ISOLATION AND SCREENING OF ENDOPHYTIC *Fusarium oxysporum* FOR BIOLOGICAL CONTROL OF NEMATODES IN TISSUE CULTURE BANANAS

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To my loving and caring wife Elizabeth together with my lovely daughter Lucy for their patience, moral support, prayers and love. Also to my parent Lucy Muthoni Mutua, my brothers Raphael, Joseph, and my sisters Jane, late Ann, Judy and Alice and all my friends and comrades for their encouragement and inspiration which have seen me through this valuable work. Also all the professionals who introduced me into the world of 'tiny worms' (Nematodes) for i have come to love the unseen. Above all is the ALMIGHTY GOD whose Grace and enduring love have seen me through the hard and trying moments.

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ABSTRACT

Two major banana growing regions namely Meru district (Altitude: 1050 Masl, 06' 34S, 037°46' 23 E) and Kilifi (17 Masl 03'4S 0" 039° 40' 0" E) district) were identified as sampling sites for banana plants to be used for endophytic fungi isolation. Healthy appearing plants in heavily infested fields were sampled for endophytic fungi isolation. Endophytic fungi were isolated from the plant roots, the corm, pseudostem and the pseudostem base. A total of 2,455 *Fusarium* spp isolates were isolated from the two regions. *Fusarium oxysporum* species was the most prevalent endophytic fungi from banana roots and corm. They constituted 15.5% and 44.6% of all fungal isolates from Meru and Kilifi districts, respectively. Twelve isolates belonging to *F. oxysporum* were selected for *in vitro* bioassays against two major species of banana parasitic nematodes in the two regions namely *Pratylenchus goodeyi* and *Helicotylenchus multicintus*. The selected species of the endophytic fungi were cultured in Potato Dextrose Broth (PDB) media for seven days. Fungal filtrates from the twelve isolates were obtained after sieving and centrifuging the PDB media. One millilitre of the filtrate was used for each treatment consisting of one hundred nematodes suspended in 1 ml of distilled sterile water in sterile 5ml Bjorn bottles. Each treatment was replicated three times and the experiment laid out in complete randomised design. Mortality and paralysis were recorded after 3, 6 and 24 hours time intervals. Corrected mortalities were calculated using Abbott's formula. The mortality significantly ($p<0.0001$) varied with the time of exposure. Culture

filtrates of *F. oxysporum* isolates significantly ($p<0.0001$) differed on their mortality effects to the nematodes. Out of the total thirteen endophytic isolates from Meru district, five isolates produced consistent mortalities and paralysis to both *P. goodeyi* and *H. multicinctus*. These isolates included 5JTOC134, 5SOPB11, 4M0C321, 4SIC132 and 5MR11. Similarly five fungal isolates from Kilifi district which demonstrated consistency in causing mortality and paralysis were 7MIC334, 8SIC334, 11MOC143, 11SR23 and 11MOC353. The isolates demonstrated potential biological activity against the major banana parasitic nematodes, and can further be investigates as biological control agents against these nematodes.

CHAPTER 1

1.1 GENERAL INTRODUCTION

Banana (*Musa* spp.) is one of the world's most important crops (Frison *et al.*, 1998). It is the world's third important starchy staple food after cassava (*Manihot esculanta* Crantz) and sweet potato (*Ipomea batatas* (L.) for millions of people in developing countries of the tropics (INIBAP, 1991; IITA, 1998). The total world production of banana is estimated at around 97 million tonnes, of which approximately one third is produced in subsistence farms in Africa (Frison *et al.*, 1998; FAOSTAT, 2006). Bananas are mainly consumed domestically with an annual per capita consumption of 660 kg in the East African Great Lakes region, which is the highest in the world, providing more than 25% of the total calories consumed (INIBAP, 1991).

Modern banana and plantain originated in the Southeast Asia and western Pacific regions (Simmonds, 1959; Haarer, 1964; Simmonds, 1966; Robinson, 1996). They belong to the genus *Musa* (Stover and Simmonds, 1986). All the edible bananas and plantains are cultivars or clones of *Musa acuminata* (A genome) and *Musa bulbisiana* (B genome) (Stover and Simmonds, 1986).

The banana plant is a large herbaceous perennial with an underground stem known as the corm, bulb or rhizome (Karugaba and Kimaru, 1999). The corm

bears eyes on its middle and upper parts which develop into suckers (Montcel, 1987). The leaf consists of three parts i.e. a sheath or enlarged petiole, a petiole, and a lamina or blade. Leaf sheaths of successive leaves closely encircle each other and form a cylindrical compact structure, which is the pseudostem (Swennen and Vulysteke, 2001). The inflorescence forms from the terminal bud of the corm, which develops and rises to the top of the pseudostem turning downwards to form a bunch (Stover and Simmonds, 1986).

The root system, besides its importance in water and nutrient uptake, provides anchorage to the plant. According to Simmonds (1966), the roots reach a depth of between 15-60 cm. Due to its shallow root system, damage by nematodes, diseases, lack of soil fertility, soil compaction or mechanical damage may jeopardise the survival of the plant (Karugaba and Kimaru, 1999). Banana grows well at altitudes ranging from 0-1800 m, with annual rainfall of at least 1000 mm (Mbwana *et al.*, 1998).

In Kenya, an estimated 2% of the total arable land is under banana production, predominantly grown by smallscale farmers (Seshu *et al.*, 1998a; ISAAA, 2001). Dessert cultivars, especially Cavendish and Gross Michel (AAA), are very popular in the highlands of the Central province, Eastern province as well as in the coastal regions of Kenya (Seshu *et al.*, 1998a). In western regions of Kenya, the East African Highland (genome group ABB) cultivars such as Matooke and Mbidde are common (Seshu *et al.*, 1998a). Production is mainly characterised by

low input application, with no or limited use of pesticides and fertilizers (Swennen and Vulysteke, 2001). However, banana production in Kenya has been on a very rapid decline over the last two decades, threatening food and income security for millions of rural Kenyans as well as reducing employment opportunities in areas where the crop is grown (Wanzala, 2005). Due to these pests and diseases, the average banana yield in Kenyan has reduced to 14 tonnes per ha, less than one-third of the crop's potential under humid tropical conditions (Karamura, 1998). Poor production has also been associated to lack of clean planting material, coupled with poor knowledge by the farmers on the technologies to improve yields as well as pest and disease control. Results from a survey conducted in Kenya to identify constraints to banana production showed that a complex of banana nematodes (*Pratylenchus goodeyi* (Sher and Allen), *Pratylenchus coffeae* (Goodey), *Radopholus similis* (Cobb) Thorne, *Helicotylenchus multicinthus* (Cobb) Golden, *Meloidogyne spp.*) (Goeldi) were common in many banana fields in Kenya (Seshu *et al.*, 1998b). A similar survey carried out in the year 1993 in Kenya, revealed that *P. goodeyi* was the most widespread nematode attacking both the exotic and the East African highland bananas (Seshu *et al.*, 2006). Estimated worldwide losses due to plant-parasitic nematodes averaged 20% (Sikora *et al.*, 2003).

Pests and diseases in banana plantations have often been transferred through the use of infected planting materials. In a public rural appraisal of farmers in Kenya, it was found that farmers get their planting material from relatives, neighbours,

parents and friends (Seshu *et al.*, 1998b). Consequently, pests and diseases have been transferred from one farm to the other.

Control of nematodes has been by use of cultural methods and use of nematicides (Swennen and Vulysteke, 2001). Sucker selection and phytosanitary measures, such as hot water treatment, have shown significant nematode reduction but their use by subsistence farmers worldwide has been limited (Stanton, 1994). Rotation-based control is not adequate enough to reduce nematode densities below threshold levels due to increased land pressures (Sikora *et al.*, 2003). Effective and commonly used nematicides are among the agrochemicals that are banned or restricted due to contamination of ground water, degradation of the atmosphere and the health risk to farm workers and consumers (Graham, 1991; Mengech *et al.*, 1995). Breeding for resistance to banana nematodes using convectional methods has been faced with obstacles such as low fertility, triploidy and lack of genetic variability specific to the biology of the preferred parthenocarpic cultivars (Swennen and Vulysteke, 2001). Efficient integrated approaches to control banana nematodes that utilize a range of biological options are needed (Kiggundu *et al.*, 2003).

Biological enhancement of tissue culture banana plantlets with beneficial fungal endophytes aimed at increasing plant resistance against banana nematodes infection has gained increased interest as an alternative to the management of these pest using pesticides (Pocasangre, 2000; Sikora and Pocasangre, 2004).

Endophytes are defined as organisms that live internally for all or at least a significant part of their life cycle, either intercellularly or intracellularly, and asymptomatically within plant tissues (Petrini, 1991; Wilson, 1995; Saikkonen *et al.*, 1998). Hundreds of fungal endophytes isolates have been isolated from apparently healthy banana plants growing in nematodes infested plantations (Griesbach, 2000; Niere, 2001).

Due to the sterile conditions under which tissue cultured plants are produced, the emerging plantlets loose naturally beneficial microorganisms such as endophytes (Pereira *et al.*, 1999). Mutualistic fungal endophytes have been shown to biologically control plant-parasitic nematodes and banana weevils. Fungal endophytes have been isolated from roots and rhizomes of healthy banana plants (Schuster *et al.*, 1995a; Griesbach, 2000; Niere, 2001). Microorganisms isolated from a particular plant part in a specific environment are better adapted to pests in that plant part, as well as that particular environment the plant was growing in (Strobel and Daisy, 2003; Strobel, 2003). Thus, such organisms are postulated to offer better biological control agents than organisms originally isolated from other crops.

1.2 PROBLEM STATEMENT

Banana production in Kenya has been on a rapid decline over the last three decades (Wambugu *et al.*, 1999). This decline has threatened food security, income generation and reduced employment to millions of rural Kenyans in banana producing areas (Wanzala, 2005). The combined effects of nematodes, banana weevils (*Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae)), panama disease and black sigatoka have cut average banana yields on traditional farms to 14 tonnes per hectare, less than one third of the crop's potential (IITA, 1998). A complex of nematodes and banana weevils (*C. sordidus*) have been classified as the major threats to banana production causing a yield loss of up to 85% (Musambiyama *et al.*, 1999). Traditional cultural practices in the control of both the nematodes and the banana weevil have been inefficient in maintaining the pests below the economic threshold levels leading to this decline in banana yields (Wambugu *et al.*, 1999). Farmers transmit unknowingly most of the banana pests and diseases through banana suckers which are the major propagation materials. Recently, there have been numerous scientific reports on the development of resistance in pests against insecticides and nematicides. Besides, these chemical control measures against banana pests have faced major challenges including risks to consumers and environment, as well as high costs of both procuring and application which is far beyond the reach of the majority of the banana producers in Kenya who are smallholder farmers. As a result, the situation demands better technologies for pest control in banana production. Use

of clean, disease and insect free planting materials produced through tissue culture helps in establishing clean plantations. However, reinfestation of young and vulnerable tissue cultured plantlets in pests infested fields remains a great concern (Pereira *et al.*, 1999; Ayyadurai *et al.*, 2005). Additionally, the axenic nature of the tissue culture plants production tends to eliminate the microorganisms naturally associated with the plants (Pereira *et al.*, 1999). Some of these microorganisms are known to have mutualistic relationship with the plants, conferring pest and disease resistance as well as improved plants physiology. Re-introduction of these microorganisms (endophytes) into tissue cultured plants in higher numbers than the naturally occurring levels have been shown to confer some benefits to the plants against plant pathogens such as nematodes. Therefore, tissue cultured plants enhanced with fungal endophytes provide a technology for sustainable banana production for Kenyan small scale farmers hence increased yields and income.

1.3 JUSTIFICATION

The plant parasitic nematodes of banana (*P. goodeyi, P. coffeae, R. similis, H. multicinctus, and Meloidogyne* Spp.), banana weevil (*C. sordidus*) have been recognized as major constraints to banana production. They may cause a yield loss of up to 85% in bananas. The high costs of chemical control and labour requirements of cultural control, favour the use of integrated pest management, centred on biological control against these pests. Recent research has documented that mutualistic fungal endophytes (non- pathogenic fungi) control burrowing nematodes and weevils that attack banana (IITA, 1998). Literature has shown that, there is lack of information on endophytes from tropical hosts, which are more severely affected by pests and diseases. The use of endophytes in enhancing tissue cultured bananas for biological control against banana weevils and nematodes over chemical control offers promise to African smallscale farmers. Mutualistic fungal Endophytes use therefore will aim at developing and disseminating improved technology for sustainable banana production in different ecological zones in Kenya and Africa, contributing to poverty reduction.

1.4 OBJECTIVES

Broad objective

To isolate, identify, and screen endophytic fungi (non- pathogenic *Fusarium oxysporum*) present within banana varieties in Kenya *for* antagonism against *Pratylenchus goodeyi* and *Helicotylenchus muticintus*.

Specific objectives

i. To isolate and identify endophytic fungi from banana tissues sampled from Meru and Kilifi districts in Kenya.

ii. To screen production of metabolites from *Fusarium oxysporum* isolates from the two sampling sites for antagonism against *Pratylenchus goodeyi* and *Helicotylenchus muticintus* under *in vitro* conditions.

1.5 REVIEW OF LITERATURE

1.5.1 Nematodes as banana pests

Plant-parasitic nematodes are soil-borne pests that cause significant damage to most if not all crops grown in the tropics and subtropics (Sikora *et al.*, 2003). The most damaging species of nematodes in bananas are those involved in the destruction of the primary roots, consequently reducing water and nutrient uptake and plant anchorage, resulting in toppling over of the plants (Luc *et al.*, 1990).

Bananas in East Africa are attacked by a complex of nematodes which include burrowing nematodes (*Radopholus similis* (Cobb) Thorne), the root lesion nematode (*Pratylenchus goodeyi* (Sher and Allen)) and the spiral nematode (*Helicotylenchus multicinctus* (Cobb) Golden) (Gowen and Quénéhervé, 1990a; Gold *et al.*, 1993; Karamura, 1993; Speijer *et al.*, 1999). However, their distribution depends on factors such as elevation and temperature (Sikora, 1992; Bridge *et al.*, 1997; Seshu *et al.*, 1998b; Niere, 2001). *Radopholus similis* and *H. multicinctus* are the predominant species between 1000-1350 m above sea level (masl) (Kashaija *et al.*, 1994) while at higher elevations and cooler temperatures *P. goodeyi* is the predominant species (Speijer *et al.*, 1994). Mixed populations occur at lower elevations. Cultivar, cropping systems, farm management and sources of planting material influence abundance of nematodes (Sarah, 1989).

1.5.2 Status of banana nematodes in Kenya

In Kenya, a complex of banana nematodes (*P. goodeyi*, *P. coffeae, R. similis, H. multicinctus* and *Meloidogyne* spp.) have been found to be common in banana fields (Gichure and Ondieki, 1977; Seshu *et al.*, 1997; Seshu *et al.*, 1998b). Surveys indicated that nematodes species and densities varied across the country (Seshu *et al.*, 1998b). The coastal region in Kenya has higher densities of *H. multicinctus* and *Meloidogyne* spp., although *P. goodeyi* has also been observed in cultivar Matooke in Kilifi. In central and western Kenya (higher than 1000 masl) the lesion nematode *P. goodeyi* was the dominant species. In Muranga district, central Kenya, where coffee is being replaced with banana, *P. coffeae* was the dominant species of nematodes. Vihiga and Nyeri districts, in Western and central Kenya, respectively also showed high numbers of *P. coffeae* (Seshu *et al.*, 1998b; Seshu *et al.*, 2006). *Rotylenchus clavicaudatus* (Linford & Oliveira, 1940), *Scutellonema* spp., *Criconema* spp., *Xiphinema* spp., *Hemicycliophora* spp. and a new *Trophorus* spp. have also been recorded in banana plantations on Kenya (Seshu *et al.*, 1997; Seshu *et al.*, 1998b).

1.5.3 Banana lesion nematode (*Pratylenchus goodeyi*)

Pratylenchus goodeyi (Filipjev, 1934) is a migratory endoparasite of the root cortex, with both sexes and all juvenile stages being invasive (Luc *et al.*, 1990; Ferraz and Brown, 2002). The life cycle comprises the egg stage, four juvenile

stages (J_1-J_4) and an adult stage (male and females) (Prasad *et al.*, 1999; Ferraz and Brown, 2002). *Pratylenchus goodeyi* is diverse in pathogenicity with root populations and associated damage varying greatly among *Musa* cultivars, while differences also occur among geographic isolates (Gichure and Ondieki, 1977; Bridge *et al.*, 1997).

A generation is completed within 4-6 weeks under suitable conditions (Prasad *et al.*, 1999). However temperature, soil type and host plants directly affect the life cycle (Ferraz and Brown, 2002). Soil type also considerably influences species distribution and population densities, with most of the *Pratylenchus* spp. reaching highest densities in light sandy soils (Ferraz and Brown, 2002). Females lay eggs inside infested roots, but the eggs are subsequently released into the soil from the decaying roots, where they hatch, and the newly formed second-stage juveniles re-invade young roots.

Damages caused by *P. goodeyi*, are similar to those caused by *R. similis* (Gowen and Quénéhervé, 1990a; Bridge *et al.*, 1997). They enter the root, migrating between and within the cells, feeding on the cytoplasm and eventually damaging the cortical parenchyma cells of banana plant. The nematode causes extensive black or purple necrosis of epidermal and cortical root tissues, resulting in lesions and snapping of roots. Necrotic lesions can also be found on the corm (Bridge *et al.*, 1997). The plants exhibit stunting, lengthened vegetative cycles, a reduction in number of leaves, bunch weight, and longevity, and eventual toppling over.

Toppling of plants is higher in soils with low nutritional content (Bridge *et al.*, 1997). Occurrence of *P. goodeyi* in banana roots lesions has been correlated with infection by *Fusarium oxysporum* pv. *cubense*, the causal agent of Fusarium wilt in bananas (Bridge et al., 1997).

The use of suckers from the same field or from neighbouring field is the main source of *Pratylenchus* spp. infestation in banana fields (Gowen and Quénéhervé, 1990b). Besides banana, *Pratylenchus* spp. has a wide host range, including grasses, and crops such as groundnut (*Arachis hypogaea* Linn) and sweet potato (*Ipomea batatas* (L.) Lamb), which may be found in banana plantations (Bridge *et al.*, 1997). Other examples of alternative hosts to *Pratylenchus* spp. include weeds such as *Alternanthera sessilis* L., and *Portulaca oleracera* L. In Ivory Coast, *P. coffeae* is associated with *Asystasia gangetica* L., *Amaranthus viridis* L, *Commelina benghalensis* L, *Phyllanthus amarus* L, *solenostemon monostachys* L. and *Borreria chartophyla* L. (Gowen and Quénéhervé, 1990b). This demonstrates that the nematode situation in Kenya is potentially serious, where banana production is carried out by smallholders in mixed cropping systems (Qaim, 1999a; Wambugu *et al.*, 1999). Despite the use of clean planting material, the rates of reinfestations in the field may be enhanced by the presence of these alternate hosts.

The presence of *P. goodeyi* in high populations damages about 50-70% of the plant roots, eventually leading to loss of the entire plant after toppling (Machon

and Hunt, 1985). Bridge, (1993) found that highland bananas in Tanzania showed 39.5% necrosis due to attack by *P. goodeyi* and 37.5% necrosis in roots where mixed populations of *P. goodeyi* and *H. multicinctus* were present.

1.5.4 Control of banana lesion nematodes

Because *P. goodeyi* and *H. multicinctus* have a wide host range, their elimination from field soil prior to planting is very difficult. However various methods have been employed to try and reduce dissemination and re-infestation into new fields. Control of lesion nematodes in established commercial banana plantations usually is accomplished using systemic nematicides which inactivate the nematodes within the host tissue or in the soil for a limited length of time (Sikora and Pocasangre, 2004). Repeated use of nematicides has lead to rapid microbial breakdown in some areas, leading to an increase in the number of treatments per cycle (Sikora and Pocasangre, 2004). Fumigant nematicides (primarily halogenated hydrocarbons) are among the agricultural chemicals that are banned or restricted due to contamination of ground water; degradation of the atmosphere and the health risk to farm workers and consumers (Mengech *et al.*, 1995). The efficacy of the remaining nematicides, such as aldicarb (Temik), carbofuran (Furadan), fenamiphos (Nemacur), oxamyl (Vydate), ethoprophos (Mocap), isazophos (Rugby), is compromised by accelerated microbial breakdown (Sikora and Pocasangre, 2004).

1.5.5.0 Cultural control strategies

In smallscale farmers' fields, management of nematodes is based on crop rotation, which is rarely applied when traditional mixed cropping systems are being used (De Waele, 1992). Nematicides are often prohibitive for the small-scale farmer, due to high costs, extreme toxicity for non-target organisms, including the user, and the environment. To curb this problem, cleaning of the planting material is practiced, which involves corm paring and hot water treatment (at 54ºC for 20 min) of the pared corms (Speijer *et al.*, 1995a). Though effective, the method has its constraints, such as lack of heating facilities and most importantly, difficulties of maintaining water temperatures at the desired levels, resulting in the method not being widely adopted (Speijer *et al.*, 1999).

Other management practices in the farms include mulching and manure application, which improve the growth of the plant and thereby deter the influence of nematodes (Speijer *et al.*, 1999). A widely adopted post-planting measure is the propping of the banana plants helps limit chances of toppling or snapping over in smallscale-holder farms (Niere, 2001). Nematode damage has increased as a result of these changes and nematode related crop losses are frequently associated with other biological or physical plant stress factors (Roberts *et al.*, 1992). With phasing out of nematicides for economic or environmental reasons, new approaches to nematode control especially in perennial crops are needed (Sikora and Pocasangre, 2004).

1.5.5.1 Musa resistance to lesion nematodes

From an economic point of view, cultivars with pest and disease resistance offer the most appropriate means to prevent crop losses. However, up to date, there are no lines of East Africa highland banana cultivars with resistance to the lesion nematodes (Niere, 2001). Research indicates that Yangambi Km5 (AAA) appears to be resistant to *R. similis* (Fogain and Gowen, 1998). However, there has been no confirmation of this resistance towards the lesion nematodes in either screen house or field conditions. Breeding for resistance against migratory endoparasitic nematodes has been difficult, perhaps due to high costs of maintaining a long term breeding programme, combined with the low number of plant breeders and trained nematologists pursuing this goal (Pinochet, 1995; Sagi *et al.*, 1995; De Waele, 2000; Tripathi, 2003a). Efforts to breed *Musa* for disease and pest resistance using classical breeding methods are fraught with obstacles (such as low sterility of most edible cultivars, triploidy, lack of genetic variability and long-generation time) specific to the biology of the preferred parthenocarpic cultivars (Vuylsteke and Swennen, 1992; Tripathi, 2003a). Complicating the matter more, is the presence of different nematode pathotypes of a nematode species , which hampers the identification of nematode resistance (Pinochet, 1995; De Waele, 2000). Adoptable nematode resistant varieties are yet to be developed as the search for *Musa* germplasm intensifies.(De Waele and Speijer, 1999).

Compared to conventional breeding, genetic transformation of *Musa* has been an attractive option for enhancing banana with pest and disease resistance. Relative success has been achieved in genetic engineering of bananas and plantain by transfer of foreign genes into plant cells (Sagi *et al.*, 1995; Tripathi, 2003a). However, genetic improvement of plants against nematodes has generally focused on resistance against sedentary endoparasitic nematodes, such as the root-knot nematode *Meloidogyne* spp., the cyst nematodes *Globodera* spp. and *Heterodera* spp. (Wuyts *et al.*, 2003). The development of strategies against migratory nematodes is thus essential. Lectins are proteins other than enzymes and antibodies that bind carbohydrate. They have been regarded as valuable toxins for the control of insect pests in various crops. Lectins for control of *M. incognita* nematodes have been tested under laboratory conditions and controlled root-knot nematodes of tomato (*Lycopersicon esculentum* L.) by 75% (Burrows *et al.*, 1998; Wuyts *et al.*, 2003). However, the strategy requires the presence of lectins in the vicinity of the rhizosphere. Lectin expression inside the plant cell was found not insufficient and signal peptides were found to be necessary for the transport of the proteins to the plant's apoplast and rhizosphere (Wuyts *et al.*, 2003).

Other possible approaches for developing transgenic plants with improved nematode resistance is through the use of proteinase inhibitors, which act as nematode antifeedants (Tripathi, 2003a). Cysteine has been expressed transgenically and provides control against the cyst nematodes and root-knot

nematodes (*Meloidogyne* spp.) (Tripathi, 2003a; Atkinson *et al.*, 2004). Other strategies for the development of transgenic nematode resistance include the use of natural resistance commonly referred as R-genes, and *Bacillus thurigiensis* (Berliner) genes (Tripathi, 2003a). Though some genes show some biological activity against nematodes, some have toxic effects against insects and mammals which limit commercial use (Tripathi, 2003a).

Though transgenic resistance has been found against *R. similis*, concurrent work has to be carried out to combine transgenic plants it with acceptable consumer traits. Also, pyramiding of resistance genes is needed, since *Pratylenchus* spp. causes more losses as opposed to *R. similis* (Tripathi, 2003b). Additionally, *Pratylenchus* has more species (pathotypes). They normally attack the plant more at the same time, necessitating the need for a broad spectrum resistance to be able to combat the pathotypes effectively and effeciciently (Tripathi, 2003a; Atkinson *et al.*, 2004).

1.5.5.2 Tissue cultured banana plants

Widespread use of planting materials infected by pests and diseases has resulted in yield losses, making banana an expensive commodity for consumers, reducing the cash earnings of producers as well as threatening the potential of the crop to contribute to the food security of rural households in Kenya (Qaim, 1999a; Qaim, 1999b). The problem has so far been identified by various public and private research organizations in Kenya. In 1991, Jomo Kenyatta University of

Agriculture and Technology (JKUAT) and Kenya Agricultural Research Institute (KARI) started producing tissue culture banana plantlets with the aim of providing smallscale farmers with pathogen-free banana planting material through the use of tissue culture (TC) biotechnology (Qaim, 1999b; Dubois *et al.*, 2006b).

Tissue culture plants are produced axenically, making them pest and disease-free. Besides the advantages of rapid and mass multiplication, higher yields and uniformity, they give earlier and higher yields as well as vigorous sucker production (Robinson, 1996). They are uniform available all year round, thereby facilitating distribution of improved cultivars. According to Pereira *et al.*, (1999), the axenic nature of the plants production eliminates beneficial microorganisms such as the endophytes, which may confer some resistance to nematodes in the field. The plants may therefore become easier targets for nematodes attack in the field.

TC plantlets require higher levels of care compared to the conventional suckers. Where management practices are not optimal, such as in the many smallholder farms in Kenya, the benefits of the plantlets are not achieved due to reinfestation by pests such as plant-parasitic nematodes and banana weevils. The acceptance and the use of TC banana is very high in Kenya (Wambugu and Kiome, 2001). However, based on rural appraisals conducted by KARI, there is a great need for pest and disease control in TC bananas (Wambugu *et al.*, 1999). Though tissue

cultured banana offer pests and disease free planting material, the rate of re-infestation by these pests in the farms remain a great concern (Ayyadurai *et al.*, 2005).

There is need for improving the plantlets in order to increase or boost their resistance to pests and diseases once in the field. Reintroduction of the beneficial endophytic fungi in to TC banana plantlets shortly after deflasking or before potting can lower the number of root invading parasitic nematodes (Sikora *et al.*, 2003). This method is considered a novel approach in biological control of banana parasitic nematodes.

1.5.5.3 Biological control

Withdrawal from the market of several nematicides due to health and environmental problems associated with their production and use has elucidated increased scientific research focused on the biological control of plant parasitic nematodes (Kerry, 2005). Natural control of plant-parasitic nematodes has been in existence for a long time. Numerous antagonists against the nematodes have been described (Kerry, 1987; Stirling, 1991).

Decline of the populations of the cereal-cyst nematode, *Heterodera avenae* (Wollenweber, 1924) under monocultures of susceptible cereals in many soils in Europe has been the most studied example of natural nematodes control (Kerry,

2005). The decline was caused by two major parasitic fungi, *Nematophthora gynophyla* and *Verticillium chlamydosporium* (Kerry, 2005). Besides fungi, other organisms such as bacteria, viruses, predatory nematodes, insects and mites have been found to parasitize on vermiform stages of nematodes or females and eggs of root-knot nematodes and cyst nematodes (Stirling, 1991). In recent years the ecology, biology and potential of biological control agents have been reviewed and published in various contexts (Stirling, 1991; Sikora, 1992; Kerry, 2000).

1.5.5.4 Endophytes as an alternative biological control agent

The recent past has seen an increased growth of scientific studies and information on the use of endophytic microorganisms as a valuable tool for plant protection against pests and diseases. An endophyte is an organism that, at some time during its life cycle, lives symptomlessly within plant tissues (Petrini, 1991; Saikkonen *et al.*, 1998; Azevedo *et al.*, 2001; Saikkonen *et al.*, 2004). Almost, if not all plants are naturally associated with endophytes (Petrini, 1991; Bills, 1996). These endophytes have been isolated from different plants after surface sterilization. Endophytic relationship may be described as a physiological interaction of organisms of different species, which can further be characterised as either neutral, antagonistic or mutualistic (Saikkonen *et al.*, 1998; Saikkonen *et al.*, 2004). However, the term "endophyte" has become synonymous with "mutualist".

Endophytes have been demonstrated to be present in many plants. They have been shown to be harboured in orchids (*Lepanthes*) (Bayman *et al.*, 1997), wild triticum (*Triticum dichasians* (Zhuk.) Bowden (Marshall *et al.*, 1999) , *Quercus robur* L. and *Q. cerris* trees (Gennaro *et al.*, 2003); and in crops of agricultural importance such as rice *(Oryza sativa)* (Fisher and Petrini, 1992), cabbage *(Brassica oleraceae)* (Mennan *et al.*, 2004), maize (*Zea mays* L.) (Fisher *et al.*, 1992) and tomato (*Lycopersicon esculentum* L.) (Hallman and Sikora, 1994; Cao *et al.*, 2004). More recent has been the isolation of fungal endophytes from bananas (Pocasangre *et al.*, 2000).

Most of the literature on endophytes has demonstrated the effect of endophytes on most groups of plant-parasitic nematodes. Perhaps the most widely used agents of biological plant protection are fungal endophytes of forage and turf grasses. These are fungi of the family *Clavicipitaceae* (Schardl and Phillips, 1997). The endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams), found in the tall fescue grass (*Festuca arundinacea* L.), and the endophyte *Neotyphodium lolii* L., found in perennial rye grass (*Lolium perenne* L.), have been the most intensely studied symbionts (Schardl and Phillips, 1997; Cook and Lewis, 2001). Their numerous benefits to the plants have been documented as enhanced tillering and root growth, protection against nematodes, fungal pathogens, insect herbivores, and mammalian herbivores (Schardl and Phillips, 1997). Accumulation of toxic compound has been shown to be the mode of protection offered by these endophytes against grazing mammals (Raisbeck *et*

al., 1991; Cook and Lewis, 2001). Numerous information on the use of endophyte on the control of insect-pests has been reviewed and published (Azevedo *et al.*, 2001). The benefits conferred by endophytes to the host plants, has by far demonstrated the protection afforded by the host plants due to such an association. However, literature shows that there is lack of information on endophytes from tropical hosts, which are more severely affected by pests and diseases (Azevedo *et al.*, 2001).

1.5.5.5 Use of endophytes in nematode management

Earliest reports on the effects of endophytes on nematodes were documented in tall fescue grass. This association showed reduced numbers of various ectoparasitic and endoparasitic nematodes in field and greenhouse experiments (Pedersen and Rodriguez-Kabana, 1984; Schardl and Phillips, 1997). In perennial ryegrass, the presence of mutualistic *N. lolii* endophytes has also been reported to reduce numbers of ectoparasitic nematodes referred to as *Paratylenchus* (Eerens *et al*., 1998). *Meloidogyne marylandi*, (Jepson & Golden) a root knot nematode causing significant damage in forage and turf grasses, has been found to be affected by endophytes, with its reproduction reported to be greatly reduced in endophyte-inoculated tall fescue grass roots (Kimmons *et al.*, 1990; Elmi *et al.*, 2000).

With rapid growth of scientific interest on endophyte antagonism to nematodes, more crops have been studied. In such a study, *M. incognita* in tomato roots were shown to be affected by endophytic *F. oxysporum* isolated from tomato roots,

leading to less infection by the nematode (Hallman and Sikora, 1994). A wide range of plant parasitic nematodes exist hence many factors affect their susceptibility to parasitism and predators (Graham, 1991). The diversity includes sedentary endoparasites, (the root-knot and cyst nematodes), ectoparasite, and the migratory endoparasite such as *Radopholus* and *Pratylenchus.* Previously, lack of sufficient information on the migratory habit of endoparasitic nematodes made it difficult to develop a biological control agent , since the agent was not expected to have an impact on the nematode's critical multiplication stage (Graham, 1991). Plant parasitic nematodes have a number of development stages, representing a varied and ever changing target for biological control target. Therefore the biological control strategy in question must target the stages that are completed in the plant tissues. Consequently, endophytic fungi originating from the roots and able to colonize the roots tissues are better biological agents for root parasitic nematodes since they occupy the same ecological niche with the nematodes and are in constant contacts (Siddiqui and Mahmood, 1995). Moreover, the fact that endophytes complete their life cycle inside the plants gives them protection from variation in environmental factors which may otherwise affect their performance (Siddiqui and Shaukat, 2003).

Mechanisms through which endophytic fungi control nematodes have been controversial. Some of the ways that endophytes have been found to protect plants include: improvement in the plant physiology such as enhanced tillering, roots growth and increase in drought tolerance (Malinowski *et al.*, 1997; Elmi *et*

al., 2000), induction of systemic resistance (Kimmons *et al.*, 1990; Fuchs *et al.*, 1997), and production of nematicidal metabolites (antibiosis) (Hallman and Sikora, 1994; Cook and Lewis, 2001).

Compared to other methods of nematodes management such as the use of nematicides which may have complete nematode eradication, endophytes have been documented to have varied effects on nematodes populations in plants and in the soils. Akinsanmi and Adekunle (2003) showed reduction in the population of *M. incognita* in soybean due to endophytes innoculation. Similar reports have been documented in other crops (Kimmons *et al.*, 1990; Elmi *et al.*, 2000; Mennan *et al.*, 2005). In pot experiments with TC bananas nematodes numbers were shown to be less in endophyte treated plants (Sikora *et al.*, 2003).

With such a background, the International Institute of Tropical Agriculture (IITA) initiated an intensive fungal endophytes research in 1997 aimed at management of microorganisms to enhance plant health for sustainable banana production in East Africa (IITA, 1998). Since then numerous research has been carried out on the potential of endophytes to control banana pests. In a study of healthy asymptomatic banana roots and rhizome tissue of Pisang Awak (*Musa* ABB), a large number of fungi were isolated (Schuster *et al.*, 1995b; Niere, 2001). Most frequently isolated was the genus *Fusarium.* The species *F. oxysporum* was frequently isolated and seems to form an essential component of the endophytic mycobiota of banana (*Musa* spp.) in Uganda (Schuster *et al.*,

1995a; Griesbach, 2000; Sikora *et al.*, 2003). Presence of fungal isolates has also been documented from Central America (Pocasangre *et al.*, 2000).

An overview of the potential of endophytic non-pathogenic *F. oxysporum* for biological control of plant parasitic nematodes has been documented (Schuster *et al.*, 1995a; Niere, 2001). Isolates of non-pathogenic *F. oxysporum* from different locations and cultivars in Uganda showed more than 90% inactivation of *R. similis* in *in-vitro* experiments (Niere, 2001; Dubois *et al.*, 2004). Additionally, non-pathogenic *F. oxysporum* led to reduced root attack and penetration by the nematode *P. goodeyi* in two different banana cultivars in Kenya (Speijer, 1993).

Inoculation of the fungal isolates onto one month old tissue cultured bananas at one month old plantlets has lead to colonization of the plants by the endophytes (Paparu *et al.*, 2004). This is a crucial step in the utilization of the biological control since the plants will be inoculated before they are released into fields. Use of maize bran containing fungal inoculum has been shown as a very effective way of introducing the endophytes into the plants (Paparu *et al.*, 2004). Numerous results on banana endophytes have demonstrated positive interaction of banana endophytes (non-pathogenic *F. oxysporum*) with TC banana plants such as reduction on the number of *R. similis* in banana roots (Pocasangre, 2000), increased shoot and root weights in endophytes treated banana plants (Speijer, 1993; Pocasangre, 2000; Niere, 2001). The isolate V5W2 has been shown to be

one of the best and potential endophytic *F. oxysporum* isolate with promising application for the control of *R. similis.Reference*

The need for exploring the potential use of endophytic fungi isolated from Kenyan bananas as biologican contro agent against nematodes is a research priority. Consequently the utilization of endophytic fungi (non-pathogenic *F. oxysporum*) by incorporating promising fungal isolates into tissue culture plantlets before hardening and release to the farmer's fields is the objective of the study. The ability of the fungal isolates to colonise, grow and confer resistance in tissue cultured plants to nematodes will play a critical role is selection of the best candidate isolate. After distinctive colonization ability of the fungal isolates is understood, effective isolates will be mass-produced and integrated with the production of tissue-cultured plantlets. The biological enhancement of the plant will be evaluated in the field, by determining whether the plants will be protected from parasitic nematode attack and conclusively evaluate the sustainability and adoption of the technology in banana production in Kenya.

CHAPTER 2

ISOLATION AND IDENTIFICATION OF FUNGAL ENDOPHYTES FROM BANANAS IN KENYA

2.1 Introduction

Endophytes are defined as fungi colonizing healthy plant tissue without causing any symptoms or injury to the host (Bills, 1996). Most plants, if not all harbour endophytes. It's however difficult to detect endophytes since they can only be successfully studied by plating them out carefully on surface-sterilized tissues. Outward growth of hyphae from internal tissues of surface sterilised plant tissues has been regarded as one of the main evidence of endohytisim (Petrini *et al.*, 1990). Endophytes are generally not considered organ-specific, and it is likely that many of the species isolated from stems and roots also occur in leaves (Dix and Webster, 1995).

Various methods have been employed in isolation of endophytes (Fisher and Petrini, 1992; Edel *et al.*, 1997; Schulz *et al.*, 1999; Lori *et al.*, 2004; Ayyadurai *et al.*, 2006; Olatinwo *et al.*, 2006). A standard method for endophyte isolation uses dips in both ethanol (EtOH) and bleach (NaOCl), where the time of immersion in NaOCl varies with the tissue and the host (Bills, 1996).

A study by Niere (2001) documents that, after standard surface sterilization of banana tissues and subsequent identification, the most frequently isolated fungi from healthy banana rhizome and roots was *Fusarium oxysporum* which consisted 15% of all the fungi isolated from the rhizome cortex and around 7% from the roots and the central cylinder. *F. oxysporum* has also been shown to be the mostly frequently isolated species in other studies associated with banana (Niere, 1999; Mateille and Folkertsma, 1999; Battle-Viera and Perez-Vicente; 2000).

Fungal endophytes isolates intended for developing a biological control agent should be non pathogenic to the host or target and non target plants. The use of vegetative compatibility grouping (VCG) technique has been employed as a technique to differenciate between non-pathogenic and pathogenic isolates of *F. oxysporum* (Niere, 2001). However, pathogenicity tests are equally done by use of susceptible cultivar (Niere, 2001).

Standardizing the isolation procedure is of paramount importance in order to obtain a true picture of the fungal biodiversity in banana roots and corms (Niere, 2001). The fungal spectrum has also been shown to vary with the interval of sampling, the plant tissue and plating (Niere, 2001). Frequency of isolation of endophytes has also been reported to increase with the age of the tissue with, higher numbers of endophytes expected in older banana roots and rhizomes (Niere, 2001). In the search for a novel biological control agent against banana

pests such as nematodes, isolation of fungal endophytes was considered as an important point in understanding the frequency of endophytes distribution in bananas in Kenya.

The objective of this chapter was to isolate and identify fungal endophytes from the roots, corms, pseudostem base and pseudostems of healthy banana plants and suckers sampled from two agro-ecological zones in Kenya.

2.2 Materials and methods

2.2.1 Sampling of banana for isolation of endophytes

Plant samples for this study were taken from recently flowered banana plants in Meru district in Eastern province (06' 34S, 037°46' 23 E, 1050 masl,) and Kilifi district (03'4S 0" 039° 40' 0" E, 17 masl) in the Coast province of Kenya. The cultivars selected were Cavendish varieties and local cooking cultivars such as “Mutagato” in Meru district and “Mkono tembo” in Coast province. Questionnaires were developed to facilitate gathering of information regarding the banana plantation in terms of age of the mat, production trends, size of the farms and farmers’ perception of pests’ problem (Appendix 1).

Plants selected for sampling were visibly healthy banana plants showing no nematodes/weevil attack or symptomatic fungal infection in field that was infested by the nematodes. In total five plants were selected from each of the two sampling regions. The height, girth and number of fully open leaves for both the mother plant and the sucker were assessed. Plants were dug out carefully with the sucker attached. Pseudostems of the mother plant and the sucker were cut at the height of 30cm from the pseudostem base. Samples were kept in a cool box for transportation to the laboratory. In the laboratory, the samples were cleaned with tap water to remove soil before isolation of endophytes.

2.2.2 Isolation of endophytes

Suckers were detached from mother plants and plant tissue obtained from the joint between the two Plants. Ten cylindrical blocks 5 mm in diameter and 50 mm in height were transversally drilled using a sterile 5 mm diameter cork borer. The corms were pared to remove the root system using a knife. Five healthy roots were randomly sampled from both the mother plants and the sucker. From each of the five roots, 5 cm long blocks were cut 3 cm from the corm.

From each of the mother plant and the sucker, the pseudostems were transversally cut at 5 cm and 20 cm height from the pseudostem base respectively to obtain 5 cm thick discs. In total ten 50 mm × 5 mm cylindrical blocks were obtained from the outer pseudostem and stele of each mother plant (five from each part). Only five 50 mm × 5 mm cylindrical blocks were obtained from the sucker pseudostems since none had formed the stele. Corm tissues for endophytes isolation were made by making a cut at the collar of the pseudostem base and the corm to separate the two plant parts. Corms were separated into three 5 cm thick disks cut from below (lower parts of the corm) using a knife. From each of the corm disc, ten cylindrical blocks (5 mm in diameter, and 50 mm height) were drilled from the inner and the outer corm using a 5 mm diameter cork borer.

In total, 125 blocks were obtained from the outer corm, 125 from the inner corm, 50 from the inner pseudostem base, 50 from the outer pseudostem base, 25 blocks from the stele, 50 blocks from the pseudostem, 50 bocks from the roots, and 50 blocks from the part joining the mother plant and the sucker. In total 525 blocks were sampled from Meru district while samples from Kilifi district yielded 525 blocks. Since all the samples could not be handled the same day, they were put into polythene bags and were stored at -4°C for fungal isolation.

Throughout primary fungal isolation process, aseptic conditions were observed. All the cylinder blocks obtained from the various plant parts were individually surface sterilised. Surface sterilisation was carried out by dipping the cylindrical blocks from the samples in 75% EtOH and flaming once until all the ethanol was completely burnt off. These procedures were carried out under the laminar flow cabinet. After sterilisation, the cylinders were then placed on sterile paper and cut transversally into five 2 mm x 2 mm pieces. The same procedure was applied to the root samples. For each block only five cut pieces were randomly picked and plated on their flat sides in 90 mm diameter Petri dishes containing sterile synthetic nutrient agar (SNA) medium (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCL, 0.2 g glucose, 0.2 g sucrose, 0.6 ml 1 M NaOH , 15 g agar in 1 L distilled water). To prevent bacterial contamination, the media was supplemented with antibiotics (0.1 g penicillin G, 0.2 g streptomycin sulphate, 0.05 g chlorotetracycline in 1 L distilled water).

After plating the plant tissues on the Petri dishes, they were sealed with parafilm and put into sterile basins. The Petri dishes were incubated in the laboratory (± 26 ^{o}C and 12: 12 L: D hrs photoperiod). After four days, the Petri dishes were observed for any fungal growth, and whenever necessary, the likely endophytic fungal outgrowths were rescued from the fast growing saprophytic fungi and other contaminants. Rescuing of the endophytic fungi involved picking of the hyphal tips from areas without contaminants using an inoculation needle, under a microscope at a magnification of 200 x. Cultures were discarded if contamination was extensive.

Fungal outgrowth was carefully monitored six to ten days after plating the plant tissues on SNA. Distinct colonies emerging from the pieces were picked under a dissecting microscope and the hyphal tips were sub-cultured into 90 mm plates containing half strength potato dextrose agar (PDA) medium (19.0 g potato dextrose agar, 10 g agar / L distilled water) and SNA in 90 mm diameter Petri dishes. The plates were incubated for seven days in the laboratory (± 26 ^{o}C and 12: 12 L: D hrs photoperiod).

2.2.3 Identification of the fungal isolates

To identify isolates belonging to the genus *Fusarium*, all fungal endophytic cultures on SNA were observed under a compound microscope at magnification of 400 x. The genus *Fusarium* was identified if the isolate under observation had produced macro-conidia with foot-based basal cell in sporodochia.

2.2.4 Preparation of single spores for identification to the *species* level

Using a flame sterilised wire loop, hyphal tips were aseptically picked from the top of the colony of fungal cultures of each PDA plates from (2.2.2) above under the laminar flow cabinet. The hyphal tips were transferred into sterile 2 ml eppendorf tubes containing 1 ml distilled water. In order to release the conidia, the eppendorf tube was hand shaken for approximately 30 s. From the conidial solution, 50 µl was pipetted into 2 ml eppendorf tube containing 1 ml distilled water. Equally this eppendorf tube was hand shaken for approximately 30 s. From the resulting conidial solution 400 µl was pipetted onto a 90 mm diameter Petri dish containing water agar (20 g agar / L distilled water) and the plate were shaken slightly to evenly spread the conidial solution over the medium.

Observation of conidia germination was carried out after 15 h. 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 flame sterilized wire loop. Together with the spore the media block was transferred to the centre of a 90mm diameter Petri dish containing SNA medium. The Petri-dishes were sealed with parafilm and incubated for 6-13 days under laboratory conditions. After a pure culture has been obtained, further sub-culturing was carried out on half strength PDA (for macroscopic observation) and SNA media (for microscopic

characteristics) simultaneously using flame sterilised inoculation needle and the plated incubated in the laboratory for 5-7 days.

2.2.5 Identification of endophytic *Fusarium* to species level

Macroscopic features such as growth and culture characteristics were observed from half strength PDA media (from section 2.2.2) was used. Colony diameter (mm) was measured using a ruler from three different positions on day five, seven and ten. The values were used to obtain average colony diameter (mm) for each isolate across the days. From the same plates other culture characteristics were observed which included: colour of colony observed from below the Petri dish, colour of aerial mycelium, colour of sporodochia, and presence or absence of aerial mycelia. Microscopic features were observed from SNA (from section 2.2.2). Features observed under the microscope (magnification 400 x) included conidiophores and conidia (Table 1).

Table 1: Features of fungal structures and their relevant attributes that were used during the identification of *Fusarium* species.

Fungal structure under observation*	**Key features observed**
1. *Macroconidia produced on sporodochia*	Shape of the basal and apical cells, size and the number of septa
2. *Macroconidia produced in aerial mycelium*	Presence or absence, shape, number of septa and formation (Pairs, groups or singly).
3. *Chlamydospores production*	Presence or absence and their formation (Pairs, singly or pairs).
4. *Microconidia produced in aerial mycelium*	Presence or absence, shape and number of septa
5. *Conidiophore*	Type of conidiophores as either monophiliades or polyphiliades. Their length (classified as short, medium or long), and their type (Microconidia or macroconiadia).
6. *Microconidia produced in aerial mycelium*	Size was obtained as the length and width (µm) obtained from four conidia borne in sporodochia using a micrometer in the microscope eye piece.

*Identification was done at magnifications of 400 x for fungal isolates cultured on Synthetic Nutrient Agar (SNA) for one week.

A checklist form for identification of the species was used for the *Fusarium spp.* identification (appendix 3). Identification of *Fusarium spp.* was based on (Nelson *et al.*, 1983) and (Burgess *et al.*, 1994) identification manuals. Non- *Fusarium* isolates were not identified to species level.

2.2.6 Storage of *Fusarium* isolates

After identification, *Fusarium* spp. isolates were preserved in both soil tubes and filter papers. Filter paper preservation was for short term storage while soil tubes were for long term storage.

2.2.7 Preservation of isolates on filter paper

To preserve the endophytic *Fusarium* spp. isolates on filter papers, the following procedure was used. Using a pair of scissors filter papers (Whatman No. 1 (90 mm diameter)) were cut into 25 mm x 25 mm pieces. These pieces were autoclaved in 90 mm glass Petri dishes and dried in an oven at 70 ^{o}C. SNA medium was prepared and dispensed into sterile 90 mm diameter glass Petri dishes. Five to seven pieces of sterile filter papers were aseptically placed on SNA media. Each of these Petri dishes were inoculated with a small block of SNA containing endophytic fungi cut from actively growing culture using sterile flamed inoculation needle. The block was placed in the centre of the Petri dish containing SNA. The dishes were incubated in the laboratory for one week.

Using flame sterilised forceps, the filter papers were lifted from the SNA plates and placed into sterile 90 mm Petri dishes. The Petri dishes were left on laboratory bench to dry for three days. Using flame sterilised forceps, the filter papers were lifted into sterile 2 ml eppendorf tubes. The tubes were stored at -4^0C in the laboratory.

2.2.8 Preservation of endophytic isolates in soil tubes

Three parts of sand, three parts of compost and four parts of loam soil were sieved using a 2 mm sieve and mixed thoroughly. The mixture was put into test tubes of 15 cm length and 13 mm diameter to a depth of 7 cm. A volume of 5 ml tap water was added per test tube, and the tubes plugged with cotton wool and later covered with aluminium foil. The tubes were allowed to stand for 24 h to allow water to seep through the soil. After 24 h interval, the tubes were autoclaved and allowed to cool overnight on the laboratory bench. Fungal cultures were preserved by picking a small block of agar with mycelium from SNA plates and transfering onto the soil surface inside soil tubes which were covered with cotton plug and recapped with aluminium foil. For each fungal isolate, three tubes were prepared for preservation. The tubes were them labelled using stickers with the name of the isolate and the date of preservation. The tubes were left on the laboratory benches for one week to allow colonization of the soil by the fungus. After colonisation confirmed through the production of aerial mycelia in the tube, the tubes were stored at -4^0C.

2.2.9 Pathogencity tests

Tissue cultured banana plants (Gros Michel and Cavendish varieties) were obtained from the tissue culture laboratories in Jomo Kenyatta University. Four weeks after rooting, tissue culture plants were removed from the rooting medium and their roots and rhizomes washed using tap water. The plants were suspended in a nutrient solution in 300 ml plastic cups with lids for four weeks to enhance root growth. Plants were placed singly in cups placed on trays, and kept for four weeks in a humidity chamber in a screenhouse. The nutrient solution in the cups will be topped up weekly.

Endophytes suspension was prepared from thirteen isolates from meru and Kilifi districts respectively. The suspension was prepared by culturing the isolates in PDB in the laboratory for seven days. Spore concentrations were estimated using a hymocytometer. The suspension was regulated by either diluting the suspension with SDW or concentrating the solution by removing excess media. Spore concentration was regulated to approximately 1.50-1.75 x 10^6 spores / ml. The plants were dipped in endophytes spore suspension of concentration. The plants remained in the spore suspension for 2 hours. Five plants per treatment (Fungal Isolate) were used. The control experiment contained plants dipped in sterile PDB/ Corm broth whose pH will be adjusted to be the same as that of the spore suspension. After dipping, the plants were planted in a 200ml container containing sterilised soil, and kept in humidity chambers for four weeks.

Internal and external symptoms assessment was carried out after 10 weeks. This included visual assessment for the *Fusarium* wilt symptoms. External symptoms that were evaluated on monthly basis included: Dropping of leaves, wilting, plant stem becomes spongy, yellowing of leaves, cracking of the pseudostem and new suckers arising from the cracked sites.

Internal symptoms were evaluated on the tenth week, including internal discolouration. A visual scale of 1-6 where was used to indicate severe decolouration. Only the corm base was observed. Two small discs from the corm base were obtained from each plant for re-isolation of the fungus in the laboratory.

2.3 Data collection and statistical analysis

The frequency of occurrence of endophytic fungi was described as a percentage calculated for each fungus from the number of colonised plates. The colonisation frequency of the various plant parts were grouped into four categories i.e. the inner corm, outer corm, pseudostem and pseudostem base. The total number of isolates per category was converted to percentage. These categories were matched with the source from which they were isolated from. The plant source included mother plant, sucker or the joint. Data was arcsine transformed before subjecting to ANOVA using SAS statistical package.

2.4 RESULTS

2.4.1 Sampling, isolation and identification

Fusarium spp. counts from Kilifi district were 1,803 isolates compared to 652 *Fusarium spp.* isolates from Meru. All the samples from Kilifi district had a higher frequency of occurrence of *Fusarium spp.* compared to plant samples from Meru district (Table 2). Compared to other fungal isolates obtained from the plant parts, *Fusarium* spp. had a higher rate of occurrence than any other isolate for both samples from Meru and Kilifi districts (Table 3).

Table 2: *Fusarium spp.* isolates distribution (counts per plant tissue) from banana plant samples collected from Meru and Kilifi districts in 2005 and 2006, respectively

Distribution of *Fusarium spp.* isolates per plant tissue.								
Sample source	**R**	**IC**	**OC**	**IP**	**OPS**	**IPSB**	**OPSB**	**Total**
Meru District	224	146	156	46	9	43	28	**652**
Kilifi District	239	465	572	131	70	171	155	**1803**
Total	**463**	**611**	**728**	**177**	**79**	**214**	**183**	**2455**

R = roots, **IC** = inner corn, **OC** = outer corm, **IP** = inner pseudostem, **OP** = outer pseudostem, **IPSB** = inner pseudostem base and **OPSB** = outer pseudostem base.

Table 3: *Fusarium* and non-*Fusarium* spp. isolates per plant tissues from banana plant samples collected from Meru and Kilifi districts in 2005 and 2006 respectively.

Counts of *Fusarium* spp. isolates per plant tissues.									
Sample source	***Fungal spp.***	**R**	**IC**	**OC**	**IP**	**OPS**	**IPSB**	**OPSB**	**Total**
Meru	*Fusarium* spp.	224	146	156	46	9	43	28	**652**
	Non *Fusarium* spp.	22	107	113	43	17	67	83	**452**
Kilifi	*Fusarium* spp.	239	465	572	131	70	171	155	**1803**
	Non *Fusarium* spp.	11	278	175	119	54	79	95	**811**
Total		**496**	**996**	**1016**	**339**	**150**	**360**	**361**	**3718**

R = roots, **IC** = inner corn, **OC** = outer corm, **IP** = inner pseudostem, **OP** = outer pseudostem, **IPSB** = inner pseudostem base and **OPSB** = outer pseudostem base.

The percentage occurrence of endophytic *Fusarium oxysporum* from Meru district samples was higher in the roots (79.2%) than in the outer corm tissue (11.2%), inner corm (10.5%), inner pseudostem base (7.6%), outer pseudostem base (6.0%), inner pseudostem (3.6%) and outer pseudostem (1.6%) (Table 4).

Table 4: Frequency (%) of occurrence of fungal endophytes isolated from surface sterilised banana tissues sampled from five heavily banana nematodes infested farms in Meru district, Kenya.

	Banana tissues*						
Fungal *spp.*	**R**	**IC**	**OC**	**IP**	**OPS**	**IPSB**	**OPSB**
F. oxy	**79.2** (198)	**10.5** (79)	**11.2** (84)	**3.6** (9)	**1.6** (2)	**7.6** (19)	**6.0** (15)
F. solani	**6.8** (17)	**7.3** (55)	**8.9** (67)	**14.0** (35)	**5.6** (7)	**9.6** (24)	**4.8** (12)
F. Subglutans	**2.0** (5)	**0.7** (5)	**0.0** (0)	**0.0** (0)	**0.0** (0)	**0.0** (0)	**0.0** (0)
F. avenaceium	**0.8** (2)	**0.4** (3)	**0.5** (4)	**0.0** (0)	**0.0** (0)	**0.0** (0)	**0.0** (0)
F. equiseti	**0.0** (0)	**0.3** (2)	**0.1** (1)	**0.4** (1)	**0.0** (0)	**0.0** 0)	**0.0** (0)
F.chlamydosporium	**0.4** (1)	**0.1** (1)	**0.0** (0)	**0.0** (0)	**0.0** (0)	**0.0** (0)	**0.0** (0)
F. nivale	**0.4** (1)	**0.0** (0)	**0.0** (0)	**0.4** (1)	**0.0** (0)	**0.0** (0)	**0.0** (0)
Other *Fusariums*	**0.0** (0)	**0.1** (1)	**0.0** (0)	**0.0** (0)	**0.0** (0)	**0.0** (0)	**0.4** (1)
No growth/cont	**1.6** (4)	**66.3** (497)	**64.1** (481)	**64.4** (161)	**79.2** (99)	**56.0** (140)	**55.6** (139)
Non *Fusarium*	**8.8** (22)	**14.3** (107)	**15.1** (113)	**17.2** (43)	**13.6** (17)	**26.8** (67)	**33.2** (83)
Total	**100** **(250)**	**100** **(750)**	**100** **(750)**	**100** **(250)**	**100** **(125)**	**100** **(250)**	**100** **(250)**

*Figures in bold are percentage distribution per plant part while figures in brackets are the actual number of isolates counted. Plant tissues used were abbreviated as follows- **R** = roots, **IC** = inner corn, **OC** = outer corm, **IP** = inner pseudostem, **OP** = outer pseudostem, **IPSB** = inner pseudostem base and **OPSB** = outer pseudostem base.

Samples were taken from roots, rhizome and pseudostem tissues of banana plants. Identification of the isolates from the samples were carried out as outlined in Fusarium spp identification manual by Nelson *et al,* (1983).

Similarly, the percentage occurrence of endophytic *Fusarium oxysporum* from Kilifi district samples were higher in the roots at 82.0% than in the outer corm tissue at 37.2%, inner corm at 48.4%, inner pseudostem base at 27.2%, outer pseudostem base at 36.8%, inner pseudostem 41.6% and outer pseudostem at 42.4% (Table 5).

Table 5: Frequency (%) of occurrence of fungal endophytes isolated from surface sterilised banana tissues sampled from five heavily banana nematodes infested farms in Kilifi district, Kenya.

	Banana tissues						
Fungal species	**R**	**IC**	**OC**	**IP**	**OPS**	**IPSB**	**OPSB**
F. oxy	**79.2**	**10.5**	**11.2**	**3.6**	**1.6**	**7.6**	**6.0**
	(198)	(79)	(84)	(9)	(2)	(19)	(15)
F. solani	**6.8**	**7.3**	**8.9**	**14.0**	**5.6**	**9.6**	**4.8**
	(17)	(55)	(67)	(35)	(7)	(24)	(12)
F. Subglutans	**2.0**	**0.7**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(5)	(5)	(0)	(0)	(0)	(0)	(0)
F. avenaceium	**0.8**	**0.4**	**0.5**	**0.0**	**0.0**	**0.0**	**0.0**
	(2)	(3)	(4)	(0)	(0)	(0)	(0)
F. equiseti	**0.0**	**0.3**	**0.1**	**0.4**	**0.0**	**0.0**	**0.0**
	(0)	(2)	(1)	(1)	(0)	(0)	(0)
F. chlamydosporium	**0.4**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(1)	(1)	(0)	(0)	(0)	(0)	(0)
F. nivale	**0.4**	**0.0**	**0.0**	**0.4**	**0.0**	**0.0**	**0.0**
	(1)	(0)	(0)	(1)	(0)	(0)	(0)
Other *Fusariums*	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.4**
	(0)	(1)	(0)	(0)	(0)	(0)	(1)
No growth/cont	**1.6**	**66.3**	**64.1**	**64.4**	**79.2**	**56.0**	**55.6**
	(4)	(497)	(481)	(161)	(99)	(140)	(139)
Non *Fusarium*	**8.8**	**14.3**	**15.1**	**17.2**	**13.6**	**26.8**	**33.2**
	(22)	(107)	(113)	(43)	(17)	(67)	(83)
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**
	(250)	**(750)**	**(750)**	**(250)**	**(125)**	**(250)**	**(250)**

Figures in bold are percentage distribution per plant part while figures in brackets are the actual number of isolates counted. **R**= Roots, **IC**= Inner corn, **OC**= Outer corm, **IP**= Inner pseudostem, **OP**= outer pseudostem, **IPSB**= Inner pseudostem base and **OPSB**= Outer pseudostem base.

Samples were taken from roots, rhizome and pseudostem tissues of banana plants. Identification of the isolates from the samples were carried out as outlined in Fusarium spp identification manual by Nelson *et al,* (1983).

Other major *Fusarium* species identified in high numbers included *F. solani, F. subglutans, F. avenaceium, F. equiseti, F. chlamydosporium, F. nivale* and *F. moniliforme*. Some *Fusarium* that was difficult to identify and classify formed an important part of the fungal spectra from both two sampling locations. From Meru samples other *Fusarium* spp. accounted for 0.1% frequency of occurrence, while in Kilifi samples, other *Fusarium* spp. accounted for 2.0% frequency of occurrence. Other *Fusarium* which could not be described using the morphological characteristics, had a frequency of occurrence in the range of 0.1-2% in Meru and Kilifi districts isolates respectively (Table 3 and 4). The roots gave the highest occurrence of *F.oxysporum* compared to the corm and pseudostem tissues (Table 4 and 5).

There were significant differences in the various *Fusarium* spp. isolated across the various plant tissues obtained from the mother plants, sucker and the joint between the mother plant and the sucker, for samples from Meru district (Table 6). *Fusarium oxysporum* varied significantly among the mother plant, sucker and the joint tissues. The inner corm of the sucker had a higher frequency of occurrence of *F. oxysporum* compared with the inner corms of the mother plants and the joint ($P < 0.05$) (Table 6). Isolates of *F. oxysporum* from the joint and the mother plant did not vary from each other (Table 6).

The frequency of *F. oxysporum* isolates originating from the different mother plant tissues did not statistically differ from each other ($P > 0.05$) (Table 6). However the distribution of *F. oxysporum* across the plant tissues obtained from

the sucker plant demonstrated some significant (P < 0.05) differences Isolates of *F. oxysporum* from the outer corm and the inner corms of the joints had no variations from each other (P = 0.05) (Table 6). The outer corm of the mother plant had significantly (P < 0.05) higher occurrence of *F. oxysporum* as opposed to the inner corm. However, the frequency of isolation of *F. oxysporum* from the sucker inner corm was relatively higher compared to all other plant tisses that were plated (Table 6).

Table 6: Distribution of *Fusarium oxysporum* (Means±S.E) isolates across the various plant tissues from different plant parts in samples collected from Meru district.

F. oxysporum (percentage colonisation)				
Plant part	**Inner corm**	**Outer corm**	**Pseudostem**	**Pseudostem base**
Mother Plant	6.67±1.80Bab*	11.09±1.77aA	1.80±0.88cAB	5.76±1.79bA
Sucker	10.15±1.37aA	5.01±1.39bcB	2.16±0.95cA	6.48±1.94abA
Joint	3.95±0.94bB	6.21±1.14aB	0.00±0.0bB	0.00±0.0bB

*Means (± SE) within a row or column followed by the same lower or upper case letter, respectively, are not significantly different at P = 0.05. Means and standard errors of each cell of each column are means of percentage colonisation of plant tissues obtained from five healthy banana plants.

Isolates of *F. oxysporum* originating from different plant stages (Mother plant and the sucker plant) and the joint between the two plants varied significantly (P< 0.001). The inner corm of the mother plants had a higher frequency of colonisation by the endophytic *F. oxysporum* as opposed to the sucker plant and

the joint tissues which did not vary from each other. Equally, the outer corm, pseudostem and pseudostem tissues originating from the mother plant had a higher rate of occurrence of endophytic *F. oxysporum* as opposed to the sucker and the joint tissues (Table 7).

Table 7: Distribution of *Fusarium oxysporum* (Means±S.E) isolates across the various plant tissues from different plant parts in samples collected from Kilifi district in Kenya.

F.oxysporum (percentage colonisation)				
Plant part	**Inner corm**	**Outer corm**	**Pseudostem**	**Pseudostem base**
Mother Plant	34.8±2.21bA	44.4±2.23aA	29.5±2.89bA	46.8±3.04aA
Sucker	23.5±1.77bB	30.7±1.86aB	14.0±1.87cB	34.2±2.47aB
Joint	19.2±1.60aB	20.3±1.604aB	0.00±0.0bC	0.00±0.0bC

*Means (± SE) within a row or column followed by the same lower or upper case letter, respectively, are not significantly different. Means and standard errors of each cell of each column are means of percentage colonisation of plant tissues obtained from five healthy banana plants.

Among the mother plant tissues, the inner corm tissues and the pseudostem base had almost the same frequency of endophytic colonisation whereas the inner corm and the pseudostem tissues had the same frequency of occurrence of endophytic *F. oxysporum*. Similar to the mother plant, the inner corm tissues obtained from the suckers had higher rates of occurrence of *F. oxysporum* as

opposed to the inner corm and the pseudostem. The joint between the mother plant and the sucker was also a key area of interest during the isolation process. The inner corm tissue and the outercorm tissues had no difference in their colonisation rates (Table 7).

Thirteen isolates tested did not show any pathogenic symptoms to the tester plants. It was therefore concluded that the isolates were not pathogenic, and were fit for further tests.

2.5 Discussion

The surface sterilisation method used in the currecnt study was effective in removing epiphytic micro-organisms, which made it possible to isolate and characterise endophtytic fungi from banana plants. Equally, the method was appropriate in this experiment since it enabled handling of large sample size during the endophytes isolation process. The use of antibiotics in the culture media inhibited bacterial contamination, enhancing chances of fungal growth.

The main aim of the isolation process of endophytes was to evaluate the occurrence of endophytes in banana plants with the aim of identifying isolates that can be used for pest control. Therefore the isolation started from healthy plants that were among other infested plants in fields with high levels of banana parasitic nematodes. Appropriate plant parts were sampled from these plants such as the roots which are normally attacked by the nematodes, the corm and the pseudostem. The isolates can be used to control various pests that are normally associated with banana plants and occur at the same time in the same banana mat, such as the nematodes and the banana weevil (*Cosmopolites sordidus*).

The current study used morphological identification as the first step in the classification of the endophytes. Morphological methods of identification have been used in similar studies in the past and have been regarded as the first step for fungal taxonomy and can give additional information on the isolates biological activity (Gams, 1992). However, where fungal species were hard to

identify using morphological information, further investigation is needed, utilising either genetic or biochemical traits before they can be evaluated for their potential as biological control agents.

Results of the current study demonstrate that *Fusarium* spp. is a major inhabitant of banana plants. The frequency of occurrence of *Fusarium* spp. was higher in all the plants under the study as opposed to non *Fusarium* isolates. *F. oxysporum* was the most frequently isolated endophytic fungus from healthy banana plants. These fungi constituted 15.5% and 44.6% of all the fungi isolated from the healthy banana roots, corms and pseudostems from Meru and Kilifi districts, respectively. The same species has previously been reported as the most frequently isolated endophytic fungus from bananas (Schuster *et al.*, 1995b; Pocasangre *et al.*, 2000; Niere, 2001). Equally *F. oxysporum* has also been documented to be the most frequently isolated endophyte from rice and maize (Fisher and Petrini, 1992; Fisher *et al.*, 1992).

The frequency of occurrence of endophytic fungi was higher in the roots than in the corm and pseudostem tissues. *F. oxysporum* accounted for 79.2% and 82.0% of all the *Fusarium* isolates from the roots isolated from Meru and Kilifi districts respectively. Similar observations were recorded earlier from isolation studies from banana roots, pseudostems and corm tissues (Schuster *et al.*, 1995b; Pocasangre *et al.*, 2000). The endophytes (*F. oxysporum*), were isolated in two locations (Meru and Kilifi), and were isolated from different banana cultivars

including dessert and plantains indicating that endophytes are not limited to specific cultivars.

Occurrence of higher number of isolates from the roots as opposed to other plant tissues under study may offer an explanation for lower or no nematodes attack observed in these roots and overall plants sampled. These endophytes colonising the roots may benefit from higher nutrient and water in the roots where major nutrient absorption and transport takes place. Similar reports have indicated high numbers of *Fusarium* spp. in banana tissues (Speijer, 1993; Schuster *et al.*, 1995b; Griesbach, 2000; Pocasangre *et al.*, 2000; Niere, 2001). Isolates from the corms and the pseudostems were isolated at lower frequencies than those from the roots. Though few in their occurrence, these isolates are likely to be adapted to physiological conditions pertaining to these plant parts and therefore may be used to target banana pests attacking these parts such as the banana weevil, as indicated in earlier studies (Griesbach, 2000).

Endophytic populations were higher in lower altitudes of Kilifi region compared to highlands of Meru region. Environmental factors and predisposition of the host plants is apparently important for successful infection by the endophytes as shown by the preference of *Fusarium* spp. for cultivars grown in warm and dry conditions (Kilifi isolates). This compare with the results of Strobel (2003) who reported that perennial plants growing in drier regions are host to a greater diversity than their counterparts growing in the cooler regions, which were partly attributed to environmental stresses.

The occurrence of endophytes from the joints between the sucker and the mother plants is a great indication that there is transmission of endophytes from one plant to the other or simply from the mother plant to the sucker. The presence of endophytic *F. oxysporum* colonising the joint offers promise of transmission of endophytic strains with antagonistic effects to nematodes from the mother plant to the sucker plants in the same ratoon.

In the light of the overall objective of the current study, isolation and search for a new fungal strain for biological control of banana nematodes, the isolation of endophytic fungus demonstrated that *F. oxysporum* is the most promising candidate based on the frequency of occurrence. The isolation frequency gave the indication that *F. oxysporum* is adapted to an endophytic lifestyle. It was therefore concluded that by virtue of the high frequency of occurrence in banana plants and the fact that it also occured in many other plant parts apart from the roots it has the capacity of being able to colonise and persist within banana plants. As an effective biocontrol agent, the endophytes must posses the ability to succesfully colonise the plant. *F. oxysporum* demonstrated this ability and therefore was screened for antagonisim against banana parasitic nematodes as discussed in the next chapter.

CHAPTER 3

IN VITRO SCREENING OF ENDOPHYTIC *FUSARIUM OXYPORUM* FOR BIOLOGICAL CONTROL OF TWO BANANA NEMATODES (*Pratylenchus goodeyi* and *Helicotylenchus multicintus*)

3.0 Introduction

The banana lesion nematode, *Pratylenchus goodeyi* and the spiral nematode, *Helicotylenchus multicintus* are among the major constraints to banana production in Kenya (Gichure and Ondieki, 1977; Inzaule *et al.*, 2003). In Kenya, *P. goodeyi* and *H. multicintus* have been observed in bananas in the coastal region as well as in Central and Western provinces (Seshu *et al.*, 1998b). Yield losses caused by *P. goodeyi* range between 30-69% in Kenya (Qaim, 1999a; Wanzala, 2005). At higher densities *H. multicintus* may cause toppling over and lesions in the corms just similar to those caused by *Radopholus similis* (Luc *et al.*, 1990). The majority of banana producers in Kenya are small-scale farmers, making chemical control of nematodes unaffordable due to high costs (Seshu *et al.*, 1998b).

Management of *P. goodeyi and H. multicintus* in Kenya has relied on use of cultural methods, such as the use of healthy, planting material obtained from pared and hot water-treated suckers, and tissue culture plants (Speijer *et al.*, 1995b; Speijer *et al.*, 1999). These methods have however not been effective

offering only temporary control of nematodes. As a result, nematode reinfestation in the fields occurs readily to newly planted banana plants (Stanton, 1999; Speijer *et al.*, 2001). Biological control of *P. goodeyi* and *H. multicintus* using fungal endophytes opens up a novel method towards nematode management especially for the resource poor farmers in Kenya and Africa. Previously, fungal endophytes have been shown to successfully control nematode in other crops such as tomato (Hallman and Sikora, 1994). Additionally, fungal endophytes isolated from healthy banana roots were shown to cause nematodes mortality during *in vitro* experiment and even offer protection against nematodes to tissue cultured banana plants in screenhouse trials (Pocasangre, 2000; Niere, 2001; Dubois *et al.*, 2004; Athman, 2006)

There are several mechanisms by which endophytes protect plants against nematodes. Production of a wide array of secondary metabolites that cause antibiosis has been documented as the main mode of action of fungal endophytes against plant nematodes (Alabouvette and Lemanceau, 1999; Athman, 2006; Dubois *et al.*, 2006a). In addition, literature documents that during *in vitro* trials, *F. oxysporum* endophytes from tomato (*Lycopersicon esculentum* L.) produced secondary metabolites which reduced nematode hatching and caused juvenile mortality (Hallmann and Sikora, 1996). *F. oxysporum* endophytes have been demonstrated to cause *in vitro* mortality and paralysis of *R. similis* (Athman, 2006).

The potential of *F. oxysporum* as a novel strategy in banana nematodes management has never been investigated in the past in Kenya. Therefore the objectives of this study were 1) to screen for the effects of metabolites produced by endophytic *F. oxysporum* isolates from Kenyan bananas against two nematodes species (*P. goodeyi* (Sher and Allen) and *H. multicinctus* (Cobb) Golden) and 2) to determine the efficacy of various endophytic *F. oxysporum* isolates on the two nematodes species (*P. goodeyi* and *H . multicintus*).

3.1. Materials and methods

3.1.1 Nematode inoculum

Fifty tissue-cultured banana plantlets (cv. Gross Michel) were obtained from Jomo Kenyatta University of Agriculture and Technology (JKUAT, Nairobi, Kenya), planted in 20 litre buckets (12.5 cm diameter, 13 cm depth) containing soil mixture (2: 3 (v/v) sand: forest soil) in a screenhouse (30ºC and 12: 12 L: D) and watered twice a week. After two months, they were each inoculated with 100 g cut roots from nematode-infested banana plants (cv. Gross Michel, AAA) obtained from farmers' fields around JKUAT. Plantlets were inoculated with cut roots by placing them on the plantlets' roots before covering with the soil mixture. After 10 months, ten roots with ≥ 70% root necrosis were collected from each plantlet and transported to the laboratory in polythene bags for nematodes isolation.

3.1.2 Nematode extraction

Under running tap water, all soil was washed off the roots. From each root sample, five roots were picked at random and cut transversally to assess nematode damage

Five nematodes infested roots were cut into 2 cm^3 pieces and a sample (10 g) macerated using a blender for 15 s in 75 ml sterile distilled water. The suspension from roots of each plantlet were each poured separately on to different modified

Baermann dishes and left undisturbed on the laboratory bench for 24 h. Nematodes suspensions from each Baermann dish were transferred separately into 250 ml beakers. The suspensions were left to stand for one hour and concentrated by siphoning off the excess water to a volume of 20 ml in each beaker using a pipette. The concentrated suspensions were then sieved using a 38 µm sieve and backwashed into sterile 20 ml glass bottles. One hundred microliters of the nematode suspension was put on a nematode counting slide and the number of living nematodes was determined by species (*P. goodeyi* and *H. multicinthus*) and development stage (female, male or juvenile). Nematodes were identified using various characters such as total body length, shape of the tail,location of the vulva, length of the conical part of the stylet, distance of the dorsal aesophageal gland opening behind the stylet knobs as percentage of the stylet, number of annules from vulva to the anus, total number of annules among other factors as described in nematodes identification manual used during this experiment (Mai *et al*., 1971). Where the nematodes population was low, nematodes concentrated solutions were mixed together and sieved using a 38 µm sieve to have more concentrated nematodes suspensions. The nematode suspensions of *P. goodeyi* and *H. multicinthus* at all developmental stages (female, male or juvenile) were pooled together and adjusted to 100 nematodes/100 µl.

3.1.3. Selection of *F. oxysporum* isolates

Isolates of *F. oxysporum* selected for screening in this study were obtained from Meru and Kilifi districts, Kenya as discussed in chapter two. The isolates were selected based on banana variety and plant part (Appendices 1 and 2). In total twelve fungal isolates collected from Meru isolates were selected. Thirteen fungal isolates were selected from Kilifi samples at random. The isolate V5W2, which was originally isolated and tested in Uganda and found to be highly effective against *R. similis* (Athman, 2006), was included for comparison. Two controls were also included i.e. sterile distilled water at pH 7, and potato dextrose broth (PDB) with pH adjusted to the average pH of the fungal culture isolates (Appendices 4 and 5).

3.1.3 Preparation of fungal culture filtrates

Isolates were stored in 2.5 ml Eppendorf tubes and refrigerated at 4ºC prior to the study (Chapter 2). Synthetic nutrient agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar/l sterile distilled water (SDW)) was dispensed into 90 mm diameter glass Petri dishes. Under sterile conditions, one filter paper containing endophytes from each isolate was placed on an individual Petri dish containing SNA in dublicate. Petri dishes were sealed using parafilm, labeled and incubated for 7 days in the laboratory (26°C and 12: 12 L: D h photoperiod). From each isolate three blocks of media (1×1 cm), were cut from each Petri dish and placed

together into 100 ml pyrex bottle containing 80 ml autoclaved (121ºC for 15 min) potato dextrose broth (PDB) (12 g PDB/l SDW). Control flasks containing PDB were left uninoculated. The cultures were incubated for two weeks in the laboratory with manual shaking of the flasks once a day.

3.1.4 Preparation of culture filtrates

Fungal cultures were transferred into sterile 40ml centrifuging bottles and centrifuged at 6000 rpm at centrifugal force of (2012g) (Pierce, 2005) for 15 min. The supernatant obtained was transferred aseptically into sterile 25 ml universal glass bottles. The pH of the fungal filtrates was determined by dipping sterile electrode of a pH meter into universal bottles containing the supernatants aseptic conditions. Three repeats of pH measurement were carried out per isolate and average recorded (Appendix 4 and 5). The pH of one of the control treatments (PDB) was adjusted to the average pH of all fungal culture filtrates (Tables 3 and 4). The culture filtrates were kept for 24 h at 4°C.

3.2 Bioassays

For each isolate, one milliliter of culture filtrates as described in section 3.1.5 was transferred into 7 ml sterile glass Bjorn bottles. The culture filtrates were mixed with 100 µl of SDW containing 100 mixed species and stages of two nematodes species (*P. goodeyi* and *H. multicintus*). Control treatments were similarly mixed with 100 µl of SDW containing 100 nematodes (mixed species).

The bioassay was conducted in a completely randomized design and repeated thrice. The bioassays consisted of three replicates per isolate.

For each nematode species and stage, the number of active, paralysed and dead individuals were recorded after exposure to culture filtrates at 3, 6 and 24 h. Active nematodes were considered to be those nematodes with normal sigmoid shape and exhibited active movement, while paralysed nematodes were not active and were curved (never in a straight position) whereas dead nematodes appeared straight (uncurved) with elongated bodies. For each nematode species and stage, the percentage dead and paralyzed nematodes were counted as percentages of the initial number of nematodes.

After 24 h, nematodes were concentrated through a 38 µm sieve, rinsed with SDW and transferred into sterile 7 ml Bjorn bottles containing 2 ml SDW. The nematode cultures were left in the laboratory for an additional 24 h to test for reversibility of the toxic effects of the nematodes. Nematodes were probed with a fine needle under a compound microscope (magnification $\times$ 200) and those which were elongated and remained immotile even after probing were considered dead.

3.3 Data collection and statistical analysis

Percentage mortality values were corrected from one control (water) using Abott's formula (Abbott, 1925). Levene test was used to test variances homogeneity. Percentage corrected mortality and paralysis values were arcsine-squareroot transformed prior to analysis of variance (ANOVA). ANOVA was used to determine single and factor interaction effects using PROC GLM (SAS, 2001). Where factor interaction was significant, the effects of one factor were analysed at each level of the interacting factor. Where effects of the factors were significant, means were compared using Tukey test. Corrected mortality and paralysis were analysed separately for isolates collected from Meru and Kilifi districts each on two species of nematodes (*P. goodeyi* and *H. multicinctus*).

3.4 Results

3.4.1 Effects of fungal culture filtratesisolated from banana plants sampled from Kilifi district, Kenya

3.4.1.1 Mortality of *P. goodeyi and H. multicintus* after 3hrs of exposure to fungal filtrates of endophytic isolates from Meru

The efficacy of the tested endophytes were significantly ($P < 0.0001$) higher than the controls. The endophytes caused corrected mortalities of between 43% to 59% for both *P. goodeyi* and *H. multicintus* after 3 hrs of exposure. At the same time period the corrected mortalities in the controls were upto 28%. The mortalities of the two nematode species (*P. goodeyi* and *H. multicintus*) significantly ($P < 0.0001$) varied with the exposure times and isolates. Since, the effect of the isolates on nematode mortality depended on the exposure time (df = 28, F = 2.66, $P < 0.0001$) the effects of the endophytes were analysed separately at each time of exposure. Likewise, mortality varied significantly ($P < 0.0001$) between the two nematodes species and therefore their corrected mortalities were not pooled. The Uganda isolate V5W2 did not perform any better compared to most Kenyan isolates (Fig 1). Filtrates from the isolate 5JTOC134, produced the highest percentage mortality (59.5 %) on *P. goodeyi*, while the lowest was recorded in the control (PDB) at 16.6%. The highest mortality on *H. multicinctus* after 3 h exposure to culture filtrates was recorded from isolate 5MR55 at 59.1%.

PDB (control) had the lowest mortality on *H. multicintus* at 28.9%. V5W2 caused CM of 47.1% on *P. goodeyi* and CM of 56.9% on *H. multicintus*, respectively (Fig. 1).

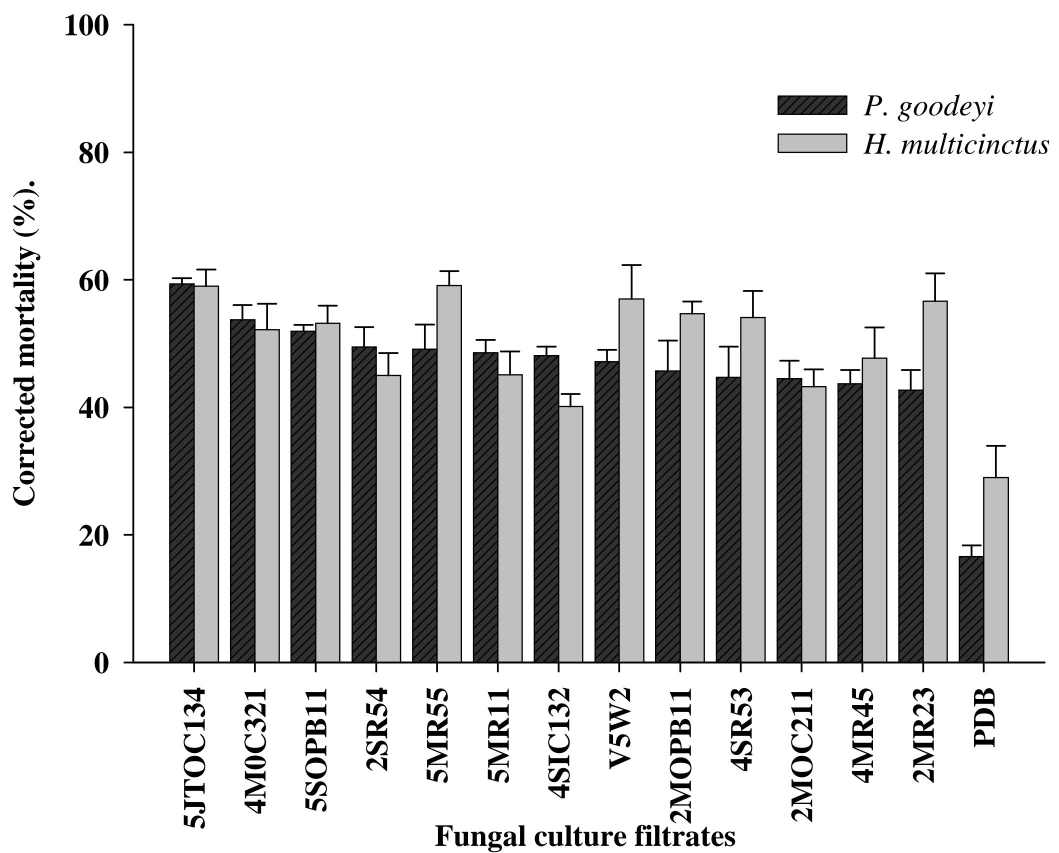

Figure 1: Mortality (%) of *Pratylenchus goodeyi* and *Helicotylenchus multicintus* after 3 hrs of exposure to culture filtrates of thirteen endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) obtained from Meru district, Kenya. Each bar represents means and standard errors of three bioassays repeated

three times. (Percentage mortality was obtained using abotts corrected mortality formula) (Abbott, 1925).

3.4.2 Mortality of *P. goodeyi and H. multicintus* after 6 hrs of exposure to fungal filtrates of endophytic isolates from Meru. (Corrected mortality using abbotts formula)

After 6 h of exposure, culture filtrates caused mortalities of upto 74.1% and 59.0% on *P. goodeyi* and *H. Multicinctus,* respectively. More of *P. goodeyi* than *H. multicinctus* succumbed to the antagonistic nature of the isolates metabolites after 6 h of exposure compared with 3 h exposure (Fig. 1 and 2). The control (PDB) still caused significantly lower Corrected mortality to *P. goodeyi* after 6 h exposure to culture filtrates ($P < 0.0002$). Isolate 5JTOC134 continued to cause the highest mortality on *P. goodeyi* (82.6%) after 6 h of exposure to culture filtrates compared to the control which caused a mortality of 24.1%. Isolate 5MR55 caused the highest mortality of 79.3% on *H. Multicintus* after 6 h (Fig. 2).

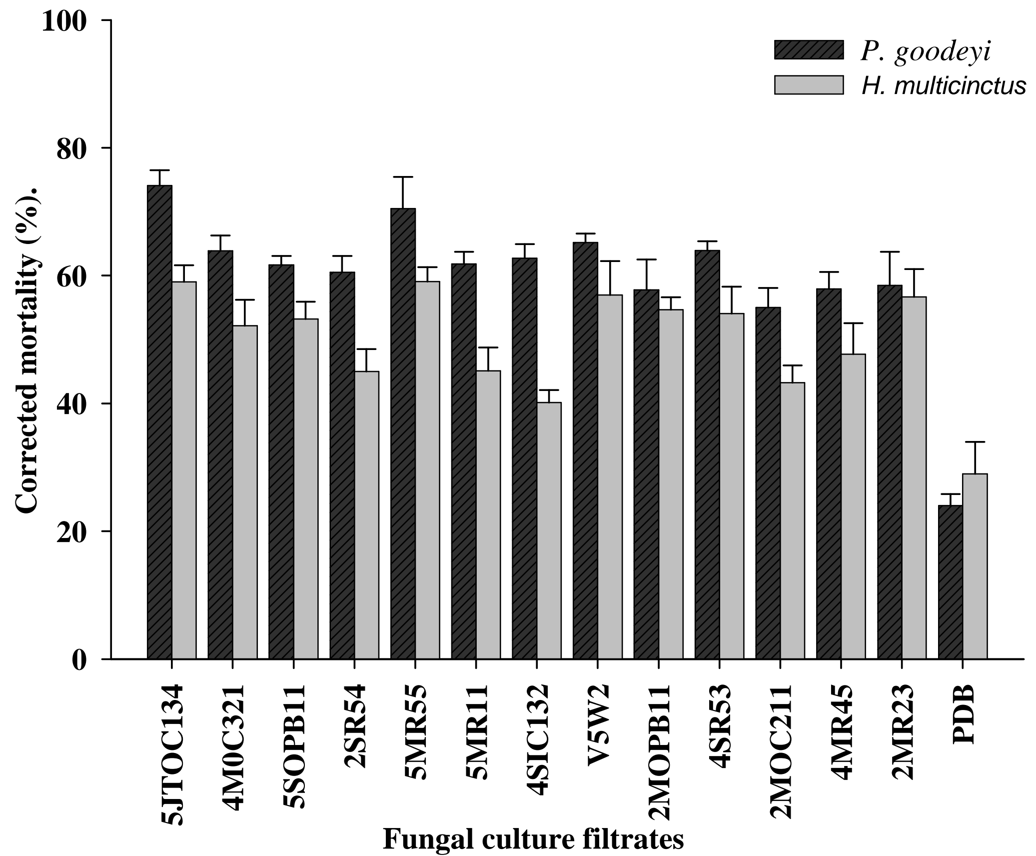

Figure 2: Mortality (%) of *P. goodeyi* and *H. multicintus* after 6 hrs exposure to culture filtrates of thirteen endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) obtained from Meru district, Kenya. Each bar represents means and standard errors of three bioassays repeated three times. (Percentage mortality was obtained using abotts corrected mortality formula) (Abbott, 1925).

3.4.3 Mortality of *P. goodeyi and H. multicintus* after 24 hrs of exposure to fungal filtrates of endophytic isolates from Meru. (Mortality corrected using abbotts formula)

After 24 h of exposure to fungal filtrates, nematodes corrected mortality rose to 83%. At this time period *P. goodeyi* appeared to be more affected by the endophyte filtrates except for those treated with fitrates isolates 2MOC211 and 2MR23. Filtrates from isolate 5MR55 continued to exhibit strong effect on *H. multicintus* causing the highest corrected mortality of 85.4%. Isolate 5JTOC134 caused a corrected mortality of 82.6% to *P. goodeyi* while V5W2 caused a corrected mortality of 79.5%. The control (PDB), maintained a significantly ($P < 0.0001$) lower corrected mortality of 28.3 and 32% for *P. goodeyi* and *H. multicintus*, respectively (Fig. 3). All the filtrates differed significantly ($P < 0.0001$) from the control (PDB).

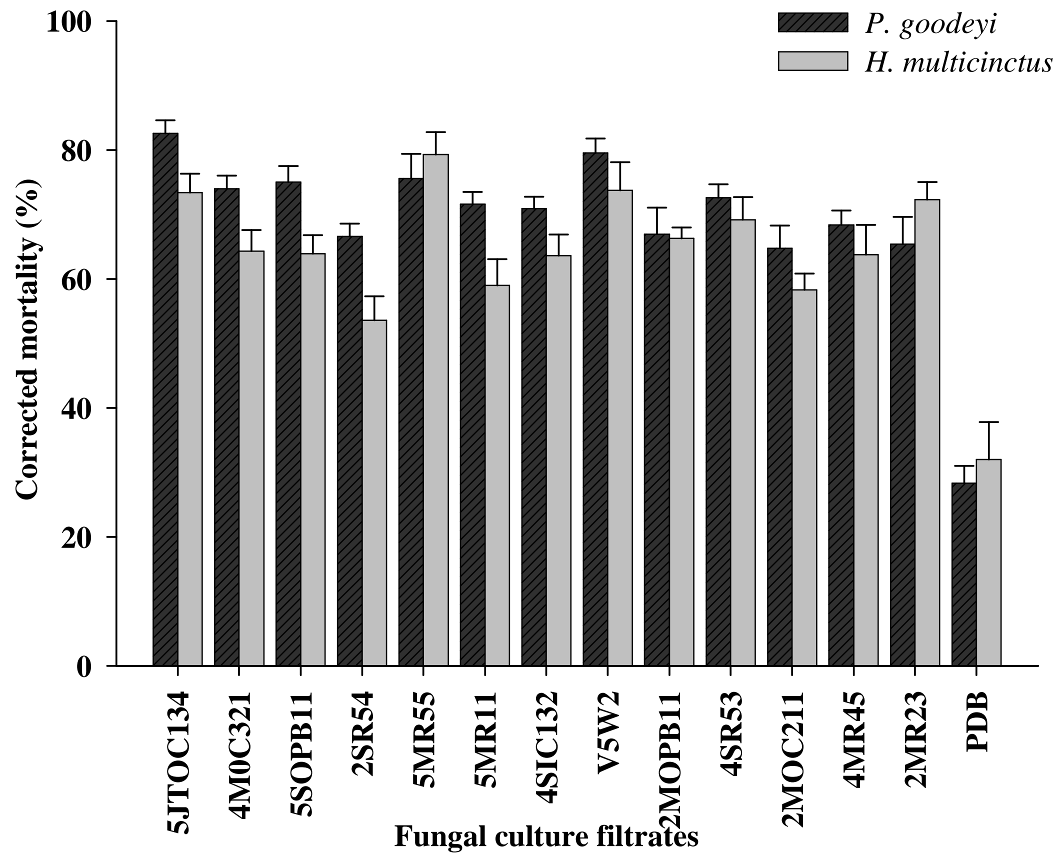

Figure 3: Mortality (%) of *P. goodeyi* and *H. multicintus* after 24 hrs exposure to culture filtrates of thirteen endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) obtained from Meru district, Kenya. Each bar represents means and standard errors of three bioassays repeated three times. (Percentage mortality was obtained using abotts corrected mortality formula) (Abbott, 1925).

3.4.3 Effects of culture filtrates of endophytic *F. oxysporum* isolates on paralysis of *P. goodeyi* and *H. multicinctus* (Isolates collected from Meru district)

The time of exposure and filtrates caused significant ($P < 0.0001$) variation in the paralysis of two nematode species (*P.goodeyi* and *H. multicintus*). Paralysis of the two nematodes species did not differ significantly from each other. The effect of the filtrates depended on the time of exposure in influencing the paralysis of the two nematodes species ($df = 28$, $F = 4.0$, $P < 0.0001$). Thus nematode paralysis caused by culture filtrate was analysed at each time of exposure.

3.4.3.1 Paralysis of *P. goodeyi* and *H. multicinctus* to fungal filtrates

After 3 h exposure, the highest percentage paralysis (30.7%) on *P.goodeyi* was recorded from culture filtrate of isolate 5SOPB11. All the isolates did not significantly ($P > 0.0001$) differ from each other, but they differed from control (PDB) (9.5%). After 6 h exposure, there was a decrease in paralysis recorded in all of the isolates to both species of nematodes. All the filtrates did not differ from each other and neither from the control after 24 h exposure ($P > 0.05$) (Fig. 4).

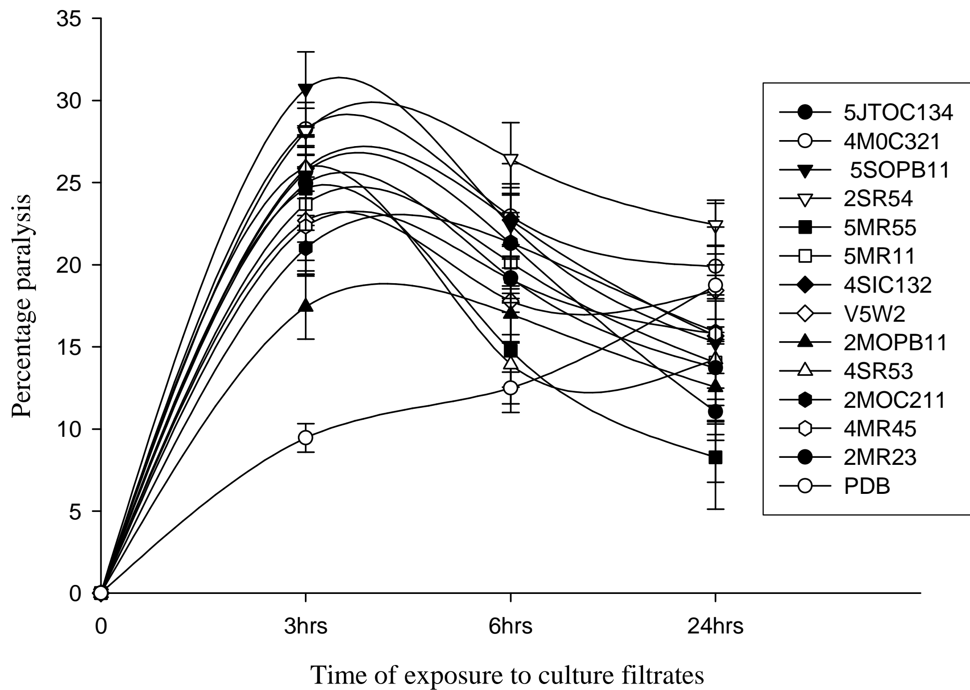

Figure 4: Paralysis (%) of *P. goodeyi* across three times of exposure to culture filtrates of thirteen different endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) sampled from Meru district, Kenya. Each line represents means and standard errors of three bioassays repeated three times.

However, for *H. multicintus* the highest paralysis after 3 h of exposure to culture filtrates was 33.4% from isolate 4MOC321 (Fig. 5). Increase in exposure time to 6 h led to decrease in percent paralysis with the highest paralysis recorded from filtrates of isolate 4MOC321 at 26.6%. All isolates did not differ from each other

in causing paralysis to *H. multicintus* after 24 h exposure. The lowest paralysis was observed in culture filtrates of isolates 5MR55 at 3.69% (Fig. 5).

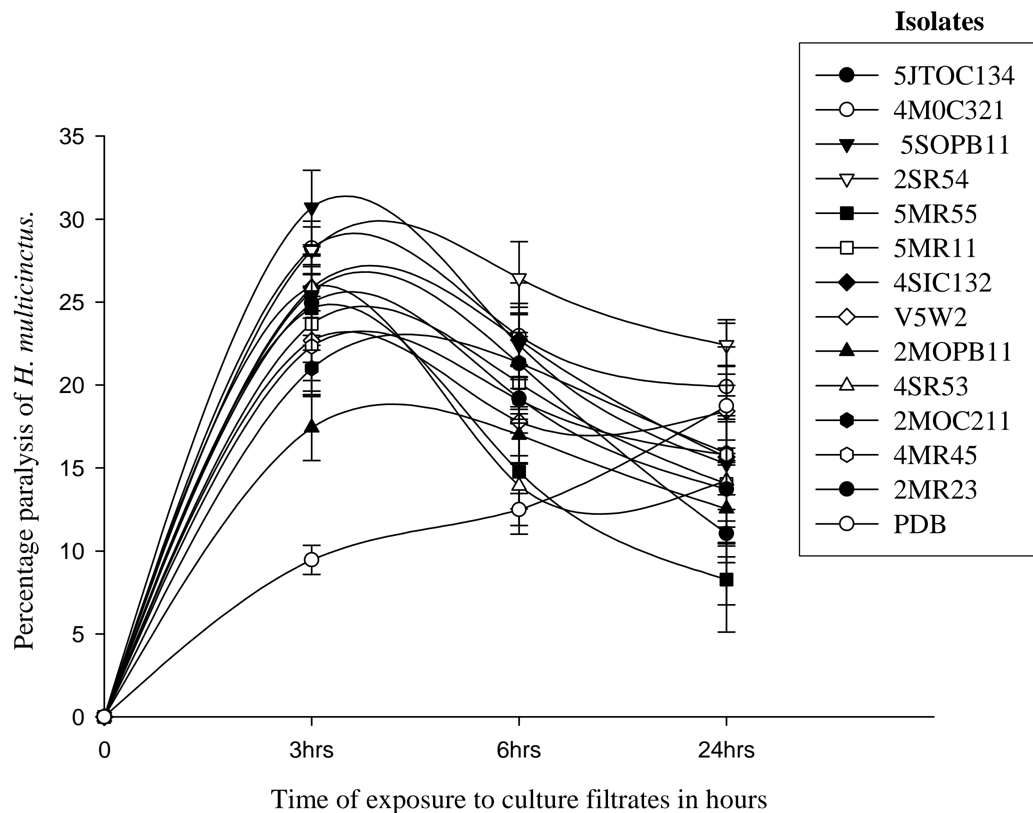

Figure 5: Paralysis (%) of *H. multicintus* across three times of exposure to culture filtrates of thirteen different endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) sampled from Meru district, Kenya. Each line represents means and standard errors of three bioassays repeated three times.

3.5 Effects of fungal culture filtrates from Kilifi district endophytic *F. oxysporum* on mortalities of *P. goodeyi* and *H. multicinctus*

Isolates from Kilifi district were tested for mortality and paralysis on *P. goodeyi* and *H. multicinctus* using culture filtrates of thirteen endophytic fungi isolated from healthy bananas. The time of exposure (df = 2, F = 597.59, P < 0.0001) and isolates (df = 14, F = 348.89, P < 0.0001) caused significant variation in the mortalities of two nematode species (P = 0.0002) (*P. goodeyi* and *H. multicintus*). The effect of the culture filtrates interacted with the length of exposure of nematodes in causing mortalities of the two nematodes species (df = 28, F = 5.28, P < 0.0001). Data was therefore analysed on the effects of filtrates effects on each nematodes species and each time of exposure.

3.5.1.1 Mortality of *P. goodeyi* and *H. multicinctus* after 3 h of exposure to fungal filtrates of isolates from plants sampled in Kilifi district

The results of culture filtrate isolates from kilifi were comparable to the ones obtained with the Meru isolates. All the filtrates differed significantly (P < 0.0001) from the control (PDB) after 3 h exposure. Filtrates differed significantly (P < 0.0001) from each other in causing mortality of *P.goodeyi* Lowest mortality was recorded in the control (PDB) which was 10.6%. Filtrates of isolate 7MIC334 caused the highest mortality at 62.6% on *H. multicinctus* after 3 hrs of exposure.

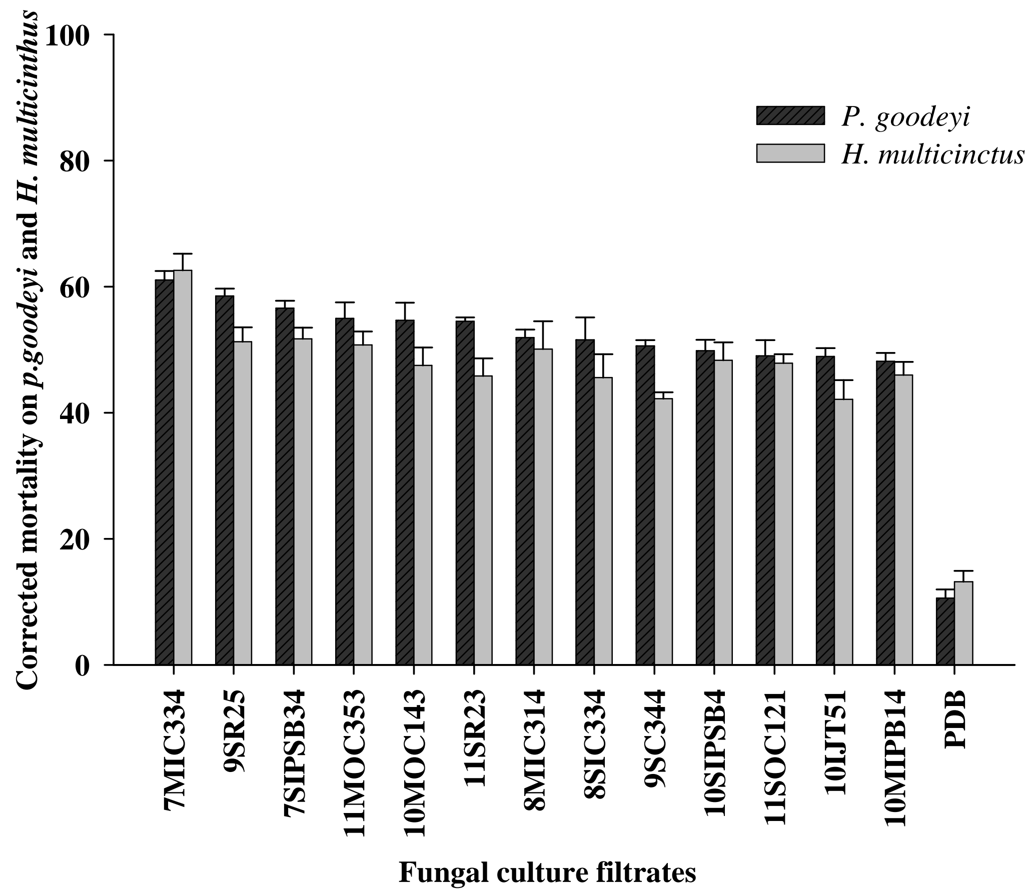

Figure 6: Percentage mortality of *P. goodeyi* and *H. multicintus* after 3 hrs exposure to culture filtrates of thirteen endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) obtained from Kilifi district, Kenya. Each bar represents means and standard errors of three bioassays repeated three times. (Percentage mortality was obtained using abotts corrected mortality formula) (Abbott, 1925).

3.5.1.2 Percentage mortality of *P. goodeyi* and *H. multicinctus* after 6 h exposure to culture filtrates

All the isolates differed significantly from the control. Filtrates of isolate 7MIC334 caused the highest corrected mortality on *P. goodeyi* and *H. Multicintus* (Fig. 7).

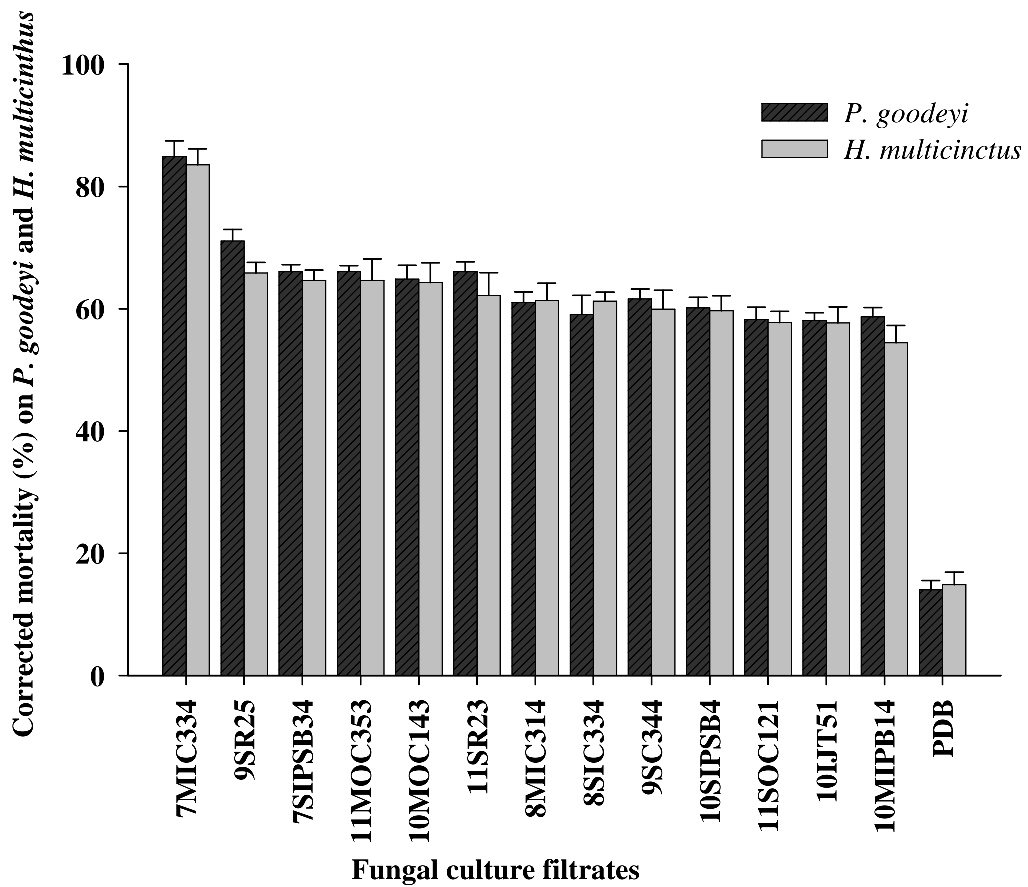

Figure 7: Percentage mortality of *P. goodeyi* and *H. multicintus* after 6 hrs exposure to culture filtrates of thirteen endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) obtained from Kilifi district, Kenya. Each bar

represents means and standard errors of three bioassays repeated three times. (Percentage mortality was obtained using abotts corrected mortality formula) (Abbott, 1925)

3.5.1.3 Percentage mortality of *P. goodeyi* and *H. multicinctus* after 24 h exposure to culture filtrates

Mortality of both nematodes species was highest after 24 h exposure to culture filtrates from all isolates. There were significant differences among the various isolates ($P < 0.0001$). The best culture filtrate after 3 h of exposure (7MIC334) continued to cause the highest mortality after 24 h of exposure at 87.8% (Fig. 8).

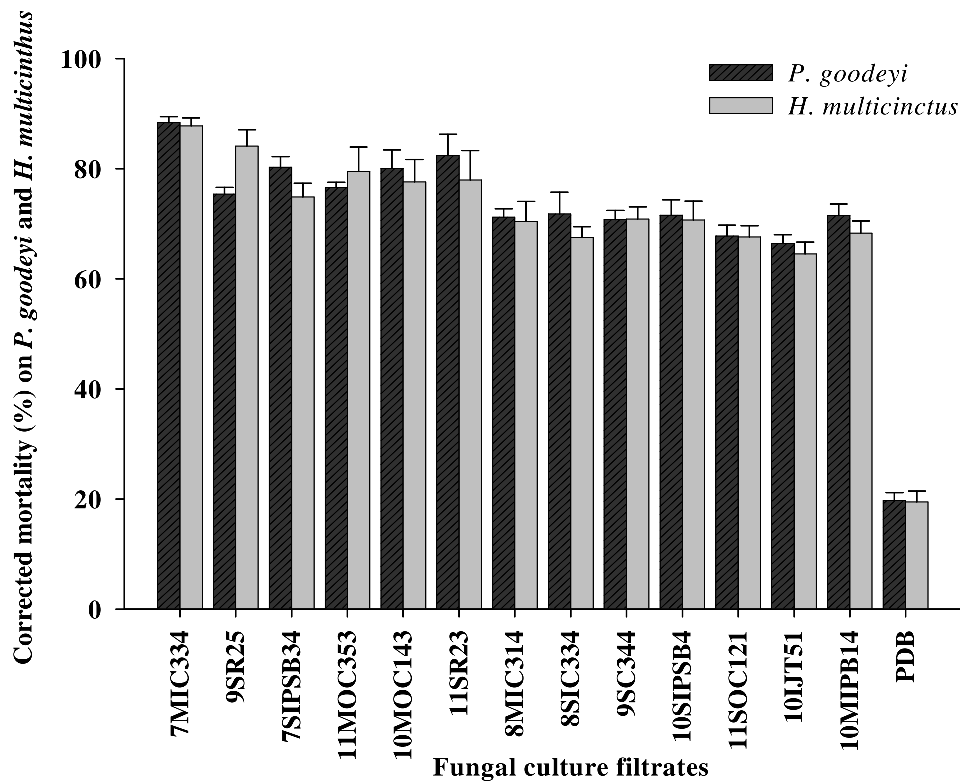

Figure 8: Percentage mortality of *P. goodeyi* and *H. multicintus* after 24 hrs exposure to culture filtrates of thirteen endophytic *F. oxysporum* isolates from banana plants (*Musa* spp.) obtained from Kilifi district, Kenya. Each bar represents means and standard errors of three bioassays repeated three times. (Percentage mortality was obtained using abotts corrected mortality formula) (Abbott, 1925)

3.5.3. Effects of culture filtrates of endophytic *F. oxysporum* isolates on paralysis of *Pratylenchus goodeyi* and *Helicotylenchus multicinctus* (Isolates collected from Kilifi district)

Paralysis of *P.goodeyi* and *H. multicinctus* was recorded after exposure to thirteen different endophytic *F. oxysporum* from Kilifi district in three exposure time intervals. There was significant difference in the time of exposure (df = 2, F = 205.52, P < 0.0001) on paralysis of *Pratylenchus goodeyi* and *Helicotylenchus multicintus*. The effect of the culture filtrates depended on the time of exposure in influencing the paralysis of the two nematode species as indicated by a two way-interaction (df = 28, 91; F= 7.58, P < 0.0001). However, paralysis of the two species of nematodes did not differ significantly (df = 1, F = 1.32, P = 0.2511).

3.5.3.2 Paralysis of *P. goodeyi* exposed to culture filtrates from Kilifi district

All the culture filtrates significantly (P < 0.0001) differed from the control (PDB) in causing paralysis to *P. goodeyi* (Fig. 9). Paralysis on *P. goodeyi* at 3 h exposure was highest at 35.2% from filtrates of isolate 7MIC334. Paralysis continued to drop after 6 h exposure to culture filtrates reaching a high of 23.7% (for isolate 9SC344). Most of the culture filtrates from different isolates did not significantly differ from each other. After 24 h of exposure, filtrates of isolate

7MIC334 recorded the lowest paralysis of *P. goodeyi* at 4.44%. The highest paralysis was recorded from culture filtrates of isolate 9SC344 at 18.4% (Fig. 9).

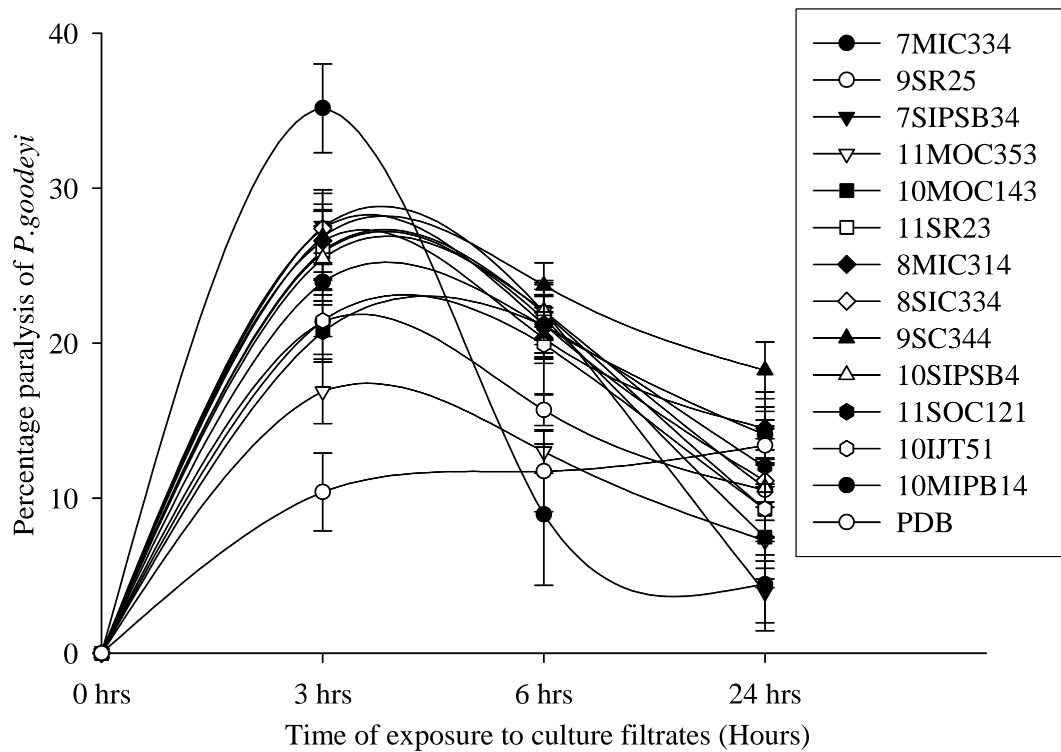

Figure 9: Paralysis (%) of *P. goodeyi* across three times of exposure to culture filtrates of thirteen different endophytic *Fusarium oxysporum* isolates from banana plants (*Musa* spp.) sampled from Kilifi district, Kenya. Each line represents means and standard errors of three bioassays repeated three times

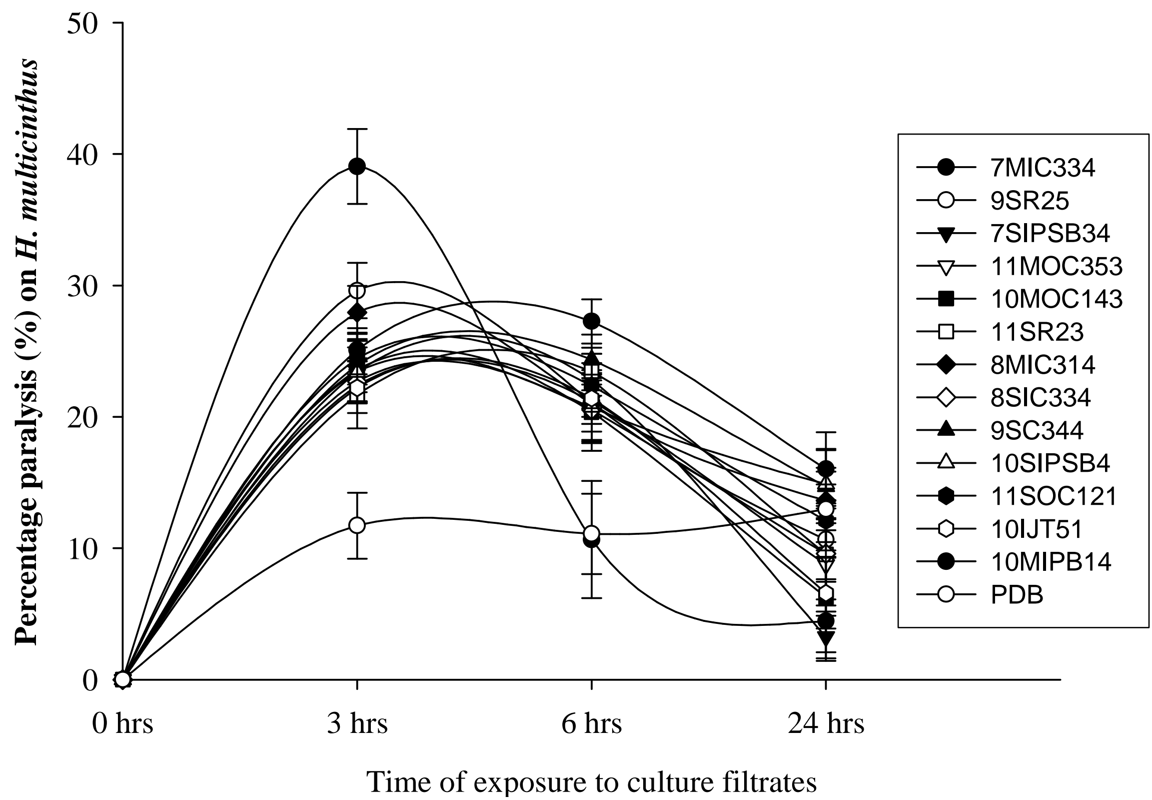

Figure 10: Paralysis (%) of *H. multicintus* across three times of exposure to culture filtrates of thirteen different endophytic *Fusarium oxysporum* isolates from banana plants (*Musa* spp.) sampled from Kilifi district, Kenya. Each line represents means and standard errors of three bioassays repeated three times

3.5 Discussion

Results from this study demonstrated an antagonistic effect of culture filtrates from all endophytic *F. oxysporum* isolates against the two nematodes species of banana (*P. goodeyi and H. multicinctus*). Out of the total thirteen endophytic isolates from Meru district, five isolates produced consistent mortalities and paralysis to both *P. goodeyi* and *H. multicinctus*. These isolates included 5JTOC134, 5SOPB11, 4M0C321, 4SIC132 and 5MR11. Equally five fungal isolates from Kilifi district which demonstrated consistency in causing mortality and paralysis were 7MIC334, 8SIC334, 11MOC143, 11SR23 and 11MOC353.

The fact that filtrates did not contain any spores after centrifugation and filtration; it was a strong indication that fungal isolates under the current study produced nematicidal metabolites that were antagonistic to the two species of nematodes. Previous studies on *Fusarium* spp have demonstrated that fungi can produce toxic secondary metabolites such as zearalanone, fumonisins, tricothecenes and fusaric acid (Vey *et al.*, 2001). In *in vitro* trials, *Fusarium* mycotoxins, such as T2-toxin, monilliformin, verrucarin A, cytochalasin B and Enniatin B, caused significant mortality of *Meloidogyne javanica* (Treub) Chitwoodi juveniles (Ciancio, 1995). Culture filtrates of non-endophytic strains of *Penicilliun oxalicum* Currie & Thom, *Penicllium anatolicum* Stolk and *Aspergillus niger* Van Tieghem were able to cause mortality to *R. similis* after 48 h immersion (Molina and Davide, 1986). Recently, culture filtrates from

endophytic *F. oxysporum* have been shown to cause *in vitro* mortality on *R. similis* supporting the concept of production of nematicidal metabolites (Athman, 2006).

Though PDB was prepared from an equal volume of sterile distilled water, there were variations in pH of the same culture filtrates over time, since new culture filtrates were prepared per replication. The isolates were cultured for similar number of days under similar lab conditions. Possible reasons for the differences in pH could have been growth rates of the fungus, which were variable among different fungi isolated from different plant parts and different regions. However, previous studies have shown that pH (PDB) had no affect on nematodes mortality or egg hatchability during *in vitro* tests on *Meloidogyne incognita* (Pike *et al.*, 2002; Meyer *et al.*, 2004), thereby ruling out any effect of pH in the current study.

Time of incubation of endophytic *F. oxysporum* was constant (14 days) and was regarded as the period when the fungus had the highest growth. However, the growth rates of the fungus varied across the different isolates under the study. It is therefore possible that the rates of growth had an effect on the production of mycotoxins and thereby affecting the levels of antagonism of individual culture filtrates to individual nematodes species. These findings are similar to those of Cayrol *et al.* (1989) who obtained highest toxins production from the fungus *Paecilomyces lilacinus* during prime growth period (10 days). Similary, Athman

(2006) and Dubois *et al.* (2004) observed that culture filtrates of endophytic *F. oxysporum* across the bioassays differed in causing mortalities despite maintaining constant experimental conditions. Therefore possible reasons for the variations in individual culture filtrates in causing mortalities on nematodes could be levels of mycotoxins production from the fungus cultured.

The time of exposure of nematodes to fungal culture filtrates had a great impact on nematodes mortality. The longer the time of exposure, the higher the *in vitro* mortality recorded. After rinsing the nematodes and further exposure of 24 h in SDW, the toxic effects were not reversible resulting to mortality of up to 90%. This concurs with findings by (Athman, 2006), who achieved nematodes mortality of up to 100% after 24 h exposure. Longer exposure times of nematodes to culture filtrates (72 h) has also been shown to produce mortality of 100% on *Meloidogyne incognita* (Hallmann and Sikora, 1996).

Nematodes paralysis decreased with increase in time of exposure for all the culture filtrates. Higher paralysis of nematodes was achieved after the first 3 h of exposure to fungal filtrates. The percentage paralysis decreased with time for most of the fungal culture filtrates. After 24 h of exposure, most of the isolates resulted to low paralysis. Decrease in paralysis was due to death of nematodes after a longer time of exposure to culture filtrates. Therefore the number of paralysed nematodes decreased with time across the experiments for all the isolates. However there were exceptions where percentage paralysis increased

with time, meaning more nematodes were paralysed by the fungal culture filtrate than causing mortality. There were instances where there was reversibility of toxic effects on nematodes that had undergone paralysis after the 24 h exposure to fungal culture filtrates. Such is demonstrated by isolates with increased paralysis after the 24 h of fungal culture exposure and rinsing in sterile distilled water. According to Cayrol *et al.* (1989), there was reversibility of toxic effects in *in vitro* tests with culture filtrates of *Paecilomyces lilacinus* to nematodes when they were exposed to culture filtrates for less than 48 h. Similarly, these results would agree with the hypothesis of a neurotrophic action upon the nervous receptors of the nematodes. However mechanisms to such neurotrophic actions need to be established.

The data obtained in the current study demonstrated mortality rates on both species of nematodes after time exposure of 24 h is the best time giving the separation among the isolates under the study. Therefore as a basis of selecting candidate endophytic *F. oxysporum* isolates for future trials the 24 h time of exposure would be regarded as the best. The results correspond with the findings of *in vitro* tests of endophytic *F. oxysporum* against *R. similis* in Uganda (Dubois *et al.*, 2004; Athman, 2006). There were few significant differences among culture filtrates in causing paralysis and therefore paralysis on nematodes was not used as a criteria in selecting isolates. This is in contrast to earlier reports where paralysis had been used as a basis of selection of the best isolates for *in vivo* test with banana plants. Mortalities across the individual times of exposure were

regarded as the best criteria for selecting candidate strains of endophytic *F.oxysporum* isolates for further trials.

Isolates from two sampling regions did not differ from each other in causing mortality or paralysis on two species of nematodes. This is a clear indication that there is similarity in biology and biochemistry of the endophytes from similar plant species despite geographical location. Similarly, the mode of action can be assumed to be the same for isolates from the two sampling locations.

All the isolates tested demonstrated mortality on two species of nematodes (*P. goodeyi* and *H. multicinctus*). Those showing high mortality on *P.goodeyi* equally demonstrated high mortality on *H. multicinctus.* The effectiveness of these single strains against both species of nematodes is a roadmap of possibility of using a single endophyte strain to target two major species of nematodes which usually occur together in banana orchards.

Nematicidal components of fungal culture filtrates and specific phytotoxins produced by such fungi have received little attention. Though literature documents that *Fusarium* spp have been shown to produce mycotoxins such as moniliformin , cytochalasin B and verrucarin A that lead to *in vitro* mortality of *meloidogyne javanica* (Ciancio, 1995), little information exist on mycotoxins produced by endophytic *F. oxysporum* leaving room for further research on the isolates tested in this study.

The study demonstrated that endophytic *F. oxysporum* isolates are a rich source of bioactive nematicides that can be incorporated in the production of tissue culture plants towards the management of *P. goodeyi* and *H. multicintus.* However, there is need for further identification of these isolates through gene sequencing to determine their similarities and possibly identification of genes responsible for the production of these nematicidal compounds. This information will open a new avenue for sustainable banana production especially for the rural small-scale farmers. These results of this study indicate the potential of endophytic fungi as biocontrol agent against banana nematodes. It will lead to fortification of tissue culture banana seedling before they are delivered to the farmer and would be an important inclusion in IPM in good agricultural practices.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

4.0 Discussion

The occurrence of *Fusarium oxysporum* in higher frequencies than the rest of the *Fusarium* species isolated from healthy banana plants during this study confirmed earlier reports (Schuster *et al.*, 1995b; Griesbach, 2000; Niere, 2001) that *F. oxysporum* is a natural inhabitant of banana plants. Apparently, although different surface sterilisation techniques were used during these previous studies, nearly identical fungal endophytic fungi distribution was observed. Outward hyphal growth from the internal tissues of surface sterilised banana plant parts was the main indicator of endophytisim in the current study as well as in the previous reports. Other similar observations of endophytisim have been documented in litrature (Petrini, 1986; Petrini, 1991).

The identification of *Fusarium* in this study involved morphological characteristics of the anamorph, which included the size and the shape of macroconidia, the presence or absence of microconidia, and the chlamydosphore, colony colour, and conidiophores structure as described by Nelson *et al.* (1983). Such morphological characteristics are the up to date basis of fungal identification (Gams, 1992). However given the shortcomings of morphological characters for delineating species and subgeneric grouping of *Fusarium*, molecular gene sequencing could in future improve the taxonomic observation. It was not the objective of this study to analyse the isolates

genetically, but a classification was achieved through macro- and microscopic observations.

Fusarium spp. was demonstrated to be a major component of banana plants due to its high frequency of occurrence. The frequency of occurrence of *F. oxysporum*, which was the most frequently isolated endophytic fungus was higher in the roots concurring with other previous studies (Pocasangre *et al.*, 2000; Niere, 2001), but also contrasting with (Griesbach, 2000) who found higher frequencies of endophytic *Fusarium* in the inner cortex of banana plants in Uganda. In his explanation, (Griesbach, 2000) documents that higher occurrence of endophytic *F. oxysporum* could have been responsible for the lower banana weevil (*Cosmopolites sordidus*) damage in the inner corm of the banana plants. Therefore similar conclusion can be demonstrated from the current isolation process where the higher occurrence of endophytic *F. oxysporum* from the roots could have been responsible for the lower or no nematodes damage in the roots sampled for isolation.

Selection of two agro-ecological zones (Meru and Kilifi districts) for plants sampling was a critical step aimed at elucidating differences in endophytic fungi colonisation based on plants predisposition to environmental factors such as different nematodes species pressures, temperature among many others as well as geographical position. Apparently it was found that there was higher endophytes occurrence in Kilifi district (low altitude and higher temperature and humidity) as opposed to Meru district (low temperature and lower humidity) conforming with

the suggestion by Strobel (2003) that perennial plants growing in drier regions are host to a greater diversity than their counterparts growing in the cooler regions. Additionally, the host endophyte symbiosis is affected by the environmental conditions under which the host is growing, especially temperature. Breen (1994) suggested that the compatibility of an endophyte and its host may be important in determining production of toxic metabolites by the endophyte.

Isolation of a new endophytic strain for biological control of nematodes focused more on the most frequently isolated mutualistic endophyte - *F. oxysporum*. Therefore screening for antagonism against banana nematodes (*P. goodeyi* and *H. multicintus*) was only carried out on *F. oxysporum* spp isolates.

Results from the screening protocol as described in chapter 3 of this thesis revealed that all the culture filtrates of endophytic *F. oxysporum* had *in vitro* activity against *P. goodeyi* and *H. muticintus*, suggesting that these isolates produced secondary metabolites that were nematicidal. Isolates screened from two regions of sampling did not differ from each other in causing mortality or paralysis on *P. goodeyi* and *H. multicintus*. This is a clear indication that there is similarity in mode of action of the endophytes from similar plant species obtained from different geographical locations.

There is limited information on the mycotoxins produced by *Fusarium* spp, especially those showing antagonism towards nematodes. Infact, there is only

scanty information that exists on mycotoxins produced by endophytic *F. oxysporum.* The current study demonstrated that the isolates that were tested could be a rich source of bioactive nematicides that can be harnessed to improve the management of *P. good*eyi and *H. multicintus* in bananas among other nematodes species. These strains could be considered a research priority towards the development of a novel strategy of nematodes management in tissue cultured bananas.

4.1 Recommendations

The current study explored the presence of endophytes in banana plants in Kenya, and conclusively found them present. The fact that the *in vitro* experiments against the major parasitic nematodes showed promising results opens a new field of research in employing the use of endohytes as biological control agent against banana parasitic nematodes. The isolates showing consistant mortalities during in vitro trials therefore should be a research priority towards the use of endophytes as biological control agent. Considering the global need for sustainable means of pests and diseases control, there is need to address the importance of endophytes as alternative to chemical control measures to nematodes. Specifically, future research should be geared towards;

- Genetic characterisation of the isolates that showed consistency in causing nematodes mortality.

- Evaluation of genes responsible for the production of metabolites that showed nematicidal effects to the nematodes using molecular techniques.

- Screenhouse and field trials are needed to determine the performance of the promising isolates recommended in the study.

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APPENDICES

Appendix 1: Plants sampled from Eastern province, Meru district Kenya for fungal isolation.

Plot No.	**Plant No.**	**Variety**	**Age (yrs)**	**Mother plant**			**Sucker**		
	No.			No. of Standing leaves	Height (M)	Girth at Pseudostem base (cm).	No. of Standing leaves	Height (M)	Girth at Pseudostem base (cm)
1	1	Giant Cavendish	6	3	3.5	70	3	50.6 cm	10
2	2	E.A. Highland Banana (*Mutagato*	4	5	2.6	53	4	79 cm	22
3	3	Giant Cavendish	4.5	8	4.3	60	3	1.4 m	20.2
4	4	Giant Cavendish	6	10	3.9	60.6	7	1.3 m	30
5	5	Giant Cavendish	5	6	3.2	75	6	1 m	20.6

Appendix 2: Plants sampled from Eastern province, Kilifi district Kenya for fungal isolation.

Plot No.	Plant No.	Variety	Age (yrs)	Mother plant			Sucker		
				No. of standing leaves	Height (M)	Girth at pseudostem base (cm).	No. of standing leaves	Height (M)	Girth at pseudostem base (cm)
1	7	Mkono tembo (EAHB)	5	8	4.7	69	5	3.6	34
2	8	Grand naine	5	6	2.3	57	6	2.3 cm	29
3	9	Giant cavendish	3	5	4.0	61	5	2.4 m	21.3
4	10	Grand naine	6	8	3.5	63	7	2 m	33
5	11	Dwarf cavendish	4	8	2.6	77	8	2.1 m	30

Appendix 3: The species identity and in-plant origin of 13 endophytic *Fusarium* isolates obtained from banana plants in Meru district, Kenya that were screened for antagonisim to motile stages of *Pratylenchus goodeyi* and *Helicotylenchus multicinctus* in the laboratory.

Isolate	spp	Cultivar	Genome	Plant No.	Stage of Plant	Plant part
5MR55	*F.oxysporum*	Giant Cavendish	AAA	5	Flowering	Roots
5JTOC134	*F.oxysporum*	Giant Cavendish	AAA	5	M-S Joint	Joint
2MR23	*F.oxysporum*	EAHB-"*Mutagato*"	ABB	2	Flowering	Roots
2MOPB11	*F.oxysporum*	EAHB-"*Mutagato*"	ABB	2	Flowering	Outer pseudostem base
4SR53	*F.oxysporum*	Giant Cavendish	AAA	4	Sucker	Roots
5SOPB11	*F.oxysporum*	Giant Cavendish	AAA	5	Sucker	Outer pseudostem base
4M0C321	*F.oxysporum*	Giant Cavendish	AAA	4	Flowering	Outer corm
4MR45	*F.oxysporum*	Giant Cavendish	AAA	4	Flowering	Roots
5MR11	*F.oxysporum*	Giant Cavendish	AAA	5	Flowering	Roots
2SR54	*F.oxysporum*	EAHB-"*Mutagato"*	ABB	2	Sucker	Roots
2MOC211	*F.oxysporum*	EAHB-"*Mutagato"*	ABB	2	Flowering	Outer corm
4SIC132	*F.oxysporum*	Giant Cavendish	AAA	4	Sucker	Inner corm
V5W2	*F.oxysporum*	Kubuzi		ISOLATE	Flowering	corm

Appendix 4: The species identity and in-plant origin of 13 endophytic *Fusarium* isolates obtained from banana plants in Kilifi district, Kenya that were screened for antagonisim to motile stages of *Pratylenchus goodeyi* and *Helicotylenchus multicinctus* in the laboratory.

Isolate	spp	Cultivar	Genome	Plant No.	Stage of Plant	Plant part
9SR25	*F.oxysporum*	Giant Cavendish	AAA	9	Sucker	Roots
9SC344	*F.oxysporum*	Giant Cavendish	AAA	9	Sucker	Inner pseudostem base
8SIC334	*F.oxysporum*	Grand nine	AAA	8	Sucker	Inner corm
8MIC314	*F.oxysporum*	Grand nine	AAA	8	Flowering	Inner corm
7SIPSB34	*F.oxysporum*	Mkono Tembo (EAHB)	ABB	7	Sucker	Inner pseudostem base
7MIC334	*F.oxysporum*	Mkono Tembo (EAHB)	ABB	7	Flowering	Inner corm
11SR23	*F.oxysporum*	Dwarf Cavendish	AAA	11	Sucker	Roots
11SOC121	*F.oxysporum*	Giant Cavendish	AAA	11	Sucker	Outer corm
11MOC353	*F.oxysporum*	Dwarf Cavendish	AAA	11	Flowering	Outer corm
10SIPSB4	*F.oxysporum*	Dwarf Cavendish	AAA	10	Sucker	Inner pseudostem
10MOC143	*F.oxysporum*	Giant Cavendish	AAA	10	Flowering	Outer corm
10MIPB14	*F.oxysporum*	Grand nine	AAA	10	Flowering	Inner pseudostem base
10IJT51	*F.oxysporum*	Grand nine	AAA	10	Joint	Joint

Appendix 5: pH of endophytic *Fusarium oxysporum* isolates measured in potato dextrose broth culture filtrates during individual bioassays for isolates from Meru district

	Bioassay 1	Bioassay 2	Bioassay 3
Isolate	**pH**	**pH**	**pH**
5MR55	3.8	4	3.9
5JTOC134	4	4.9	4.3
2MR23	3.7	4.1	3.8
2MOPB11	4.1	4	4.2
4SR53	3.8	3.8	3.5
5SOPB11	4.1	3.9	4.3
4M0C321	4.4	4.3	3.9
4MR45	4	3.7	3.1
5MR11	3.9	4.7	3.5
2SR54	4.1	4.3	4
2MOC211	4	3.3	3.9
4SIC132	3.9	4.3	3.5
V5W2	4.1	4	4
PDB	4.0	4.1	3.8
Water	6.9	7.7	7.1

Appendix 6: pH of endophytic *Fusarium oxysporum* isolates measured in potato dextrose broth culture filtrates during individual bioassays for isolates from Kilifi district

	Bioassay 1	**Bioassay 2**	**Bioassay 3**
Isolate	**pH**	**pH**	**pH**
7MIC334	4.1	4.6	4
9SR25	3.6	5	3.5
7SIPSB34	4.5	3.1	3.5
11MOC353	4.2	3.7	3.9
10MOC143	3.6	3.7	3.6
11SR23	4.2	4.3	4.1
8MIC314	3.6	5.2	3.9
8SIC334	3.8	3.5	3.2
9SC344	4	5	4.6
10SIPSB4	5	4.7	3.2
11SOC121	4	4.2	3.7
10IJT51	4.6	4.3	4.1
10MIPB14	3.6	3.9	4
PDB	4.1	4.2	3.8
Water	7.1	6.8	7