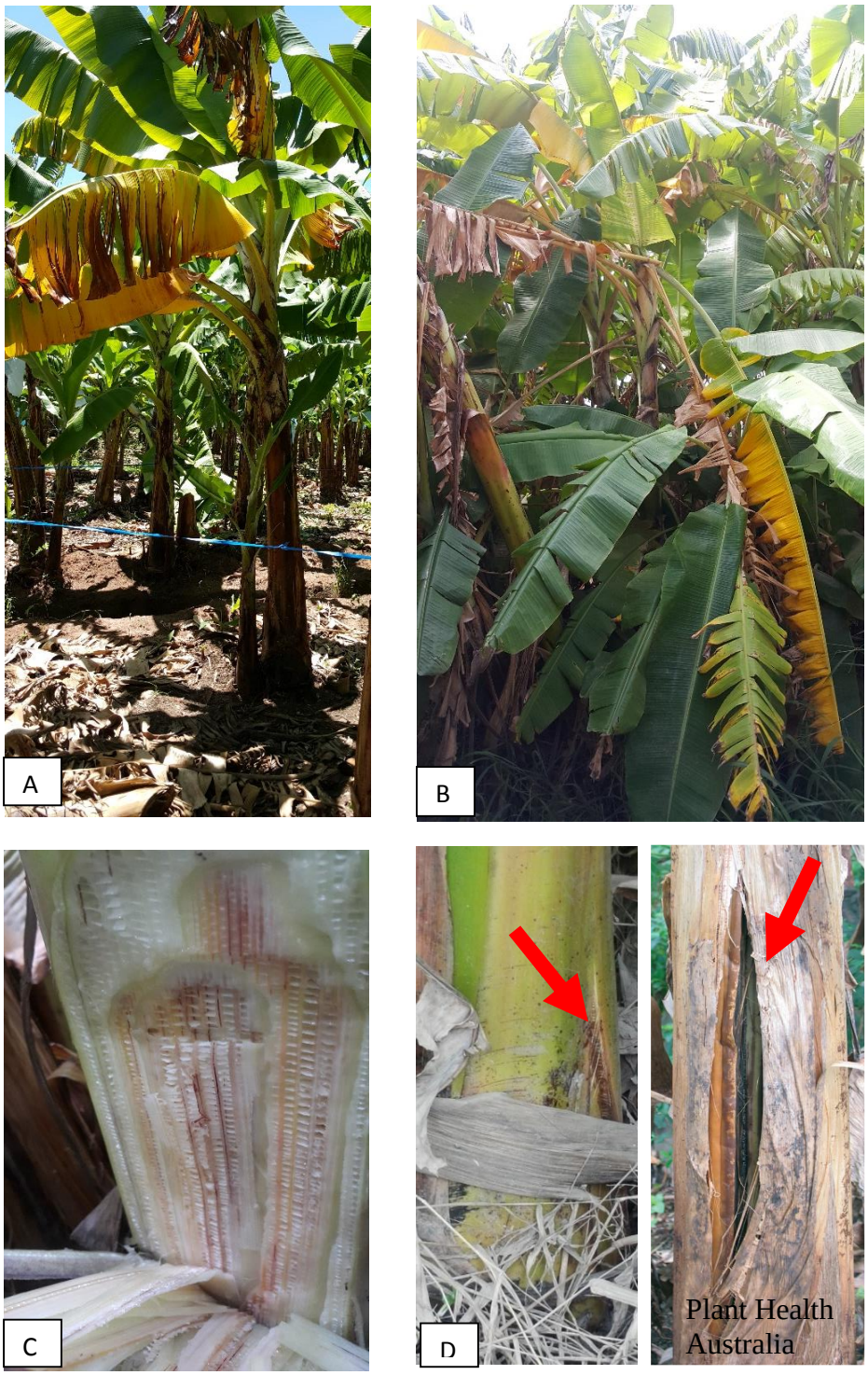

Figure 4: Signs and symptoms of Panama disease as observed in Mozambique; A – Wilting and yellowing starting with older leaves from leaf margin to midrib. B - Bending at the petiole close to the midrib forming skirt of dead leaves. C – Internal discoloration of yellow, red or brownish spots and streaks within the vascular strands. D - Longitudinal splitting of the outer leaf sheaths in the pseudostem. A – Nampula, Jacaranda. B – Maputo, Boane.

2000). There is continuous growth of the fungus in dying banana plants from outside the xylem in the surrounding tissues with resultant development of abundant chlamydospores that can persist in the soil when the plant dies (Pérez-Vicente *et al*., 2014; Gang *et al.,* 2013).

2.2.3 Management of Panama disease

Plant disease management has increasingly become necessary in modern agriculture in attempt to maintain production, quality and yield of plant products which would otherwise be affected by plant diseases. Agrios (2005), grouped methods of disease management into regulatory, cultural, biological, physical, and chemical based on the control agents used. Regulatory methods are intended to exclude a pathogen from a host or geographical area. Cultural and biological control methods often aid plants to avoid contact with a pathogen, create unfavourable environmental conditions to the pathogen or favourable conditions to the organisms antagonistic to the pathogen, reduce or eradicate pathogen inoculum and improve the resistance of the host plants. While physical and chemical methods are used when the pathogen is likely to occur, already occurring or in curing an infected plant host or area with the intention of preventing further spread of the pathogen.

Management of *Foc* is mandatory in banana farming to prevent spread of the devastating disease either within the infested sites or from diseased areas to healthy areas. *Fusarium oxysporum* f. sp. *cubense*, just as all other species in *Fusarium oxysporum*, is a soil borne saprophyte that is capable of surviving in soil organic matter and root rhizosphere for long periods of time (Garrett, 1970). The polycyclic pathogen (Ploetz, 2015) can survive in the soil under unfavourable conditions for up to 3 decades making management of Panama disease difficult (Ploetz, 2006; Stover, 1962; Simmonds, 1966).

Panama disease is spread to new areas through use of infected banana planting materials and with soil carried through human movements, water runoff, machinery, animals and contaminated farming tools (Pérez-Vicente and Dita, 2014). Agrios (2005), recognized disease management techniques that involve exclusion or reduction of the initial inoculum followed by an effective control measure such as chemicals as the most efficient in the management of polycyclic pathogens such as *Foc*. Pérez-Vicente and Dita (2014), stated that the first step in Panama disease

management is management of the risk which involves implementation of phytosanitary measures that avert the entry of the disease into disease free areas and eradication-confinement or suppression-contention measures in infected areas. These can be equated to regulatory and cultural methods described by Agrios (2005).

Regulatory measures including quarantine against Panama disease (TR4), extensive disease surveillance and public sensitization campaigns can be very effective in preventing spread of the disease when used effectively and thoroughly (Pérez-Vicente and Dita, 2014). According to MINAG (2014), a quarantine against Panama disease, TR4 is in place and generally, certification from the ministry of agriculture of Mozambique is required to move any plant material including banana sucker and corms. Additionally, inspection points have been designated by the ministry at major entry and exit points such as seaports and airports to monitor movement of planting material. To effect a quarantine, issues regarding the quarantined disease have to be effectively communicated to the public and the ministry of agriculture of Mozambique has made efforts to reach the public through television, radio, local newspapers, awareness and management leaflets and training and workshops across the country.

In Matanuska and Jacaranda, blocks suspected to bear TR4 within the plantation are immediately fenced out to restrict access and avoid direct interaction between infected blocks and the disease-free areas. The workers, machinery, tools and any other equipment used in the infected blocks have been separated from those used in disease free blocks to minimize interaction and hence spread of TR4. Both Matanuska and Jacaranda have been enclosed all round to prevent unnecessary access into the plantations. All automobiles that enter or leave the respective plantations are subjected to chemical disinfection along the entry and exit points to reduce the chances of the possibility of spread of TR4 to the surrounding or distant farms. In attempt to reduce on the TR4 inoculum, at Matanuska, severely infected plants and diseased plants whose bunches have been harvested are cut into small pieces, mixed with dry material, placed into a hole dug next to the base of the diseased banana plant and burnt in such a way that higher temperatures (over 67˚C) are attained across a relatively long period to ensure total death of the fungus. At Jacaranda, fungicides are added to the burning material to ensure maximum elimination of the TR4 pathogen. Pérez-Vicente et al. (2014), recommended the destruction of infected plants and all plants in the surrounding 7.5 m radius with fire. However, this is getting increasingly difficult with the growing

number of infected plants since the applicability is limited to relatively small scale and individual banana plants.

Cultural methods that include; crop rotation, intercropping, flood fallowing and use of fertilizers and soil amendments have also proven to be very important in management of Panama disease. These approaches are also environmentally friendly and are intended to reduce the pathogen inoculum, boost the host or pathogen natural enemies and to create unfavourable conditions to the pathogen. In China, crop rotation and intercropping of banana with Chinese chive (*Allium tuberosum* Rottler) has proven to be significant in the suppression of Panama disease (Huang *et al., 2012;* Zhang *et al.*, 2013). Zhang *et al.* (2013), identified four organosulfur compounds (dimethyl trisulfide, dimethyl disulfide, dipropyl disulfide, and dipropyl trisulfide) and 2-methyl-2-pentenal from the leaves and roots of Chinese chive as the chemical compounds responsible for the inhibitory effect on *Foc*. Rotating banana with some other crops such as paddy-rice (Su *et al.*, 1986), sugarcane or sunflowers for 3 years (Hwang, 1985) has not registered much success in the management of Panama disease. However, intercropping and crop rotation with the right crops can be very effective.

Flooding of infested areas with water for several months was reported to give short term control measures against Panama disease (Stover, 1962). This method did not effectively control *Foc* in the long run as the created anaerobic conditions had a negative effect on several microbes that could have given the soil natural suppressiveness, hence resulting in more severe re-occurrences of Panama disease (Stover and Ploetz, 1990). Earlier studies involving addition of fertilizers such as NPK into the soil showed negligible effects in the management of Panama disease (Stover, 1962). High soil fertility from organic matter which translates to a higher soil microbial index (*Bacillus* spp., *Streptomyces* spp., *Trichoderma* spp., *Aspergillus* spp., *Penicillium* spp., and *Gliocladium* spp.) was reported to have a suppressive capacity to Panama disease in Indonesia (Sudarma and Suprapta, 2011). Majority of these microbes have antagonistic traits against *F. oxysporum* which help maintain a highly suppressive soil (Garbeva *et al.,* 2004). Hence, maintenance of soil fertility through addition of organic matter is very important in the suppression of Panama disease. These microbes can also be isolated and mass produced for use as biological control agents of Panama disease.

Microbial biological control (MBC) methods are widely gaining acceptance in agriculture since the products are environmentally friendly, overcome public safety and food products' concerns and offer newer mechanisms of disease management (Thangavelu and Mustaffa, 2012; Fravel *et al.,* 2003). Woo *et al.* (2014), categorized microbial biological control agents (MBCA) in to generalists and specialists based on the mechanism of action or interaction with the pathogen and/or host plant. The generalists (*Bacillus, Pseudomonas, Streptomyces, Trichoderma, Clonostachys, yeasts* among others) use various mechanisms of action against a wide range of taxonomically different plant pathogens while the specialists (*Agrobacterium, Ampelomyces, Coniothyrium,* non-pathogenic Fusaria, atoxigenic *Aspergillus* among others) are antagonistic to only one or a few specific pathogens. MBCA can directly interact with the plant pathogen hence killing or inhibiting it through parasitism or mycoparasitism in case of physical penetration, antibiosis, production of lytic enzymes that break down the pathogens' cell walls' polysaccharides, chitin and p-glucans, and also induce competition for nutrients or niches (Junaid *et al.,* 2013; Trabelsi and Mhamdi, 2013; Hermosa *et al.,* 2013; Benítez *et al.,* 2010; Sempere and Santamarina, 2009; Mukherjee *et al.,* 2006; Woo *et al.,* 2006; Howell, 2003; Donzelli *et al.,* 2001). Indirectly, MBCA can induce the plant hosts resistance to the pathogens through activation of molecules (enzymes, proteins, secondary metabolites) that detect and counter effectors/elicitors and/or associated pathogens, activation of induced systemic acquired resistance and improve tolerance to abiotic stresses such as drought and flooding (Woo *et al.,* 2014; Junaid *et al.,* 2013; Rawat *et al.,* 2012; Hanhong, 2011; Fontenelle *et al.,* 2011; Tucci *et al.,* 2011). Most MBCAs however employ several mechanisms/modes of action to achieve effective antagonism against plant pathogens (Berg, 2009). The use of MBC in the management of *Foc* is still in the developing stages but success stories have been reported in the management of Fusarium wilts of other crops by several authors (Raza *et al.,* 2016; Naing *et al.,* 2015; Poozad and Kariminik, 2015; Gang *et al.,* 2013; Thangavelu and Mustaffa, 2012; Shishido *et al.,* 2005; Alabouvette *et al.,* 1993).

The use of synthetic chemicals has registered less success in the management of the Panama disease under filed conditions despite showing positive results under in-vitro and in vivo conditions (Nel *et al.,* 2007; Stover, 1962; Meredith, 1943). Synthetic chemicals are however very important in the management of Panama disease as they are used as disinfectants of farm tools, shoes, vehicles and any other equipment used in infected areas to prevent spread of the disease to

healthy areas. Farmcleanse, a detergent-based non-corrosive environmentally friendly degreaser with a quaternary ammonium additive, was reported in Australia as the most effective disinfectant against *Foc* and it replaced chlorine bleach, methylated spirits and copper oxychloride solutions (Moore *et al.,* 1999a/b; Nel *et al.,* 2007). The use of chemicals in the management of Panama disease has been losing prominence in the last 40 years (Nel *et al.,* 2007) and this can be attributed to the ever-rising negative public perception on use of synthetic agro-chemicals in agriculture on human health and environmental safety (Woo *et al.,* 2014). In the management of *Foc*, synthetic chemicals have been used as fungicides, surface sterilants, soil fumigants and plant activators. Over the years, Mercury, R&H-3888 (nitrile), EP-161 (methyl isothiocyanate), Vapam (sodium n-methyl dithiocarbamate), allyl alcohol, Mylone (3,5-dimethyltetrahydro-1,3,5,2H-thiadiazine-2-thione), Nabam, CP 30249 [2-chloro-3-(tolylsulfonyl) propionitrile], Formaldehyde, carbendazim, phosphonates, copper sulphate, carbolineum and methyl bromide have offered either inconsistent or short term results in the management of *Foc* (Davis *et al.,* 1994; Herbert and Marx, 1990; Lakshmanan *et al.,* 1987; Stover, 1962; Rishbeth and Naylor, 1957; Meredith, 1943; Brandes, 1919).

Cultivars resistant to Panama disease have provided the most efficient management measure (Pérez-Vicente and Dita, 2014). The capacity of Panama disease to wipe out susceptible cultivars and the difficulty faced in eliminating the pathogen using available management measures have led to strengthening of banana breeding programs in attempt to develop cultivars relatively resistant to the *Foc* pathogen. In the 1960s, the banana industry was saved by the discovery of 'Cavendish cultivars', a replacement for 'Gros Michel', the main export cultivar then that was wiped out by Race 1 (Ploetz, 2000). The Cavendish subgroup are however now susceptible to TR4. Being slow, low numbers of fertile seeds (Gros Michel) and sterility of some banana cultivars (Cavendish subgroup) have made breeding for resistant banana cultivars very difficult (Jones, 2000). Consumer taste preferences have also limited the capacity of farmers to adopt newly bred cultivars, soma-clones or hybrids resistant to Panama disease (Stover and Buddenhagen, 1986; Viljoen, 2002).

Phenolic compounds: These are a large class of secondary metabolites that range from simple phenolic acids to complex polymeric compounds involved in a variety of physiological processes in plants (Farkas and Kiraaly, 1962; Nicholson and Hammerschmidt, 1992; Cheynier, 2012;

Duodu, 2014). Unlike animals that interact with the environment through mobility, plants depend on production of a greater range of phenolic compounds for antibiosis against pathogens, UV light screening, anti-nutritional/unpalatable properties against herbivores, plant pigmentation (red, blue and purple pigments), anti-oxidation and metal chelation and as above and below ground signalling agents (Kutchan, 2001; Swain, 1977; Bell and Charlwood, 1980). Phenolic compounds exist as either preformed phenolics, those synthesized during normal plant development or induced phenolics, those that are accumulated during biotic or abiotic stress (Hammerschmidt, 2003; Lattanzio *et al*., 2006). In plant disease management, antibiotic properties of phenolic compounds are increasingly gaining prominence and various phenolic compounds have been identified and extracted. The ability of a plant host to ward off pathogen attack at its initial stages determines its level of resistance to that pathogen and this involves production of antibiotic phenolics among other modifications (Nicholson and Hammerschmidt, 1992). De Ascensao, and Dubery (2000), observed this trend of modifications in Goldfinger, a banana cultivar that was more resistant to Race 4 of Panama disease as compared to its susceptible counterpart, Williams, under similar conditions. Hence, identification of effectively antibiotic phenolic compounds and their subsequent use in management of plant diseases especially Panama disease can help boost production in diseased areas through induction of resistance to susceptible plants.

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CHAPTER THREE

Distribution and Diversity of *Fusarium oxysporum* f. sp. *cubense* in Mozambique

Abstract

Panama disease, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is a recurrent disease of banana that is considered one of the most devastating diseases in agriculture. Believed to have evolved with banana in Asia, three races (race 1, 2 and 4) of *Foc*, structured into 24 vegetative compatibility groups (VCGs) have been reported globally. Tropical race 4 (TR4), a strain that has broken down the resistance in the currently banana export dependent 'Cavendish cultivars' was recently reported in Mozambique. Samples from symptomatic banana plants were collected from three-major banana producing provinces of Manica, Maputo and Nampula. Tropical race 4 was found in the northern province of Nampula and race 1 in both Nampula and Maputo. Panama disease was not found in the leading banana producing province of Manica. VCGs 01213/16, 01213, 01216, 0125, 01220 and 0124 were found to be present in Mozambique. Currently, TR4 is restricted to two commercial banana plantations but the number of infected plants within is intensely rising despite desperate attempts to manage the disease. This calls for more effective and urgent measures that can eradicate the disease while ensuring prevention of spread to surrounding traditional banana farmers, other banana producing provinces and countries within the region.

3.1 Introduction

Banana is the fourth most important food crop in the world after rice, wheat and maize respectively (FAO, 2014). In Mozambique, it is the most consumed fruit, accounting for more than half of fruit consumption in the country (FAO, 2011). Mozambique produces an average of 321, 995 tons of banana per year from 14, 380 ha, encompassing both the private sector and small-scale farmers (MINAG, 2014). Small scale farmers produce about 122, 000 tons of banana from 10, 000 ha while the rest is from the private sector consisting of large scale (over 1000 ha) and medium scale (100 ha) producers (Calima, 2014; MINAG, 2014). Despite having some cooking and other dessert cultivars, much of banana produced in Mozambique including Williams, ASDIA, dwarf Cavendish, Grand Naine and Chinese Cavendish belong to the dessert type Cavendish subgroup (Uazire *et al.*, 2008; MINAG, 2014). Most of the banana, approximately 85% is produced for local markets and consumption while 15% (0.5% of the global market share) is exported to South Africa (more than 75% of banana exports), East Europe, Middle East and other neighbouring countries (Calima, 2014; FAO, 2014). The establishment of large banana plantations in Maputo and Nampula provinces, through encouragement of local investments and the creation of agro-partnerships with foreign agribusinesses for the development of export agriculture, led to increase in banana export earnings from USD 4.5 million in 2008 to USD 37.7 million in 2013 (Calima, 2014).

The banana industry is, however, faced with various production challenges which include, among others, pests such as the fruit flies and diseases such as Panama disease (Uazire *et al.*, 2008; MINAG, 2014; Butler, 2013). Noronha (1970) and Plumb-Dhindsa and Mondjane (1984) respectively, reported Panama disease as a significant disease of banana in Mozambique.

Panama disease, also known as Fusarium wilt of banana, is one of the most menacing diseases of the crop that has devastated commercial production throughout ages of banana export in the world (Stover, 1962; Correll, 1991; Ploetz, 2000; Visser *et al.*, 2010). The disease is caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), a soil fungus that invades the banana vascular system through the roots, corm, pseudostem and fruit stalk before growing out of the xylem into surrounding tissue and later into soil (Stover, 1962; Ploetz, 2000).

Based on virulence on banana subgroups or cultivars, three races of *Foc* have been identified. Race 1 is pathogenic to Gros Michel, Musa textilis (abacá), Maqueño, Silk, Pome, Pisang Awak, I.C.2 while Race 2 attacks Bluggoe, bred AAAA hybrids and Race 4 attacks cultivars in the Cavendish subgroup along with all cultivars susceptible to race 1 and race 2 (Fourie *et al.*, 2011; CABI, 2007; Beckman and Ploetz, 1990). Race 1 unlocked the destructive path of Panama disease when it almost ended banana export trade which was dependent on the susceptible 'Gros Michel' cultivars in the mid-20th century (Stover, 1962, Stover and Ploetz, 1990). The dessert banana export industry was saved by the discovery of Cavendish cultivars that were resistant to race 1. Currently, Race 4 has rendered Cavendish cultivars susceptible to Panama disease in absence of yet a known resistant cultivar to effectively replace the Cavendish banana (Ploetz, 2000).

From the race structure, *Foc* has been grouped further into 24 vegetative compatibility groups (Fourie *et al.*, 2011). Vegetative compatible fungi, applied to study relationships within *Foc*, are those that can form a zone of wild-type growing functionally diploid cells (heterokaryons) from haploid cells of phenotypically distinct nit mutants when paired on minimal media (Visser, 2010; Leslie, 1993; Correll, 1991; Puhalla, 1985). The distribution of VCGs across banana producing areas has been reported to be restrictive to some areas or regions, while others are known to exist in several geographic areas (Fourie *et al.*, 2011).

With the discovery of Tropical Race 4 (TR4) in 2013, Panama disease is currently the most significant disease and challenge facing banana production in Mozambique (García-Bastidas *et al*., 2016; Pérez-Vicente *et al*., 2014). TR4 was first noticed in Matanuska, a commercial farm planted with about 1,400 ha of banana before later being found in Jacaranda, a commercial farm about 150 km away from Matanuska. At Matanuska, over 2,000 new cases of infected plants are being noticed every week (MINAG, 2014).

Currently, cases of TR4 have only been reported in the two banana commercial farms in Nampula Province. With banana farming being a common practice in Nampula Province and across the entire country, the extent of occurrence of Panama disease especially TR4 has not been extensively established. Considering the rate at which new cases have been reported in Matanuska farm, this disease poses a devastating effect to future banana production in Mozambique. This current position needs to urgently be reversed to safeguard the future of banana production in the country.

To achieve a relatively safe position, the area to which the Panama disease has spread should clearly be mapped out and disease spread checked to minimize the emergence of new cases in disease free areas.

The aim of this study is to determine the diversity of *Foc* in the three-major banana growing provinces of Mozambique through identification of VCGs to establish the geographical distribution of *Foc*.

3.2 Materials and Methods

3.2.1 Survey

Three provinces; Manica, Maputo and Nampula were surveyed and a total of 56 samples (23, 12 and 21 samples respectively) were collected (Figure 5). Manica, Maputo and Nampula are the leading banana producing provinces in Mozambique.

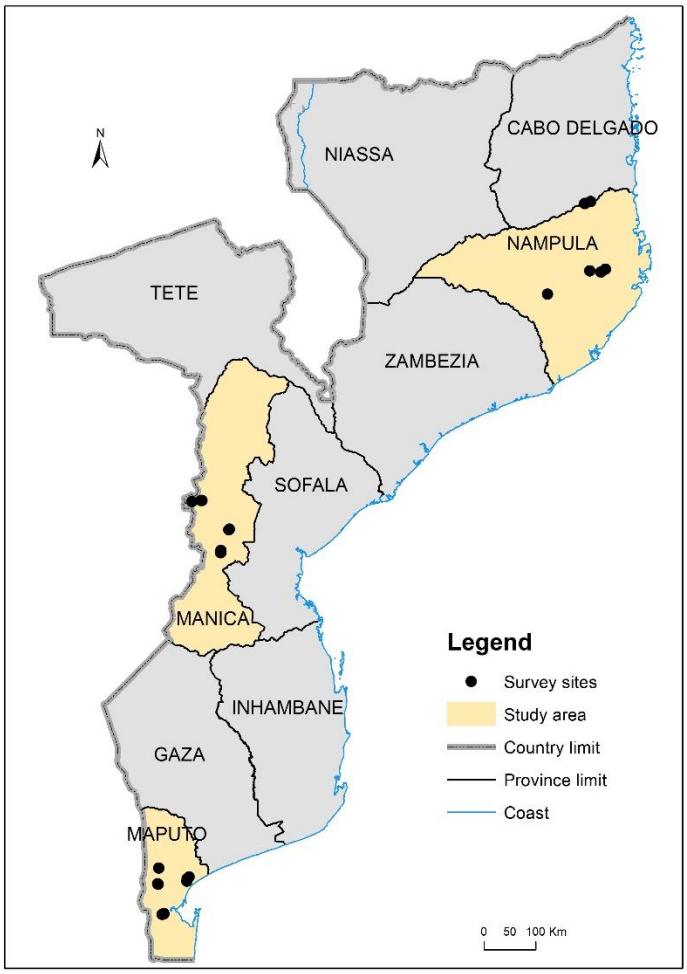

Figure 5: Map showing the study areas (Provinces).

Purposively, 3 districts were selected from each of the provinces for the survey. In each district, 2 administrative posts were considered for the study. The districts that were selected from each of the provinces include: Nampula Province (Monapo, Muecate and Erati), Maputo Province (Boane,

Manhiça and Moamba) and Sussundenga, Manica and Macate districts in Manica Province. The survey areas were chosen based on the level of banana production in which major banana producing areas were considered. Associated survey costs and time frame limitations during the study were also considered.

The survey was conducted under the guidance of province and district agricultural extension staff (DPAs). Banana plantations were selected for sampling by snowball. Snowball sampling technique was used to help speed up and ease access to banana farms known by already sampled farmers. Both private sector farms and small scale farms were visited.

3.2.2 Sampling

In the survey, *Foc* samples were obtained from banana plants bearing Fusarium wilt symptoms. *Foc* sampling was independent of banana cultivars found in the field despite the prioritization of traditionally grown banana cultivars over Cavendish cultivars. This was done to widen the diversity of *Foc* possibly found in the field since the pathogenicity of *Foc* strains is highly restrictive to respective susceptible banana cultivars or subgroups. To sample plants, a zig-zag transect across the field was used and samples were obtained from symptomatic plants found in the field. In each of the 2 administrative posts, five (5) banana plantations were visited. However, this number of plantations could not be reached in some administrative posts due to fewer numbers of accessible farmers. GPS coordinates were recorded for each plant in which *Foc* samples were extracted.

3.2.3 Sample Extraction

Symptomatic banana plants suspected to have Panama disease were selected. Using a machete, a section of a pseudostem from the outside-leaf inwards was cut from as low and close to the centre of the pseudostem as possible to expose continuous coloured vascular strands. Samples of thin strands were obtained from evident continuous coloured vascular strands while ensuring that the rotting ends were avoided. The thin strands were then wrapped in a sterile napkin (serviette) before placing inside a properly labelled envelop. The sterile napkins were used to absorb excess water from the sampled banana strands hence facilitating drying of the sample intact at room temperature and to prevent rotting of the sample. Knives, gumboots/shoes and all sample extraction materials

were sterilized with 70% alcohol prior to and after use on each symptomatic plant. New gloves were also worn between sampled plants.

3.2.4 Morphological Identification and isolation of fungal isolates:

To generate Fungal cultures from banana samples obtained during the survey, 4 pieces of about 4mm from banana strands in each envelope were cut and placed in a 90mm petri dish containing PDA media amended with streptomycin (Figure 6). The plates were left at room temperature for 7 days to allow for growth of the fungal isolates. The cultures were morphologically examined to identify the associated fungi. Each of the identified *Foc* culture isolates was then subsequently purified, single spored, re-cultivated on PDA and incubated for 7 days at room temperature before molecular identification.

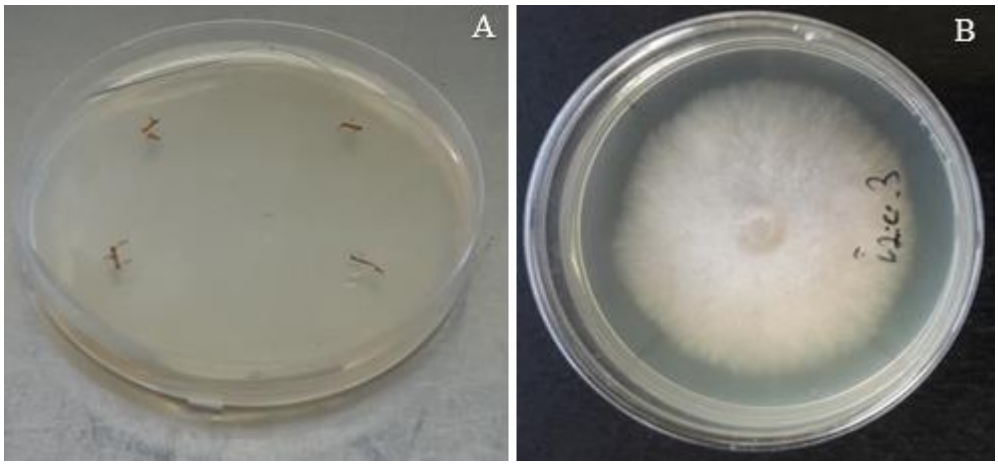

Figure 6: Isolation of *Foc* in the laboratory. A – Cultivation of coloured vascular strands. B - Morphologically identified *Fusarium oxysporum* culture.

3.2.5 DNA Extraction:

DNA extraction was done as described by Osmundson *et al.,* (2013). In short, mycelia from the morphologically identified *Foc* isolates was scrapped off into 2 ml Eppendorf micro-centrifuge tubes under sterile conditions. To the Eppendorf tubes containing mycelia, glass beads and 700ųl of 0.5 M NaOH was added. To lyse the mycelia cells in the Eppendorf tubes, a Retch was used to shake the tube contents for 5 minutes. The Eppendorf tubes were then transferred to a Spectrafuge 24D centrifuge (Labnet International, Edison NJ, USA) and spun at maximum speed (14,000 rpm)

for 1 minute to separate the supernatant from dense residue in the tubes. Five microliters (5ųl) of the supernatant (DNA) were pipetted and diluted in 450ųl of 100Mm Tris-HCL pH 8.0 in new Eppendorf tubes. Two microliters (2 ųl) of the diluted DNA was used for PCR.

3.2.6 Polymerase Chain Reaction (PCR):

With two pairs of primer sets; Lin VI F-D, Lin VI R-D, Lin 6 F-P and Lin 6 R-P (Unpublished) for Lineage VI and a pair, *Foc* TR4-R and *Foc* TR4-F (Dita *et al.,* 2010) for TR4, a PCR was performed on DNA of the morphologically identified *Foc* isolates. For Lineage VI amplification in the PCR, 12 ųl of 1 X Kapa ready mix, 0.5 ųl of each primer at 10 mM and 2 ųl of DNA were added to 9 ųl of distilled water. The PCR cycle conditions included 35 cycles at 94°C for 5 min, 94°C for 45 secs, 62°C for 45 secs, 72°C for 1 min, 72°C for 5 min and finally 4°C for infinity.

For TR4 amplification, 4 ųl of 1x Buffer, 3.5 ųl of 2 mM $MgCl_2$, 1.3 ųl of 0.32 mM dNTPs, 2 ųl of 0.8 mg.ml-1 BSA, 1 ųl of each primer at 10 mM, 0.6 ųl of Taq DNA polymerase, 2 ųl of DNA were added to 34.6 ųl of distilled water. The PCR cycle conditions consisted of 30 cycles at 95°C for 2 min, 95°C for 30 secs, 62°C for 30 secs, 72°C for 30 secs, 72°C for 5 min and finally 4°C for infinity. A positive control and a non-template control were also included for both TR4 and Lineage VI PCR assays. The PCR products (mixed with loading dye) were systematically loaded in agarose gel wells for electrophoresis to separate the DNA fragments.

3.2.7 Vegetative compatibility testing:

Generating *nit* mutants: From each of the *Foc*-PDA plates, four plugs with mycelia were obtained, planted on chlorate media (CLM) with 1.5% chlorate and left at room temperature for 7 days to generate mutants (Correll, 1991). A plug from possibly mutated fast growing cultures that resisted the chlorate in CLM were transferred to Minimal media (MM) slants for observation of *nit* mutant development. *Nit* mutants characterized by poorly sparse thin mycelia growing colonies with no aerial mycelia were then obtained from MM slants for VCG testing (Figure 7). Non-sparse wild type cultures were discarded.

VCG testing: A plug (2 × 2 mm) from the thin *nit* mutant colonies in the slants was obtained and paired with known VCG *nit*- 1 and M mutant testers on 65 mm petri dishes containing MM media.

The plates were then incubated at room temperature (25°C) and inspected after every two days for heterokaryon growth of complementary *nit* mutants. The nit mutants that formed a dense, wild-type growth (heterokaryon growth) on MM were regarded complementary to the *nit* mutant testers and were assigned to the same VCG while those that failed to form a dense wild type growth were regarded incompatible and hence belonging to different VCGs.

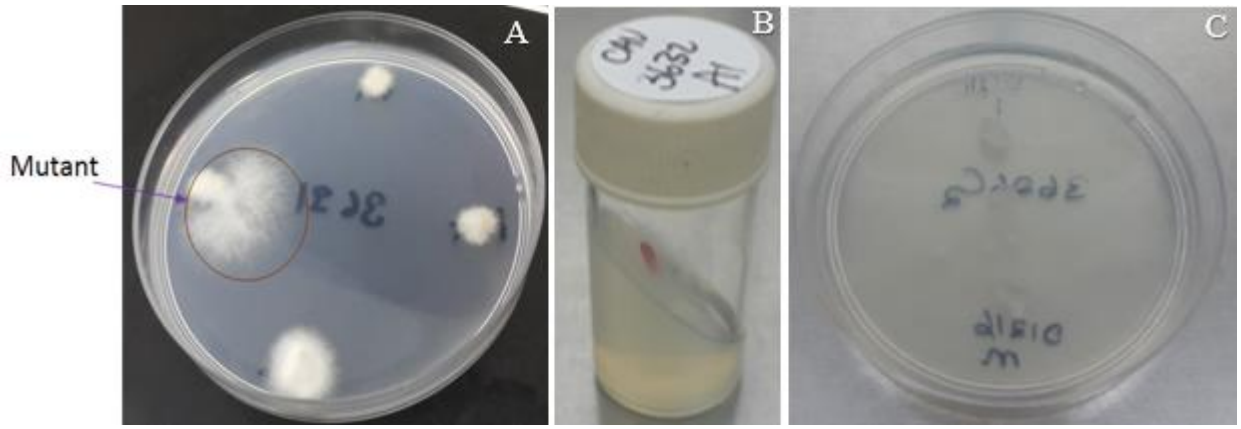

Figure 7: Steps involved in generating VCGs. A – Development of mutants on Chlorate media. B – Continued development of mutants on minimal media slants. C – Pairing of generated mutants with known nit mutants.

3.3 Results

3.3.1 Isolate identification

Forty-one percent (41%) of the samples collected during the survey were morphologically identified as *Fusarium oxysporum* based on phenotypic characteristics of the respective pure cultures grown on PDA. The phenotypic characteristics included: colony colour, growth habit and presence, septation and shape of microconidia and macroconidia. After PCR and electrophoresis, thirteen isolates were identified as *Foc* (see appendix H). Race 1 was found in Nampula (4 isolates) and Maputo provinces (3 isolates) (Figure 8). Tropical race 4 was only present in the North, within Jacaranda and Matanuska commercial farms as reported in 2013 (MINAG, 2014). *Foc* was not found in Manica province. In Nampula Province, Lineage VI was found in banana farms that were close to the TR4 infested areas. Race 1 was isolated from Apple banana in Maputo province. In Nampula, race one was recovered from Nasapato and Apple banana. TR4 was found in only Williams, the mainly grown Cavendish cultivar in the two commercial banana plantations of Matanuska and Jacaranda.

Among other banana cultivars observed in the survey were; 'Nzara-Yapera' in Manica, 'Banana Makaku' and 'Queroso in Nampula and Dwarf Cavendish, Chinese Cavendish and Grand Naine in Maputo.

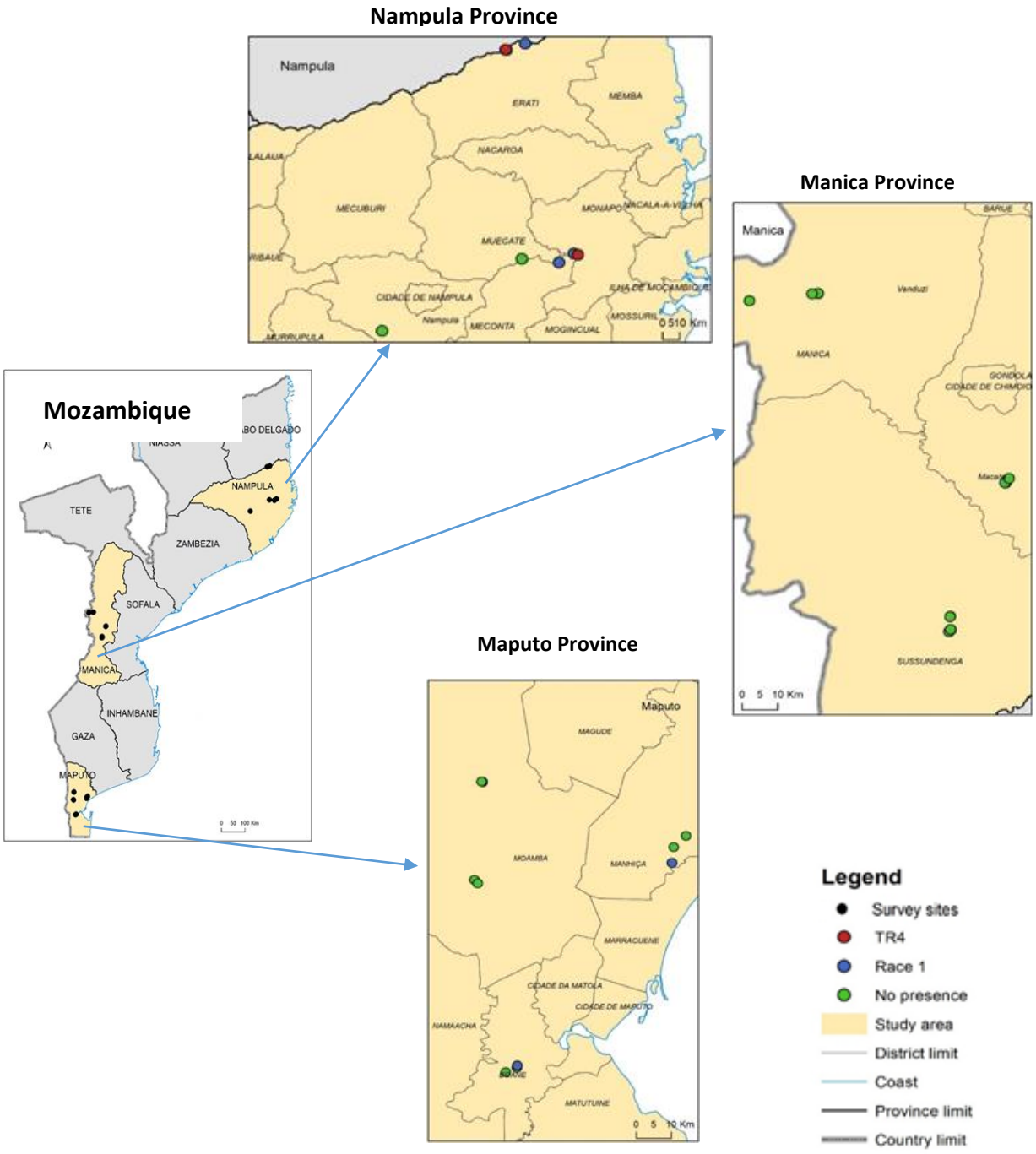

Figure 8: Survey areas and distribution of Foc Races in Mozambique.

3.3.2 Vegetative Compatibility Groups

Nit mutants were generated on chlorate media for all isolates confirmed as *Foc* except for isolates CAV 3633, CAV 3634, CAV 3755 and CAV 3761 (Table 1). Mutants were then paired with known *nit* 1 and *nit* M testers incubated at room temperature and inspected after every 2 days for formation of heterokaryons (Figure 5). On pairing with *nit* 1 and *nit* M testers, race 4 isolates formed heterokaryons with VCGs 01213/16, 01213 and 01216 while race 1 (lineage IV) isolates paired with *nit* M of VCGs 01220, 0124 and 0125.

Table 1: Isolates from respective host banana cultivars and areas and identified races and compatible nit testers.

Isolate	**Race**	**VCG**	**Nit**	**Host Cultivar**	**Location**
CAV 3755	**1**			Apple banana	Maputo, Manhiça
CAV 3760	1	0125	M	Apple banana	Maputo, Boane
CAV 3761	**1**			Apple banana	Maputo, Boane
CAV; 3624, 3625, 3626, 3627, 3628, 3629	4	01213/16	M	Williams	Nampula, Erati
		01213	M	Williams	Nampula, Erati
		01216	M	Williams	Nampula, Erati
		01213/16	1	Williams	Nampula, Erati
		01213	1	Williams	Nampula, Erati
		01216	1	Williams	Nampula, Erati
CAV 3630	1	01220	M	Apple banana	Nampula, Erati
CAV 3631	1	0124	M	Nasapato	Nampula, Monapo
CAV 3633	1			Nasapato	Nampula, Meconta
CAV 3634	1			Nasapato	Nampula, Meconta

Race 4 is responsible for TR4 on Cavendish bananas in Nampula province. VCGs 0124 and 01220 were found in Nampula province in the districts of Monapo and Erati respectively. VCG 0125 was found in Maputo province, Boane district. VCGs 01220, 0124 and 0125 belong to race 1.

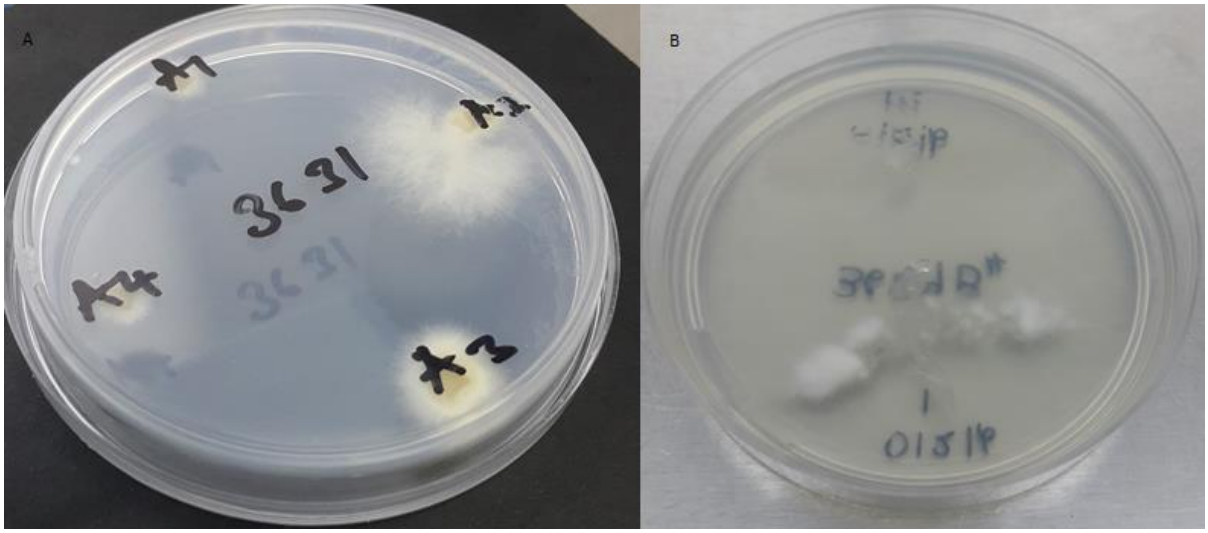

Figure 9: Development of mutants and heterokaryons on Chlorate and minimal media respectively. A – A Generated Chlorate Mutant (A2) and B - Heterokaryon Formation.

3.4 Discussion

In agreement with Ploetz (2006), who reported that Panama disease spread to all major banana producing areas except Somalia, Melanesia, areas bordering the Mediterranean and some islands in the South Pacific, this study confirms that race 1, the cause of the first Panama disease epidemic is spread across Mozambique. Race 1 was confirmed in Nampula and Maputo provinces. It was not found in Manica province. Race 1 was found in all the districts that were surveyed in Nampula and Maputo provinces except Moamba district in Maputo. For race 1, VCG 0125 in Maputo and VCGs 01220 and 0124 in Nampula were identified. Vegetative compatibility groups 0125, 01220 and 0124 are grouped into evolutionary clade B and are hence more related (Fourie *et al.,* 2009; Bentley *et al.*, 1995).

However, race 1 has been limited to few areas where farmers are still growing traditional cultivars that include 'Nasapato' and Apple banana. The widely-grown banana cultivars in Mozambique are the Cavendish cultivars (Williams, ASDIA, dwarf Cavendish, Grand Naine and Chinese Cavendish) (MINAG, 2014), a subgroup of cultivars that are resistant to race 1 (Ploetz and Pegg, 1997; Beckman and Ploetz, 1990). The absence of Panama disease in Manica province can be attributed to the apparent widespread dependency on Cavendish cultivars across the province.

The advantage of resistant cultivars is however extinguished with the confirmation of a restrictively distributed Tropical race 4 of Panama disease that is present in Nampula. Vegetative compatibility groups 01213/16, 01213 and 01216, all responsible for TR4 were identified in Nampula province. Vegetative compatibility groups 01213/16, 01213 and 01216 belong to evolutionary clade A. Tropical race 4 and sub-tropical race 4 broke down the resistance to Panama disease in Cavendish cultivars, although the latter is restricted to South Africa and only affects banana that have been pre-exposed to subtropical cold temperatures (Ploetz, 2015; Ploetz, 2000; Viljoen, 2002). The presence of TR4 in Mozambique is of greater concern since it does not only affect Cavendish cultivars, but also all cultivars that are susceptible to race 1 and race 2 (Fourie *et al.,* 2011; CABI, 2007). Previously, TR4 was reported in Asia and Australia and more recently, the Middle East and Africa (Ploetz, 2006; Pérez-Vicente *et al*., 2014; García-Bastidas *et al.,* 2016).

Since Panama disease is primarily spread through infected sucker and rhizome pieces (Ploetz and Pegg, 2000), the presence of TR4 in Nampula is a threat to quarantine measures effected by the Ministry of Agriculture of Mozambique since traditional farmers share conventional planting material. However, locally, Panama disease can spread through contaminated soil or spores attached to farm machinery, tools, footwear, running or irrigation water and any other mobile aid (Pérez-Vicente *et al*., 2014). At Matanuska and Jacaranda, measures including restriction of movement within the farms, planting of resistant clones, destruction by burning, fungicides and burying of dead banana plants and extensive use of chemical sterilants have been adopted to contain the spread of TR4 into new plots within and out of the farms.

However, there has been a continuity in the rise of infected plants within Matanuska and Jacaranda which could be attributed to inefficient means of controlling water runoff and irrigation water. This calls for adoption of more effective disease management measures and cultural practices that could contain and eradicate the disease within infected sites in the long run while preventing spread to neighbouring fields in the short run. The certainty of Panama disease spreading to other countries within the region should also be considered.

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CHAPTER FOUR

In vitro response of *Fusarium oxysporum* f. sp. *cubense* to fungicides, biocontrol-agents and phenolic compounds

Abstract

Fusarium oxysporum f. sp. *cubense* (*Foc*), the pathogenic fungi responsible for Panama disease is among plant pathogens that are difficult to eradicate once introduced in an area except for the adoption of resistant banana cultivars to maintain production. The objective of this study was to evaluate the effect of phenolic compounds, biological control agents, and fungicides on mycelial growth of *Foc* race 1 and race 4 isolates from Mozambique in vitro. Trans-ferulic acid at 2.5 mM concentration was the most suppressive phenolic compound (51.2%). Sinapic acid promoted growth of *Foc* isolates at all concentrations (0.5 mM, 1.5 mM and 2.5 mM). Prochloraz was the best fungicide that completely inhibited *Foc* mycelia growth on both the inoculation plug and amended PDA at concentration 1 ppm. Propiconazole at 100 ppm was also completely suppressive to *Foc* isolates on amended PDA. Sporekill, a disinfectant, was effective against the *Foc* isolates. *Bacillus subtilis* was the most effective biological control product that formed a clear inhibitory zone against the *Foc* isolates. Further field evaluations and studies on how to integrate *Foc* suppressive phenolic compounds into available Panama disease management measures are required.

4.1 Introduction

Panama disease also called Fusarium wilt of banana is one of the most destructive diseases in agriculture that has periodically disrupted the global banana industry (Moore *et al.*, 2001; Ploetz, 2000; Simmonds, 1966). The disease is caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), a fungus whose spores (chlamydospores) can last for up to 3 decades in infested soil under unfavourable conditions (Stover, 1962). When these dormant spores perceive chemical signals from primary and secondary root exudates of newly planted proximal banana plants, they adhere to and grow through root tips' epidermis (also wounded rhizome surfaces) into the cortex and finally the banana plant's vascular system (Pérez-Vicente et al., 2014; Li *et al*., 2013; Beckman and Ploetz, 1990; Sequeira *et al.*, 1958). In susceptible banana cultivars, the *Foc* continue to sporulate, clogging the plant vascular system at the sieve cells as manifested in a progressive internal discoloration that initiates from the rhizome to the pseudostem (Ploetz, 2000; Jeger *et al.*, 1995). Thus, the infected banana plant wilts, first with older leaves, then young leaves before the entire plant finally dies. In some cases, splitting at the lower leaf sheath occurs (Ploetz, 2000).

Globally, three races (race 1, 2 and 4) and 24 vegetative compatibility groups (VCGs) have been identified for *Foc* (Pérez-Vicente *et al*., 2014; Visser *et al.*, 2010; Ploetz and Pegg, 2000). The catastrophe of Panama disease was first noticed in the mid-20th century when race 1 threatened to wipe out commercial banana production which was dependant on the susceptible Gros Michel cultivars (Ploetz, 1992; Stover, 1962). The discovery and replacement of Gros Michel cultivars with the resistant Cavendish banana cultivars ensured the survival of the banana industry. Race 4 however, now found in Asia, Australia, Middle East and Africa, has rendered the Cavendish cultivars susceptible to Panama disease, hence pushing the banana industry into fear for another catastrophe due to its dependence on almost entirely Cavendish cultivars (García-Bastidas *et al.*, 2016; Bastidas *et al.*, 2014; Pérez-Vicente *et al*., 2014; Butler, 2013; Ploetz, 2005).

In Africa, Tropical Race 4 (TR4) has been reported to occur in only Mozambique (Pérez-Vicente *et al*., 2014; MINAG, 2014). Coincidently, having been ranked among the top ten economically growing African countries in recent years, Mozambique had earmarked the banana industry domestically for the development of export agriculture and as an agro-partnerships with foreign agribusinesses (Calima *et al.*, 2014). This was reflected in the growing number of commercial

banana farming enterprises in Nampula and Maputo Provinces. However, among other challenges such as fruit flies' invasions, the promising banana industry was overwhelmed by the finding of the virulent Tropical Race 4 of Panama disease in 2013. Tropical race 4 (TR4) was found in Matanuska and later in Jacaranda, both commercial banana producers in Northern Mozambique, Nampula Province. With a quarantine in place, little success has been achieved in the effort to eradicate TR4 given the increasing number of infected plants within the two diseased plantations.

Except for resistant cultivars, the use of various management measures in the control of Panama disease has given inconsistent results (Thangavelu and Gopi, 2015; Thangavelu *et al.,* 2004; Ploetz, 2000; Ploetz, 1990). Thus, research on management of Panama disease has been skewed towards discovering more effective alternatives such as novel fungicides and sterilants, exploring resistance mechanisms existing in resistant cultivars, novel biological control agents, etc. (Huang *et al.,* 2012; Nel *et al.,* 2007; Van den Berg *et al.,* 2007; Nel *et al.,* 2006; De Ascensao and Dubery, 2000; Thangavelu *et al.,* 2003).

Since plants are immobile, they interact with their surrounding and other living organisms through production of a wide range of secondary compounds (phenolic compounds) for various physiological processes such as antibiosis, UV light screening, anti-nutritional properties, plant pigmentation, signalling agents, etc. (Lattanzio *et al*., 2006; Kutchan, 2001). Phenolic compounds can occur as a normal constituent of the plant or can be produced as a response to a condition as in pathogen attacks (Nicholson and Hammerschmidt, 1992; Matern and Kneusel, 1988). The later results in modifications in plant metabolism, the cell wall and plasma membrane which are characteristic to resistant plants including cultivars resistant to Panama disease. (De Ascensao and Dubery, 2000; Ewané *et al.,* 2012; Van den Berg *et al.,* 2007; Bailey and Mansfield, 1982; Ecker and Davis, 1987; Roby *et al.,* 1985).

Despite having critics regarding public and environmental health, the correct and systematic use of fungicides can efficiently suppress *Foc* (Duniway, 2002; Gullino *et al.,* 2000). Also, having been used in stem injections, soil fumigation and banana sucker dips among other application forms, fungicides have been inconsistent, short lived and/or less successful under field conditions regardless of success under in vitro and green house conditions (Herbert and Marx, 1990; Deacon, 1984; Meredith, 1943; Lakshmanan *et al.,* 1987; Stover, 1962). The existence of new fungicides

and chemicals (surface sterilants) in the market has however increased the possibility of having better alternatives to the inconsistent past choices of fungicides used in various field studies against Panama disease (Nel *et al.*, 2007; Gullino *et al.*, 2000).

Recently, biological control has been identified as an option that can offer new alternatives to the management of Panama disease (Thangavelu and Mustaffa, 2012; Fravel *et al.*, 2003). *Trichoderma spp., Bacillus spp., Pseudomonas spp.* and non-pathogenic *F. oxysporum* represent some of the most common biological control agents used in disease management (Woo *et al.*, 2014; Junaid *et al.*, 2013; Alabouvette, 1986; Larkin *et al.*, 1996; Larkin and Fravel, 1998, 1999). Biocontrol agents supress pathogenic organisms in a way that may include one or more of production of antibiotic compounds, competition for niches with the pathogens, mycoparasitism/ hyper-parasitism, degradation of pathogen cell walls and stimulation of growth and induced resistance in the plant host (Junaid *et al.*, 2013; Pal and Gardener, 2006; Harman, 2000; Dennis and Webster, 1971). Complementary to fungicides, biological control agents continue to grow and multiply after the applied fungicides breakdown (Rowe and Farley, 1978). When inoculated on banana plant roots, biocontrol agents have the potential to trigger the accumulation of phenolics, phenylalanine ammonia lyase (PAL) and peroxidase (POX) in banana plants as resistance mechanisms against Panama disease as observed by Thangavelu *et al.* (2003) with *Pseudomonas fluorescens.* Since they are reported to be more susceptible to Panama disease than conventionally grown plants (Smith et al., 1998), inoculation of tissue culture plants with biocontrol agents prior to planting in the field can also boost individual plant resistance to the disease.

The objective of this study was to ascertain the potential of, first, selected commercial fungicides, secondly, phenolic compounds, and thirdly, identified antagonistic biocontrol products to inhibit the growth of *Foc* isolates (Race 4 and 1) from Mozambique under laboratory (in vitro) conditions. Ability of Trichoderma isolates to produce toxic metabolites (non-volatile antibiotics) with inhibitory effects against *Foc* isolates was also evaluated.

4.2 Materials and Methods

4.2.1 Fungal Isolates:

Four *Foc* isolates obtained from diseased banana plants during the survey of Nampula, Manica and Maputo Provinces for Panama disease, were tested in respective experiments against each commercial biological control product, phenolic compound and fungicide. The *Foc* isolates CAV 3625 and CAV 3628 representative of Tropical Race 4 (TR4) and CAV 3631 and CAV 3634 representative of Race 1 were randomly chosen from the *Foc* cultures for the experiment. These isolates were all grown for 7 days at room temperature on half-strength potato dextrose agar (PDA) before being evaluated against biological control products, phenolic compounds and fungicides.

4.2.2 Experimental design:

A completely randomized design was used in all the experiments. Controls of each *Foc* isolate on non-amended PDA media were also included in each of the experiments (biological control, phenolics and fungicides) for comparison to isolates grown on phenolic-amended PDA, fungicide-amended PDA and *Foc* pathogen vs biocontrol-agent plates. Each of the treatment was replicated five times and the experiment performed twice.

4.2.3 In vitro evaluation against biological control agents:

Two commercial bacterial biological control agents, Extrasol (*Bacillus subtilis strains*) and BS and 2 Trichoderma isolates, *Trichoderma atroviride* (WVJ-T1) and *Trichoderma atroviride* (WVJ-T8) from the WVJ culture collection at Stellenbosch University were evaluated in vitro for antagonistic activity against soil borne pathogenic *Foc* isolates CAV 3625, CAV 3628, CAV 3631 and CAV 3634 from Mozambique.

The biological control agents were evaluated for ability to inhibit growth of the four *Foc* isolates (CAV 3625, CAV 3628, CAV 3631 and CAV 3634) on PDA media in 90mm Petri dishes. The *Foc* isolates were initially grown on PDA for 7 days at room temperature (25°C). For the bacterial biological control agents, ten microliters (10 µl) of a respective commercial product were inoculated at 4 relatively equidistant spots towards the edge of the PDA containing petri dish and incubated at 25°C for 24 hours (Figure 10). A plug of *Foc* mycelia about 5.2 mm wide from the

selected isolates was then taken and placed at centre of the growing 24 hour old bacterial colonies in the 90 mm Petri dishes. Antagonistic activity was assessed after 6 days on a 1-4 scale as described by Nel *et al* (2006): 1=bacterial colony completely overgrown by Foc, 2=overgrown but bacterial colony visible, 3=grown to the edge of the bacterial colony and 4=visible inhibition zone.

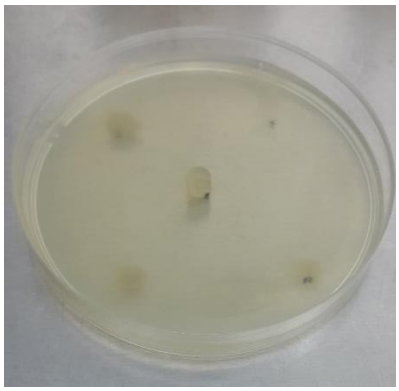

Figure 10: A plug of Foc placed at the centre of 24-hour old bacterial colony.

To evaluate the ability of *Trichoderma* isolates to produce toxic metabolites (non-volatile antibiotics) with antagonistic activity against the *Foc* isolates, an agar layer technique was used as described by Dennis and Webster (1971). Eighty-five millimetre (85 mm) discs of autoclaved cellophane were placed on PDA in 90 mm petri dishes. A plug from a 3-day old culture of the *Trichoderma* isolates was then placed at the centre of cellophane in the petri dishes. The plates were then incubated in the dark for 42 hours before carefully removing the cellophane while ensuring that *Trichoderma* spores do not fall on the PDA. A plug from a 7-day old *Foc* culture was placed at the centre of the petri dish at the point where the Trichoderma was growing. The plates were incubated at 25°C in the dark for 6 days. The diameter of each colony was then measured after 6 days using a digital calibre (Mitutoyo Crop, Model no. CD-6”C).

4.2.4 In vitro evaluation against phenolic compounds:

Phenolic compounds vanillic acid, trans-ferulic acid, caffeic acid, p-coumaric acid, sinapic acid and protocatechuic acid (Sigma-Aldrich) were selected and evaluated for potential of antagonistic activity against *Foc* isolates. The phenolic compounds are commercial available naturally plant extracts. Each phenolic compound was dissolved in 100% ethanol except for sinapic acid (100%

methanol) to a stock concentration of 100 mM (see appendix G). The phenolic compounds were evaluated at three concentrations (0.5 mM, 1.5 mM, and 2.5 mM). For the controls, unamended PDA was used.

PDA was amended with a respective phenolic compound at the 3 separate concentrations and 20 ml of the amendment dispensed out on 90 mm plastic petri dishes under aseptic conditions. This was done on relatively hot autoclaved PDA and left in a 55°C oven overnight for the ethanol or methanol to evaporate. Mycelial plugs from the margins of 7-day old *Foc* isolate cultures (CAV 3625, CAV 3628, CAV 3631 and CAV 3634) were then placed at the centres of the amended PDA in Petri dishes (Figure 11) and incubated at 25°C for 6 days. The diameter of each colony was measured on the 2nd, 4th and 6th day using a digital calibre (Mitutoyo Crop, Model no. CD-6”C).

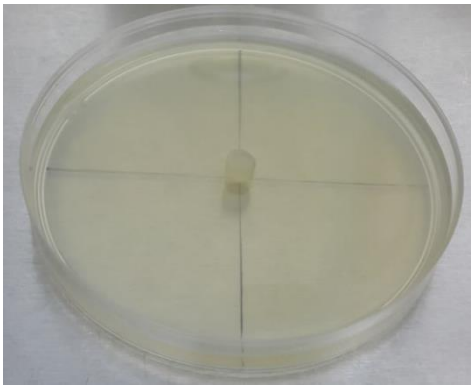

Figure 11: A plug of Foc placed at the centre of a marked petri dish containing amended PDA.

4.2.5 In vitro evaluation against fungicides:

Commercially available fungicides with expected potential to control Panama disease along with newly available fungicide products were selected for the study. The fungicide products were all tested against the selected *Foc* isolates (CAV 3625, CAV 3628, CAV 3631 and CAV 3634) from Mozambique. Fungicide concentrations were derived from manufacturers recommended application rates for various crops and/or related diseases and from previous studies in which specific fungicides were reported effective against *Foc* (see appendix A).

The fungicides were added to cooling autoclaved PDA according to manufacturer's application rates except for phosphonate that was added to Ribeiro's medium according to Fenn and Coffey (1984). The fungicide-amended PDA was then circulated to 90mm Petri dishes (20 ml per plate) under aseptic conditions. Mycelial plugs (5.2 mm cm wide) from the margins of the 7 day *Foc* isolates (CAV 3625, CAV 3628, CAV 3631 and CAV 3634) were placed at the centres of the fungicide-amended media in Petri dishes. The *Foc* isolate- fungicide amended PDA dishes were then incubated for 6 days at 25°C. The diameter of each colony was measured on the 2nd, 4th and 6th day using a digital calibre (Mitutoyo Crop, Model no. CD-6"C).

4.2.6 Data Analysis

Experimental data from *Foc* mycelial growth studies involving phenolic compounds, fungicides and *Trichoderma* isolates was collected by taking two perpendicular diameter measurements of the *Foc* colony. Surface area ($SA = \frac{1}{4}\pi\, D^2$) was calculated from the average diameter (D) of the 5 repeats in a respective experiment. Percentage suppression (PS) of mycelial growth was then calculated from SAs and used to evaluate the performance of the fungicides, *Trichoderma* isolates and phenolic compounds against the *Foc* isolates.

Where $\mathrm{PS} = 100 - \left([\frac{SA\ amended\ PDA}{SA\ control}] * 100 \right)$. Modified from Cassiem (2015).

The area of the *Foc* plug used for *Foc* inoculation was subtracted from all observations made in the experiment.

PS of 100% indicated that the *Foc* isolate was effectively suppressed and no growth occurred on PDA- fungicide amended media. A negative PS indicated that the PDA amendment promoted growth of the *Foc* pathogen relative to the controls.

The data from both experiments was collectively subjected to analysis of variance (ANOVA) using XLSTAT version 2016.1. Mean differences (Pairwise multiple comparisons) were separated per Fisher's LSD test ($P \leq 0.05$). Part of the data was analysed and graphs generated with Stata version 12.0. All the experimental data was normally distributed and differences between all treatments in respective experiments were significant at $P \leq 0.05$.

4.3 Results

4.3.1 In vitro evaluation against biological control agents:

Percentage suppression of *Foc* isolates CAV 3625, CAV 3628, CAV 3631 and CAV 3634 by toxic metabolites (non-volatile antibiotics) produced by the two *Trichoderma* isolates, *Trichoderma atroviride* (WVJ-T1) and *Trichoderma atroviride* (WVJ-T8) were not significantly different from each other (see appendix E). However, there was a significant difference among race 1 and race 4 representative *Foc* isolates hence isolates CAV 3625 and CAV 3628 were not significantly different from each other and so were CAV 3631 and CAV 3634 (Figure 12). WVJ-T1 slightly suppressed mycelial growth in CAV 3631 (0.5%) but not at all in CAV 3634 (-8.6%).

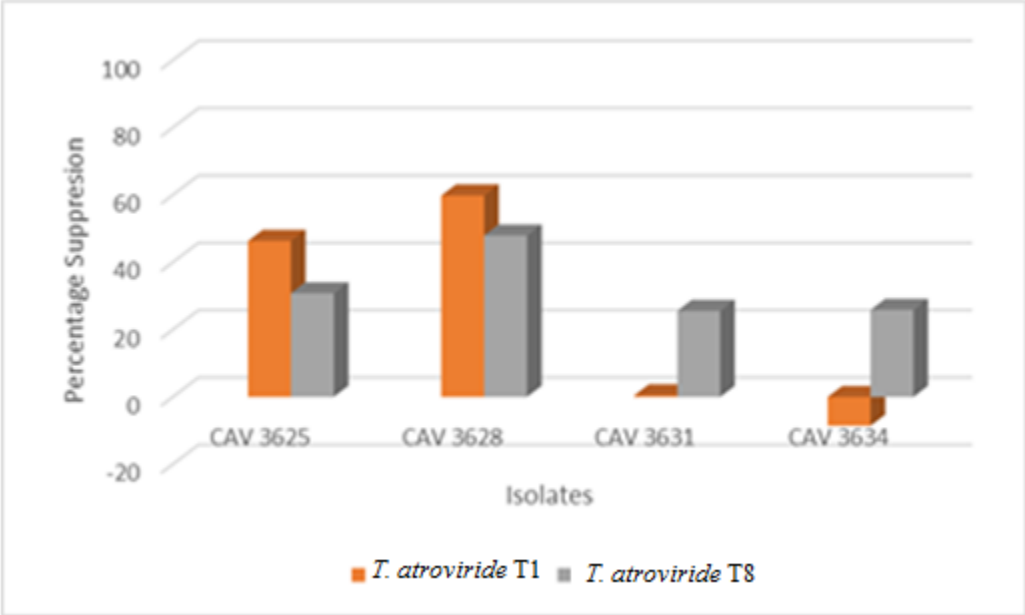

Figure 12: Percentage Suppression of *T. atroviride* isolates on Foc Isolates. Margins sharing a letter in the row labels are not significantly different at the 5% level.

However, for CAV 3625 and CAV 3628, WVJ-T1 (46.3% and 59.8% respectively) was most suppressive compared to WVJ-T8 whose percentage suppression was 30.8% and 47.9% for the respective *Foc* isolates. WVJ-T8 was suppressive to all isolates. Both WVJ-T1 and WVJ-T8 were more suppressive to TR4 representative isolates CAV 3625 and CAV 3628 compared to race 1 isolates CAV 3631 and CAV 3634 (Figure 12).

With an average score of 4 in both experiments, Extrasol (*Bacillus subtilis strains*), a commercially available biocontrol product, produced a visible inhibition line against all the *Foc* isolates after 6 days of incubation (Figure 13). For BS, a commercially available biocontrol product, the mycelia of the *Foc* isolates could only grow to the edge of the bacterial colony hence average score 3. Extrasol was the most inhibitory bacterial biological control compound.

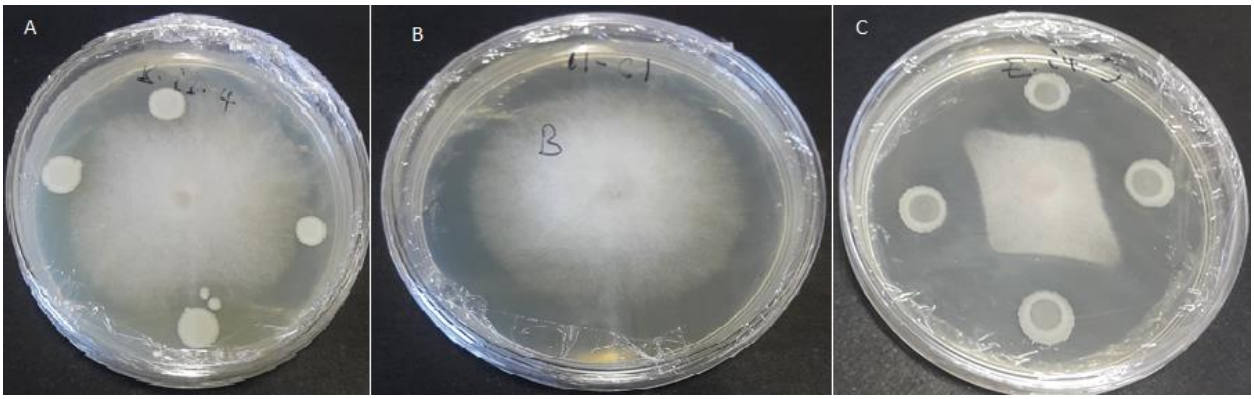

Figure 13: Effects of the bacterial biocontrol agents; A - Foc grew to the edge of BS colony, B - experimental control and C - Visible inhibitory zone created by *Bacillus subtilis.*

4.3.2 In vitro evaluation against phenolic compounds:

Mycelial growth was significantly different among *Foc* isolates, CAV 3625, CAV 3628, CAV 3631 and CAV 3634 (see appendix D). Phenolic compounds and the concentration of phenolic compounds also had a significant effect on *Foc* mycelial growth (see appendix F). Trans-ferulic acid at concentration 2.5 mM was the most effective phenolic compound against *Foc* isolates (51.2% suppression) and it vividly suppressed *Foc* isolates at concentrations 0.5 mM (10.9%) and 1.5 mM (20.9%) compared to other phenolic compounds (Figure 14).

All phenolic compounds were suppressive against *Foc* isolates at concentration 2.5 mM except Sinapic acid. Relative to the controls, sinapic acid promoted growth of *Foc* isolates at all concentrations 0.5 mM, 1.5 mM and 2.5 mM. At concentration 0.5 mM, p-coumaric acid, caffeic acid and vinilic acid promoted growth of *Foc* isolates. However, at concentrations 1.5 mM and 2.5 mM p-coumaric acid, caffeic acid and vinilic acid were relatively suppressive to *Foc* isolates. Protocatechuic acid was relatively suppressive to *Foc* at all the 3 concentrations despite

suppression at concentrations 1.5 mM (14.1%) and 2.5 mM (13.9%) not being significantly different from each other (see appendix F).

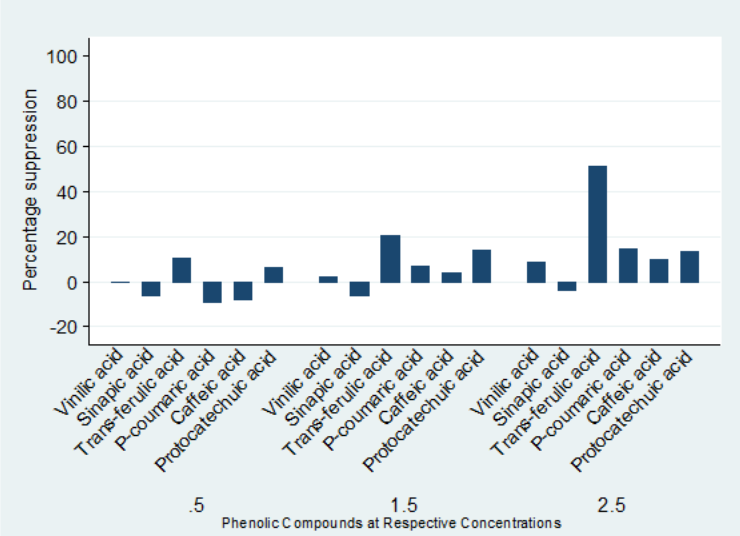

Figure 14: Mean percentage suppression of various concentrations of phenolic compounds on *Foc* isolates.

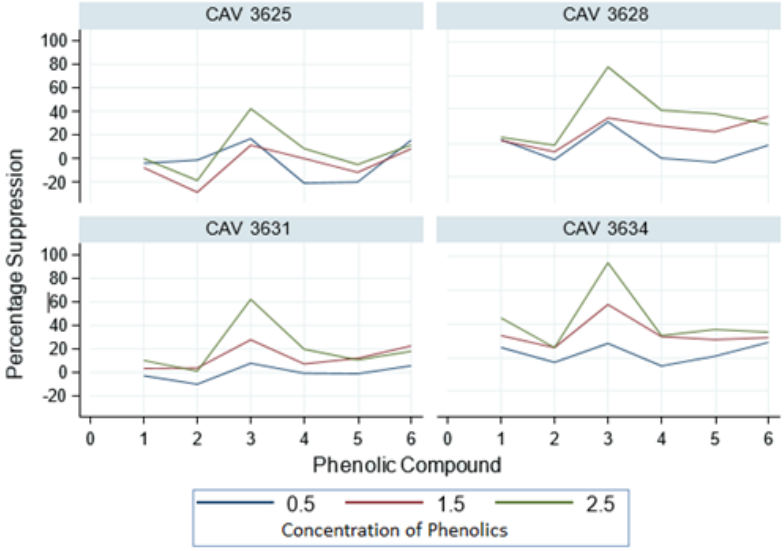

Figure 15: The effect of phenolic compounds on *Foc* by isolate. Phenolic compounds: 1 = Vinilic acid, 2 = Sinapic acid, 3 = Trans-ferulic acid, 4 = P-coumaric acid, 5 = Caffeic acid and 6 = Protocatechuic acid.

Sinapic acid did not show any suppressiveness against *Foc* at all concentrations among the phenolic compounds, but instead increased mycelial growth at a decreasing rate with increase in concentration except for isolate CAV 3625 where mycelial growth was highest at concentration 1.5 mM (Figure 15). Both experimental results were similar and Trans-ferulic acid emerged as the most suppressive phenolic compound (Figure 16).

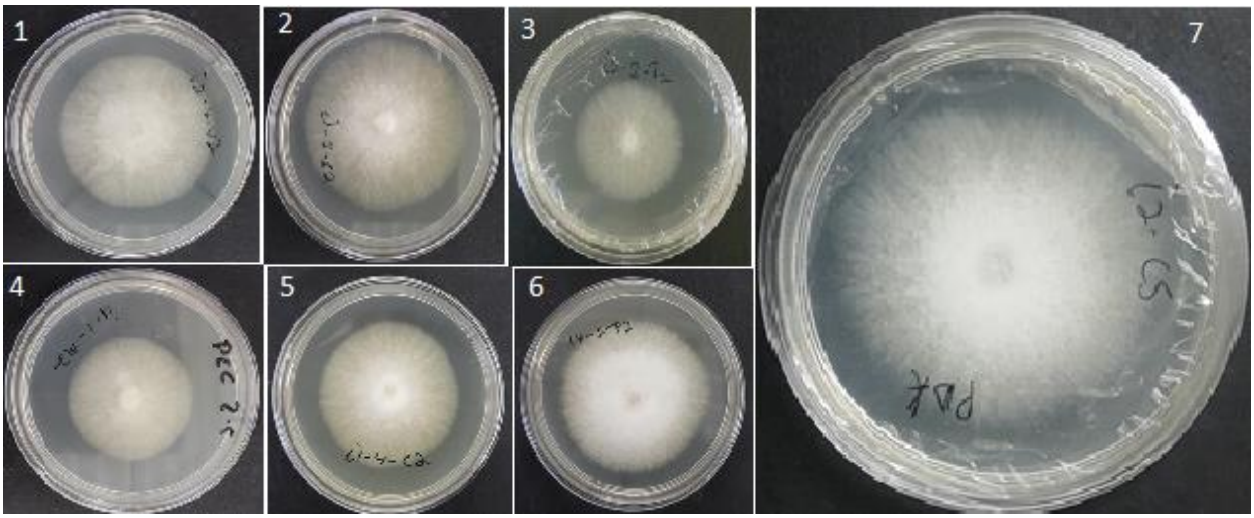

Figure 16: Effects of phenolic compounds on *Foc* at 2.5 mM concentration. 1 = Vinilic acid, 2 = Sinapic acid, 3 = Trans-ferulic acid, 4 = P-coumaric acid, 5 = Caffeic acid, 6 = Protocatechuic acid and 7 = experimental control.

4.3.3 In vitro evaluation against fungicides:

Mycelial growth in all the isolates, CAV 3625, CAV 3628, CAV 3631 and CAV 3634 selected for the study was significantly reduced by all fungicides tested at respective concentrations and the *Foc* isolates were not significantly different from each other (see appendix B). Didecyldimethyl-ammonium chloride (Sporekill), a disinfectant included in the study also significantly reduced mycelial growth in all the isolates. Mycelial growth was not registered (100% suppression) on media amended with Prochloraz, Propiconazole and Sporekill (Figure 17).

However, on the plugs of all isolates, sparse mycelial growth occurred for Propiconazole and Sporekill (CAV 3634 formed slightly denser growth for Sporekill). Prochloraz completely inhibited mycelial growth on both the amended PDA and inoculation plug. Carboxamide and

Benomyl were the least effective against the *Foc* isolates and showed poor mycelial growth inhibition at the considered concentrations.

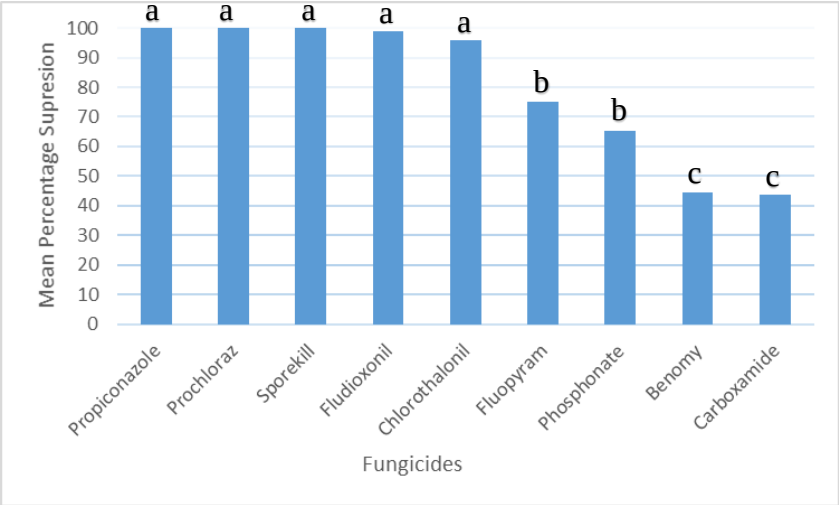

Figure 17: Mean percentage suppression of fungicides on Foc isolates. Bars sharing the same letters are not significantly different from each other at $P \leq 0.05$.

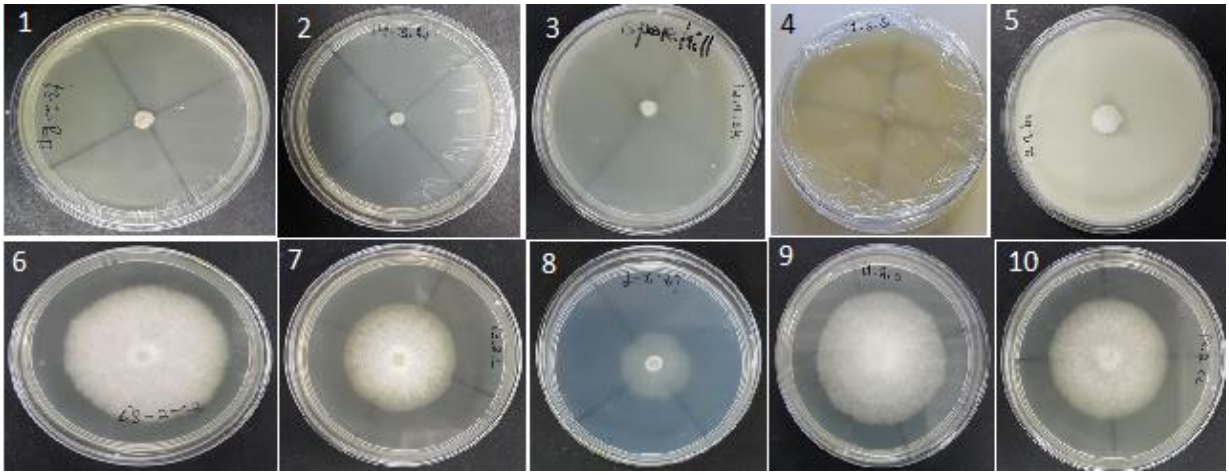

Figure 18: Effects of amended fungicides on *Foc*. 1 - Propiconazole, 2 - Prochloraz, 3 - Sporekill, 4 – Fludioxonil, 5 – Chlorothalonil, 6 – Control, 7 – Fluopyram, 8 – Phosphonate, 9 – Benomyl and 10- Carboxamide.

Fludioxonil completely inhibited growth of isolates CAV 3625 and CAV 3628 (representative of TR4) but slight growth occurred for isolates CAV 3631 and CAV 3634 (representative of race 1). On PDA amended with Chlorothalonil, dense mycelial growth occurred around the plug. Results

were similar in both experiments and Prochloraz was the most effective fungicide, completely inhibiting growth of mycelia while Carboxamide and Benomyl were least effective (Figure 18). With Fisher (LSD) at 95% confidence interval: Prochloraz, Propiconazole, Sporekill, Fludioxonil and Chlorothalonil; Fluopyram and Phosphonate; Carboxamide and Benomyl, were not significantly different from each other (See appendix C).

4.4 Discussion

Both the commercially available bacterial biocontrol products, Extrasol (*Bacillus subtilis* strains) and BS tested in the study showed a significant inhibitory effect against *Foc* isolates. However, *Bacillus subtilis* was the most outstanding biocontrol agent that formed a visible inhibitory line against the *Foc* pathogen in vitro. Among other studies, Poozad and Kariminik (2015), identified some strains of *Bacillus subtilis* that were effectively antagonistic to *Fusarium oxysporum* from palm dates. *Bacillus subtilis* as a biocontrol agent against pathogens is involved in direct competition for the niche and nutrients, stimulation of plant host defensive mechanisms and mycoparasitism with the aid of a series of lipopeptide antibiotics in the families of surfactin, iturin and fengycin (Junaid *et al.,* 2013; Chen *et al.,* 2008; Compant *et al.,* 2005).

The toxic non-volatile metabolites produced by both *Trichoderma* isolates, *T. atroviride* T1 and *T. atroviride* T8 were more suppressive to TR4 isolates especially CAV 3628 compared to race 1 isolates. In fact, *Foc* isolate CAV 3634 on T1 metabolites containing plates outgrew the experimental controls. Dennis and Webster (1971), noted the significant resistance of *Fusarium oxysporum* Schlecht. ex Fr. to non-volatile antibiotics that were active against a range of fungi tested in their experiment. In addition to mycoparasitism and competition for nutrients and niches with plant pathogens, *Trichoderma* species are the most widely studied and used biocontrol agent due to their ability to produce large volumes of viable spores, stimulate plant host defences against pathogens, enhance plant host growth and promote soil fertility build up through biodegradation and decomposition (Harman *et al.,* 2004; Junaid *et al.,* 2013; Woo *et al.,* 2014). However, as reported by Thangavelu and Gopi (2015) on rhizospheric and endophytic *Trichoderma* isolates, combinative use of biocontrol agents both within or across species can have a much more significant effect compared to use of individual microorganisms.

Unlike animals that interact with the environment through mobility, plants depend on production of a greater range of secondary chemical metabolites or phenolic compounds for antibiosis against pathogens, anti-nutritional properties, plant pigmentation, above and below ground signalling to other organisms, anti-oxidation, etc. (Kutchan, 2001; Swain, 1977; Bell and Charlwood, 1980). Phenolic compounds exist as either preformed phenolics; those synthesized during normal plant development or induced phenolics; those that are accumulated during biotic or abiotic stress

(Hammerschmidt, 2003; Lattanzio *et al*., 2006). Of the 6 phenolic compounds (vinilic acid, sinapic acid, trans-ferulic acid, p-coumaric acid, caffeic acid and protocatechuic acid) tested in this study for antagonism against *Foc*, trans-ferulic acid emerged at all concentrations (0.5 mM, 1.5 mM and 2.5 mM) as the most inhibitory compound against *Foc* mycelial growth. At 2.5 mM, trans-ferulic acid achieved over 50% *Foc* mycelial inhibition more than any of the other phenolic compounds.

Trans-ferulic acid and p-coumaric acid are both hydroxycinnamic acids found as cell wall bound phenolic acids and/or as esters (Lattanzio *et al*., 2006). Hydroxycinnamic acids are involved in the commencement of lignification, acting as phenylpropanoid reserve units and possibly in regulation of cell expansion particularly as feruloyl pectin from radically dimerized trans-ferulic acid (Hatfield *et al.,* 1999; Fry, 1983). De Ascensao and Dubery (2000), observed that strong lignin deposition on cell walls and accumulation of phenolic acids especially ester-bound and cell wall-bound phenolic acids were more prominent characteristics of the race 4 tolerant Goldfinger compared to the susceptible Williams cultivar. Similar results were reported by Van den Berg *et al*. (2007).

Sinapic acid tended to promote mycelial growth of the *Foc* isolates. This was also true for p-coumaric acid, caffeic acid and vinilic acid at 0.5 Mm. The tendency to promote growth in some phenolic compounds can be attributed to the existence of an evolutionary relationship between the *Foc* pathogen and the banana plant host (Ploetz and Pegg, 1997; Stover 1962; Stover and Buddenhagen, 1986) that could have worked in favour of the *Foc* pathogen. Since germination of dormant *Foc* spores or chlamydospores is initiated by primary and secondary root exudates from proximal banana roots (Li *et al*., 2013), the *Foc* mycelial growth promoting phenolic compounds could be involved in the process as signalling compounds that are detected by *Foc*. However, phenolic compounds with the tendency to inhibit *Foc* growth can be exploited as a management strategy against Panama disease since they also have the potential to boost plant host defences in less tolerant cultivars or tissue culture plants.

Prochraz, a demethylation inhibitor (DMI) fungicide of the imidazole group, effectively and consistently completely inhibited mycelial growth of all isolates at 1 ppm both on the amended PDA and around the *Foc* plug. Demethylation inhibitor fungicides function through inhibition of demethylation in sterol (a component of fungal cell walls) biosynthesis (Uesugi, 1998).

Comparatively, Propiconazole at 100 ppm, also a DMI but in the triazoles group, performed as Prochraz except for sparse mycelial growth on the *Foc* plugs. In addition to Baldwin and Rathmell (1988), who described DMI fungicides as one of the most important groups of fungicides, Nel *et al.* (2007), reported Prochraz and Propiconazole as the best in vitro and in vivo suppressive fungicides against *Foc* race 4 isolates from South Africa.

Ability of a fungicide to completely suppress *Foc* at recommended concentrations is very significant in the management of *Foc* since the pathogen is characterized by extensive survival time periods in the soil once introduced in an area. Fludioxonil at 100 ppm and Chlorothalonil at 500 ppm were comparatively suppressive but did not achieve 100% suppression on amended PDA at recommended concentrations. However, Amini and Sidovich (2010) with Fludioxonil and Dar *et al.* (2013) with Chlorothalonil on *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *pini* respectively, reported an ineffective effect of the fungicides on mycelial growth.

Contrary to Nel *et al.* (2007), Benomyl did not completely inhibit mycelial growth at 50 ppm in this study and it was the least effective fungicide against *Foc* isolates along with Carboxamide. As observed in previous studies, Sporekill is still an effective, cheaper and easier to handle disinfectant that can be used to sterilize field equipment, tools, foot wear and machinery in attempt to prevent spread of Panama disease across Mozambique. (Nel *et al.,* 2007; Tanner, 1989).

Generally, except for prevention and adoption of resistant cultivars, no individual measure has achieved ultimate control of Panama disease in the field (Ploetz et al., 2003; Viljoen, 2002; Ploetz and Pegg, 2000; Moore et al., 2001; Nelson, 1981). Therefore, most studies have aimed at developing novel and more effective measures that could be used together with the existing techniques to control Panama disease. Also, following increasing consumer health and environmental concerns regarding the increasing use of chemicals especially fungicides, an integrated management approach involving differing applicable control measures can result into much more effective and safer plant disease management (Woo et al., 2014; Junaid et al., 2013). This study supposes the possibility of combinative use of phenolics, biocontrol agents and fungicides among other measures for the management of Panama disease. Particularly, the concept of phenolic compounds in the management of Panama disease has not been extensively studied.

This concept could however lead to the development of novel systemic antifungal agents and identification of highly resistant banana cultivars that would rather bolster the currently threatened banana industry.

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CHAPTER FIVE

5.1 Conclusions and Recommendations

The banana industry in Mozambique is a substantial contributor to food security, employment and local trade. The industry had also been identified by the government of Mozambique along with its partners as an export earner to the very fast growing African nation, hence a drive to increase commercial banana plantations in the country. The growth of the banana industry has however been back-pedalled by reports of Panama disease (TR4) in the country at Matanuska and Jacaranda commercial banana farms. Currently, there is no satisfactory method that can be adopted to effectively manage TR4 despite the overcoming of the earlier races, 1 and 2 by the replacement of susceptible cultivars with resistant ones. This research document attempted to establish the distribution of *Foc* in Mozambique and to evaluate chemicals and biocontrol products with antagonistic activity against *Foc* for further investigation at field level.

In this study, race 1 of Panama disease and TR4 were found. Race 1 was found in the provinces of Nampula and Maputo. Tropical race 4 was only found in Nampula. Panama disease was not found in Manica, the leading banana producing province in Mozambique. In Maputo, VCG 0125 was identified. In Nampula, VCGs 01220 and 0124 were identified for race 1 while VCGs 01213, 01216 and 01213/16 were identified for TR4. In Africa, TR4 has been only reported in Mozambique. Without an effective management measure to significantly reduce the increasing number of infected plants in the diseased areas, TR4 remains a big threat not only to the local banana industry but also the regional top banana producing countries like Tanzania and Uganda.

Therefore, collective measures involving political, regulatory and scientific applications should be adopted in the fight against Panama disease. Surveillance, regulated movement of planting material, sensitization of the public and extensive training of farmers and institutions involved in banana production should be adopted at both local and regional level.

The study identified *Bacillus subtilis*, trans-ferulic acid and prochloraz as the most antagonistic products against *Foc*. *Bacillus subtilis*, a biological control agent formed a visible inhibitory zone against *Foc* on PDA. Trans-ferulic acid achieved over 50% suppression of *Foc* mycelial growth at concentration 2.5 mM and it was the most suppressive phenolic compound. Prochloraz, a

fungicide, completely inhibited mycelial growth for all *Foc* isolates. Propiconazole and Sporekill (a disinfectant) equally suppressed *Foc* mycelial growth, however, sparse mycelia were observed on the inoculation plug. Total suppression of *Foc* is significant in the management of Panama disease since the pathogen can potentially last for decades in infected sites. This is especially important for fungicides since these rapidly breakdown after application.

Once used in integration with fungicides, biological control agents can continue multiplying after breakdown of fungicides hence further inhibiting the activity of the *Foc* pathogen. The biocontrol agents can also stimulate a banana host to produce antagonistic phenolic compounds that can inhibit multiplication of *Foc* spores in the plant. Further field and in vivo investigations should be carried out to identify possible combinations of fungicides and biological control products that can be adopted in the long-term management of Panama disease.

Understanding the significance of plant secondary metabolites (phenolics) vis-à-vis *Foc* can also create new avenues for Panama disease management. Phenolic compounds with antagonistic activity against *Foc* can potentially be used as antifungal compounds or identified as chemical markers for the breeding of Panama disease resistant banana cultivars. Since individual technics have often failed against *Foc*, an integrated approach involving the above antagonistic products should be undertaken at field level before recommending to farmers.

APPENDIX

Appendix A.

Table 2: Fungicides selected for the study and respective concentrations used to amend into 1 litre of PDA media.

Chemical group	Active ingredient	Product name	Type	Concentration (ppm)	Action
Benzimidazole	Benomyl	Benomyl 500	WP	50	Systemic
DMI-Imidazole	Prochloraz	Chronos 450	SC	1	Contact
DMI-Triazole	Propiconazole	Bumper	EC	100	Systemic
Phosphonate	Phosphate	NA	NA	800	NA
Nitrile	Chlorothalonil	Bravo 500	SL	500	Contact
Phenylpyrrole	Fludioxonil	Scholar	SC	100	Contact
SDHI group	Carboxamide	Cantus WG	WDG	50	Systemic
Pyridinyl ethylbenzamides	Fluopyram	Luna Privilege 500	SC	50	Systemic

Appendix B.

Table 3: ANOVA; Percentage Suppression, Isolate, Fungicide and Isolate#Fungicide.

	Number of obs =	72	R-squared	=	0.8882
	Root MSE	= 12.1524	Adj R-squared	=	0.7795

Source	Partial SS	df	MS	F	Prob > F
Model	42237.022	35	1206.77206	8.17	0.0000
isolate	484.379431	3	161.45981	1.09	0.3644
fungicide	37033.7678	8	4629.22097	31.35	0.0000
isolate#fungicide	4718.87477	24	196.619782	1.33	0.2140
Residual	5316.54001	36	147.681667		
Total	47553.562	71	669.768479		

Appendix C.

Table 4: Table 4: Mean Percentage Suppression of Fungicides on Foc.

Fungicide	*LS means*	*Standard error*	*Lower bound (95%)*	*Upper bound (95%)*	*Groups*		
Propiconazole	100.000	4.297	91.286	108.714	A		
Prochloraz	100.000	4.297	91.286	108.714	A		
Sporekill	100.000	4.297	91.286	108.714	A		
Fludioxonil	99.035	4.297	90.321	107.748	A		
Chlorothalonil	95.849	4.297	87.135	104.563	A		
Fluopyram	75.211	4.297	66.497	83.925		B	
Phosphonate	65.163	4.297	56.449	73.877		B	
Benomyl	44.611	4.297	35.898	53.325			C
Carboxamide	43.645	4.297	34.931	52.359			C

Fisher (LSD) mean comparisons of fungicides with a confidence interval of 95% (percentage suppression).

Appendix D.

Table 5: ANOVA; Percentage Suppression, Isolate, Phenolic, Concentration, Isolate#Phenolic, Isolate#Concentration, Phenolic#Concentration and Isolate#Phenolic#Concentration.

Source	*DF*	*Sum of squares*	*Mean of squares*	*F*	*Pr > F*
Isolate	3	3804.548	1268.183	7.235	0.000
Phenolic	5	15346.232	3069.246	17.511	< 0,0001
Concentration	2	6998.777	3499.389	19.965	< 0,0001
*Isolate*Phenolic*	15	1458.565	97.238	0.555	0.899
*Isolate*Concentration*	6	1008.591	168.098	0.959	0.459
*Phenolic*Concentration*	10	4574.649	457.465	2.610	0.009
*Isolate*Phenolic*Concentration*	30	1842.237	61.408	0.350	0.999

Appendix E.

Table 6: ANOVA; Percentage Suppression, Isolate, Trichoderma and Isolate#Trichoderma.

	Number of obs =	16	R-squared	=	0.9266
	Root MSE =	8.74008	Adj R-squared	=	0.8624

Source	Partial SS	df	MS	F	Prob > F
Model	7714.9498	7	1102.13569	14.43	0.0006
isolate	5515.93216	3	1838.64405	24.07	0.0002
trichoderma	256.903733	1	256.903733	3.36	0.1040
isolate#trichoderma	1942.11391	3	647.371302	8.47	0.0073
Residual	611.112399	8	76.3890499		
Total	8326.0622	15	555.070813		

Appendix F.

Table 7: Fisher (LSD) mean comparisons of phenolic compounds at respective concentrations with a confidence interval of 95%.

Phenolic compound	LS means	Standard error	Lower bound (95%)	Upper bound (95%)	Groups								
Caffeic acid*Conc-0.5	-8.289	4.681	-17.620	1.042								H	I
Caffeic acid*Conc-1.5	4.175	4.681	-5.156	13.506			C	D	E	F	G	H	
Caffeic acid*Conc-2.5	9.842	4.681	0.511	19.173		B	C	D	E				
P-coumaric acid*Conc-0.5	-9.205	4.681	-18.536	0.126									I
P-coumaric acid*Conc-1.5	7.057	4.681	-2.274	16.388			C	D	E	F			
P-coumaric acid*Conc-2.5	15.107	4.681	5.776	24.438		B	C						
Protocatechuic acid *Conc-0.5	6.703	4.681	-2.628	16.034			C	D	E	F	G		
Protocatechuic acid *Conc-1.5	14.138	4.681	4.807	23.469		B	C						
Protocatechuic acid *Conc-2.5	13.868	4.681	4.537	23.199		B	C						
Sinapic acid *Conc-0.5	-6.327	4.681	-15.658	3.004							G	H	I
Sinapic acid *Conc-1.5	-6.089	4.681	-15.420	3.242						F	G	H	I
Sinapic acid *Conc-2.5	-3.169	4.681	-12.500	6.162					E	F	G	H	I
Trans-ferulic acid*Conc-0.5	10.927	4.681	1.596	20.257		B	C	D					
Trans-ferulic acid*Conc-1.5	20.884	4.681	11.553	30.215		B							
Trans-ferulic acid*Conc-2.5	51.165	4.681	41.834	60.496	A								
Vinilic acid*Conc-0.5	-0.228	4.681	-9.558	9.103				D	E	F	G	H	I
Vinilic acid*Conc-1.5	2.257	4.681	-7.074	11.588			C	D	E	F	G	H	I
Vinilic acid*Conc-2.5	9.192	4.681	-0.139	18.523		B	C	D	E				

Appendix G.

Table 8: Volume of ethanol or methanol used to dissolve a given amount of phenolic compound.

Compound	*Solvent*	*Volume (ml)*	*Concentration (mM)*	*Amount Grams*
Vanillic acid	Ethanol	6	100	0.425
Trans-ferulic acid	Ethanol	6	100	0.485
Caffeic acid	Ethanol	10	100	0.45
P-Coumaric acid	Ethanol	6	100	0.41
Sinapic acid	Methanol	10	100	0.56
Protocatechuic acid	Ethanol	5	100	0.39

Appendix H.

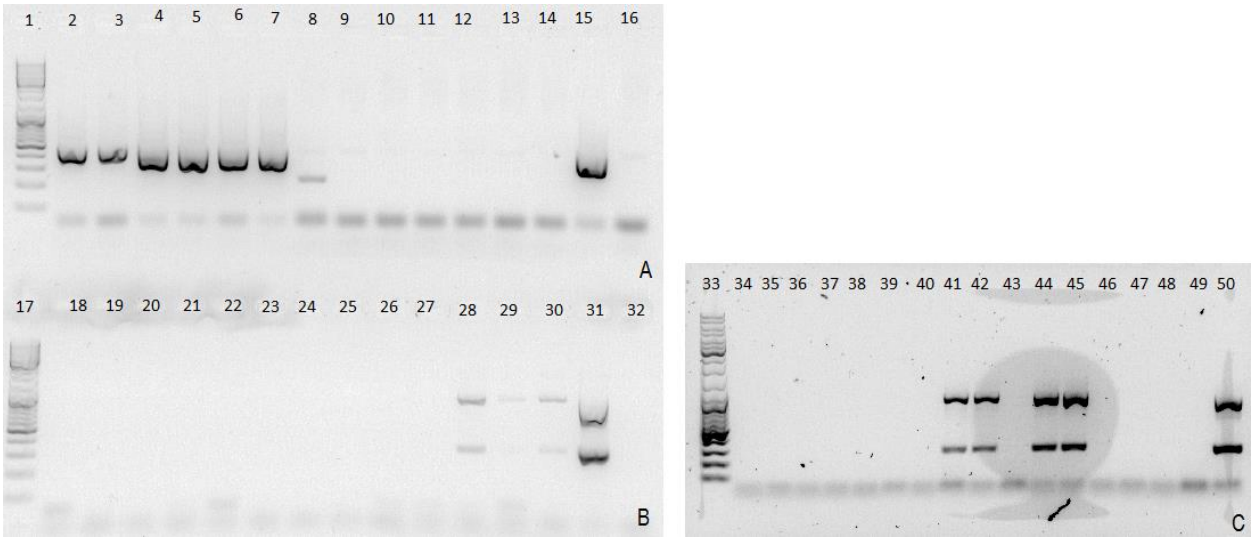

Figure 19: PCR amplification of Foc races with the primer sets Lin VI F-D, Lin VI R-D, Lin 6 F-P and Lin 6 R-P for lineage VI (B and C) and Foc TR4-R and Foc TR4-F for TR4 (A). Lines: 1, 17 and 33 - Ladder; 2, 3, 4, 5, 6, and 7 – TR4; 28, 29, 30, 41, 42, 44, and45 – race 1; 15, 31 and 50 – Positive controls; and 16, 32 and 49 – No template controls.