Extra-cellular Enzymes Assay and Genetic Identification of Banana (*Musa spp*) Endophytic Fungal Isolates

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DECLARATION

This is my original work and has not been presented elsewhere for the award of a degree.

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DEDICATION

This is a dedication to my parents Mr and Mrs Ng'ang'a. Mum and Dad, your support was enormous and all round. May Almighty God bless you in abundance.

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ACRONYMS AND ABBREVIATIONS

dNTPs	Deoxynucleoside triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EF	Elongation factor
IGS	Inter- Genic Spacer
ITS	Internal transcribed spacer
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
rDNA	Ribosomal Deoxyribonucleic acid
SNA	Synthetic Nutrient Agar
s	Seconds
TAE	Tris Acetic acid buffer
Taq	Thermusaquaticus

ABSTRACT

Banana fungal endophytes are known to produce extracellular enzymes that hydrolyze polymeric compounds, including chitin, proteins, lipids cellulose and hemicelluloses that are primary components of pest cuticle. It is the hydrolytic activity of the extracellular enzymes that enables nematode antagonistic fungi to accomplish direct parasitism.

Fusarium spp occur in different forms and lack of clear morphological traits separating different species in this genus. This is often confusing making morphological observations alone unable suffice for complete identification of this group. DNA-based identification techniques have had often been greatly useful in distinguishing such closely related species. The objective of the study was to screen production of extra-cellular enzymes to appraise the suggested mode of action by endophytes and to use DNA sequence analysis to confirm the morphological identification.

Seventeen Fungal endophytic isolates were used in screening for extra-cellular enzymes activity in substrate amended solid media. Gelatin agar, tween20 and chitin powder were used to amend the media so as to mimic nematodes cuticle structural components. All the isolates produced protease as a zone of inhibition starting from 24 hours after inoculation and lasted until the entire gelatin was hydrolyzed. There was significant difference on area of inhibition among the isolates ($P \leq 0.05$) isolates 4MR45,7SIPB34, 5JTO22 and 2MR24 had marginally higher protease activity rate than other tested isolates while isolate 10IJT43, 5MR52, 5IC112 and 2MR23 had the lowest rate. No

lipase and chitinase activities were observed on the isolates used in the study. There was zone of inhibition around the isolates that were inoculated on the control plates.

The results suggested that the fungal endophytes have the ability to degrade collagen, which is a major component of exoskeleton thus employing protease to attack nematodes cuticle to gain penetration. The absence of lipolytic and chitinolytic activities suggest the fungal endophytes banana isolates are incapable of attacking the nematodes eggs.

The phylogenetic analysis of the isolates 18s rDNA gene grouped all the isolates in the genus Fusarium under four species; *Fusarium oxysporium, F solani, F aquiseti* and *F culmorum*. Thirteen isolates grouped together with Fusarium *oxysporum* and formed the major clade of specie (clade 1). Clade 2-4 contained isolates with species similar or closely related to *Fusarium oxysporium*. The molecular phylogenetic analysis confirmed the isolates to be fusarium isolate with scores of 99% to 100% in similarity.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Bananas

Bananas (*Musa* spp.) are among the most important tropical fruits in Sub-Saharan Africa, providing the bulk of dietary carbohydrates and daily calorie intake for millions of people (Frison and Sharock, 1998). In Kenya the crop covers around 1.7 percent of Kenya's total arable land and is predominantly grown by small scale farmers for home consumption and for the domestic market (MOALD, 1997). However, there are very few commercial farms which produce bananas for export. Apart from being the most popular eating fruit in the country, the banana cooking varieties also serve as an important staple food (Wabule, 1998). The year-round fruiting habit of the crop ensures food security at household level with a potential of sustaining food supply to urban markets especially in periods between cereal crop harvests. It's also a source of income for the majority of small-scale growers (Qaim, 1999). Prevalence of smaller farms is higher in the western parts of Kenya, whereas large-scale farmers are mostly found in the Central, Coast and Eastern Provinces

The average banana yields in Kenya are meager with 5.7 tons per acre, which is less than one-third of the crop's potential under the favorable conditions of the humid tropics (Qaim, 1999).Yield potential are mainly affected by pests and disease; among the major pest are nematodes and cause yield loss of up to 50% . The economically most important banana pests in Kenya are nematodes and weevils (Wambugu and kiome, 2001).

1.2 Banana Nematodes

A complex of banana nematodes (*Pratylenchus goodeyi*, *P. coffeae* , *Radopholus similis*, *Helicotylenchus multicinctus*, *Meloidogyne* spp.) are common in many banana fields in Kenya; *H. multicinctus*, *Meloidogyne* spp are common at the Coast, up to 200 meters above sea level (masl), while *H. multicinctus* is common in the Western region, *Radopholus similis* is in the Central and Western regions. The lesion nematode, *P. goodeyi* is the dominant species at altitudes higher than 1000 masl and is also at Kilifi, Coast region. In Muranga district, where coffee is being replaced with banana, *P. coffeae* is commonly found. (Seshu Reddy *et al.,* 1997). These attack the root and corm, reducing the stability of the banana plant and interfering with the uptake of nutrients and water. Their combined damage results in plant loss through toppling, snapping reduced and bunch weights and shortened plantation life.

Control measures against *nematodes* include the use of clean planting material such as pared and hot water treated suckers and tissue culture plantlets (Stanton, 1999; Speijer *et al*., 1999).However the problem of re-infestation of plants in the field is a major problem (Speijer *et al*., 1995; Speijer *et al*., 2001). Host plant resistance offer a safe and long-term intervention strategy against nematodes. However, resistance is yet to be identified in East African highland banana cultivars. Thus, research is currently focusing on several alternative ways, including the use of microbial antagonists such as endophytes, for controlling banana pests and diseases (Sikora and Schuster, 1999; Pocasangre, 2000; Niere, 2001).

1.3 Endophytes

Endophytes are microorganisms that spend some time in their life cycle living symptomless within plant tissues (Petrini, 1991). Endophytes develop mutualistic relationships with plants, acting as antagonists to pests and diseases (Clay 1991, Azevedo, 1998). In banana systems, endophytes have the advantage of targeting the destructive stages of banana nematodes that occur within the plant. The influence of fungal endophytes on herbivores was demonstrated by Barker *et al*. (1998) where he observed that several insects prefer endophyte-free perennial rye grass (*Lolium perenne*). Besides acting as antagonists to pests and diseases, fungal endophytes have been reported to increase biomass production in inoculated plants. Latch *et al*. (1993) reported a significant increase in leaf area for perennial ryegrass inoculated with the fungal endophyte *Acremonium lolii*. Griesbach (2000) reported increased banana biomass production in field plants inoculated with the fungal endophyte *Fusarium concentricum*. Griesbach (2000) obtained 200 isolates of endophytes from highland banana (AAA-EA) and Kayinja (Pisang awak subgroup, ABB). In a related study, Mutua , (2008) isolated Fungi from healthy looking banana plant of Kilifi and Meru District of Kenya identified them using morphological traits and tested for their nematode controlling ability *in vitro* using culture filtrates which had a positive result. However the gap on the endophytes mode of action still remains.

Although endophytes have long been known to protect plants from both biotic and a biotic stresses, little is known on how they suppress pests and diseases. A thorough

understanding of the mechanisms of action by endophytes, is needed to maximize the use, efficiency and consistency of biological control. Various mechanisms of action by fungal endophytes have been suggested (Clay, 1991). These include production of nematicidal metabolites (Li *et al*., 2002), changes in the host plant physiology, induction of general plant defense responses and direct parasitism which is accomplished through the hydrolytic activity of extra cellular enzymes produced by the nematode-antagonistic fungi (Stirling, 1991). For direct parasitism of nematodes to occur, the fungus must penetrate the nematode cuticle, a rigid and flexible exoskeleton composed mainly of proteins (Inglis, 1983). The nematode egg shell consists mainly of a chitinous and lipid layer (Perry and Trett, 1986; Bird and Bird, 1991). Production of extra cellular enzymes by nematode parasitic fungi has been demonstrated mainly for nematophagous fungi like *Arthrobotryis oligospora* Fresenius (Minglian *et al*., 2004) and *Verticillium chlamydosporium* Goddard (Segers *et al*., 1994).

Several researchers have reported association between endophytic *Fusarium* and banana plants (Pereira *et al.,* 1999; Pocasangre *et al.,* 1999; Dubois *et al.,* 2004). *Fusarium oxysporum* has been identified as the predominant species establishing endophytic relationships with banana plants.

1.4 Identification

The isolates identification to taxa like species and genus is subsequently accomplished by studying phenotypic characteristics such as Gram stain, morphology, culture requirements, and biochemical reactions along with a combination of intuition and stepwise analysis of the results (Relman, 1999). In today's laboratory, the ability to identify pathogens has undergone major changes (Dubois *et al.,* 2004).

Morphological identification of plant fungi is the first and the most difficult step in the identification process. This is especially true for fusarium fungi which is a cosmopolitan genus comprising of non-pathogenic and pathogenic strains, some of which occur as endophytes colonizing plant tissues (Niere, 2001; Tan and Zhou, 2001; Sikora *et al.,* 2003). Although morphological observations may not suffice for complete identification, a great deal of information is usually obtained on the culture at this stage (Mohamed *et al*., 2004). However, for species that cannot be reliably identified this way, especially for members of the *Fusarium* genus complex, additional analysis is required to avoid species name misapplication (Geiser *et al.,* 2004).

Complex molecular methods have emerged as the confirmatory method for identification in many taxonomic applications (Iwen *et al*., 2002). The development of molecular methods that rely on the detection of genomic elements (DNA or RNA) with or without culture has led the way in this charge (Stefani and Berube, 2006). Some of the main reasons for this change from phenotypic to molecular testing include such issues as the slow growth of microbes, the detection of organisms that exhibit

biochemical characteristics that do not fit patterns of known species, and the inability to detect non-cultivatable organisms (Relman,1999). Although culture-based methods are still considered the standard for identification diagnosis, molecular methods have emerged as the confirmatory method for identification.

The basic principle of any molecular analysis is the detection of a specific nucleotide sequence (signature sequence) within the organisms' genome which is then hybridized to a labeled complementary sequence followed by a detection mechanism (Iwen *et al*., 2002). Sequence-based identification requires the recognition of a molecular target that is large enough to allow discrimination of a wide variety of microbes. One such target area that has been recognized is the rDNA gene complex which is present in all microbial pathogens. Within fungi there are three genes (18S, 5.8S, and 28S) with spacers located between the genes (ITS1 and ITS2). Located in the rDNA gene complex are highly variable sequences that provide unique signatures for the identification of species and also conserved regions that contain genomic codes for the structural restrains that are present within organism groups (Mohamed *et al*., 2004). The availability of these variable sequence regions (ITS) surrounded by conserved sequences (18S/5.8S/28S) allows for the utilization of an amplification system using fungal primers. Once amplification has occurred using the primers, the sequence is determined and comparison analysis of the unknown sequence to known sequences contained within a large database (such as the National Center for Biological Information (NCBI), GenBank databases) can be done to determine similarity and subsequently may lead to species identification (Meyer *et al*., 2010).

The most widely used method for nucleic acid hybridization is the polymerase chain reaction assay i.e., PCR. This assay includes a specific primer pair to amplify a unique genomic target nucleotide sequence for analysis. Following PCR, a variety of post-amplification methods is used to evaluate the product such as direct sequence analysis (Meyer *et al*., 2010).

Even though all these post-amplification methods have been shown to be useful for the evaluation of microbes, sequence analysis is considered a particularly useful method for the identification of microbial species due to its wide range application to a variety of species (Mohamed *et al*., 2004). One drawback to this methodology is that access to sequencing facilities is not readily available for many laboratories, limiting the ability of most laboratories to conduct routine sequence analysis testing (Stefani and Berube, 2006).

Association between endophytic *Fusarium* and banana plants has been reported (Pereira *et al.,* 1999; Pocasangre *et al.,* 1999; Dubois *et al.,* 2004). *Fusarium oxysporum* has been identified as the predominant species establishing endophytic relationships with banana plants. The ability of endophytic *F. oxysporum* isolates to protect banana plants against pests and diseases has been demonstrated in laboratory and screen-house experiments (Pocasangre *et al.,* 1999; Dubois *et al.,* 2004; Gold and Dubois 2005, Nel *et al.,* 2006, Mutua *et al.,* 2008).

Molecular methods have emerged as the confirmatory method for identification in many taxonomic applications. The basic principle of any molecular test is the detection of a

specific nucleotide sequence (signature sequence) within the organisms' genome which is then amplified using non-selective means. Sequence-based identification requires the recognition of a molecular target that is large enough to allow discrimination of a wide variety of microbes. One such target area that has been recognized is the rDNA gene complex which is present in all microbes (Mulé *et al.,* 2004).The rDNA gene complex has become the choice as a molecular identification tool in *Fusarium* (Geiser *et al.,* 2004). Geiser *et al.,*.(2004) created the first generation of FUSARIUM- ID v.1.0, a readily available database currently consisting of 441 sequences from the genus and placed in a local BLAST server.

The 18s gene region that shows considerable divergence within closely related species is of particular importance (Appel and Gordon 1994). The use of PCR amplified and sequenced 18s of rDNA regions for comparison in the data base is one of the widely used methods for identification of *Fusarium spp* populations.

This includes use of a specific primer pair to amplify a unique genomic target nucleotide sequence for analysis. Following PCR, a variety of post-amplification methods is used to evaluate the product such as direct sequence analysis. Even though all these post-amplification methods have been shown to be useful for the evaluation of microbes, sequence analysis is considered a particularly useful method for the identification of microbial species due to its wide range application to a variety of species (Iwen *et al*., 2000). Universal PCR assay has been used to identify some *Fusarium* species in the *G. fujikuroi* species complex. (Murillo *et al.,* 1998; Moeller *et al.,*1999). One drawback to this method is that access to sequencing facilities is not readily available for many

laboratories, limiting the ability of most laboratories to conduct routine sequence analysis testing.

Despite the known association of banana plants with endophytic *Fusarium spp*. and availability of molecular techniques, information on the genetic of Kenyan endophytic isolate remain scanty.

1.5 Screening for Production of Extra Cellular Enzymes by Fungal Endophytic Isolates

Biological control of *Pratylenchus goodeyi* and *Helicotylenchus multicinthus* using endophytic *Fusarium oxysporum* Schlecht: Fries, is a promising management option that can be used to complement other nematode management strategies (Mutua, 2008). Endophytic fungi suppress the nematodes in a number of ways by utilizing mechanisms that may act alone or in combination. One of the main mechanisms for *in vitro* inhibition of nematodes is the production of antagonistic compounds that cause nematode paralysis and mortality (Niere, 2001; Dubois *et al*., 2004, Mutua, 2008). The suppression of nematodes by endophytes could be as results of production of secondary metabolites however there are no evidences to ascertain this postulation (Mutua, 2008). Assaying for extra-cellular enzymes that the endophytes produce help in getting the knowledge on mode of action of endophytic fungi. Extracellular hydrolytic enzymes target the external and internal structures of nematodes and their eggs (Wuyts *et al*., 2004) thus direct parasitism can occur through the hydrolytic activity of extra cellular enzymes produced by the antagonistic fungi (Stirling, 1991). For direct parasitism of nematodes, the fungus

must penetrate the nematode cuticle, a rigid and flexible exoskeleton composed mainly of proteins (Inglis, 1983). The nematode egg shell consists mainly of a chitinous and lipid layer (Perry and Trett, 1986; Bird and Bird, 1991). Production of extra cellular enzymes by nematode parasitic fungi has been demonstrated mainly for nematophagous fungi like *Arthrobotryis oligospora* Fresenius (Minglian *et al*., 2004) and *Verticillium chlamydosporium* Goddard (Segers *et al*., 1994).

The major component of the nematode cuticle extracellular matrix (ECM) is collagen, a protein that represents over 80% of the soluble protein released following its extraction in reducing agents (Mcgraw, 2007). Collagens are ubiquitous structural proteins with a characteristic Glycine-X-Y tripeptide repeat, where X is frequently proline and Y hydroxyproline (Page and Johnstone, 2007)). The pro-collagens are synthesised, modified and trimerize in the endoplasmic reticulum (ER) (Sebastiano et al., 1991). In addition to collagens, a novel highly cross-linked insoluble class of proteins called cuticlins are associated with the cuticle (Sebastiano et al., 1991). The outermost layer, the epicuticle is lipid-rich and this in turn is overlaid by a loosely associated glycoprotein-rich surface coat.

1.6 PROBLEM STATEMENT

Previous research results have shown that endophytic fungal isolates tested against *P goodeyi* and *H. multicintus* possessed *in vitro* nematicidal activity, causing over 80% nematodes mortality (Mutua, 2008). However little is known on how they suppress nematodes. An understanding of the mode of action of endophytes is needed to maximize the use, efficiency and consistency of nematodes biological control

Suggestion that suppression was as a result of production and excretion of extracellular enzymes and/ or metabolites by the isolate lacks evidences to ascertain the hypothesis. The type of extracellular enzymes or metabolites produced by the endophytes has not been studied. This problem was addressed by finding out the extra-cellular enzyme the isolates produces. Amending the solid media with enzymes specific substrates helped to induce extra-cellular enzymes production and mimic the nematodes cuticle component as well as nematodes egg shell.

Fusarium genus of toxigenic fungi has had a confusing and unstable taxonomic history. Lack of clear morphological characters separating genus species, lead to species concepts that are too broad creating taxonomic systems that poorly reflect species diversity. The result of this confusion is the rampant misapplication and inconsistent application of species names to the isolates unless a more accurate method is used to supplement morphological identification. Molecular technique offers more accurate and highly reliable identification which was exploited in this study.

1.7 JUSTIFICATION

Various modes of action by fungal endophytes have been suggested which includes production of metabolites (Clay, 1991), secretion and release of enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, and DNA (Li *et al*., 2002), changes in the host plant physiology and the induction of general plant defense responses all which interferes with pathogen growth and/or activities (Hallmann and Sikora, 1994). Production of extra cellular enzymes by fungi that are parasitic on nematodes has been demonstrated using *Athrobotryis oligospora fresenius* and *verticillium chlamydosporium* (Minglian et al., 2004). Priest (1984) showed that use of substrate amended solid media is one of the useful regulatory mechanisms for the extra-cellular enzymes production screening.

Sequencing of the ribosomal DNA genes has emerged as a useful identification and diagnostic tool for the rapid detection of fungi, regardless of whether or not morphologically distinct structures are produced (Hoorfar *et al.,* 2004). The gene has combination of conserved and variable regions offers great flexibility for PCR sensitivity and specificity. The conserved sequences at the flanking ends of the regions allow for universal PCR priming sites, while the variable internal regions provide species-specific sequence in many cases (Balajee *et al.,* 2007). The18S and ITS rDNA region gene sequences have always been ideal targets during fungal isolates genetic identification due to their ability to allow discrimination to species and intra-specific level (Borman *et al*., 2008).

1.8 HYPOTHESIS

Endophytic fungal isolates act by secreting extra-cellular enzymes that digest collagen and other primary components of nematodes exoskeleton.

1.9 General objective

To determine genetic identity of the local bananas endophytic fungi isolates using molecular techniques and screening for the production of extra-cellular enzymes

1.10 Specific objectives

- To determine the production of extracellular enzymes; protease, chitinase and lipase by endophytic fungi isolates on a solid media.
- To identify endophytic fungal isolates antagonistic to banana nematodes using molecular techniques.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Production of Banana and it constraints.

Africa produces nearly 30 million tons (34% of the world production) of bananas yearly, which is mostly consumed locally (Gold et al., 1999). In East Africans highland bananas are the staple food for over 20 million people and this region alone produces nearly 15 million tons annually (Inibap, 1986). The country with the highest per capita consumption in the world is Uganda (243kg/cap/yr) (Frison and Sharrock, 1998).

In Kenya an estimated 1.7% of the total arable land is under banana production, predominantly grown by small scale farmers (Seshu *et al*., 1998, ISAAA, 2001). Banana production is characterized by low input application, with no or limited use of pesticides and fertilizer (Swennen and Vulysteke, 2001). Banana is vulnerable to attack by pest and diseases, such as, nematodes, black and yellow Sigatoka (Ploetz, 2001). In Kenya banana production has been on a very rapid decline over the last decades, threatening food and income security (Wanzala, 2005). The decline has been attributed to pest and disease reducing yield levels to about 14 *tons* per ha (Karamura 1998). The crop yield losses due to nematodes pest attack can be up to 50% (Speijer and Kajumba, 2000).

The banana burrowing nematodes (*R. similis*) is one of the major constraints to banana production in the world (Sarah, 1989). They feed on plant roots, and the damage it cause leads to necrosis and impaired water and nutrients uptakes (Davide, 1996). Root

necrosis, in turn, reduces the plant's anchorage in the soil and plants can easily topple. A survey conducted in Kenya to identify constraints to banana production indicated a complex of banana nematodes (*Pratylenchus goodeyi* (Sher and Allen), *Radopholus Similis* (Cobb) Thorne, *Helicotylenchus multicinthus* (Cobb) Golden, *Meloidogyne spp* (Goeldi), were common in many banana fields (Seshu *et al.*, 1998). *Pratylenchus goodeyi* is the most wide spread nematode attacking bananas (Seshu *et al.*, 1998). The spread of banana Pest and diseases in banana plantations have often been through the use of infected planting materials.

Control measures against nematodes include the use of hot water treated suckers and tissue culture plantlets (Stanton, 1999; Speijer *et al*., 1999). Clean planting material may provide adequate control in the first crop cycle, but the problem of re-infestation of plants in the field is a major disadvantage (Speijer *et al*., 1995; Speijer *et al*., 2001). Host plant resistance would offer a safe and long-term intervention strategy against nematodes. Therefore studies have focused on the use of microbial antagonists such as endophytes, for controlling banana pests and diseases (Niere, 2001; Pocasangre, 2000; Sikora and Schuster, 1999).

Endophytes are microorganisms that spend some time or their entire life cycle living symptomless within plant tissues (Petrini, 1991). Biological control of nematodes using endophytic fungi offers a novel and promising nematodes management option. In the past fungal endophytes have been successfully used to control nematodes in crops such as tomatoes (Hallman and Sikora 1994). It has been demonstrated that fungal

endophytes isolated from roots of banana in Kenya were able to cause nematodes paralysis and mortality *in vitro (*Mutua, 2008). Fungal endophyte protects plants against nematodes in several ways. The main method by which nematodes are inhibited *in vitro* is through antibiosis. Antibiosis is the antagonism resulting from production of secondary metabolites by one microorganism that are toxic to another microorganism (Alabouevette and Lemanceau, 1999) various non-endophytic Fusarium isolate have been shown to produce filtrate toxic to plant parasitic nematodes *in vitro* (Ciancio, 1995; Anke and Sterner, 1997; Nitao *et al*., 2001; Meyel *et al*., 2004; Mennal *et al.,* 2005). Hallman and Sikora (1996) demonstrated production of toxins by an endophytic isolate of *Fusarium oxysporum* Schecht from tomato plant against *Meloidogyne* spp. Similarly various endophytic *Fusarium* isolate obtained from banana produce secondary metabolites in culture that immobilized and caused mortality of *R. similis* (Pocasangre, 2000; Niere, 2001; Dudois e *et al*., 2004).

Although tissue culture plants may provide healthy and nematode-free planting materials, the effects may offer only a temporary solution to nematode problems in banana. This necessitates the search for affordable, sustainable and environmentally friendly management strategies that complement the benefits of clean planting material. Due to the sterile conditions under which tissue culture plants are produced, the plants loose naturally beneficial microorganisms such as endophytes (Pereira *et al*., 1999). The artificial introduction of endophytic fungi in these sterile plants at the hardening phase may offer protection against pests and diseases to the young plants in the early growth

stages and extend the life of planting material (Sikora and Schuster, 1999). This strategy would form part of an integrated nematode management approach consisting of biological control and clean planting material.

2.3 Biological control of Nematodes

It has been shown that nematophagous fungi play a crucial role in suppressing the populations of plant-parasitic nematodes in nature and has great potential as biological agents which can be integrated in intergrated pest management (IPM) (Nordbring-Hertz *et al.,* 2000).

Research on the use of endophytes for the biological control of nematodes in banana in Kenya has been in progress. The isolation of *Fusarium oxysporum* from healthy banana and screening for the antagonism against banana nematodes *P. goodeyi* and *H. muticintus* suggested that the isolate produced metabolites that caused mortality or paralysis on the two species of nematodes (Mutua, 2008).

Though endophytic fungi is viewed as nematodes biological control agent less literature is available regarding the genetic and phylogenetic identity of endophytic *Fusarium* spp. from banana plants in Kenya.

2.3 Mechanisms of action of endophytes against nematodes

Although endophytes have long been known to protect plants from both biotic and a biotic stresses, little is known on how they suppress pests and diseases. A thorough

understanding of the modes of action is needed to maximize the use, efficiency and consistency of biological control. Various mechanisms of action by fungal endophytes have been suggested (Clay, 1991). These include production of nematicidal metabolites (Hallmann and Sikora, 1996), changes in the host plant physiology and the induction of general plant defense responses. Diverse microorganisms secrete and excrete other metabolites that can interfere with pathogen growth and/or activities. Many microorganisms produce and release extra-cellular enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, and DNA. Expression and secretion of these enzymes by different microbes can sometimes result in the suppression of plant pathogen activities directly. For example, control of *Sclerotium rolfsii* by *Serratia marcescens* appeared to be mediated by chitinase expression (Ordentlich *et al*., 1988).

2.4 Detecting Genetic Identity and Diversity

Great deal of species diversity which had vastly been under-estimated by all previous morphological treatments (O'Donnell 1998; O'Donnell *et al*.,2000;).

Molecular techniques are available to accurately identify organisms, and laboratories are becoming increasingly available throughout world to undertake accurate identification. Multi-locus phylogenetic methods which allows for the objective identification of species boundaries in the Fungi. Relationships among well-defined Fusarium species have been inferred.

Genetic diversity exists between and within pathogenic and non-pathogenic populations of *F.oxysporum* and various molecular methods can be used to detect such variation (Gordon and Okamoto 1991; Edel *et al*., 1995). The amplification of variable ribosomal DNA (rDNA) regions allows for discrimination at the genus, species and intraspecific level (Edel *et al*., 1995). Intergenic spacer (IGS) region which shows considerable divergence within closely related species is of importance in (Appel and Gordon, 1995). Some researchers have used species-specific PCR assay to identify some *Fusarium* species in the *G. fujikuroi* species complex (Murillo *et al*., 1998; Moeller *et al.,* 1999). For example, Mulé *et al.* (2004) used species-specific primers to identify *F. subglutinans*, *F. proliferatum* and *F. verticillioides* from maize based on partial calmodulin gene sequences.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Source of fungal endophytic isolates

Sixteen randomly selected *Fusarium oxysporum* endophytic isolates that had previously been isolated from healthy looking bananas in both Meru (60`34S, 037°46`23 E, 1050 Meters above sea level) and Kilifi (03`4S 0" 039°40`0" 17 Meters above sea level) and preserved in sterile soil and filter paper at 4°c in JKUAT plant physiology laboratory and one isolate from Uganda (V2W5) were used for this study. All the isolates (**Table 1**) were assayed for the production of extra cellular enzymes on solid medium amended with enzyme-specific substrates and used for molecular identification.

3.2 Isolates activation

The isolates were restarted on Synthetic Nutrient Agar (SNA) (1g KH2PO4, 1g KNO3, 0.5g MgSO47H2O, 0.5 g KCl, 0.2 g glucose, 0.2g sucrose, 0.6ml NaoH (1 M) and 13.2g agar/L distilled water) under laboratory conditions (room temperature of 25±2°C and a photoperiod of 12 hrs light and darkness) for 1 week. The SNA medium was supplemented with 10mg chlortetracycline, 100mg penicillin G and 50 mg streptomycin sulphate/ L to prevent bacterial contamination. This was followed by series of hyphal tips sub culturing until pure cultures were obtained which were used for various extra-enzymes assay.

Table 1: Endophytic fungus isolates that were tested for extra-cellular enzymes potential

Isolate code	Isolates area of origin	Cultivar isolated from	Plant part isolated from
1SR55	Meru	EAHB-Mutagato	Roots
1MR24	Meru	Giant Cavendish	Roots
2SR24	Meru	EAHB-Mutagato	Roots
2SR23	Meru	EAHB-Mutagato	Roots
3MR45	Meru	Giant Cavendish	Roots
3MR43	Meru	EAHB-Mutagato	Roots
4MR45	Meru	Giant Cavendish	Roots
5MR52	Meru	Giant Cavendish	Roots
5IC112	Meru	Giant Cavendish	Roots
5SOPB13	Meru	Giant Cavendish	Outer pseudo stem
7SIPB34	Kilifi	Mkono Tembo (EAHB)	Inner pseudo stem
7MIC334	Kilifi	Mkono Tembo(EAHB)	Inner pseudo stem
10IJT43	Kilifi	Dwarf Cavendish	Joint
10IC121	Kilifi	Grand nine	Inner corm
5JTO22	Meru	Grand nine	Joint
9SIC334	Kilifi	Giant Cavendish	Inner corm
V_5W_2	Uganda	Kubuzi	

Joint- link between banana parent plant and sucker

3.3 Chitinase activity

All the selected isolates were assessed for chitinase activity using 0.4% chitin agar media (4g chitin powder (Sigma-Aldrich St. Louis MO, USA), 0.7g K_2HPO_4, 0.3g KH_2PO_4, 0.5g $MgSO_4.5H_2O$, 0.0 g $FeSO_4.7H_2O$, 0.001g $ZnSO_4$, 0.001g $MnCl_2$ and 20g agar per liter distilled water) (Hsu and Lockwood, 1975). One-week-old fungal isolates growing on SNA was point inoculated in the middle of Petri dishes containing chitin agar. A 5mm-diameter flame sterile cork borer was used to remove a disc of agar at the middle of the chitin agar plates and the hole replaced with a similar sized mycelia agar disc of the 1week old fungal cultures. All experiment was done in triplicate and organized using completely randomized block design.

3.4 Production of lipases

Fungal isolates from 1-week-old cultures on SNA were point inoculated on solid medium (5g NaCl, 1g $CaCl_2$, 10 mL Tween20, 15g Agar, pH 7.0) used to assay for lipase enzyme. A zone of clearance around the colony was observed for a period of 10days.

3.5 Protease activity

Fungal isolates were point inoculated on gelatin medium comprising of (16g gelatin agar (Sigma) and 9g agar in 600 ml distilled water). Six Petri dishes were used for each of the isolates and enzymes; three with amended media and three with non-amended media (controls). All cultures were incubated for 1 week under laboratory conditions (room

temperature of 25±2 °C and photoperiod of 12 hrs of light and darkness). Cultures were examined for the presence of a clear zone (halo) around the fungal colony. Those that produced a halo were considered to be positive for the extra- cellular enzyme assayed while those which exhibited no halo were regarded not to be producing the tested extra-cellular enzyme. For the positive one the diameters of the clear zone and of fungal colonies was measured, and the difference between the areas of the clear zone and the fungal colony calculated to provide an estimate of the levels of enzyme production by the different isolates (Alves *et al*., 2002). Extra-cellular enzyme production ratio which is the ratio of halo area ($\prod r^2$) to that of colony area was calculated.

3.6 Data Analysis

For the enzyme production assays, statistical analysis was performed on the averages of the size of the clear zones (calculated from the difference in size between the fungal colony and the clear zone) using one-way ANOVA.

3.7 Preparation of single spores for molecular identification

Using a flame sterilized wire loop, hyphal tips were aseptically picked from colonies of fungal cultures of each PDA plates under the laminar flow cabinet. The hyphal tips were transferred into sterile 2ml eppendorf tubes containing 1 ml distilled water. In order to release the conidia, the eppendorf tube was vortexed for approximately 30 seconds. From the conidial solution, 50 µl was put into 2ml eppendorf tube containing 1ml

distilled water. Equally this eppendorf tube was vortexed for approximately 30 seconds. The resulting conidial suspension 400 µl was put into Petri dish containing water agar (20g agar / L distilled water) and the plate were shaken slightly to evenly spread the conidial suspension over the medium.

Observation of conidia germination was carried out after 15hours. Presence of germ tube indicated spore viability and therefore picked singly under a dissecting microscope (magnification 100 ×). This was done by cutting a 0.1 × 0.1 mm block of medium surrounding the germinating spore using a sterilized inoculation needle. Together with the spore the media block was transferred to the centre of a 90mm diameter Petri dish containing Synthetic Nutrient Agar (SNA) medium (1g KH_2PO_4, 1g KNO_3, 0.5g $MgSO_47H_2O$, 0.5g KCl, 0.2g glucose, 0.2g sucrose, 0.6 ml NaOH (1 M) and 13.2g agar/L sterile distilled water .The Petri-dishes were sealed with parafilm and incubated for 10 days under laboratory conditions temperature of 25±2°C and photoperiod of 12 hrs of light and darkness. After a pure culture has been obtained, further sub-culturing was carried out onto new PDA plates in order to allow more vegetative growth.

The isolates were cultured by taking hyphal tips from small block of media from the SNA plates and culturing onto the PDA plates. Isolates were incubated for 10 days.

3.8 DNA Extraction

Fungal mycelia was scrapped from the surface of the medium using a flame-sterilized scalpel and transferred into eppendorf tubes. rDNA was extracted according to

Sambrook and Russel (1989) protocol. The mycelia was frozen and ground in liquid nitrogen using sterile mortar and pestle then transferred to the eppendorf tube where 300µl of solution A (25% sucrose, 100mM tris-HCl and 100mM EDTA at pH 8.0), 30µl of lysozyme (20mg/ml) and 15µl of RNAse (20mg/ml) were added followed by 2 hours incubation at 37°c with occasional gentle shaking. 600µl of solution B (10% SDS, 20mM Tris-Hcl and 10mM EDTA pH8.0) and 20µl of proteinase K (20mg/ml) was added and the mixture incubated at 60°c for 1hour with occasional gentle shaking. This was followed by cooling to the room temperature 23°C, proteins were extracted by adding equal volume of phenol-chloroform (ratio 1:1) mixed thoroughly and centrifuged for 15 minutes at 10,000 revolutions per minute (rpm), the supernatant was transferred into a new eppendorf tube this step repeated once, the phenol was washed by adding equal volume of chloroform isoamyl alcohol (24:1) then centrifuged at 10,000 rpm for 10 minutes, the supernatant was transferred to a new eppendorf tube, this was repeated once. DNA precipitation was done by adding to the final supernatant 40µl of 3M sodium acetate and 1ml of absolute ethanol this was inverted to mix and incubated at -20°C overnight then centrifuged for 30 minutes at 10,000 rpm, the supernatant was poured off and the DNA pellet was twice washed with 70% ice cold ethanol, the pellet was air dried for 30 minutes at room temperature after which it was dissolved in 50µl of T.E buffer (10 mM Tris/HCl (pH 8.0,1 mM EDTA (pH 8.0) and dH2O) (pH 8). The resulting DNA was electrophoresis in 1 % agarose gels and visualized using ethidium bromide staining and UV light to check the DNA quality. The DNA concentrations were determined using a spectrophotometer and diluted to a final working concentration of 20ng/ µl for

PCR (polymerase chain reaction). The DNA was used as templates for subsequent PCR amplification.

3.9 PCR Amplification of 18S rDNA gene.

The full length 18s rDNA gene was amplified using fungal primer pair Ef4 forward (5'GGGGAACCAGGACTTTTA3') and FF390r reverse (5'AGGTCTCGTTCGTTATCG 3') primers (O'Donnell *et al.,* 1998).). Amplification was carried out in a 40µl mixture containing 5µl of PCR buffer [100 mMTris–HCl (pH 9)], 1µl of each primer at a concentration of 5pmol, 3µl of de-oxynucleoside triphosphate at a concentration of 2.5mM, 1µl of 2.0mM $MgCl_2$, 1.5µl bovine serum albumin (BSA), 1.5µl of template DNA, and 0.3 µl of Taq DNA polymerase (Roche). The volume was adjusted to a final volume of 40µl with sterile PCR water. The control contained all the above except the DNA template. Peqlab primus 96 PCR machine (peqlab, Germany) was used. The reaction mixture was subjected to 5 minutes initial activation step at 96°C followed by 40 cycles of a denaturation step at 95°c for 45sec, a 40sec of primers annealing at 48°c, and a 90sec of elongation at 72°c and a final extension at 72°c for 8min. The presence of PCR amplicon was confirmed by analyzing 5µl of PCR products on 2% agarose gels after stained with ethidium bromide. The products were compared with a molecular weight marker 10,000bp (Smartladder; Eurogentec) (**Fig 3**).

3.10 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Germany)

Five volumes of buffer PB (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column in a 2ml collection tube; the sample was applied to the QIAquick column to bind the DNA, and was centrifuged for 60 seconds at 13000 rpm.The flow-through was discarded, and the QIAquick column was placed back into the same tubes. To wash the DNA, 740 µl buffer PE was add to the QIAquick column and centrifuged for one minute. The flow-through was discarded and the column centrifuged again for an additional one minute at 13000rpm to remove residual ethanol from buffer PE. The Qiaquick column was placed in a 1.5ml micro centrifuge tube and adds 30µl of buffer EB (10mM Tris-Cl, pH 8.5) to elute DNA. Tubes were centrifuged for one minute, spin column was removed and DNA was stored at –20 ^{o}C for application (Sambrook *et al.,* 1989).

3.11 Sequence analysis

Partial sequences were generated at the sequencing facility at ILRI, (BecA-ILRI Hub Services, Segoli laboratory as follows;

10µl master mixture each containing; 4µl of purified PCR products, 1µl of FF390r (5'AGGTCTCGTTCGTTATCG 3') primer, 2µl of Big dye III, 1µl 5x dilution buffer and 2µl dH2O.

The master mix was then placed under PCR conditions of 25 cycles for 10 s at 96 ºC, 5 s at 50 ºC and 4 min at 60 ºC. Sequenced products are precipitated and electrophoresed using an ABI Prism™ 3100 Genetic Analyzer.

3.12 Phylogenetic Investigation

Partial sequencing of purified PCR products was done at ILRI, (BecA-ILRI Hub Services, Segoli lab using the reverse primer FF390r. The CHROMAS-LITE program (http://www.technelysium.com.as/chromas) was used to check for the presence of possible chimeric artifacts. Alignments were checked and corrected manually where necessary, based on conserved regions. The sequences were compared with GenBank sequence databases using nucleotide Blast on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) to determine similarity to sequences in the Gene bank database (Tamura *e tal.,* 2007).The 18S rDNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results.

Phylogenetic placement of the fungus and its possible evolutionary relationship with other that exist as endophytes, saprophytes and pathogens was carried out using the

Neighbor-Joining method . The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,*2007). Phylogenetic analyses were conducted in MEGA4.0 software (Tamura *et al.,*2007).

CHAPTER FOUR

4.0 RESULTS

4.1 Production of Extra Cellular Enzymes

The *F. oxysporum* isolates tested did not show chitinase or lipase activity in solid medium, as no clear zone formed around the fungal colonies 1 week after incubation. All the isolates, however, showed positive protease activity (**Table 2**). A clear zone formed around the fungal colonies starting from 24 hours after the fungus was placed on gelatine-amended medium **(Fig. 1**). The measurements of the fungal colony diameter and the diameter of the clear zone were conducted on the third day of incubation. Measurements could not be done on the second day since the distinction between the fungal colony and the clear zone could not be easily discerned. From the fourth day onwards the clear zone was not visible anymore. No clear zones were observed in the control plates in which the fungus was grown without the enzyme substrates.

Based on the size of the clear zone, isolates *4MR45, 3MR43,2MR23* and *5IC112* had marginally higher levels of protease activity compared to isolates *1SR55*(**Table 2**).

The area of the clear zone had significant different between the different fungal isolates ($P \geq 0.05$).

Ugandan isolate (V_5W_2) had similar level of protease activities with 10IC121 and 3MR45 showed no significant difference with all other Kenyan isolates except 4MR45 and 1SR55 which had higher and lower protease activities respectively (**Table 2**).

Table 2: Isolates response to various extra-cellular enzymes screening Mean area covered by protease extra cellular enzyme activity and average halo to colony area ratio.

Isolate code	chitinase	protease	lipase	Hola Area (cm^2)	Average Ratio of Halo to colony size
4MR45	-	+	-	8.4±4.0a	0.6
3MR43	-	+	-	2.9±3.6ab	1.4
5SOPB13	-	+	-	2.7±3.8ab	1.6
2MR24	-	+	-	2.2±3.8ab	1.5
2MR23	-	+	-	1.6±3.4bc	1.2
5IC112	-	+	-	0.8±3.4bcd	1.7
7MIC334	-	+	-	0.2±3.1bcd	2.3
1MR43	-	+	-	.9±3.0bcde	0.9
V5W2	-	+	-	.6±2.3bcdef	1.4
10IC121	-	+	-	.6±2.3bcdef	1.5
3MR45	-	+	-	.6±2.2bcdef	0.8
9SIC334	-	+	-	.3±2.2bcdefg	1.4
5JT022	-	+	-	.8±1.8cdefg	1.1
5MR52	-	+	-	.2±1.7defg	1.3
10IJT43	-	+	-	.1±1.1efg	1.0
7SIPB34	-	+	-	.9±1.3g	1.5
1SR55	-	+	-	.8±0.2g	1.6

The (+) signs indicates presence of extra-cellular enzymes activities, (–) signs indicate presence of extra-cellular enzymes activities while mean values (±SE) within a column followed by the same letter are not significantly different by Duncan’s multiple range test (*P≥0.05*)

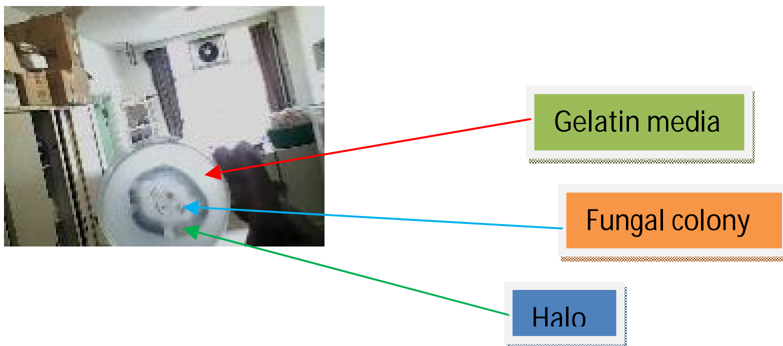

Figure 1: Protease activities of endophytic *Fusarium oxysporum* (isolate 5MR52) in 80mm Petri dishes 2 days after inoculation on gelatin amended media. Clear zones (halo) indicate positive protease activity

[Figure 2; Plate of Isolate 9sic334 (30R2) and it replicates in assay to screen for lipase on tween 20 amended media. Isolate grew without zoning.

4.2 DNA extraction and PCR amplification.

The amplification of 18s rDNA gene with primers ef4 and FF390Rr resulted in approximately produced fragments of 550 bp of fragment (fig3)

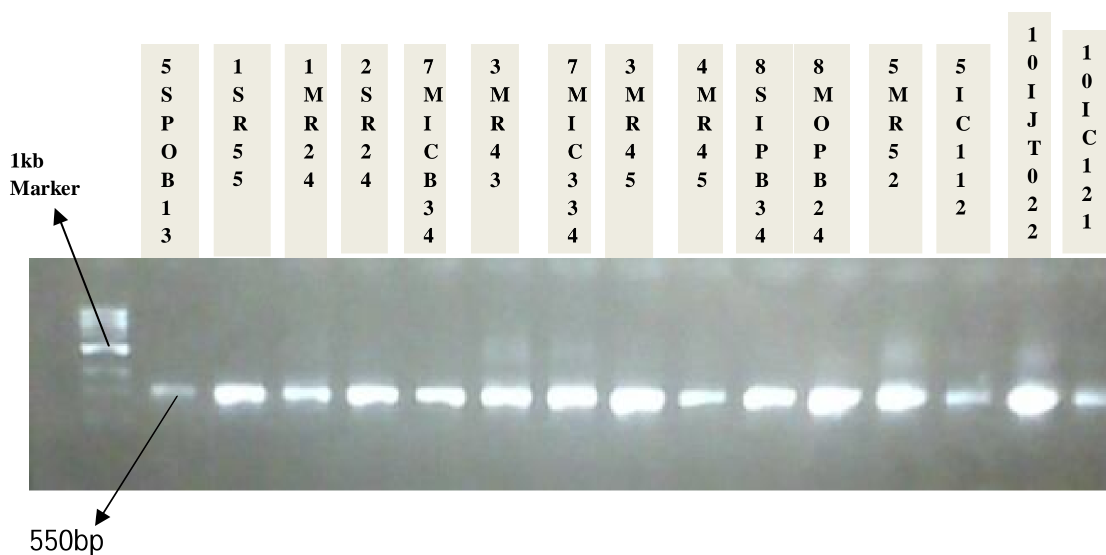

Figure 3: A 2% agarose gel showing PCR amplification of 18S rDNA of the isolates visualized after ethidium bromide staining.

4.2 Phylogenetic analysis of sequences

The sequence data was then assembled and submitted at the NCBI Genbank with accession number GenBank, accession numbers (HQ174214, EF397944, and HM165488) **(Table 3).** Based on BLAST search of ribosomal RNA gene sequence, the

fungus was found to belong to the Fusarium genus with closest homolog to species members *Fusarium spp* (**Fig 3**).

Phylogenetic tree was generated using neighbor joining method. The generated tree showed four clades of species and one lineage which are all from genus Fusarium **(Fig 4**).

Thirteen isolates clustered together with one major group which were found to be closest homolog to *Fusarium oxysporum* species (HQ174214) (**Fig 4**) with a score of between 99 % and 100 % similarity (**Table 3**).

Isolate (5IC112) clustered together with *Fusarium solani* species with similarity score of 99% (EF397944) clade 2 (**Fig. 4**).

Isolate (3MR43) grouped together with *Fusarium culmorum* (AF548073) in Clade 3.*Fusarium culmorum*is also a soil borne facultative parasite that exists both as pathogenic, saprophytic forms and endophytic form. In coastal dune grass (*Leymusmollis*), *F. culmorum* is a non-pathogenic and confers both salt and drought tolerance to the plant (Rodriguez *et al.,* 2008). It Colonies grow rapidly and has the following trait among others ; Aerial mycelium is whitish to yellow, tan or pale orange, but become brown to dark brown to red-brown with age. Under alternating conditions of light and temperature, rings of spore masses may be formed by some isolates (Leslie and Summerell 2006).

Isolate J (3MR45) grouped together with *Fusarium equiseti* (AF141949) *(***Fig 4**) in clade 4 with a score of 100 % similarity (**Table 3**).

Table 3: Blast result of endophytes fungal isolates from Kenya Bananas and close relatives

isolate	Bp	Acc No.	Next neighbor in BLAST	% similarity
5SPOB13	527	HQ174215	*Fusarium oxysporum clone P10*	100%
1SR55	536	HM067111	*Fusarium sp*. LNUF014	100%
1MR24	532	AB473810	*Fusarium solani*	100%
2SR24	545	HQ174215	*Fusarium oxysporum clone P10*	99%
7MICB34	541	AF141949	*Fusarium equiseti*	99%
2SR23	533	AF141951	*Fusarium oxysporum*	100%
3MR43	536	GQ166777	*Fusarium sp. EF1 18S*	100%
7MIC334	538	FN666092	*Fusarium oxysporum*	100%
3MR45	537	HM067111	*Fusarium sp. LNUF014*	100%
4MR45	547	EF590326	*Fusarium oxysporum f. cubense strain ATCC 96285*	99%
8SIPB34	566	GQ120172	*Fungal sp. FCASAn-2*	99%
8MOPB24	544	EF397944	*Fusarium solani strain 421502*	99%
5MR52	540	AF219122	*Fusarium oxysporum f. sp. vasinfectum isolate Ag149-I*	99%
51C112	548	EF397944	*Fusarium solani strain 421502*	99%
10IJT022	539	EU710825	*Fusarium sp. 16010*	99%
10IC121	434	HQ174215	*Fusarium oxysporum clone P10*	99%
V2W5	539	FN666090	*Fusarium oxysporum*	100%
8MR24	538	AB332408	*Fusarium oxysporum*	100%
10MIPB23	544	AB332408	*Fusarium oxysporum*	100%

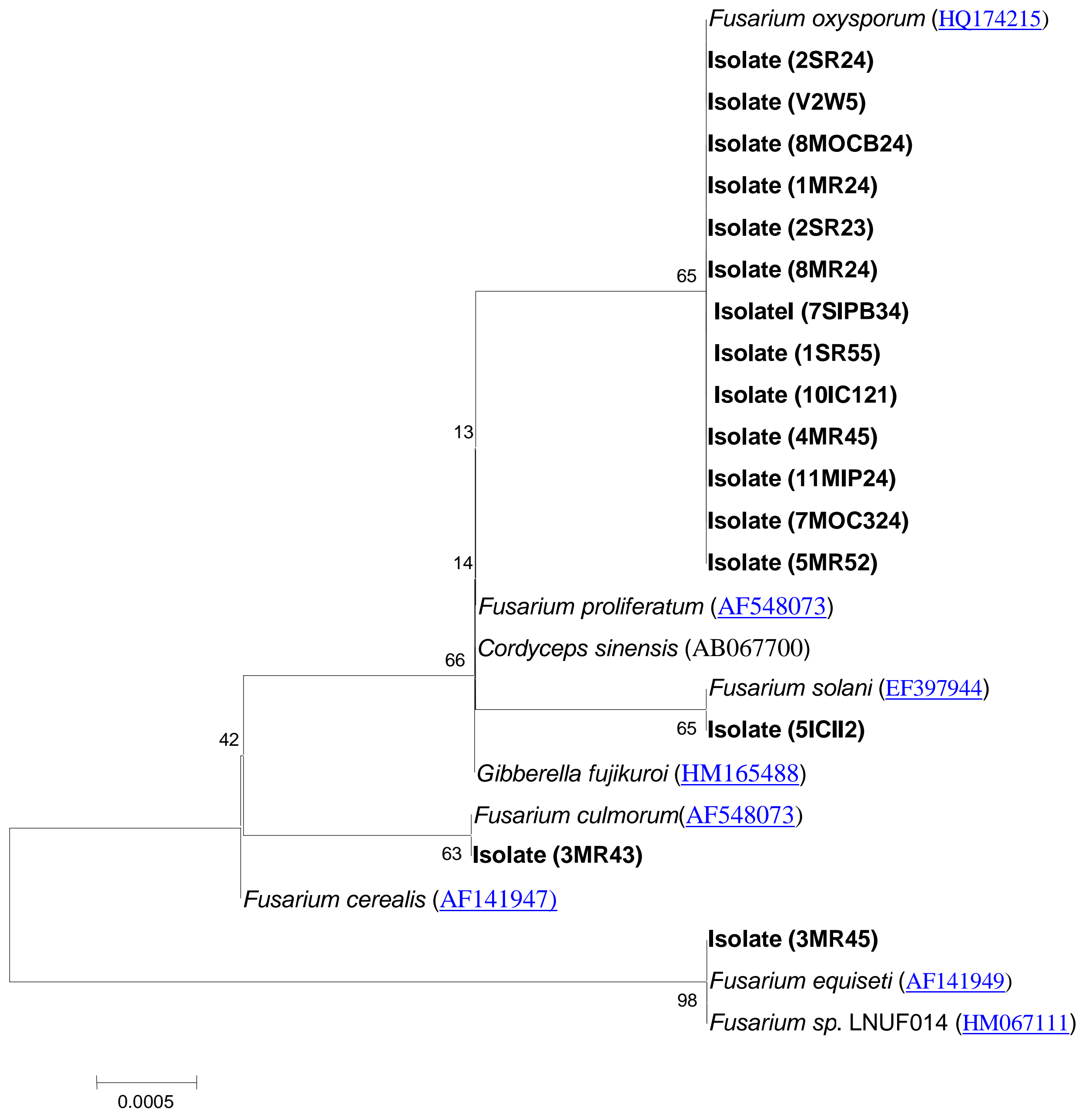

Figure 4: Tree showing phylogenetic relationship of 20 isolates from Kenya bananas and the closest relatives from blast analysis.

CHAPTER FIVE
5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

In this study chitin powder, tween20 and gelatin were used as substrates to mimic chief component of both nematode cuticle and eggshell. The substrates were mixed with low nutrient content agar that had just enough nutrients for mycelia to spread across the plates. Priest (1984) showed that there are several possible regulatory mechanisms in enzyme production and enzyme induction is one of most useful.

Extracellular hydrolytic enzymes target the external and internal structures of nematodes and their eggs (Wuyts *et al*., 2004). In this study, all *F. oxysporum* isolates produced proteases, but none showed chitinolytic or lipolytic activity. The production of proteases might be partly responsible for mortality of the motile stages of *P.goodeyi* and *H. multicinthus* nematodes when they were treated with fungal culture filtrates (Mutua *etal.,* 2008), since the nematode cuticle matrix (ECM) is mainly composed of collagen, a protein that represents over 80% of the cuticle soluble protein released following its extraction in reducing agents which compare to gelatin collagen (Johnstone, 1994). The gelatin agar is an irreversibly hydrolyzed form of collagen derived from the collagen inside animals' skin and bones. The protease production enables the fungi to penetrate into the nematode through degradation of cuticle and gain entry to the pest internal structures or expose it to other harmful biotic and a biotic factors; this is in agreement with Wuyts *et al*., 2004 finding that extracellular hydrolytic enzymes target the external and internal structures of nematodes and their eggs Ward and Courts (1977) and. Vu *et*

al. (2004) previously demonstrated a lack of direct parasitism of *R. similis* by endophytic *Fusarium* isolates, suggesting that other secondary metabolites might be involved in the killing of nematodes.

The lack of chitinolytic and lipolytic activity suggests that direct parasitism of nematode eggs, composed mainly of chitin and lipids (Bird and Bird, 1991), by *F. oxysporum* is unlikely.

For direct parasitism of the nematodes by fungal hyphae to occur, contact between the nematode and the fungus for a sufficient duration is required. Paparu (2005) demonstrated that colonization of banana roots by endophytic *F. oxysporum* isolates was extensive in the hypodermal cells and cortex. All developmental stages of banana nematode also occur in the cortex of plant roots (Araya and De Waele, 2001; Gowen and Quénéhervé, 2005). Despite occupying the same niche inside roots, direct parasitism at adult stages and eggs of nematodes by endophytic fungi may not happen due to the migratory nature of the nematode.

The previous morphological identification revealed the isolated fungus to be *Fusarium* sp. in taxonomy (Mutua *et al* 2008). Genotypic identification which was carried out in this study by considering the 18S rDNA also found out that the isolates belong to the genus fusarium with over 80 percent of them 99-100% similarity to *Fusarium oxysporum* species.

Fusarium is a large genus of filamentous fungi widely distributed in soil and in association with plants e.g. bananas, grass, legumes among others. Most fusarium

species are harmless saprophytes, and are relatively abundant members of the soil microbial community. Some species produce mycotoxins in cereal crops that can affect human and animal health if they enter the food chain, while some are said to cause beneficial attack to the plants.

Pathogenic and endophytic strains of *F. oxysporum* might have evolved from a saprophytic ancestor and pathogenic strains have given rise to endophytic forms. Such findings led us to conclude transformation in *F. oxysporum* into various forms seems to be natural phenomenon which may be governed by different cultural and environmental conditions. This also supports the assumption that *F. oxysporum* is phylogenetically diverse and genetic variations within this species complex could arise based on different geographical locations and ecological habitats

F. oxysporum is a soil borne facultative parasite that exists as pathogenic, endophytic and saprophytic. Recently, it has also been isolated as endophyte from several plant species with diverse biological activity (Bashyal *et al.,* 2006 and Kour*et al.,* 2008,) in the present investigation the extra-cellular producing endophytic *F oxysporum* was isolated from Banana. This compare favourably to results of earlier investigation in South Africa, *Fusarium oxysporium* isolates were obtained from the banana roots, pseudo stems and corms were found to be highly diverse and had sub-clades (Shahasi, 2006). Xu *et al.,* (2008) reported similar findings from endophytic *F. oxysporum* isolated from *Dioscorea zingiberensis* rhizomes (Griesbasch, 2000 reported high levels of fungal diversity from recently harvested plants of East African Highlands Banana,

which showed little or no weevil attack from western part of Uganda. Occurrences of *F. oxysporum* in different forms suggest existence of genetic diversity within this species and molecular methods can be used to detect such variation (Edel *et al.,* 1995). Several researchers have reported genetic diversity within pathogenic and non pathogenic isolates of *F. oxysporum* and of endophytic isolates from various plants (Woo *et al.,* 1996).

Fusarium solani is one of the most frequently isolated fungi from soil and plant debris (Booth, 1997) The species ubiquitous in soil and decaying plant material, where they act as decomposers, but they are also host-specific pathogens of a number of agriculturally important plants, including pea, cucurbits, and sweet potato. This species, as defined based on morphology, is actually a diverse complex of over 45 phylogenetic and/or biological species (O'donnell, 2000)

Fusarium equiseti (corda) Sacca 1886 is an Ascomycete fungi which is wide spread in soil and can be hosted by wide range of plants. It also exists as pathogen, saprophyte and endophytes. It Bionial name is *Gibberella intrican* wollen (1930) while the *synonyms F. equiseti, F falcatum, F.gibbosum* and *Selenosporium gibbosum.*

5.2 Conclusion

The hypothesis that endophytic fungal isolates secretes extra-cellular enzymes for their mode of action against plant pest like nematodes was proved to be acceptable.

The protease activities on gelatin amended media demonstrated the ability of the local bananas endophytic fungal isolates to produce protease extracellular enzyme within the first 24 hrs of contact with collagen rich substrate.

This is a clear indication that the microorganism release the enzyme to directly parasite the nematodes during their interaction.

Direct parasitism may not necessarily represent a substantial part of nematode control by endophytes, the association with other modes of action may improve their efficacy and levels of plant protection. Thus there is need to analyze other possible mode of action like systematic resistance induction so as to have a thorough understanding of the mechanisms of action by endophytes, which is needed to maximize the use, efficiency and consistency of it use in biological control.

Nematophagous fungi, a group of natural enemies of nematodes, have commercial potential for developing into effective biopesticides (Siddiqui and Mahmood, 1996). These fungi mostly live in the soil environment where nematodes are found. Among these fungi, some have trapping devices (such as adhesive branches, adhesive nets, constriction rings.) to capture nematodes, endophytes do not possess trapping devices

but proved to secrete proteases enzymes to kill nematodes, and still others parasitize nematodes.

Like most host-pathogen interactions, the following steps are involved in a typical nematode infection by endophytic fungi: ***1***) recognition and adhesion of fungi to the cuticle of nematodes; ***2***) degradation and penetration of nematode cuticle/eggshells by enzymes secreted by fungi; and ***3***) immobilization and digestion of nematodes by fungi (Tunlid and Jansson, 1991). Nematode cuticles are complex, composite structures with a high percentage of proteins and are the major barrier against fungal infection. The penetration of the cuticle of nematodes or their eggshells has been assumed to be the consequence of mechanical forces exerted by the endophytic fungi, in combination with cuticle-degrading enzymes (such as proteases) produced by the fungi. The proteases play a critical role during host infection (Nordbring hertz *et al.,* 2000).

From the genetic identification, it is clear that the isolate in question resembles the *Fusarium oxysporium* with similarity range of 99 to100% .Based on the previous experiment on VGA test the selected isolates are non pathogenic . The 18S rDNA gene was considered to derive phylogenetic relationship of *F. oxysporum* isolates, which is believed to be more conserved evolutionarily (Duggal *et al.,* 1997). However, consideration and analyses of other genes from different loci may provide more information on phylogenetic relationship of *F. oxysporum* isolates those exist as pathogens, saprophytes and endophytes.

5.3 Recommendation

During the past several years, several cuticle-degrading proteases have been purified and characterized from different entomopathogenic fungi, including *Arthrobotrys oligospora* , *Pochonia chlamydospora* (syn. *Verticillium chlamydosporium*) , *Beauveria bassiana* , and *Metarhizium anisopliae* . The endophyte fungi protease need to be purified, characterized and compared with other Nematophagous fungi protease which in turn will facilitate protein engineering study aimed at improving nematicidal activity of fungi.

To confirm the role of protease extracellular enzymes in biological control protease need to be purified from fungal cultures and assayed against nematodes and their eggs, and observations made by means of scanning electron microscopy.

Based on the 18S rDNA gene phylogenetic analyses of the local banana endophytic isolates were confirmed to be fusarium oxysporium. However, consideration and analyses of other genes from different loci may provide more information on phylogenetic relationship of *F. oxysporum* isolates those exist as pathogens, saprophytes and endophytes.

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