EVALUATION OF ENDOPHYTIC *Fusarium oxysporum* ISOLATES FOR CONTROL OF LESION NEMATODES *Pratylenchus goodeyi* IN TISSUE CULTURE BANANA PLANTS

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Evaluation of Endophytic *Fusarium oxysporum* Isolates for Control of Lesion Nematodes *Pratylenchus goodeyi* in Tissue Culture Banana Plants

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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

This thesis is dedicated to my best friend, Matthew, my parents and siblings.

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ABSTRACT

Pratylenchus goodeyi is one of key pests of bananas in Kenya. Although management of this pest has mainly relied on use of clean planting material produced through tissue culture, re-infestation of plants in the field remains a critical concern. Fungal endophytes may be used to provide protection and extend plant life. In this study, the effect of selected *Fusarium oxysporum* endophytic isolates on *P.goodeyi* damage, population and growth of tissue culture bananas and the effect of different endophyte inoculation methods on root colonization of tissue culture banana plants were evaluated. Tissue culture banana variety Giant Cavendish and Grand naine were inoculated with fungal endophytes by sprinkling the roots with the solid substrate containing the fungal inoculum during planting. The plants were inoculated with 1200 *P.goodeyi* in mixed developmental stages and nematode damage and population evaluated sixteen weeks later. Plant response to endophyte treatment was assessed from plant height, girth, number of functional leaves, length and width of the youngest leaf, fresh and root and shoot dry weights for the entire duration of the experiments. Two post flask weaning techniques, plants potted before inoculation and plants placed in nutrient solution before endophyte inoculation and three inoculum delivery methods namely use of solid substrate, dipping plants in fungal inoculum and drenching plants with the fungal inoculum were evaluated. *F.oxysporum* endophytic isolates significantly suppressed nematode damage and population densities in inoculated plants compared to uninoculated controls ($P<0.001$). Reduction in nematode damage and population in

inoculated plants over the controls ranged between 28.9% to 66.4% and 46.1% and 65.2% respectively. Improved growth was observed for plants inoculated with endophytes when compared to the control treatment for most growth parameters, though the differences were not significant ($P>0.05$). Up to 14.4% increase in plant height, 12.9% increase in girth, 38.6% increase in shoot weight and a 36% increase in root weight was observed for endophyte inoculated plants when compared to the control treatment. Percentage root colonization did not differ significantly between plants potted before inoculation and plants put in nutrient solution prior to inoculation ($P>0.05$). Percentage root colonization for the two techniques ranged between 47.6% to 54.7% for the three endophytic isolates. There were no significant variations in percentage root colonization between the three inoculation methods ($P>0.05$), however the use of solid substrate gave relatively higher rates of colonization. The results demonstrate the potential of fungal endophytes for the biological control of banana nematode *P.goodeyi*, potential to enhance plant growth and ability to colonize tissue culture banana plants.

CHAPTER 1

1.0 GENERAL INTRODUCTION

Banana (*Musa* sp.) is the fourth most important global food commodity after rice, wheat and maize in terms of gross value of production with total world production estimated at 86 million tons (INIBAP, 1998). In Eastern Africa it is a major staple crop and source of income for over 20 million people most of whom are resource poor women farmers (ISAAA, 1996). The region accounts for 17% of the world total banana production with an annual production of 3.6 million tonnes in Uganda, 2.5 in Tanzania and 0.4 in Kenya (Speijer and Sikora, 1993).

Bananas are large herbaceous perennials with short underground rhizomes or corms and are cultivated in the humid and sub-humid regions of the world where the average temperature is 27 - 30^{o}C and the yearly rainfall exceeds 1250 mm per year with moist and well drained soils (Karugaba and Kimaru, 1999). They belong the genus *Musa* and are considered to be derived from intra and inter specific hybridization of two diploid ancestor species *Musa acuminata* (AA) and *Musa balbisiana* (BB) originating from India and Malaysia, respectively (Robison, 1996). This gave rise to the present day commonly cultivated triploid banana varieties that comprise of the desert and beer bananas (AAA), the plantains (AAB) and the cooking varieties (ABB) (Tenkouano, 2000; Gold *et al*., 2002). In most parts of the world bananas are grown as a permanent crop or on a system of replacing every 3-8 years or longer with most being produced for

local consumption in mixed cropping or as a subsistence crop in home gardens (Stover and Simmonds, 1986).

In Kenya, banana covers an estimated 2% of the country's total arable land, (74,000 hectares) and is predominantly grown by small-scale farmers for home consumption and domestic market (Qaim, 1999; ISAAA, 2001; Mbogoh *et al*., 2003). The crop is grown in various agro-ecological zones from coast up to an altitude of 2000 metres with a variation in varieties grown (Kungu, 1995; Qaim, 1999). In the highlands of Central, Eastern and Coastal region dessert cultivars that include the Cavendish and Gros Michel are the most commonly cultivated, while in the higher Western region, the East African highland bananas such as cv. Mbidde are the most common (Kungu, 1995; Seshu-Reddy *et al*., 1998).

The potential yield of bananas in Kenya stands at 40 t/ha against the actual yield of 10 t/ha, which is steadily declining (Qaim, 1999). The decline has been attributed to infestation by diseases and pests of which Fusarium wilt (*Fusarium oxysporum* fsp *cubense*), black and yellow sigatoka (*Mycosphaerella fijiensis* (Morelet) and *Mycosphaerella musicola* (Leach), nematode and weevils (*Cosmopolites sordidus*) complexes are the most important. (Wambugu *et al.*, 1999; Mbogoh *et al*., 2003). Following the rapid decline in banana production food, employment and income security in the banana producing areas of the country has been threatened. Traditional cultural practices in banana production have been a major cause of the problems in banana

production in Kenya. Through such traditional cultural practices, the farmers transmit unknowingly most of the banana pests and diseases through banana suckers as they search for and procure suckers as planting material from one farm to another. The spread of pests and disease through this practice can reduce banana yields by up to 90% (Mbogoh *et al*., 2003). Therefore, there is need to encourage farmers to use clean, disease and pest free planting materials of which tissue culture propagation techniques can provide such planting materials.

Plant parasitic nematodes are important pests of bananas in Kenya. A complex of nematodes *Pratylenchus goodeyi*, *Pratylenchus coffeae*, *Radopholus similis*, *Helicotylenchus multicinctus* and *Meloidogyne* spp. are reported on bananas in Kenya. Damage due to nematodes leads destruction of the root system thus reduced anchorage and water and nutrient uptake by the plant, reduced yields, lengthening the vegetative cycle and increasing plant toppling (Gowen and Quénéhervé, 1990; Sarah *et al.*, 1996; Gold and Merrianen, 2000; Brooks, 2004). The global annual banana yield loss due to nematodes has been estimated at 30-60% (Speijer and Fogain, 1998; Sikora *et al.*, 2003; Brooks, 2004). On East African Highland bananas *R. similis* has been reported to cause yield losses of up to 50% (Niere, 2001). Where nematodes occur in combination with banana weevils yield loss of up to 90% has been reported (Wambugu *et al.*, 1999).

Management of banana nematodes has been based on several strategies which have generally proved inadequate at the subsistence farm level. Chemical nematicides though

effective, the feasibility for use is beyond the economic means of most small scale farmers. Cultural management practices such as crop rotation, flooding and fallowing, which require the farmer to uproot and replant their fields after a period of time have not been applicable to small scale farmers, who have small holdings and depend on the crop as a source of livelihood (Whitehead, 1998; Sikora *et al.*, 2003). Development of nematode resistant cultivars would offer a long-term intervention against nematodes for resource poor farmers in Kenya. To date only a few sources of resistance to nematodes are available while maintaining a long term breeding programme is costly and fraught with difficulties (Pinochet, 1995; Whitehead, 1998; De Waele and Speijer, 1998; Swennen and Vulysteke, 2001). Biological control of plant parasitic nematodes may offer an alternative to nematicides due to environmental and health concerns associated with chemicals. Several biological control agents of nematodes that include nematode trapping fungi, endoparasitic fungi, and parasites of nematode eggs have shown potential for control of nematodes but research for exploiting them on field scale has so far yielded little success (Hopper and Evans, 1993; Coosemans, 1993; Sikora, 2000).

At present the use of clean planting material for establishment of new banana plantations and expansion of established ones is the most important measure for management of banana nematodes. Traditionally bananas are propagated by suckers which in most cases are infested by nematodes facilitating the spread of nematodes to new areas (Speijer *et al*., 2000; Sikora *et al*., 2000; Niere, 2001). Paring, followed by hot water treatment of suckers, significantly reduces nematode infestation of planting material. However, the

method is labour intensive and requires careful monitoring of water temperature to minimize negative effects of heat on the plant which has limited adoption of the method by farmers (Kashaija *et al*., 1998; Speijer *et al*., 2000). Provision of clean planting material through the tissue culture technique is a major step forward in an attempt to reduce the impact of nematodes.

The Kenyan farmers' practice of using pest and disease-infected sucker material for banana propagation is one of the main constraints for improving the crop's yield performance (Qaim, 1999). Tissue cultured (TC) planting material is the best means of providing pest and disease free planting material. TC plants are produced axenically making them sterile. Additional advantages of TC banana plants over the use of suckers are higher production, rapid multiplication, uniformity and more simultaneous plantation development, which facilitates orchard management (Athman, 2006). Another major advantage associated with the use of TC plants is the possibility of introducing new and superior germplasm in large quantities in a relatively short amount of time owing to the rapid multiplication rates (Niere, 2001). The use of TC banana plants also permits the introduction of beneficial microorganisms prior to release into farmer's fields.

In Kenya the use of TC banana for establishment of plantations has been widely promoted by the government and non-governmental organizations that have established efficient production systems and channels for distribution of plantlets to farmers. Over the previous ten years five hundred thousand farmers have planted TC banana plants,

which represent an average of fifty thousand farmers per year (Mbogoh *et al.*, 2003; Wambugu, 2004). The biggest limitation for use of the plantlets from the point of view of farmers is the high price of the material when compared to conventional suckers. A sucker for planting is usually provided free or at minimum cost while on average, a TC plantlet costs around KSh. 80 (Qaim, 1999). This implies higher cash outlay for plantation establishment, which is beyond the economic means of resource-poor small scale farmers. However, the higher yields in the crop and the ratoon have been shown to balance the initial investment (Robison, 1996). The price of TC plants is also likely to decrease when more skills in banana biotechnology are gained and when competition is created by other providers entering the market. Aid donors have also been disseminating free TC banana plants to farmers over an initial period. Non-governmental organizations have also played an important role as links between the TC laboratories and small scale farmers.

Despite the benefits offered by TC banana planting material, the material is highly susceptible to re-infestation by pest and diseases in the field, probably due to a lack of sufficient defense mechanism. Due to the sterile conditions under which the plantlets are produced they lose naturally beneficial microorganisms, such as endophytes, which increase host plant resistance against nematode attack in the field (Speijer *et al.*, 2000; Sikora and Pocasangre, 2004; Athman, 2006). There is therefore a need to develop new strategies that will improve the resistance of tissue culture banana plants for protection

against infestation in the field. Artificial introduction of beneficial microorganisms such as endophytes into sterile TC plants is a new approach that seems a promising option for sustainable and ecologically sound nematode management. The approach is necessary for offering protection to young plants against pest and diseases before being transferred to the field (Sikora and Schuster, 1998).

Endophytes are defined as micro-organisms that spend a portion of their lifecycle symptomlessly within the plant tissue but demonstrate antagonism against one or more pest or disease affecting the crop (Sikora *et al.,* 2003). Evaluation of endophytes for control of banana nematodes has been ongoing at the International Institute for Tropical Agriculture (IITA) in Uganda, where a number of endophytic *Fusarium oxysporum* isolates antagonistic to banana nematode have been identified and tested for their activity against the banana nematode *R. similis.* Results show the potential for endophytic *F. oxysporum* to kill *R. similis in vitro* and also reduce *R. similis* population and damage in endophyte-inoculated tissue culture plants (Niere, 2001; Athman, 2006). The fungal isolates when inoculated into TC banana plants have been able to colonize the plants roots, which has led to enhanced plant growth. For the endophytic isolates to provide sustained protection of bananas against the nematodes there is need to evaluate their efficacy under different environmental conditions and nematode species. In addition, their utilization in plant nematode management will require an understanding

of their effect on plant growth and their ability to colonize and persist in a wide diversity of banana cultivars.

1.1 PROBLEM STATEMENT

Nematodes are major pest affecting bananas worldwide. Control remains difficult with most control measures being inadequate at the subsistence farm level. The use of clean planting material produced through tissue culture is the most important measure for management of banana nematodes. The material however, is highly susceptible to re-infestation in the field. There is therefore a need to develop new strategies to enhance the resistance of the clean planting material. *Fusarium oxysporum* endophytic isolates are potential microorgainisms for biological enhancement of tissue culture banana plants. Preliminary results involving *F.oxysporum* endophytic isolates for control of banana nematodes have been promising (Niere, 1999; Athman, 2006). The isolates tested have shown potential to immobilize *R.similis in vitro* and *in vivo*. However, information on effect of the isolates on other nematode species attacking bananas, their effect on plant growth and appropriate inoculation techniques remains limited.

1.2 RESEARCH OBJECTIVES

Main objective

The main objective of the current research was to evaluate the efficacy of selected endophytic *F. oxysporum* isolates for control of banana nematode *Pratylenchus goodeyi* under screenhouse conditions.

Specific objectives

- To evaluate the effect of endophytic *F.oxysporum* isolates on growth of tissue culture bananas
- To screen endophytic *F.oxysporum* for control of the lesion nematode *Pratylenchus goodeyi* in tissue culture banana plants
- To evaluate the effects of different inoculation methods on root colonization of tissue culture banana plants by endophytic *F. oxysporum* isolates.

1.3 HYPOTHESES

- There are significant differences in growth between tissue culture banana plants inoculated with endophytic *Fusarium oxysporum* isolates and non inoculated plants
- There are significant differences in nematode damage and population between tissue culture banana plants inoculated with endophytic *Fusarium oxysporum* isolates and non inoculated plants

- The endophyte inoculation method has a significant effect on root colonization of tissue culture banana plants by endophytic *Fusarium oxypsorum* .

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 NEMATODES AS PESTS OF BANANAS

The species of nematodes found to be most detrimental to bananas are those involved in the destruction of primary roots, disrupting the anchorage system and resulting in the toppling of the plants (Luc *et al*., 1990). The most wide spread and important are *Pratylenchus goodeyi* and *Pratylenchus coffeae* (lesion nematodes*), Radopholus similis* (burrowing nematode*)* and *Helicotylenchus multicinctus*. It is also common to find some sedentary endoparasites such as *Meloidogyne* spp and *Rotylenchulus reniformis* parasitizing in the root system (Gowen and Queneherve, 1990). The root lesion nematode *P.goodeyi* is a major pest of *Musa.* It is considered to be indigenous to Africa where it is limited to the higher elevation zones of Central, Eastern and West Africa. It is an important pest of highland bananas (*Musa* AAA, Matooke and Mbidde groups) in Uganda, Tanzania, Kenya, Rwanda and Burundi; of both highland bananas and plantains in Cameroon, *Ensete* in Ethiopia and the main nematode affecting Cavendish bananas in Canary Islands (Bridge *et al.*, 1997).

Occurrence, abundance and distribution of banana nematodes in Kenya as in most places is influenced by several factors including elevation, soil characteristics and cropping history. At altitudes above 1000 masl, which include Central and Western regions *P. goodeyi* and *P. coffeae* are the most dominant while below 1000 masl (Coast and Eastern

region) *R. similis* and *H. multicinctus* are the dominant species (Gichure and Ondieki, 1977; Kungu,1995; Seshu Reddy *et al.*, 1998). In Uganda at elevations of 1500 masl and above *P. goodeyi* is often the only nematode species found in banana roots in densities that easily exceed 100,000 per 100 g fresh roots. In East and West Africa, *P.goodeyi* represents a pest almost exclusively of smallholder cultivation (Bridge *et al.*, 1997; Speijer and Fogain, 1998)

P.goodeyi is migratory endoparasitic nematode completing its life cycle in less than 30 days in roots and corm tissue at a temperature of 25^{o}-30^{o}C. Penetration of nematodes in the roots occurs along the root apex though invasion can occur at any point along the root length. The nematodes after penetration occupy the intercellular position in the cortical parenchyma and migrates in and between the cells in the root cortex feeding on the cell cytoplasm. This results in collapsed cell walls, cavities and tunnels. On the corm, lesions begin to develop at the point where infested roots are attached and extend outwards. The females lay eggs inside the roots and all development stages occur within the root. All life stages and sexes are infective and cause damage. Dissemination of nematodes to new areas occurs through vegetative propagation using infested suckers or corms (Sarah *et al.*, 1996).

Damage due to nematodes occurs as a result of their migration and feeding activity inside the plant roots. Symptoms of damage are identified on longitudinal section of roots as reddish brown necrotic lesions confined in the root cortex, extending from the

root surface to the centre (Speijer and De Waele, 1997). Nematode feeding causes destruction of the root system and corm tissue, reducing water and mineral uptake hence reduction in plant growth and development. This causes a severe reduction in bunch weight and lengthening the vegetative cycle. Due destruction of the root system, plant anchorage is weakened thus the plants are easily blown over by wind a condition referred to as toppling which is a major cause of yield loss in bananas and plantains (Gowen, 1993; Jones, 2000; Gowen and Queneherve, 2005). The roots infested by nematodes are also easily colonized by soil borne pathogens become necrotic, atrophy, and may finally die (Gowen and Queneherve, 1990).

P. goodeyi has been shown to cause yield losses at elevations above 1350 masl (Elsen *et al.* 1988; Speijer *et al.* 1998). Surveys of East Africa highland bananas in Tanzania have shown 39.5% necrosis due to attack by *P. goodeyi* and 37.5% necrosis in roots where mixed populations of *P.goodeyi* and *Helicotylenchus multicinctus* were present (Bridge, 1993). Nematodes and banana weevils frequently occur together on the same plant thus their damages are highly associated which aggravates the loss of banana plants. Due to the association between banana nematodes and weevils it's often difficult to estimate the actual yield loss caused by nematodes in farmers' fields and at times snapping caused by banana weevils is confused with toppling (Gold *et al* ., 2000). The nematode induced losses are a result of an increase in number of dead roots, root necrosis, reduced number

of standing leaves, reduced flower production, increased plant toppling and reduction in bunch weights (Athman, 2006).

2.2 MANAGEMENT OF NEMATODES IN BANANAS

Several control methods have been suggested to reduce the production losses caused by the nematodes. However, banana nematodes are a difficult group of organisms to control as they generally live well protected in the roots and rhizomes (Gowen and Quénéhervé 1990). Conventional methods of establishing plantations using nematode infested suckers as planting material are the main avenues of disseminating nematodes to new fields (O'Bannon, 1977). Several nematode management options are however available.

2.2.1 Cultural control

The most important cultural control method is use of healthy planting material. The clean planting material is obtained through several ways that include pairing and hot water treatment and use of tissue culture techniques. Paring procedure and hot water treatment which involves removal of nematode infested roots and corm tissue have been found to be effective. The corm-pared suckers are treated by dipping in hot water at a temperature of 54^{o}C for twenty minutes (Speijer *et al*., 1995). Speijer *et al*. (1995) reported up to 30% crop yield improvement when the method was used. Additionally the material has been found to have faster rate of development and lower pest infestation rates (Seshu -Reddy *et al*., 1998). However, technique is quite difficult to manage by the small-scale farmers due to the critical balance in administering the required temperature

at 54°C for 20 minutes. The method though effective provides temporary control as re-infestation readily occurs in the field (Speijer *et al.,* 1995). Crop rotation can also mitigate the effects of nematodes on banana plants. The method operates on the principle of starving nematodes to death, therefore inducing a decline in nematode populations in a field, so that the next banana crop starts with a low initial inoculum. Adoption of the method is however constrained by land pressure and cultivation of bananas as a perennial crop (Kashaija *et al*., 1998). In addition, where crop rotation is practiced some yield loss still occurs because most rotations are seldom broad enough to reduce nematode densities below damage levels (Sikora *et al.,* 2003).

2.2.2 Chemical control

Nematicides are widely used by growers producing fruits for export trade, a number of organophosphates and carbamates are used (Gowen, 1993). However, their use is often prohibitive for many resources poor small scale farmers, registered products are highly toxic, expertise is required for application and most of them have been phased out of the market (Gowen and Queneherve, 1990). The pesticide usually inactivates the nematode within the plant tissue or in the soil, which after microbial degradation the nematode recovers and damage continues (Sikora and Pocasangre, 2004).

2.2.3 Biological Control

Identification and use of biological control organisms against nematodes and of the factors that favour their development would be an important step in management of

banana nematodes. Various organisms have shown potential as biocontrol agents of banana nematodes. Isolates of fluorescent bacteria *Pseudomonas fluorescens* and *Pseudomonas putida* showed repellent effects on *R.similis in vitro* and lower nematode invasion *in vivo (*Aalten *et al.,* 1998). Antagonistic fungi like *Arthrobotrys* spp., *Paecelomyces pilacinus,* and rhizobacteria (*Pseudomonas* spp.) are potential control agents of nematodes. These micro organisms need to be studied for efficacy under the various agrosystems so as to contribute to reducing nematode damage on bananas and plantains (Kashaija *et al*., 1998).

2.2.4 Host Plant Resistance

Plant resistance is probably the best form of nematode control, especially for resource-poor farmers who cannot afford the high cost of nematicides (Sikora *et al.,* 2003). Several evaluations of plantains (AAB), Cavendish (AAA) and East African highland bananas (AAA-EA) have been carried out to look for clones with lower susceptibility level that could be recommended to farmers to replace the susceptible ones. Strong resistance to *R.similis* has been identified in Pisang jari buaya clones (Pinochet and Rowe, 1979) and in the banana cultivar Yangambi Km5 (Fogain and Gowen, 1998). There are no widely confirmed sources of resistance to the banana lesion nematodes *P.goodeyi* and *P.coffae.* There are however, indications that Calcutta 4, a diploid AA variety which is used as a female parent in the *Musa* breeding programme at FHIA, is resistant to *P. coffeae* (Viaene *et al.,* 1998) while Yangambi Km5 appears also to be resistant to *P. goodeyi* (Fogain and Gowen, 1998). Resistance to nematodes has also not

being identified in East Africa Highland cooking cultivars. Improvement of bananas through conventional plant breeding remains a challenge due to the genetic complexity of the crop and the long period required to evaluate crossings for resistance to different nematode collections (Pinochet, 1995; Swennen *et al.*, 2001; Tripathi, 2003).

2.3 TISSUE CULTURE BANANAS

Banana nematodes are dispersed mainly through infested planting material. Adoption of clean planting material would help reduce infestation to new plantations and thus delay pest population build up (Seshu-Reddy *et al.*, 1998). Tissue cultured planting material is the best means of providing pest and disease free planting material (Mateille *et al.*, 1994; Sarah, 2000). Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant on artificial media (Vuylsteke, 1989). Micro propagation of banana has been achieved by use of standard shot-tip protocols as described by Vulysteke (1998).

The shoot cultures are initiated from any plant part that contains a shoot meristem mainly the parental pseudostem, small suckers, peepers and lateral buds (Vuylsteke, 1989). The apex of the inflorescence and axillary flower buds are also suitable explants for tissue culture initiation (Cronauer and Krikorian, 1985). The initiation media is based on the Murashige and Skoog (1962) mineral salt mixture (MS) with some modifications as suggested by Vulysteke (1998). Plant multiplication begins eight weeks after initiation by sub-culturing the emerging buds every four weeks to modified MS media in

sterile jars. Formation of multiple shoots and buds is promoted by supplementing the medium with relatively high concentrations of cytokinins. The multiplication stage takes between twelve to sixteen weeks, after which root growth is induced. After rooting, plants are hardened *in vitro* for 2-4 extra weeks on the regeneration/rooting medium prior to weaning on soil. The process leads to production of plantlets that are pest and disease free (Cronauer & Krikorian, 1984; Vulysteke 1998).

The absence of nematodes and other diseases in tissue cultured banana plants is considered important long-term advantages of tissue cultured plants. However, if the plants are transplanted to fields with a history of nematodes they have been found to be susceptible than the sucker derived materials (Daniells, 1997; Smith *et al.*, 1998). Smith *et al.* (1998) hypothesized that the loss of beneficial such as endophytes during the culture initiation may be responsible for the grater susceptibility of tissue culture banana plants to nematodes.

2.4 ENDOPHYTES AS BIOCONTROL AGENTS

The term endophyte generally refers any organism that lives inside plant tissue for all or part of their life cycle without causing any disease symptom or any apparent injury to the host plant (Petrini, 1991). The relationship between the two species can be neutral, antagonistic or mutualistic. Endophytes are contained entirely within plant tissues, are asymptomatic and be described as mutualistic (Clay, 1990). A mutualistic relationship

refers to an interrelationship in which the fitness of both partners is maintained or even increased due to presence of the partner (Griesbach, 1999).

Presence of endophytes has been demonstrated in many plants, including important crops such maize (Fisher *et al*., 1992), rice (Fisher and Petrini, 1992) and tomato (Hallman and Sikora, 1994). Among the best studied endophytes are intercellular symbiotes in the family Clavipitaceae found in many cool season grasses, which are known to benefit the host with improved tolerance to heavy metals, increased drought resistance, systemic resistance to pests and pathogens and enhanced growth (Arnold *et al.,* 2003). Presence of endophytes has been reported in banana plants. Endophytic fungi have been isolated from healthy banana plants growing in nematode and banana weevil infested plantations in Uganda (Griesbach, 1999; Niere, 2001) and Central America (Pocasangre, 2000) of which *Fusarium oxysporum* has been documented as the dominant species.

F. oxysporum is a cosmopolitan anamorphic fungus occurring in soils. Majority of strains are non- pathogenic soil inhabitants (Kistler, 1997) while a number of specialized forms cause vascular wilt and root rot on many important crops plants (Gordon *et al.*, 1989). It is one of the most variable species with regard to microscopic and macroscopic characters. Shape and size of the micro- and macroconidia, the formation of chlamydospores and short lateral monophialides distinguish *F. oxypsorum* from other

species (Kistler, 1997). Non–pathogenic isolates of *F.oxysporum* are one of the best studied microrganisms used for biological control of soil borne-plant diseases (Mandeel and Baker, 1991; Alabouvette *et al.,* 1996; Fuchs *et al*., 1997; Duijff *et al.,* 1999) and nematodes (Hallmann and Sikora, 1994). Isolates of *F.oxysporum* have also been shown to produce plant growth- promoting substances (Clay and Schardl, 2002; Spiering *et al*., 2006).

Endophytes have been reported to be antagonistic to plant parasitic nematodes. This has been widely studied in grasses where, for example, in tall fescue (*Festuca arundinacea*) endophytic fungi *Acremonium coenophialium* adversely affected populations of *Meloidogyne marylandi* in inoculated plants when compared to non- inoculated plants (Kimmons *et al.,* 1989). Reproduction of *Pratylenchus scribneri* was hindered in tall fescue plants inoculated with endophytic fungi *Neotyphodium coenophialium* (*Kimmons et al.,* 1990). In food crops similar observations have been made. In tomato Hallman and Sikora (1994) reported the antagonistic effect of endophytic *Fusarium oxysporum* on *Meloidogyne incognita.* Inoculation of tomatoes with endophytic *F. oxysporum* isolates resulted in 60% reduction of *M. incognita* infection. Endophytic *F.oxysporum* isolates have been evaluated for control of banana nematode *R.similis*. Culture filtrates have been shown to have inactivating effects on *R.similis* both *in vitro* and *in vivo (*Niere, 1999; Athman, 2006).

Mechanisms of action of endophytes against nematodes as well as other pests and diseases are not well understood. However, various mechanisms have been suggested that include competitive exclusion, parasitism, metabolites production and induced resistance (Azevedo *et al*., 2003). The presence of endophytes makes the plant tissue unacceptable and unpalatable to the pest and also gives rise to toxic effects on the pest causing poor growth, development reduction, and reduced reproduction capacity or death (Isaac, 1992). Kimmons *et al.* (1989) reported reduced juvenile emergence from eggs and number of eggs per egg mass of *Meloidogyne marylandi* in tall fescue inoculated with endophytic *Acremonium coenophialium.*

Production of toxic compounds is another important mechanism of action of endophytes against plant parasitic nematodes. Production of alkaloids toxic to insects and herbivores by grasses harbouring endophytes has been documented (Breen, 1994; Yue *et al*., 2001). In bananas Griesbach (1999) recorded the production of secondary metabolites by endophytic *F.oxysporum* that were toxic to the banana weevil larvae and eggs *in vitro*. Secondary metabolites in culture filtrates of endophytic *F.oxysporum* isolated from tomato roots were found to cause inactivation of infective juveniles of *Meloidogyne* sp. and *Heterodera* sp. The same metabolites were reported to cause inactivation of adults and juveniles *Pratylenchus zeae* and *R. similis* (Hallmann and Sikora, 1995).

Induced resistance has been proposed as a possible mechanism of endophytes against plant parasitic nematodes. It is enhancement of plant defensive capacity against a broad spectrum of pathogens or pests that are acquired after appropriate stimulation (Stone *et al.*, 2004). Endophytes have been shown to induce resistance in inoculated plants. The bacterial endophyte *Pseudomonas fluorescenes* was reported to induce resistance in sugarbeet that inhibited penetration of cyst nematodes *Heterodera schachtii* (Ramamoorthy *et al.*, 2001). Fungal endophytes may induce resistance responses in plants by means of structural and physiological/biochemical changes. Studies indicate that presence of endophytes in the root induces fortification of cell walls by increased production of polysaccharides and increased lignification, which could hamper penetration of plants by plant parasitic nematodes (Elsen *et al*., 2003). Biochemical responses include synthesis of defense related chemicals, such as phenolics, which are recognized as plant resistance factors against nematodes (Fogain and Gowen, 1996).

Biological control of pests and diseases is related to establishment of the antagonistic organisms at the target site (Larkin and Fravel, 1998). Thus the ability of an ideal endophyte to effectively and persistently colonize the host plant is important. Colonization of plant tissue by endophytes is a complex process that involves host recognition, spore germination, penetration and growth of the endophyte within the tissues. Penetration occurs through natural openings or wounds or actively using hydrolytic cellulases and pectinases forming inconspicuous infections within plant tissue for all or part of their life cycle (Siegel *et al.*, 1987; Rutherford *et al.*, 2002). To detect

endophytic colonization in plants several methods have been developed. The simplest method involves microscopic examination of differently stained samples of endophyte – infected plants (Saha *et al.,* 1988). Other methods for *in situ* detection of endophytes have been developed for example the use of monoclonal antibodies (Hiatt *et al.,* 1999) and electron microscopy (Sardi *et al.,* 1992). Colonization and persistence of introduced fungal endophytes in tissue culture banana plants has widely being studied through re-isolation from infected root and corm tissue (Pocasangre, 2000; Paparu, 2005). Although different methods of introducing fungal endophytes into tissue cultured banana plants have been evaluated (Paparu, 2005), a cost effective method has so far not being developed.

Research on the control of nematodes suggests that no single control strategy will provide complete control (Sikora *et al.,* 2003). A broad integrated pest management (IPM) approach including new components of pest control is necessary to safe guard sustainable banana production. The use of endophytes for management of banana nematodes is a new approach that offers potential for economically and environmentally sustainable nematode management system. Endophytes share the same ecological niche with endoparasitic nematodes, insect borers and fungal pathogens thus they are effective at the exact site of the pest or disease attack, which gives them an edge over other biological control agents (Sikora and Pocasangre, 2004). They spend most of their life inside the plant tissues where the host provides a relatively uniform and protected environment enabling the endophytes to avoid microbial competition and extreme

environmental conditions (Ramamoorthy *et al.*, 2001). Endophytes are easy to culture *in vitro* and can be applied on TC banana plantlets thus reducing the inoculum level required (Gold *et al.,* 2003). Once developed farmers will not be required to apply the control product themselves as this may be done by private and public organizations engaged in commercial TC production (Athman, 2006). However, use of fungal endophytes for management of nematodes in banana production systems remains largely unexploited.

CHAPTER 3

3.0 GENERAL MATERIALS AND METHODS

3.1 SITE DESCRIPTION

All the experiments were carried out in a screenhouse at the Kenya Plant Health Inspectorate Service Plant Quarantine Station (KEPHIS), approximately 25 km west of Nairobi ($0^{o}10^{'}$S, $36^{o}40$'E). The station is situated at approximately 2095 masl with a mean daily temperature of 16.8^{o}C.

3.2 TISSUE CULTURE PLANTS

Tissue culture banana plants used for the experiments were obtained from Jomo Kenyatta University of Agriculture and Technology commercial tissue culture laboratory. The plants were micro propagated using a standard shoot-tip culture protocol for banana according to Vuylsteke (1998).

Production of tissue cultured plants

The production of tissue culture plants involved four stages namely; initiation of an aseptic culture, multiplication of propagules, rooting of plantlets and hardening of the plantlets in the nursery. Banana suckers from healthy mother plants with bunches were obtained from Jomo Kenyatta University of Agriculture and Technology field collection. The roots of the suckers were removed and plants washed thoroughly with water. The leaf bases were removed and cubes of 2-4cm^3 of healthy plant tissue containing the

shoot tip were obtained. The shoot tips were surface sterilized by washing in 70% ethanol for 30-60 seconds followed by a 20 minutes treatment in a 2% hypochlorite solution. The surface sterilized shoot tips were washed three times in sterile water, dried shortly on sterile tissue paper and cut into pieces of about $1cm^3$ before being transferred onto initiation media. The initiation media was based on the Murashige and Skoog (1962) mineral salt mixture as shown in Table 1

Table 1. Composition of Murashige and Skoog (1962) medium for growing tissue culture banana plants *in vitro*

Major salts	**Quantity (mg/l)**	**Minor Salts**	**Quantity (mg/l)**
KNO_3	1900	Mn SO_4. 4 H_2O	22.3
NH_4NO_3	1650	H_3BO_3	6.2
$CaCl_2$. 2 H_2O	440	Zn SO_4. 4 H_2O	8.6
$MgSO_4$.7 H_2O	370	KCL	0.83
KH_2PO_4	170	Na_2MoO_4. 2 H_2O	0.25
$FeSO_4$.7 H_2O	27.8	$CuSO_4$. 5 H_2O	0.025
Na_2EDTA. 2 H_2O	37.3	$CoCl_2$. $6H_2O$	0.025
Organic supplements	**Quantity (mg/l)**	**Growth regulators**	**Quantity (ml/l)**
Glycine	2	Indole-3- Acetic acid	0.5 ml/l
Thiamine- HCL	0.5	Benzylaminopurine (BAP)	5ml/l
Pyridoxine -HCL	0.5		
Nicotinic acid	0.5		
Ascorbic acid	20		
Sucrose	30 g/l		
Phytagel	2.3g/l		

Stock solutions of the major salts (KNO_3, NH_4NO_3, $CaCl_2$. 2 H_2O, $MgSO_4$.7 H_2O, KH_2PO_4) and minor salts (Mn SO_4. 4 H_2O, H_3BO_3, Zn SO_4. 4 H_2O, KCL, Na_2MoO_4. 2 H_2O, $CuSO_4$. 5 H_2O, CoCl2. $6H_2O$) were prepared by dissolving the salts in distilled water. Iron stock solution was prepared using $FeSO_4$.7 H_2O and Na_2EDTA. 2 H_2O, which were heated to dissolve in distilled water. Vitamins and Amino acids (Glycine, Thiamine, Pyridoxine, and Nicotinic acid) were dissolved in distilled water. Growth regulators were dissolved in 1N NaOH and final volume adjusted with distilled water. The stock solutions were stored at 4^oC.

The Murashige and Skoog basal salt was mixed with sucrose in distilled water. The mixture was placed on hot- plate stirrer and the phytagel, vitamins and the phytohormones added while heating. The pH was adjusted to 5.8 using 1N NaOH and 1N HCL. The media was autoclaved for 20min at 121^oC and 1 bar pressure. The media was dispensed in culture vessels. The cultures were incubated at 27-28^oC and 14 hrs of light for a period 3 weeks before being transferred to fresh media

Subculturing for multiplication was done by subdividing shoot cultures under sterile conditions and transferring the propagules to fresh medium. This was usually done every 4 weeks until sufficient numbers of plantlets were obtained for regeneration. Shoots at this stage do not have roots and were transferred to rooting medium for root initiation (Plate 1). The plants were maintained in the rooting media for a period of 4 to 6 weeks after which they were ready for hardening (Plate 2).

Plate 1. Tissue culture banana plants at multiplication stage.

Plate 2. Tissue culture banana plants at hardening stage.

3.3 FUNGAL ISOLATES

Three *F. oxysporum* endophytic strains *V5W2, Emb 2.4o* and *Eny 7.11o* were used for the experiments. The isolates were obtained from the International Institute of Tropical Agriculture (IITA) Uganda where they were isolated from roots and corms of healthy East African highland banana (*Musa* spp. EA-AAA) plants. They selected for their ability to immobilize and kill nematodes *in vitro* (Niere, 2001).

3.3.1 Preparation of fungal inoculum

The *F. oxysporum* endophytes preserved on pieces of filter paper in ependorf tubes at 4^{o}C were sub cultured by plating a piece of filter paper containing mycelium of each strain on synthetic nutrient agar (SNA) (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 NaOH (1 M) and 13.2 g agar, 0.1 g penicillin G, 0.2 g streptomycin sulphate and 0.05 g chlortetracycline / L distilled water) in 90 mm diameter Petri dishes under sterile conditions. The plates were maintained ± 25°C with a natural photoperiod of 12: 12 L: D hrs for 7-10 days to enable sufficient growth and sporulation for preparation of a suspension. The fungal suspension was obtained by taking four cubes of media (approximately 0.5 cm^3) containing spores and mycelia from the culture and inoculating into 100 ml sterile potato dextrose broth (PDB) (4 g potato infusion obtained from 200 g potatoes and 20 g glucose /L distilled water) in 200 ml Erlenmeyer flasks. The flasks were incubated in the laboratory for 7 days. During incubation, the flasks were shaken once daily to ensure even distribution and fungal

growth. For each treatment, 200g of maize bran was weighed, placed in a 500 ml Erlenmeyer flask, moistened by adding water and autoclaved for 30 min at 121^{o}C. The substrate was allowed to cool overnight before re-autoclaving for 30 min. A sterile pipette tip was used to transfer 30 ml of the fungal suspension into each of the flasks with maize bran. Non-inoculated maize bran was used as a control. The solid substrate was maintained at room temperature for 10 days. Frequent observation of mycelia growth was performed. The concentration of the endophytes in the solid substrate was determined by adding two grams of the solid substrate into 98 ml of water. Fungal spores were harvested by filtering the suspension through a 1-mm- diameter sieve to remove mycelial fragments. Spore densities were estimated using a haemocytometer. The spore concentration in the solid substrate was standardized to provide a final spore concentration of 1.65 x 10^{6}spores/2g. The solid substrate was either diluted by adding sterile maize bran or concentrated by adding solid substrate with the fungal inoculum Two gram of the solid substrate were weighed and used to inoculate the plants.

3.3.2 Inoculation of plants with endophytes

Banana plants after deflasking were washed of adhering media with tap water and selected for uniformity in size. The plants were planted in weaning trays (60cm x 30cm) and allowed to grow for one month before being transplanted into three litre potting bags (5 ×9 ×4 cm) where they were allowed to grow for one month before inoculation.

The potting bags were filled with steam sterilized sandy loam soil and were watered to field capacity. A hole was made in the middle a banana plant was placed on the hole and the roots sprinkled with the two grams maize bran containing the fungal inoculum. Control treatment plants were inoculated with sterile maize bran.

CHAPTER 4

EFFECT OF ENDOPHYTIC *Fusarium oxysporum* ON GROWTH OF TISSUE CULTURE BANANA PLANTS

4.0 ABSTRACT

The effects of three endophytic *Fusarium oxysporum* isolates (*V5W2, Emb 2.4o* and *Eny 7.11o*) on the growth of tissue culture banana plants was evaluated in screen house trials. Two experiments were conducted to determine the effect of the fungal isolates on banana plant growth. Dessert banana cv. Giant Cavendish and cv. Grand Naine were inoculated with the endophytic isolates two months after weaning and evaluated six months later. Plant response to endophyte treatment was assessed from plant height, girth, number of functional leaves, length and width of the youngest leaf, fresh and dry root and shoot weights. Improved growth was observed for plants inoculated with endophytes when compared to the control treatment for all the growth parameters, though the differences were not significant. Up to 14.4% increase in plant height, 12.9% increase in girth, 38.6% increase in shoot weight and a 36% increase in root weight was observed for endophyte inoculated plants when compared to the control treatment. The study shows that endophytes may have potential to enhance growth of tissue cultured banana plants.

4.1 INTRODUCTION

Endophytes are mutualistic symbionts that live symptomlessly for a whole or part of their life cycle in plant tissues, receiving nutrition and structural refuge from the host, while benefiting the host with enhanced growth and health (Faeth and Fogain, 2002; Sikora *et al.*, 2003; Paparu *et al.*, 2004; Ju, 2006). A wide diversity of endophytic species have been isolated from a variety of healthy plants in various plant parts including seeds, roots, stems and leaves (Yates *et al.*, 1997; Faeth, 2002; Bandara *et al.*, 2006). The effect of endophytes on plant growth has been widely studied in grasses, where improved growth, size and quality of the host plant has been documented. Baker *et al.* (1984) reported increased biomass production in perennial ryegrass infected with endophytic *Lolium perenne*, while Latch *et al.* (1984) reported a significant increase in leaf area of perennial ryegrass inoculated with fungal endophyte *Acremonium lolii*. Clay and Schardl (2002) showed that endophyte infected plants of *Lolium multiforum* had more vegetative tillers and allocated more biomass to roots and seeds than endophyte-free plants. Schardl and Phillips (1997) reported enhanced tillering and root growth in tall fescue inoculated with endophytic *Neotyphodium coenaphialum*.

The positive effect of endophytes on plant growth has been thought to result from a number of mechanisms, the most important being production of phytohormones and enhanced uptake of nutrients from the soil (Costa Pinto *et al.*, 2000; Clay and Schardl, 2002; Spiering *et al*., 2006; Bandara *et al.*, 2006). For example, Nassar *et al.* (2005)

documented the production of indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPYA) by yeast endophytic *Williopsis saturnus* colonizing maize roots. Despite the strong evidence for improved growth in endophyte-infected grass, information on the effect of endophytes on growth of other crops is rather rare and in most cases research has failed to provide conclusive results.

Bananas have been found to host a wide diversity of endophytic species of which *F. oxysporum* has been documented as the dominant species (Griesbach, 1999; Pocasangre, 2000; Niere, 2001). Research on the potential of endophytic *F. oxysporum* to control pests, mainly the nematodes and weevils constraining banana production, has yielded positive results (Griesbach, 1999; Pocasangre, 2000; Niere, 2001; Athman, 2006). However, results on the effect of endophytic *F. oxysporum* on the growth of banana plants have not been conclusive. Pocasangre (2000) and Niere (2001) reported higher root and shoot weights of tissue culture plants treated with *F. oxysporum* endophytes when compared to the control, although results were not consistent for all isolates. There is also need to test these isolates on different banana cultivars and under different environmental conditions.

The objectives of the current study were: 1) to evaluate the effect of endophytic *F. oxyporum* isolates on the early post-flask weaning development of tissue culture banana

plants under and 2) to evaluate the response of different banana cultivars to endophytic fungi inoculation.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

The experiment comprised two factors: treatment and cultivar. Four treatments included the three *F. oxysporum* endophytic strains *V5W2, Emb 2.4o* and *Eny 7.11o* and an uninoculated control. Two dessert banana cultivars Giant Cavendish (*Musa* spp. AAA) and Grand Naine (*Musa* spp. AAA) were used. The experiment was replicated three times with a total of eighteen plants per treatment. The experiment was laid down in a split plot design with cultivars as the main plot factor and treatments as the subplot factor. The experiment was conducted two times. Propagation of tissue culture banana plants, preparation of fungal inoculum and inoculation of plants with endophytes followed the protocol described in Chapter 3.

4.2.2 Assessment of plant growth parameters

Plant height, girth, number of functional leaves (NOFL), length and width of the youngest leaf were assessed. The growth parameters were taken every two weeks for all plants for the duration of the experiment. Plant height was measured as the distance from the pseudostem base to the point where the youngest leaf emerges from the pseudostem. Leaves were considered functional when three quarters of the leaf was green as opposed to yellow or dry leaves. Width of the youngest leaf was measured from the widest part of the young leaf. At conclusion of the experiments shoot and root fresh

weights were recorded, before oven drying at a temperature of 70^{o}C for 48 hours and the shoot and root dry weights recorded.

4.2.3 Data analysis

All data were first checked for normality and variance homogeneity using the Lervene option of the Hovetest prior to analysis (SAS institute 2002). Factor effects and factor interaction effects were determined using the Generalised Linear Model procedure of the SAS programme (SAS Institute, 1999) and means compared using the Tukey test at α = 5%. With significant factor interaction analysis for the effects on one factor was performed at each level of the other factor.

4.3 RESULTS

Significant differences (P<.0001) for most plant growth parameters were observed between the two experiments, hence experiments was analyzed separately. Significant variations in growth (P<0.0001) were also observed between cultivars in both experiments hence data were further separated by cultivar. Two-way interaction between treatments and banana cultivar was not significant (P>0.05) for any growth parameter.

4.3.1 Plant Growth Parameters

In experiment one, Giant Cavendish plants treated with endophytic isolate *Emb2.4o* grew relatively taller than those treated with *V5W2* and *Eny 7.11o* (Table 2). The control plants were relatively shorter (P=0.4573) than *Emb2.4o*, *V5W2* and *Eny 7.11o* by 14.4%, 11.3 % and 7.2% respectively but not significantly so. Grand Naine plants with endophytic isolate *V5W2, Emb2.4o* and *Eny* 7.11o. were relatively taller than the control treatment plants (P=0.5051) by 11.3%, 5.2% and 2.6 % respectively though this was not significant (Table 2). Inoculation of Giant Cavendish plants with endophytic isolates *V5W2, Emb2.4o* and *Eny 7.*11o led to a relative non significant (P=0.2172) increase in plant girth by 12.9 % and 6.4 % respectively when compared to the control treatment. Treatment of Grand naine plants with endophyte isolate *V5W2* led to a non significant (P=0.8482) 2.4 % increase in plant girth when compared to the control treatment (Table 2). The difference in the number of functional leaves between endophyte inoculated plants and the control treatment plants varied in both varieties (Table 2). Endophytic

Giant Cavendish plants with isolates *V5W2, Emb2.4o* and *Eny* 7.11o had relatively more (P=0.1377) leaves compared to control plants , while Grand naine endophytic plants with isolate *Emb2.4o* produced relatively more functional leaves than the controls (P=0.2959). Following inoculation of plants with endophytic isolates the youngest leaf was consistently but non-significantly longer than control plants for Giant Cavendish plants (P=0.3854). For Grand naine plants though, plants treated with isolate *Emb2.*4o and *Eny* 7.11o produced relatively shorter leaves than the controls but non significantly so (P=0.6207) (Table 2). The youngest leaves of plants inoculated with endophyte isolates were generally wider than control plants but non-significantly for both Giant Cavendish (P= 0.2457) and Grand naine (P= 0.6078) (Table 2).

Table 2. Plant growth parameters of tissue culture banana plants cv. Giant Cavendish and Grand naine, 24 weeks after inoculation with endophytic *Fusarium oxysporum* isolates in the screen house **(Experiment 1)**

Treatments	**Growth Parameter**				
Giant Cavendish	**Height (cm)**	**Girth (cm)**	**NOFL**	**Leaf Length (cm)**	**Leaf width (cm)**
V5W2	10.8 ± 0.63 aA	3.5 ± 0.14 aA	6.2 ± 0.14 aA	17.2 ± 0.70 aA	8.1 ± 0.37 aA
Emb2.4o	11.1 ± 0.74 aA	3.5 ± 0.16 aA	5.8 ± 0.18 aA	18.1 ± 0.80 aA	7.9± 0.43 aA
Eny 7.11o	10.4 ± 0. 67 aA	3.3 ± 0.14 aA	5.9 ± 0.17 aA	16.9 ± 0.78 aA	8.4 ± 0.44 aA
Control	9.7 ± 0.61 aA	3.1 ± 0.14 aA	5.7 ± 0.16 aA	16.1 ± 0.69aA	7.2 ± 0.38 aA
Grand Naine	**Height (cm)**	**Girth (cm)**	**NOFL**	**Leaf Length (cm)**	**Leaf width (cm)**
V5W2	12.8 ± 0.66 aA	4.2 ± 0.17 aA	6.5 ± 0.15 aA	19.0 ± 0.70 aA	9.6 ± 0.37 aA
Emb2.4o	12.1 ± 0.59 aA	4.1 ± 0.16 aA	6.6 ± 0.13 aA	18.1 ± 0.60 aA	9.3 ± 0.34 aA
Eny 7.11o	11.8 ± 0.64 aA	4.1 ± 0.16 aA	6.2 ± 0.15 aA	18.0 ± 0.66 aA	9.4 ± 0.36 aA
Control	11.5 ± 0.58 aA	4.1 ± 0.16 aA	6.5 ± 0.14 aA	18.2 ± 0.64 aA	9.0 ± 0.35 aA

NOFL- Number of functional leaves. In columns within a variety means followed by the same small case letter are not significantly different at $P<0.05$. Between varieties means followed by the same capital letter for each parameter are not significantly different at $P<0.05$. Values represent the mean ± standard error for three replicates (n=18).

In the second experiment, Giant Cavendish plants inoculated with *V5W2*, and *Eny 7*.11o were relatively taller than the control plants by 6% and 4.3% though non significant (P=0.7172). Grand naine plants with endophytic isolates *Emb2.4o* and *Eny 7*.11o grew relatively taller than the control plants by 2.2% but not significantly so (P=0.8681) (Table 3). Treatment of Giant Cavendish plants with endophytic isolates *V5W2*and *Eny 7*.11o led to a non significant 6.6% and 4.4% increase in plant girth respectively (P=0.5243), while for Grand naine plants treated with endophytic isolates *Emb2.4o* and *Eny 7*.11o plant girth was 2.4% higher when compared to the control treatment but not significantly (P=0.9344) (Table 3). The difference in the number of functional leaves between endophyte inoculated plants and the control treatment plants varied in both varieties. Giant Cavendish plants with endophytic isolates *V5W2* and *Eny 7*.11o had relatively more (P=0.6901) leaves compared to control plants while Grand naine plants with isolate *Emb2.4o* produced relatively more functional leaves (P=0.3253) (Table 3). The youngest leaves of plants inoculated with endophyte isolates were generally longer and wider than control plants but non-significantly and not consistently for both Giant Cavendish (P=0.5969, P= 0.9765) and Grand naine (P=0.7396 P= 0.8154) (Table 3).

Table 3. Plant growth parameters of tissue culture banana plants cv. Giant Cavendish and Grand naine, 24 weeks after inoculation with endophytic *Fusarium oxysporum* isolates in the screen house **(Experiment 2**)

Treatments	**Growth Parameter**				
Giant Cavendish	**Height (cm)**	**Girth (cm)**	**NOFL**	**Leaf Length (cm)**	**Leaf width (cm)**
V5W2	19.3 ± 1.1aB	4.8 ± 0.21 aB	7.2 ± 0.13 aB	25.1 ± 0.94 aB	11.8 ± 0.49 aB
Emb2.4o	17.8 ± 1.0aB	4.5 ± 0.21 aB	7.1 ± 0.15 aB	24.0 ± 0.99 aB	11.7 ± 0.49aB
Eny 7.11o	19.0 ± 1.0aB	4.7 ± 0.20 aB	7.2 ± 0.13 aB	25.3 ± 0.91 aB	11.8 ± 0.43 aB
Control	18.2 ± 1.1 aB	4.5 ± 0.20 aB	7.0 ± 0.14 aB	24.3 ± 0.97 aB	11.6 ± 0.52 aB
Grand Naine	**Height (cm)**	**Girth (cm)**	**NOFL**	**Leaf Length (cm)**	**Leaf width (cm)**
V5W2	13.4 ± 0.83 aB	4.2 ± 0.18 aB	7.2 ± 0.15 aB	19.2 ± 0.94 aB	11.6 ± 0.80 aB
Emb2.4o	14.1 ± 0.78 aB	4.3 ± 0.17 aB	7.4 ± 0.13 aB	20.5 ± 0.92 aB	10.6 ± 0.43 aB
Eny 7.11o	14.1 ± 0.78 aB	4.3 ± 0.19 aB	7.6 ± 0.13 aB	20.6 ± 0.91 aB	10.7 ± 0.41 aB
Control	13.8 ± 0.85 aB	4.2 ± 0.20 aB	7.4 ± 0.15 aB	20.2 ± 1.05 aB	10.5 ± 0.47 aB

NOFL- Number of functional leaves. In columns within a variety means followed by the same small case letter are not significantly different at P<0.05. Between varieties means followed by the same capital letter for each parameter are not significantly different at P<0.05. Values represent the mean ± standard error for three replicates (n=18)

4.3.2 Plant Fresh and Dry Weights

Shoot fresh and dry weights did not differ significantly between the two experiments (P= 0.0475) and the between the two banana varieties (P=0.6663). Treatment of Giant Cavendish plants with endophytic isolates *V5W2* in the first experiment led to a non significant 38.6% increase in shoot fresh weight when compared to the control (P=0.1372) (Table 4). Similar trend was observed for shoot dry weight. Inoculation of plants with endophytic isolates *V5W2* led to a relative 9.5 % increase in shoot dry weight when compared to the control (P=0.4116) (Table 4). Plants treated with endophytic isolates for both varieties had non-significant relatively lower root fresh and dry weights than the control (Table 4).

In the second experiment, inoculation of Giant Cavendish plants with endophytic isolate *Emb2.4o* and *V5W2* led to a relative non significant 14.6% and 5.6% increases in shoot fresh weight over the controls respectively (P=0.7899) (Table 5). For Grand plants a relative non significant (P=0.4469) increase in shoot fresh weight by 17.4% and 14.5% over the controls was recorded for plants inoculated with endophytic isolates *Emb2.4o* and *V5W2* respectively (Table 5). Root fresh weight of Grand naine plants inoculated with endophytic isolates *Emb2.4o, V5W2* and *Eny 7.11o* was relatively but non significantly (P=0.9910) higher than the control by 36%, 16% and 13.3% respectively (Table 5).

Table 4. Shoot and root weights of tissue culture banana plants cv. Giant Cavendish and Grand naine, 24 weeks after inoculation with endophytic *Fusarium oxysporum* isolates in the screen house **(Experiment 1)**

	Shoot weight (g)		**Root weight (g)**	
Giant Cavendish	**Fresh Weight**	**Dry weight**	**Fresh Weight**	**Dry weight**
V5W2	178.8±12.4 a	41.5± 5.0 a	52.08 ±7.7 a	8.8±0.9 a
Emb2.4o	143.2±21.2 a	34.7± 6.7 a	34.9 ± 5.0 a	7.8±0.7 a
Eny 7.11o	129.0±8.3 a	29.1±3.8 a	44.3± 10.9 a	9.2±1.5 a
Control	129.0 ±17.6a	37.9±5.2 a	52.8± 12.4 a	9.9±2.0 a
Grand Naine	**Fresh Weight**	**Dry weight**	**Fresh Weight**	**Dry weight**
V5W2	174.9±17.7a	31.1±3.4 a	52.8±7.5 a	11.4±1.4 a
Emb2.4o	193.2±19.1a	43.6±4.2a	53.8±9.8 a	9.7±1.5 a
Eny 7.11o	150.7±13.7a	36.8±5.5a	62.3±9.8 a	10.9±1.3 a
Control	178.8±19.5a	34.2 ±3.7a	66.8± 13.0 a	11.4±1.8 a

NOFL- Number of functional leaves. In columns within a variety means followed by the same small case letter are not significantly different at $P<0.05$. Between varieties means followed by the same capital letter for each parameter are not significantly different at $P<0.05$. Values represent the mean ± standard error for three replicates (n=18).

Table 5. Shoot and root weights of tissue culture banana plants cv. Giant Cavendish and Grand naine, 24 weeks after inoculation with endophytic *Fusarium oxysporum* isolates in the screen house. **(Experiment 2)**

	Shoot weight (g)		**Root weight (g)**	
Giant Cavendish	**Fresh Weight**	**Dry weight**	**Fresh Weight**	**Dry weight**
V5W2	167.5±21.1 a	36.6 ± 3.4 a	39.2±6.6	6.9 ±0.8
Emb2.4o	181.5±22.2 a	33.2±4.3 a	39.7±6.7	7.4±1.1
Eny 7.11o	137.0±17.7 a	31.6±3.3 a	41.4±7.4	6.9±0.9
Control	158.5± 20.2a	30.9±2.8 a	41.4±6.6	7.2 ±0.9
Grand Naine	**Fresh Weight**	**Dry weight**	**Fresh Weight**	**Dry weight**
V5W2	163.4±16.2a	31.3±6.5 a	30.5±6.5	5.8 ± 0.9
Emb2.4o	166.9±14.9a	32.2±7.5 a	35.8±7.5	6.9±0.9
Eny 7.11o	152.9±23.5a	29.4 ±3.7 a	29.8±3.7	5.6 ±0.5
Control	142.1±23.8a	29.4 ±3.4a	26.3 ±3.4	5.6 ±0.5

NOFL- Number of functional leaves. In columns within a variety means followed by the same small case letter are not significantly different at P<0.05. Between varieties means followed by the same capital letter for each parameter are not significantly different at P<0.05. Values represent the mean ± standard error for three replicates (n=18)

4.4 DISCUSSION

Mutualistic association between plants and endophytes has been demonstrated to be beneficial to plant growth (Latch *et al.,* 1984; Baker *et al.,* 1984; Schardl and Phillips, 1997; Clay and Schardl, 2002). The current study examined the effects of endophytic *F. oxysporum* on growth of tissue culture banana plants. Positive effects of endophytes on growth of bananas have also been made by Ting *et al.* (2007) who reported an increase in height, pseudostem diameter, and number of leaves in plants inoculated with *F. oxysporum* endophytes. Pocasangre (2000), reported plant growth promotion by endophytic *F. oxysporum 00*manifested in greater shoot and root weight. Enhancement of growth by endophytes may be a result of phytohormone production by fungal endophytes as described by Nassar *et al.* (2005) in maize and also increased nutrient uptake as reported by Zabalgogeazcoa *et al.* (2006), who observed an increased phosphorus content in *Festuca rubra* inoculated with the fungal endophyte *Epichloë festucae,* when compared to non-inoculated plants.

Effect of endophytes on growth of plants was not cultivar dependent. Significant differences in growth were observed between cv. Giant Cavendish plants and cv. Grand Naine plants, but within each cultivar no significant differences were observed between endophyte inoculated plants and the control treatment plants. This indicates that differences in growth between the two cultivars can be attributed to inherent differences in growth rate and vigour. In contrast Griesbach, (1999) and Niere, (2001) reported

interaction between banana cultivar and fungal isolate whereby inoculation of tissue culture bananas with *F. oxysporum* endophytes resulted in enhanced growth of some cultivars and reduced growth for other cultivars. In the current study, the two banana cultivars used were dessert (*Musa* AAA), while there remains a need to evaluate the effect of endophytic *F. oxysporum* on growth of plantains (*Musa* ABB).

Lack of significant differences in growth between endophyte inoculated plants and control treatments may be due to the duration in which the experiment was conducted. Considering that banana is a perennial, the significant effect of endophyte on growth is likely to be observed after a longer period of time than used in the current study. There is probably need to evaluate the effect of endophytic *F. oxysporum* on growth of bananas over a longer period of time and under field conditions. However, these preliminary results from the current study on dessert bananas show that endophytes appear to have potential to enhance growth of tissue cultured banana plants. Furthermore, even though significant differences in growth parameters were not observed, there was a consistent trend of increased growth of endophytic plants at this early stage. The use of *F.oxysporum* endophytes therefore would appear to stimulate early, post-flask development of bananas, including dessert bananas. Further research into this area, however, is warranted in order to better understand the inter species and cultivar variabilities.

CHAPTER 5

SCREENING ENDOPHYTES FOR CONTROL OF *Pratylenchus goodeyi* IN TISSUE CULTURE BANANAS

5.0 ABSTRACT

Screen house experiments were conducted to assess the potential of endophytic *Fusarium oxysporum* isolates *V5W2, Eny 7.11o* and *Emb2.4o* as biocontrol agents of banana nematodes. Endophytes were inoculated into tissue culture banana plants of two cultivars Giant Cavendish and Grand Naine, two months after post-flask weaning. Four weeks after endophyte inoculation, plants were inoculated with 1200 *Pratylenchus goodeyi* per plant in mixed developmental stages and then harvested after sixteen weeks. At termination of experiments endophyte inoculation into tissue culture bananas significantly suppressed nematode damage and population densities in inoculated plants, compared to uninoculated controls ($P<0.001$). Percentage reduction in root necrosis in inoculated plants ranged between 28.9% to 66.4% for the three isolates and nematode population densities suppressed by 46% to 65.2% in endophyte inoculated plants, compared to the controls. The number of dead roots were significantly lower, and the number of functional roots significantly higher in endophyte inoculated plants, when compared to the controls. Results indicate the potential of the three endophytic isolates for biological control of banana nematodes on dessert bananas.

5.1 INTRODUCTION

Among the biotic constrains affecting banana production, nematodes are a major problem in Kenya (Gichure and Ondieki, 1977; Kungu, 1995; Seshu Reddy *et al.*, 1998). The use of nematode-infested suckers as planting material is the main avenue for introducing these bests to new banana fields (O'Bannon, 1977). Management of nematodes is based on both chemical and non-chemical methods, which are not necessarily appropriate at subsistence farm level due to cost and availability, while they also have a negative impact on the environment (Gowen, 1994; Chabrier and Queneherve, 2003). Use of clean planting material, produced through tissue culture is viewed as a viable option for nematode management at the subsistence farm level, although it offers only a temporary control mechanism, as re-infestation readily occurs in the field (Sikora *et a*l., 1994; Speijer *et al.,* 2000; McIntyre *et al.,* 2000). The strategic use of naturally occurring organisms, such as endophytes to enhance resistance of tissue culture banana plantlets to nematodes represents a new and exciting promising option (Marshall *et al*., 1999).

Results from previous studies have shown potential for endophytes for biological control of banana nematodes (Pocasangre *et al*., 2000; Dochez *et al*., 2000; Niere, 2001; Athman, 2006). Culture filtrates for a number of isolates of *F. oxysporum* screened for *in vitro* activity against nematodes have shown high nematicidal activity inflicting mortality rates of 82-100% (Niere *et al*., 2004; Dubois *et al*., 2004; Athman, 2006).

Various isolates, when inoculated into tissue culture plants have demonstrated reduced nematode damage and reproduction in a number banana clones (Pocasangre *et al*., 2000; Dochez *et al*., 2000; Niere, 2001; Athman, 2006)

However, screening of endophytic *F. oxysporum* isolates for control of banana nematodes has primarily focused on the burrowing nematode *R. similis* (Pocasangre, 2000; Niere, 2001; Athman, 2006). The objective of the current study was to screen endophytic *F.oxysporum* isolates for control of lesion nematodes *Pratylenchus goodeyi*. The study was specifically aimed at evaluating the effect of endophytic *F. oxysporum* isolates on nematode damage, nematode populations and the influence of banana cultivar on endophyte performance.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

Three *F. oxysporum* endophytic isolates *V5W2, Emb 2.4o* and *Eny 7.11o* and two banana cultivars Giant Cavendish (*Musa* AAA) and Grand Naine (*Musa* AAA) were used throughout the study. Experiments consisted five treatments: inoculation with *V5W2*, *Emb 2.4o* and *Eny7.11o* separately; a maize bran control treatment and a complete control treatment. The maize bran control treatment was inoculated with sterile maize bran and nematodes, the complete control consisted of plants not inoculated with endophytes or maize bran but inoculated with nematodes. The experiment was replicated three times with six plants per treatment per replicate providing a total of eighteen plants per treatment. The experiment was conducted twice and laid down in a split plot design with cultivars as the main plot factor and fungal isolates as the sub-plot factor. The experiment was terminated 24 weeks after endophyte inoculation and sixteen weeks after nematode inoculation. Propagation of tissue culture banana plants, preparation of fungal inoculum and inoculation of plants with endophytes followed the protocol described in Chapter 3.

5.2.2 Extraction of Nematode Inoculum

To obtain *P. goodeyi* inoculum nematode infested banana roots were obtained from high altitude area, Kenya Agriculture Research Institute (KARI) banana plantation in Muguga (2095 masl). Nematodes were extracted using a modified Baermann funnel method

(Hooper *et al.*, 2005). The nematode extraction apparatus consisted of a sieve of *ca*. 2 cm deep and 15 cm in diameter made out of a plastic tubing, the base of which was covered with a plastic netting with *ca*.1 mm diameter mesh. The sieve was placed in a shallow plastic plate of 20 cm in diameter overlaid with tissue paper. The root samples were macerated in a Waring® Blender (Waring, Connecticut, USA) at high speed for 15 seconds and the suspension poured onto the tissue paper in the sieve. Tap water was added to a level just covering the macerated roots pieces. Nematodes were extracted overnight, during which they migrated into the water. The nematode suspension was rinsed into 100 ml glass sample bottles and stored in the fridge at 4^{o}C until counting and processing. Prior to counting, the nematode suspension was reduced to 25 ml. Nematode density and species composition was determined from 3 x 2 ml aliquots under a light microscope (magnification ×40)

5.2.3 Inoculation of plants with nematodes

Plants were inoculated with nematodes at eight weeks after endophyte inoculation for all plants treated with fungal isolates, plants treated with maize bran and complete control plants. Each plant was inoculated with 1200 nematodes *P. goodeyi* in mixed stages, into three equal sized holes ~3 cm deep spaced equally around the plant in a 2 ml suspension using a pipette. The plants remained un-watered for two days.

5.2.4 Assessment of Nematode Damage and Density

At termination of the experiments plants were removed from the plastic bags and rinsed free of soil using tap water. For each plant the number of functional and dead roots was recorded. Nematode damage was expressed as percentage of the necrotic root tissue as described by Speijer and Gold (1996). To estimate the root necrosis five functional roots were randomly selected from each plant and trimmed to 10 cm long segments. The root segments were sliced lengthwise and the visible necrotic region determined from one half of each segment. Each root segment represented a maximum percentage root necrosis of 20% a combined total for all the five roots per plant adding up to a maximum 100% root necrosis (Figure 1).

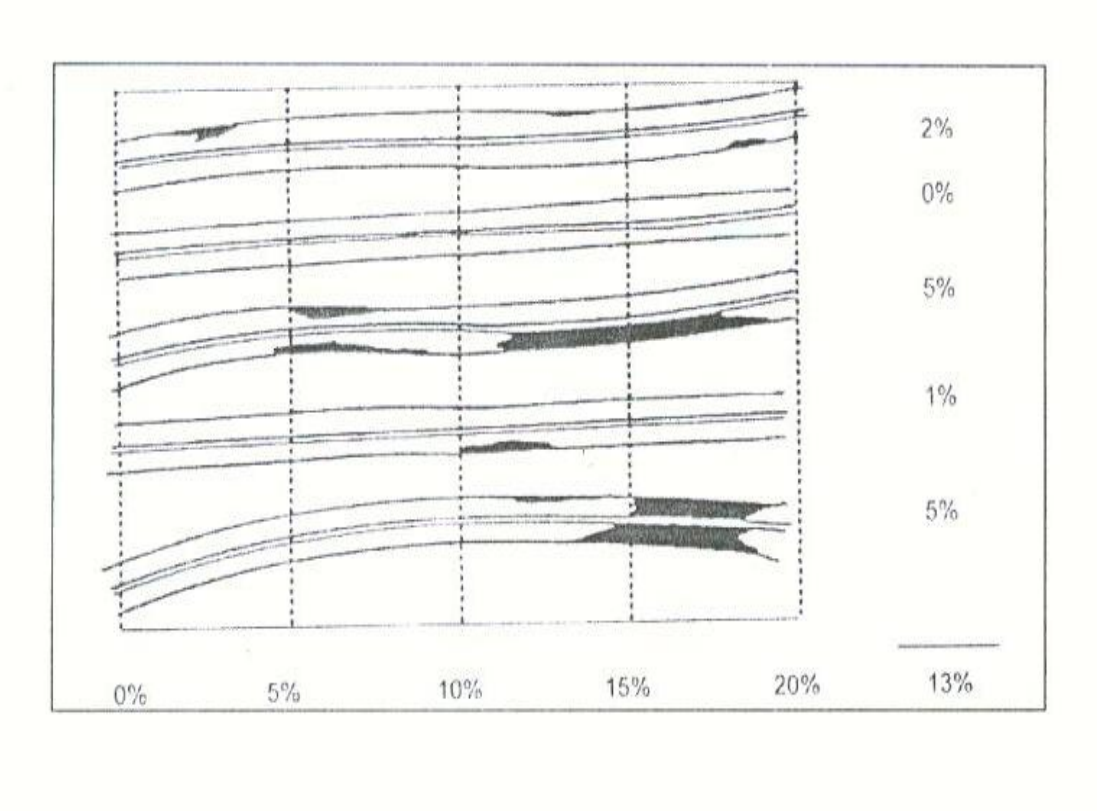

Figure 1. Example of the root necrosis assessment procedure for the estimation of percentage necrotic root tissue of longitudinal sections of five 10-cm root pieces (Source: Speijer and Gold, 1996)

The five roots used for assessment of damage were used for quantification of nematode numbers. The roots were chopped into ~0.5 cm length pieces with a knife, thoroughly mixed and a 5 g sub-sample removed for nematode extraction. Nematodes were extracted as described earlier (3.2.6). The population density of *P.goodeyi* female, juveniles and males was determined.

5.2.5 Data analysis

All data were first checked for normality and variance homogeneity using the Lervene option of the Hovetest prior to analysis (SAS institute 2002). Percentage root necrosis and nematode counts were arcsine-square root transformed prior to analysis. Factor effects and factor interaction effects were determined using the Generalised Linear Model procedure of the SAS programme (SAS Institute, 1999) and means compared using the Tukey test at $\alpha = 5\%$. With significant factor interaction analysis for the effects on one factor was performed at each level of the other factor.

5.3 RESULTS

Recorded data for nematode damage, number of functional and dead roots and nematode population differed between the two experiments ($P<0.001$), thus data from each experiment was analyzed independently. Two-way interaction between treatments and banana cultivar was not significant for nematode damage parameters ($P>0.05$). The effect of endophyte on nematode damage parameters did not differ between the two banana varieties ($P>0.05$).

5.3.1 Nematode Damage

Nematode damage to banana roots quantified as percentage root necrosis was significantly affected by endophyte inoculation. Endophyte inoculated plants for all isolates had lower percentage root necrosis, compared to control plants ($P<0.001$) in both experiments. In the first experiment inoculation of Giant Cavendish plants with endophytic isolates *V5W2, Eny 7.11o* and *Emb2.4o* suppressed percentage root necrosis by 36.9%, 37.4% and 28.9% respectively, compared with the maize bran control (Figure 2). Treatment of Grand naine plants with endophytic isolates *V5W2, Eny7.11o* and *Emb2.4o* resulted in 45.9%, 36.4% and 36.3% suppression of nematode damage respectively (Figure 3).

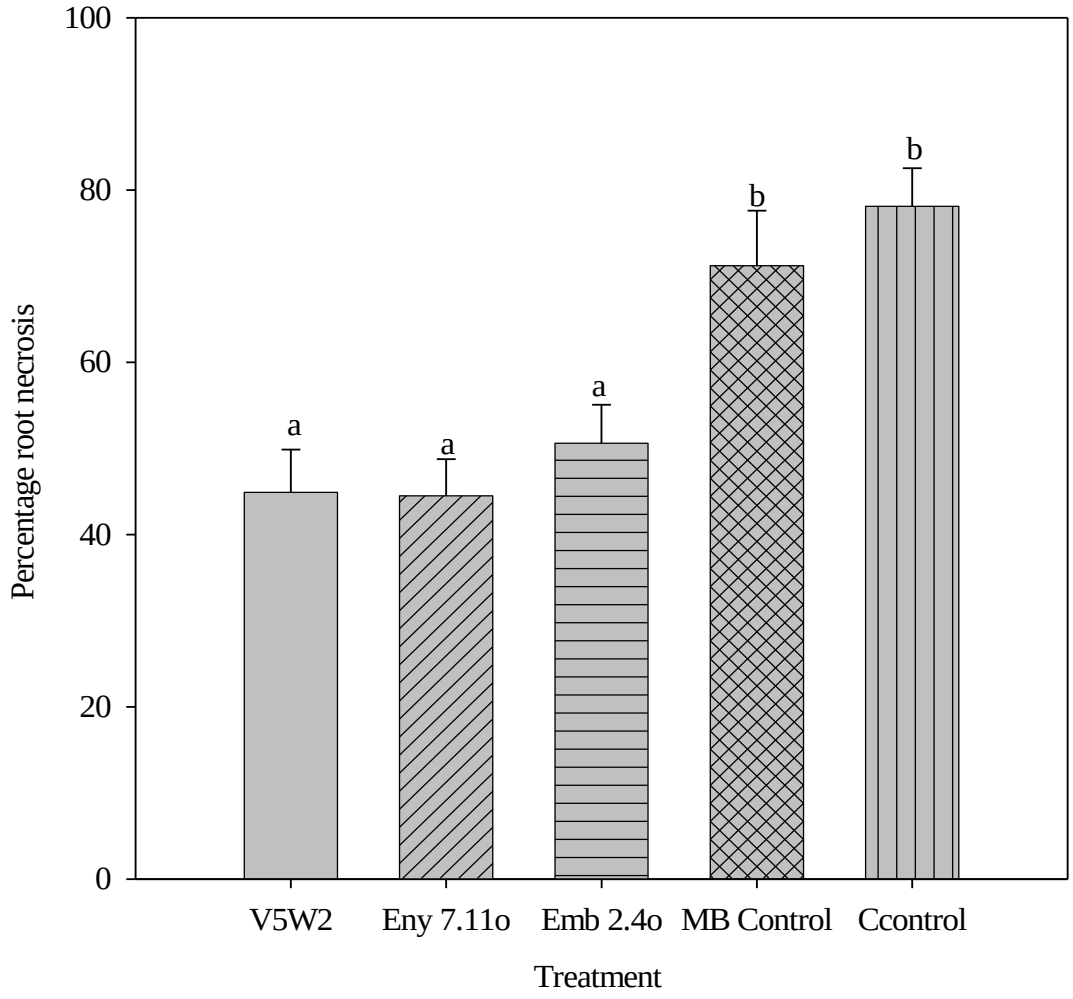

Figure 2. Percentage root necrosis of 32 weeks old tissue culture banana var Giant Cavendish inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi*. (Experiment 1)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

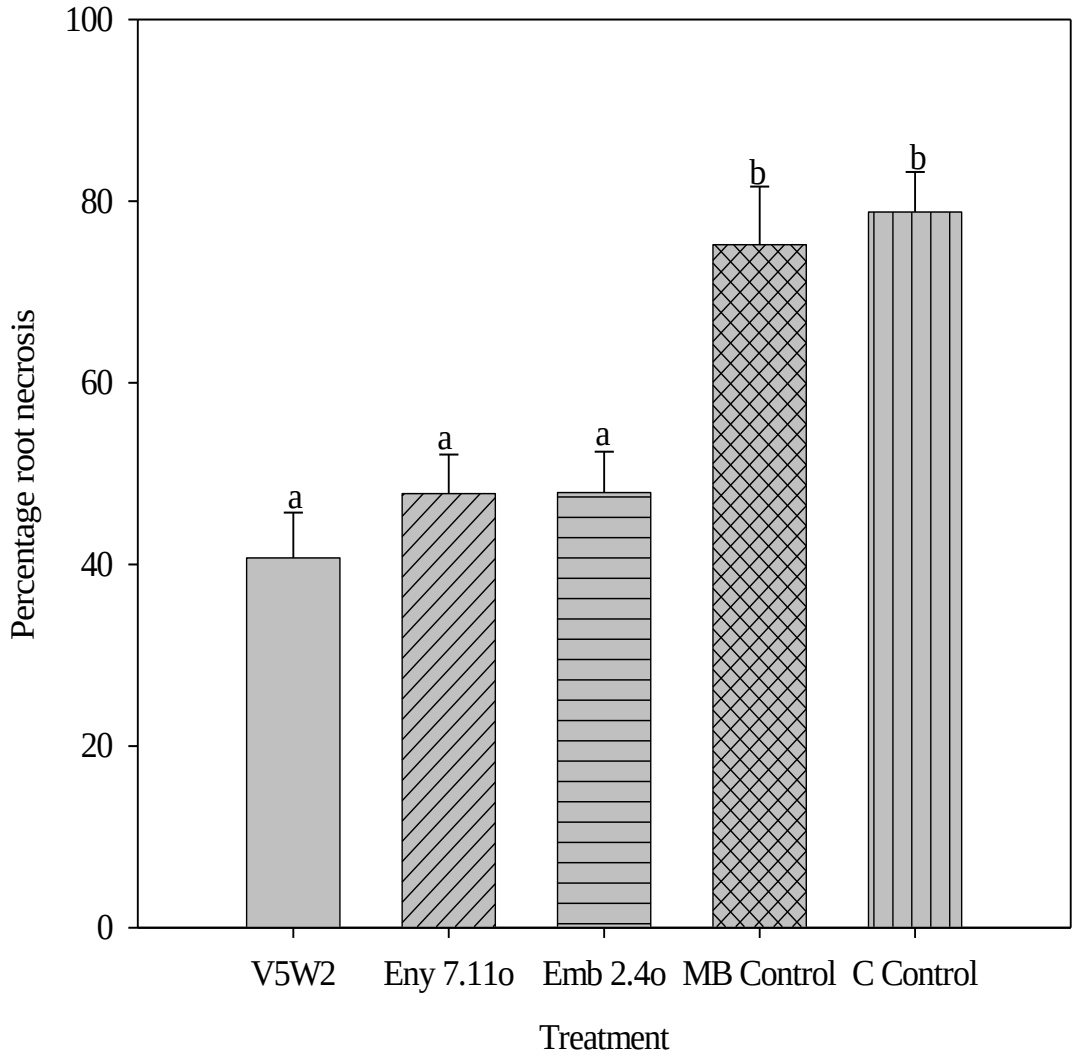

Figure 3. Percentage root necrosis of 32 weeks old tissue culture banana var Grand naine inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi.* (Experiment 1)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

In the second experiment isolate *Eny 7.11o* suppressed percentage root necrosis most for Giant Cavendish and Grand naine, by 66.4 % and 51.3 % respectively, then *V5W2* (53.6% and 45.1% respectively) and *Emb2.4o* (53.6% and 35.6% respectively), compared to the maize bran control. The three endophytic isolates similarly ($P<0.05$) suppressed percentage root necrosis over both experiments for both varieties (Fig. 4 and 5). There was no significant variation in percentage root necrosis between the maize bran control and the complete control treatment in either experiment for both varieties.

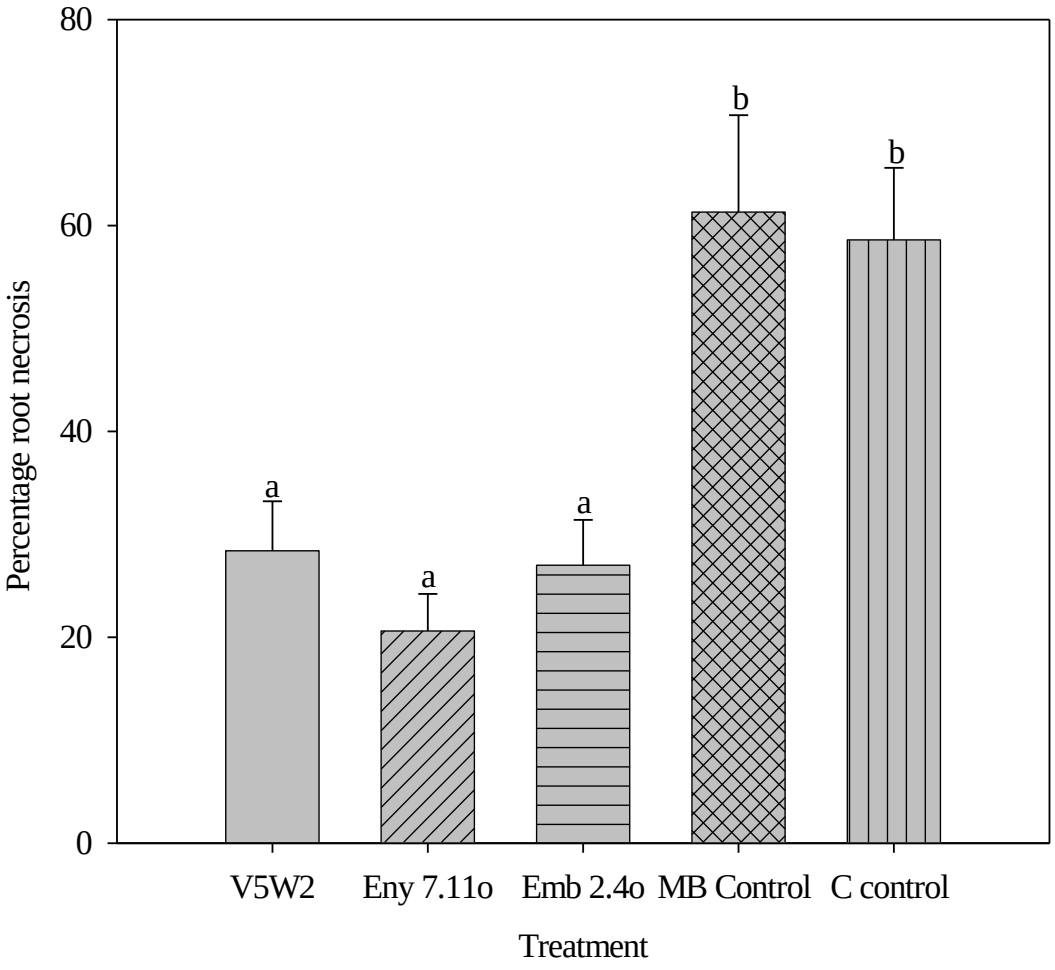

Figure 4. Percentage root necrosis of 32 weeks old tissue culture banana var Giant Cavendish inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi*. (Experiment 2)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

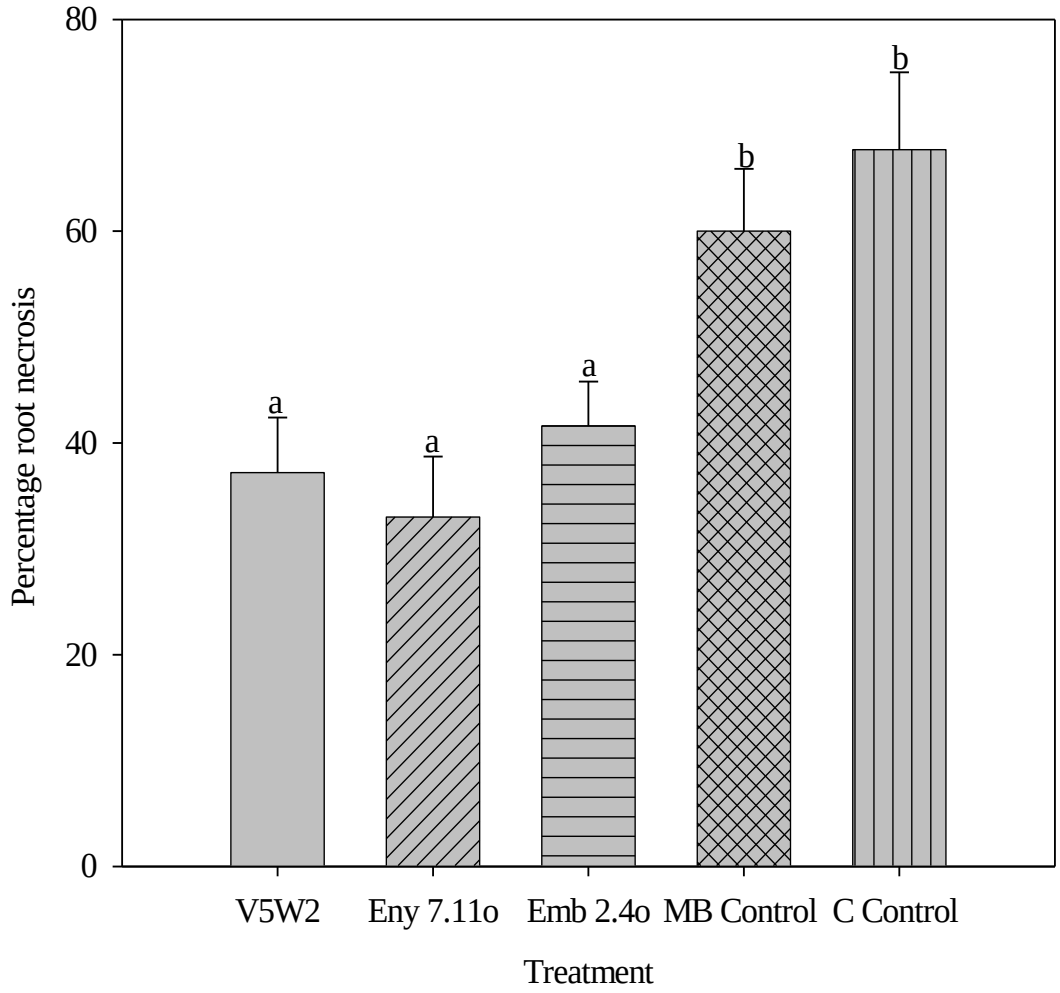

Figure 5. Percentage root necrosis of 32 weeks old tissue culture banana var Grand naine inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi*. (Experiment 2)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

5.3.2 Number of dead and functional roots

The number of functional roots differed between the two experiments ($P<0.001$). In the first experiment the number of functional roots between plants inoculated with endophytic isolates for the two varieties and the control treatments were similar ($P=0.9265$) (Table 6). In the second experiment, Giant Cavendish and Grand naine plants inoculated with the three endophytic isolates had more functional roots than the control treatments plants ($P= 0.0083$) but not significantly so, while endophytic plants for both varieties also had fewer dead roots than the control plants in both experiments ($P<0.005$) (Table 7). In the first experiment however, fewest dead roots were recorded from Giant Cavendish plants inoculated with *Eny 7.11o* followed by *Emb2.4o* (Table 6), while in the second experiment *V5W2* inoculated plants had fewest dead roots. No differences in the number of functional roots or dead roots were observed between the maize bran control and the complete control treatment plants for both varieties in either experiment (Table 6 and 7).

Table 6. Number of functional and dead roots in 32 week old tissue culture banana plants (Musa. AAA) inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after inoculation with 1200 nematodes. (**Experiment 1**)

	Number of roots per plant			
	Functional roots		**Dead roots**	
Treatment	**Giant Cavendish**	**Grand naine**	**Giant Cavendish**	**Grand naine**
V5W2	22. 3 ± 1.6 aA	23. 8 ± 2.5 aA	7.3 ± 0.9abA	6.2± 0.8 abA
Eny 7.11o	19.2 ± 2.1 aA	27.7 ± 1.8 aA	4.4 ± 0.4 aA	6.7± 1.3 abA
Emb2.4o	18.6± 1.8 aA	25.3 ± 1.5 aA	5.5 ± 0. 9aA	5.9 ± 0.7 aA
MB control	19.7± 2.0 aA	24.9 ± 1.1 bA	7.3 ± 1.1abA	7.5 ± 1.7 abA
Complete control	22. 2 ± 1.7 aA	22.1 ±1.2 bA	8.7 ± 1.5bA	10.1 ± 1.4 bA

MB- Maize bran control

In columns within a variety means followed by the same small case letter are not significantly different at P<0.05. Between varieties means followed by the same capital letters for each parameter are not significantly different at P<0.05. Values represent the mean ± standard error of three replicates, n=18

Table 7. Number of functional and dead roots in 32 week old tissue culture banana plants (Musa. AAA) inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after inoculation with 1200 nematodes. (**Experiment 2**)

	Number of roots per plant			
	Functional roots		**Dead roots**	
Treatment	**Giant Cavendish**	**Grand naine**	**Giant Cavendish**	**Grand naine**
V5W2	31.1± 2.3aA	27.0± 3.4aB	4.0 ± 0.7aA	5.5± 0.8 aB
Eny 7.11o	31.1± 2.1 aA	26.6 ± 1.8 aB	5.5 ± 0.6 aA	5.1 ± 1.3 abA
Emb2.4o	28.0± 3.0aA	29.1 ± 2.5 aB	5.4 ± 0. 8aA	6.4 ± 0.7 aA
MB control	24.2±3.2.0 aA	21.9± 2.4abA	8.9 ± 0.9aA	9.7 ± 1.7 bA
Complete control	27. 4 ± 2.2 aA	17.0 ±2.1 bA	6.2 ± 1.04bA	10.8± 1.4 bA

MB- Maize bran control

In columns within a variety means followed by the same small case letter are not significantly different at P<0.05. Between varieties means followed by the same capital letters for each parameter are not significantly different at P<0.05. Values represent the mean ± standard error of three replicates, n=18

5.3.3 Nematode population

The population density of *P. goodey*i females after 16 weeks was significantly different between endophyte inoculated plants and both control treatment plants and varieties in the two experiments ($P<0.0001$). Among the three isolates tested, lowest densities of *P. goodeyi* females were recorded for plants inoculated with *V5W2* for both Giant Cavendish and Grand naine in both experiments, though the differences between endophytic treatments were not significant (Table 8 and 9). Endophyte treatment for the two varieties affected the density of *P. goodeyi* juveniles in both experiments, compared to the control treatments ($P<0.005$). Fewer juveniles were recovered from roots of plants inoculated with *V5W2* for both experiments followed by plants inoculated with *Eny 7.11o* and *Emb2.4o*. This was consistent for the two banana varieties (Table 8 and 9). The population density of *P. goodeyi* males was also affected by inoculation of plants with endophytes in the two experiments with the lowest densities recorded for Giant Cavendish plants inoculated with *Eny 7.11o* when compared control plants (Table 9).

Table 8. Number of *Pratylenchus goodeyi* female, juvenile and male in roots of 32 week old endophyte inoculated tissue culture banana plants (*Musa*. AAA), 16 weeks after inoculation with 1200 nematodes in pots under the screen house. (**Experiment 1**)

	Number of nematodes /5 g roots					
	Females		**Juveniles**		**Males**	
Treatments	**Giant Cavendish**	**Grand naine**	**Giant Cavendish**	**Grand naine**	**Giant Cavendish**	**Grand naine**
V5W2	45.7 ± 11.3 aA	37.8± 7.8aB	18.3± 4.1aA	19.2± 5.9aB	7.3 ± 1.2 aA	7.0 ± 1.6 abA
Eny 7.11o	47.6± 10.6aA	62.1± 14.9aB	18.9 ± 3.8aA	24.4 ± 8.0 aB	6.0 ± 1.7 aA	7.6 ± 1.3 aB
Emb2.4o	57.0± 10.2aA	51.1± 8.4aB	30.9± 5.5aA	27.0± 6.0aB	11.1 ± 2.9 abA	10.0± 2.4aB
MB Control	136.7 ±21.5 bA	109.0 ± 19.6 bB	52.1± 13.0 bA	47.2.± 13.6 bB	15.9± 2.9 bA	13.1 ± 3.1aA
C control	135.9± 23.8bA	147.7 ± 34.1bB	62.3 ± 10.7 bA	48.6± 13.1bB	17.4± 3.5 bA	11.6 ± 1.9 aB

MB- Maize bran control; C Control- Complete control

In columns within an experiment means followed by different small case letters are significantly different at P<0.05. Between varieties means followed by different capital letters for each parameter are significantly different at P<0.05. Values represent the mean ± standard error of three replicates, n=9.

Table 9. Number of *Pratylenchus goodeyi* female, juvenile and male in roots of 32 week old endophyte inoculated tissue culture banana plants (*Musa*. AAA), 16 weeks after inoculation with 1200 nematodes in pots under the screen house conditions. **(Experiment 2)**

	Number of nematodes /5 g roots					
	Females		**Juveniles**		**Males**	
Treatments	**Giant Cavendish**	**Grand naine**	**Giant Cavendish**	**Grand naine**	**Giant Cavendish**	**Grand naine**
V5W2	23.7±6.3 aA	26.6± 7.0aB	8.1 ± 2.3 aA	5.8± 1.2 aB	4.3 ± 0.7aA	5.8 ± 2.9aA
Eny 7.11o	25.1 ± 5.3 aA	29.2 ± 6.1 aB	8.3 ± 1.4aA	6.8 ± 1.4aB	4.4± 1.2 aA	3.3 ± 0.7aB
Emb2.4o	27.3 ± 4.4 aA	29.1± 5.0 aB	10.4 ± 2.7aA	9.4 ± 1.6aB	5.4± 1.1aA	5.2 ± 1.5aB
MB Control	66.2 ± 18.6bA	77.8± 16.6 bB	20.3± 7.5 bA	16.6 .± 4.9bB	9.7 ± 3.7 aA	9.7 ± 3.3aA
C control	56.7 ± 8.6 bA	55.2 ± 6.7 bB	17.1 ± 3.2 bA	17.0 ± 2.5 bB	6.2 ± 2.0aA	4.8 ± 1.2 aB

MB- Maize bran control; C Control- Complete control

In columns within a variety means followed by different small case letters are significantly different at P<0.05. Between varieties means followed by different capital letters for each parameter are significantly different at P<0.05. Values represent the mean ± standard error of three replicates, n=9.

The total population density of *P. goodeyi* (female + juvenile + male) was consistently lower in endophyte inoculated plants, compared to both control treatment plants for Giant Cavendish and Grand naine in the two experiments ($P<0.0001$). Inoculation of Giant Cavendish plants with the endophytic isolates *V5W2, Eny 7.11o* and *Emb2.4o* resulted in lower total population densities of *P. goodeyi* by 65.2 %, 59.2% and 52.1% respectively (Fig 6), while for Grand naine plants 62.1%, 44.3% and 47.9% reduction in nematode density was recorded in the first experiment than the maize bran control plants (Fig 7).

In the second experiment a similar pattern was observed, where inoculation of Giant Cavendish plants with endophytic isolates *V5W2, Eny 7.11o* and *Emb2.4o* resulted in lower total population density by 54.9%, 52.7% and 46.1% respectively than the maize bran control plants (Fig 8). Nematode density was significantly lower for Grand naine plants treated with endophytic isolates *V5W2, Eny 7.11o* and *Emb2.4o* by 50.3%, 48.8% and 43.0% respectively over the maize bran control (Fig 9). There were no significant differences in total nematode population between the maize bran control and the complete control treatment for either experiment (Fig 8 and 9).

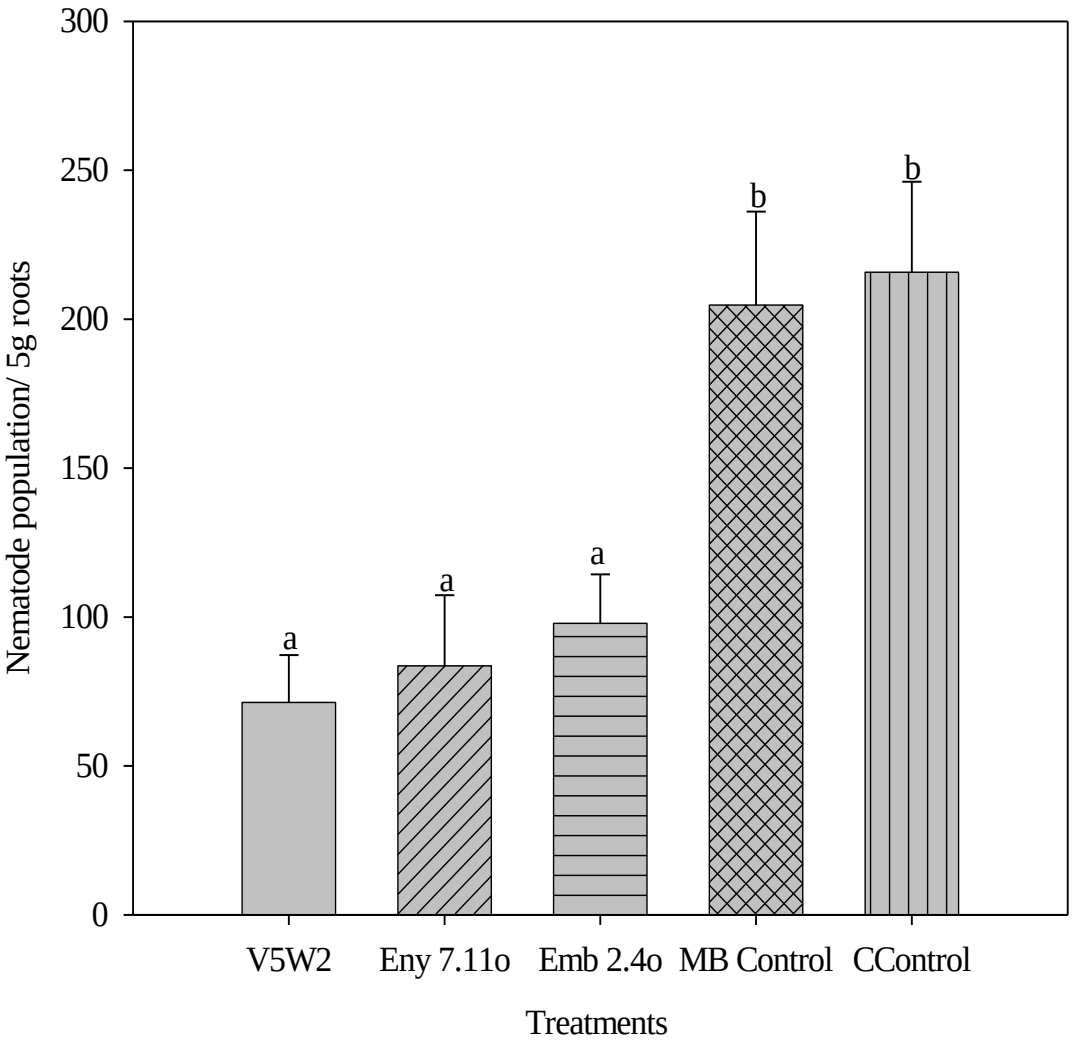

Figure 6. Total number of nematodes in 32 weeks old tissue culture banana var Giant Cavendish inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi.* (Experiment1)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

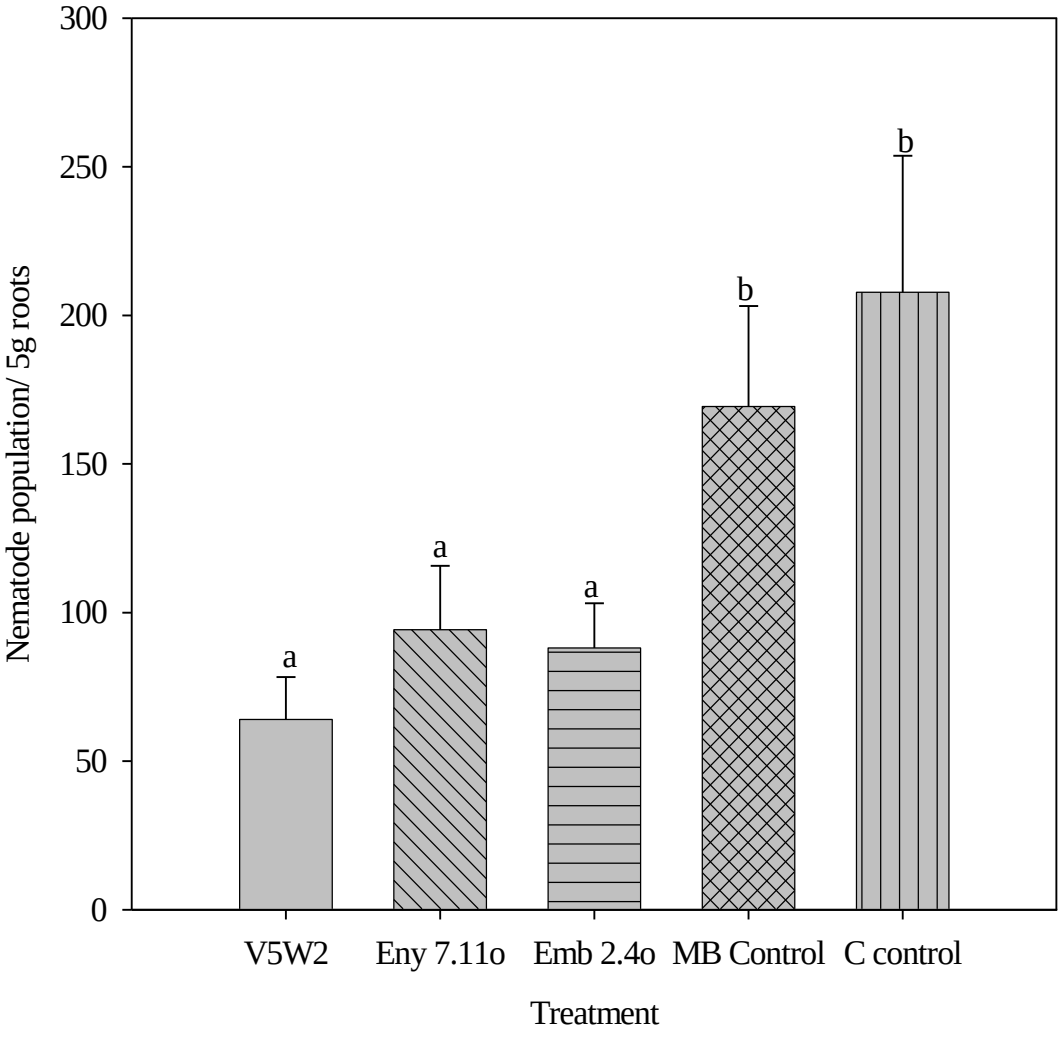

Figure 7. Total number of nematodes in 32 weeks old tissue culture banana var Grand naine inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi*. (Experiment1)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

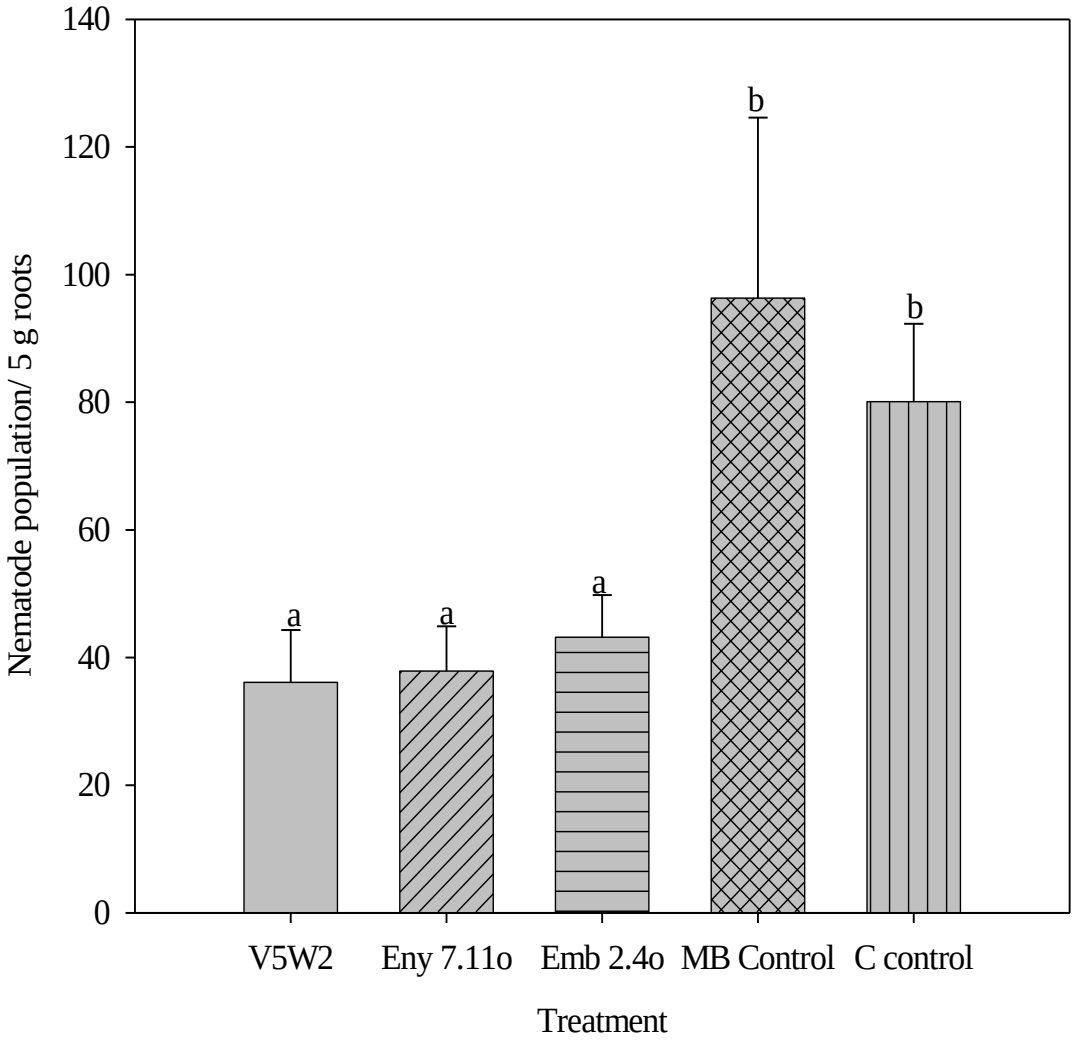

Figure 8. Total number of nematodes in 32 weeks old tissue culture banana var Giant Cavendish inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi*. (Experiment 2)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

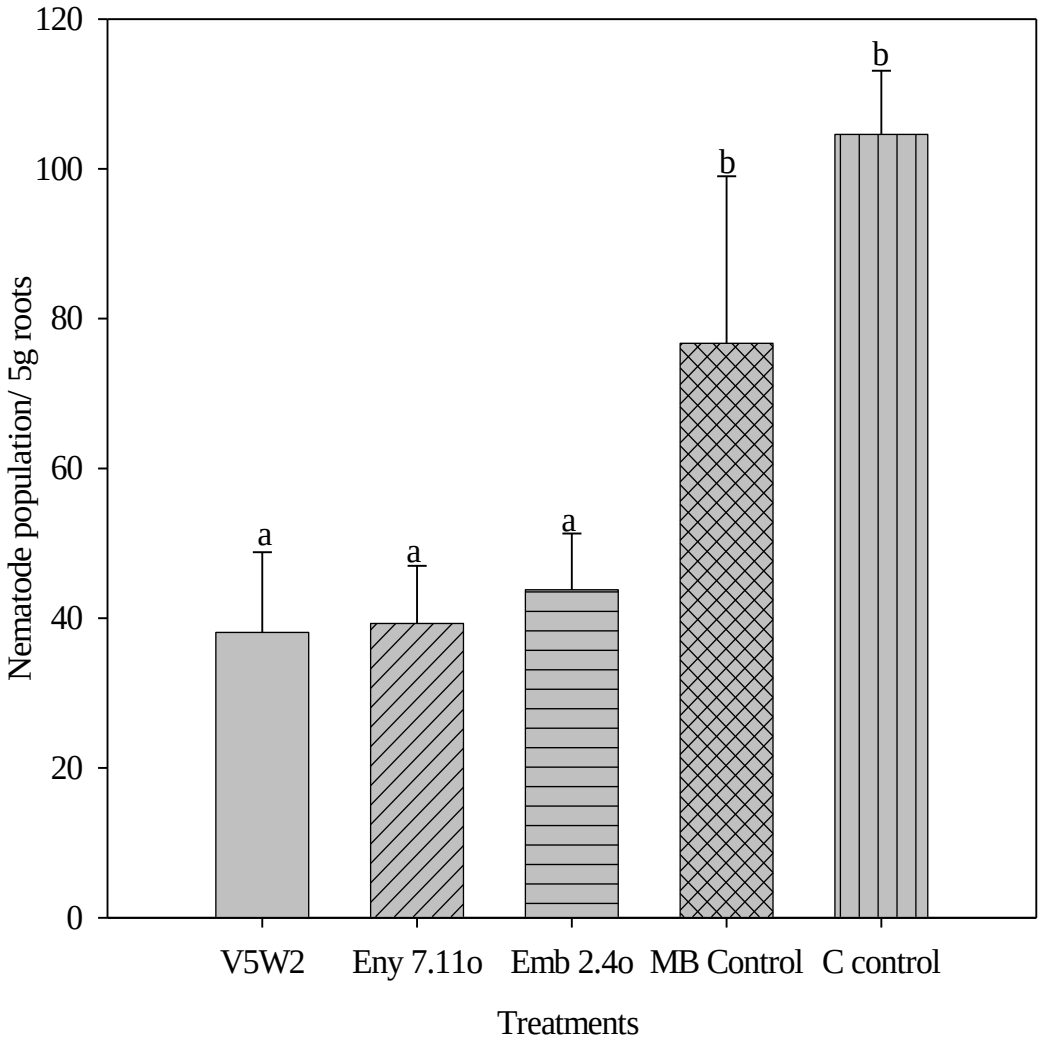

Figure 9. Total number of nematodes in 32 weeks old tissue culture banana var Grand naine inoculated with endophytic *Fusarium oxysporum* isolates, 16 weeks after challenge with *Pratylenchus goodeyi*. (Experiment 2)

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of three replicates n=9: MB- Maize bran control: C control- Complete control

5.4 DISCUSSION

The primary objective of the current study was to determine the effect of *Fusarium oxysporum* endophyte isolates on *Pratylenchus goodeyi* in banana roots and on root damage associated with nematode infection, under screenhouse conditions. Results demonstrated that inoculation of *F. oxysporum* onto tissue cultured banana plants prior to nematode challenge led to lower nematode populations and associated damage in banana roots. The three isolates tested reduced nematode damage to banana roots and suppressed population densities. Similar results were obtained by Niere (2001) for *R. similis*, who reported lower nematode population and damage in different EA highland cooking banana cultivars following inoculation with endophytic *F. oxysporum* isolates.

Nematodes feed on banana roots, causing root necrosis which leads to rotting and reduced number of functional roots (Kashaija *et al.*, 1998). In the current study, it was demonstrated that *F. oxysporum* endophytic association with bananas would result in lower nematode damage of banana roots. Root necrosis damage due to nematode infection was significantly reduced in endophyte inoculated plants by between 28.9% to 66.4 % over the controls. The effect of endophytes on nematode damage was further evidenced by the lower number of dead roots and significantly higher number of functional roots in endophyte inoculated plants than the control plants. Similar observations were made by Niere (2001), who reported a 30% to 40% reduction in root necrosis in the EA highland banana cv. Enyeru inoculated with endophytic *F. oxysporum*

isolates prior to challenge with *R. similis.* Results also corroborate findings from studies on other crops. In tomato, Sikora *et al.* (2003) reported a 75% reduction in galling by *Meloidogyne incognita* of tomato plants harbouring endophytes. Mennan *et al.* (2005) reported reduced cyst nematode damage on cabbage plants inoculated with non-pathogenic *F. oxysporum.*

All development stages of *P. goodeyi* invade and feed on banana roots, where the eggs are laid (Bridge *et al.,* 1997). In the current study, inoculation of tissue culture banana plants with endophytic *F. oxysporum* isolates had an affect on all stages of the nematode, with the total nematode density in the roots being ultimately lower for all endophyte treated plants than controls. In addition to the fact that there were fewer females to lay eggs, lower densities of *P. goodeyi* juveniles, in relation to females, could have been due to endophyte inhibition of egg hatching inside the plants. Though this is difficult to demonstrate inside the plant, it has been demonstrated *in vitro* by Athman (2006), who demonstrated hatching inhibition of *R. similis* eggs by culture filtrates of endophytic *F. oxysporum* isolates. All the isolates tested in the current study affected the overall final density of *P. goodeyi* in tissue culture banana plants, with between 46% to 65.2 % suppression over the controls. Pocasangre (2000) observed a 50% to 90% reduction in total *R. similis* populations in roots of tissue cultured bananas cultivars belonging to Cavendish subgroup inoculated with a number of unspecified fungal endophytes, originally isolated from healthy banana roots. Niere (2001) in his studies on the effect of

endophytic *F. oxysporum* isolates on *R. similis* reported 40% to 50% fewer nematodes in endophyte inoculated plants over the control treatment. Nel *et al.* (2006) also recorded endophytic effects on other pathogens, with between 87.4% and 75% reduction in Fusarium wilt incidence in bananas inoculated with non-pathogenic *F. oxysporum* isolates. In other crops similar results have been documented. In maize, for example, the presence of *Fusarium moniliforme* in roots led to significant levels of lesion nematode, *Pratylenchus zeae;* control (Sikora *et al.*, 2003).

The ability of endophytic *F. oxysporum* to reduce nematode damage and populations in inoculated plants may be attributed to various mechanisms. Lower nematode populations and damage levels in inoculated plants however, may be through a disturbance of nematode reproduction. Athman (2006) reported lower reproduction rates of *R. similis* in second and third generations in bananas (cv. Enyeru) inoculated with *F. oxysporum* endophytes. This could have also resulted from inhibited nematode root penetration, inhibition of nematode migration inside the plant roots and/or interference with the feeding process (Athman, 2006). Pocasangre (2000) also reported reduced root penetration by *R. similis* in Cavendish bananas treated with endophytic *F. oxysporum*. Production of secondary metabolites toxic to nematodes is additionally believed to among the most common mechanisms of nematode antagonism (Dubois *et al*., 2004), that can cause inactivation and mortality of *R. similis* (Pocasangre, 2000; Dubois *et al*., 2004; Athman, 2006). Although toxic metabolites by endophytes in culture filtrates have

been shown to be antagonistic to nematodes *in vitro*, the role of these compounds in nematode reduction *in vivo* has not been conclusively determined. Nematode population and damage suppression also results from systemic induced resistance (Sikora *et al.*, 2003; Sikora and Pocasangre, 2004; Athman, 2006). In banana, induction of resistance to *R. similis* by endophytic *F. oxysporum* isolate *V5W2* has been demonstrated by Athman (2006). In this study enhanced production of phenolic compounds in roots and rhizomes as an indication of induced resistance following inoculation of plants with endophytes was observed. However, the study did not conclusively determine the ability of endophytic *F. oxysporum* to induce resistance to *R. similis* in tissue culture banana plants.

Previous studies on the use of endophytic *F. oxysporum* for the management of banana nematodes indicate that banana genotype will influence endophyte performance (Niere, 2001). In the current study, genotype effect on endophyte performance on nematodes was not significant. Therefore, there is need to screen different cultivars belonging to the same and to different sub-groups to verify the interaction and performance of endophytes. Significant variation between the two experiments in the current study for the measured parameters may be attributed to variation in environmental conditions during the periods in which the experiments were conducted, vigour of nematode inoculum and possibly differences in colonization of plants by endophytes in the two experiments. The results obtained indicate however, that endophytic *F. oxysporum*

isolates *V5W2, Eny 7.11o* and *Emb 2.4o* offer the potential as a biological control mechanism for the banana nematode *P. goodeyi*. There remains the need to test endophyte efficacy of these isolates under field conditions though, where a wide diversity of nematode species, other micro-organisms and climatic conditions prevail. There is furthermore, need to compare the performance of endophytes against other nematode control strategies.

CHAPTER 6

EFFECT OF DIFFERENT INOCULATION TECHNIQUES ON ROOT COLONIZATION OF TISSUE CULTURE BANANA PLANTS BY ENDOPHYTIC *Fusarium oxysporum*

6.0 ABSTRACT

Two separate experiments were conducted to evaluate the effect of different post flask weaning techniques and inoculum delivery methods on root colonization of tissue culture bananas by three endophytic *Fusarium oxysporum* isolates. In the first experiment two techniques were evaluated. For the first technique de-flasked plants were planted in weaning trays to grow for one month before transplantation into 3l potting bags for endophyte inoculation one month later. In the second technique de-flasked plants were placed in nutrient solution for a period of two months before endophyte inoculation. In the second experiment three inoculum delivery methods were compared: solid substrate, dipping and drenching. Results showed non significant differences in percentage root colonization between plants potted before inoculation and plants placed in nutrient solution prior to endophyte inoculation. Percentage root colonization for the two techniques ranged between 45% to 54.7% for the endophytic isolates. In the second experiment there were no significant differences in root colonization for plants inoculated with the three methods. However, plants inoculated with the solid substrate gave relatively higher rates of colonization. Percentage root colonization ranged between

59.1% to 79.8% for the three isolates. This study from the techniques tested indicates that the ability of endophytic *F.oxysporum* isolates to colonize tissue culture banana plants appears independent of inoculation technique or method used.

6.1 INTRODUCTION

Biological enhancement of tissue cultured banana plants with endophytic *F. oxysporum* isolated from banana tissues to increase plant resistance to nematode infestation has shown potential as an alternative nematode management strategy (Sikora and Schuster, 1998; Niere *et al*., 1999). A number of selected isolates have been shown to inflict high mortalities of nematodes in the laboratory (Athman, 2006). When inoculated into tissue cultured banana plants they have been successful in reducing nematode damage and populations (Pocasangre, 2000; Niere, 2001; Athman, 2006). The strategy aims at incorporating active fungal isolates into the plants before they reach the field. Thus the ability of endophytic isolates to colonize and grow in the young plant tissue plays an important role and is a vital criteria for selection of effective isolates.

Successful inoculation and colonization of tissue culture banana plants by endophytic *F. oxysporum* isolates has been demonstrated in various studies (Griesbach, 1999; Niere, 2001; Paparu *et al.,* 2006; Athman, 2006). Different methods of inoculating endophytes into tissue culture banana plants have been developed. Root and corm dip of tissue culture banana plants in endophyte fungal suspension resulted in successful colonization (Niere, 2001; Paparu *et al.,* 2006). Introduction of endophytes into tissue culture banana plants by use of solid substrate has also been demonstrated to be successful (Griesbach, 1999; Paparu *et al.,* 2004). For an endophytic strain to be a successful pest antagonist, it needs to occur at high frequencies and be able to persist in the plant after inoculation.

Therefore there is need to compare the effect of different inoculation techniques on plant tissue colonization so as to optimise cost-effective and efficient inoculation techniques that will yield high colonization rates.

The objectives of the current study were to: 1) determine the ability of three endophytic *F. oxysporum* isolates to colonize tissue culture banana plants. 2) evaluate the effect of different post flask weaning techniques and inoculum delivery methods on root colonization of tissue cultured banana plants by endophytic *F. oxysporum*; 3) evaluate the effect of banana cultivar on root colonization of tissue cultured banana plants by endophytic *F. oxysporum.*

6.2 MATERIALS AND METHODS

6.2.1 EFFECT OF DIFFERENT POST FLASK WEANING TECHNIQUES ON ROOT COLONIZATION

6.2.1.1 Experimental design

The experiment comprised three factors: post flask weaning techniques, treatment and cultivar. In the first technique (hereafter referred to as 'potted') de-flasked plants were planted in weaning trays before transplanting one month later into 3l potting bags (5×9×4 cm). Plants were inoculated with endophytes one month later (two months after de-flasking). In the second technique (referred to as 'nutrient solution') de-flasked plants were weaned by suspending their roots in a nutrient solution in plastic cups for a period of two months before endophyte inoculation. For each inoculation technique the treatments were the three *F. oxysporum* endophytic isolates (*V5W2, Emb 2.4o* and *Eny 7.11o*) and two banana cultivars Giant Cavendish (*Musa* spp.AAA) and Grand Naine (*Musa*.spp.AAA). In addition to the three endophyte treatments, experiments included an uninoculated maize bran control which (autoclaved maize bran) and a complete control (without endophytes or maize bran). The experiment was laid down in a split plot design with varieties as the main plot factor and treatments as the subplot factor. The experiment was replicated three times with six plants per treatment per replicate per variety giving a total of eighteen plants per treatment for each of the techniques.

6.2.1.2 Preparation and inoculation of plants with endophytes

Tissue culture banana plants were propagated using a standard shoot-tip culture protocol for banana as described as described in Chapter 3. Four weeks after rooting the plants were removed from the rooting medium, washed off adhering media with tap water and selected for uniformity in size. For the first technique (potted plants), the plants were planted in weaning trays (60 cm x 30 cm) filled with steam sterilized sandy loam soil where they were left to grow for one month before being transplanted into three 1-litre potting bags (5×9×4cm) and left to grow for one month before endophyte inoculation. In the second technique (nutrient solution), de-flasked plants were suspended in a nutrient solution in 300 ml plastic cups with lids for two months before endophyte inoculation. The nutrient solution comprised 1 g of water soluble fertilizer Poly-feed (Haifa Chemicals, Haifa Bay, Israel) (Appendix 1) dissolved in 1 litre sterile tap water. Plants were suspended through a hole at the centre of the cup lid, with their roots submerged in the nutrient solution and the shoots being held outside the cup. Strips of sterile sponge were wrapped around the base of the pseudostem to prevent damage to the pseudostem base and to prevent the plant from falling through the hole. The plants were maintained in a humidity chamber for a period of two months, during which the nutrient solution was renewed weekly.

After two months plants were removed from the soil or nutrient solution and washed with tap water and transplanted into 3 l potting bags that were filled with steam sterilized

sandy loam soil and watered to field capacity. A hole was made centrally for the plant and the roots sprinkled with 2 g maize bran containing the fungal inoculum. For both inoculation techniques the control treatment plants were inoculated with sterile maize bran. After 24 weeks endophyte root colonization was determined.

6.2.2 EFFECT OF DIFFERENT INOCULUM DELIVERY METHODS ON ROOT COLONIZATION

6.2.2.1 Experimental design

Each inoculation method was assessed using three *F. oxysporum* endophytic isolates (*V5W2, Emb 2.4o* and *Eny 7.11o*) and two banana cultivars Giant Cavendish (Musa AAA) and. Grand Naine (Musa AAA) and a control treatment which for the dipping and drenching methods were plants treated with sterile distilled water and plant treated with sterile maize bran for the solid substrate method. The experiment was laid down in a split plot design with cultivars and inoculation method as main plot factors and treatments as the subplot factor. Experiment was replicated two times with six plants per replicate per variety for each inoculation method. Preparation of fungal inoculum as conducted as described in Chapter 3.

6.2.2.2 Inoculation of plants with endophytes

Solid substrate methods

Banana plants after deflasking were washed of adhering media with tap water and selected for uniformity in size. De-flasked plants were planted in weaning trays filled with steam sterilized sandy loam for one month before endophyte inoculation. During inoculation the plants were carefully removed from the trays, rinsed under a tap to wash off adhering soil. 3l potting bags were filled with steam sterilized sandy loam soil and watered to field capacity. A hole was made in the middle. A banana plant was placed on the hole and the roots sprinkled with the 2 g maize bran containing the fungal inoculum (Plate 3). Control treatment plants were inoculated with sterile maize bran. The plants were left to grow for eight weeks after which percentage root colonization was determined.

Plate 3. Inoculation of tissue culture banana plants with endophytes using the solid substrate method

Dipping Method

Banana plants after deflasking were treated as described above under the solid substrate method. During inoculation the plant roots were immersed in the spore suspension for 2 hrs, after which they were planted in 3l potting bags filled with steam sterile sandy loam soil (Plate 4). Plants dipped in sterile distilled water represented the control treatment For the dipping method the plant roots were immersed in the spore suspension for 2 hrs, after which they were planted in 3l potting bags filled with steam sterile sandy loam soil. Plants dipped in sterile distilled water represented the control treatment. Root colonization was determined after 8 weeks.

Plate 4. Inoculation of tissue culture banana plants with endophytes by dipping in the fungal suspension

Drenching method

Weaning trays were divided into six planting hole sections. The trays were filled with steam sterilized sandy loam soil. Six tissue culture banana plantlets were planted per tray for each treatment with two replicates per treatment. The plants were inoculated with endophytes one month later. Prior to endophyte inoculation the plants remained unwatered for two days. Endophytes were then inoculated by placing the trays with the plants in a container with the spore suspension until the soil was completely drenched (Plate 5). The plants were carefully removed from the trays with soil still intact and transplanted into 3l potting bags as above. Control treatment plants were drenched with sterile distilled water. The plants were removed for assessment eight weeks afteR inoculation.

Plate 5. Inoculation of tissue culture banana plants with endophytes by drenching with the fungal suspension

6.2.3 Determination of fungal colonization

At termination of the experiments plants were removed and debris gently rinsed from roots under a running tap. Three healthy roots were randomly selected from each plant and surface sterilized by dipping in 70% ethanol, followed by flaming. For each root, six x 0.5 cm pieces were measured and cut in equal numbers from the base, mid and tip portions. These were plated onto synthetic nutrient agar (SNA) in 90 mm Petri dishes and incubated in the laboratory for 7-10 days, during which emerging fungi was viewed under a light microscope (100 x and 400 x magnification) (Plate 6).

Plate 6. *Fusarium oxysporum* emerging from roots pieces cultured to determine percentage root colonization

F. oxysporum was identified based on its characteristic macroconidia (sickle-shaped with attenuated apical cell), short phialides and chlamydospores (Plate 7).

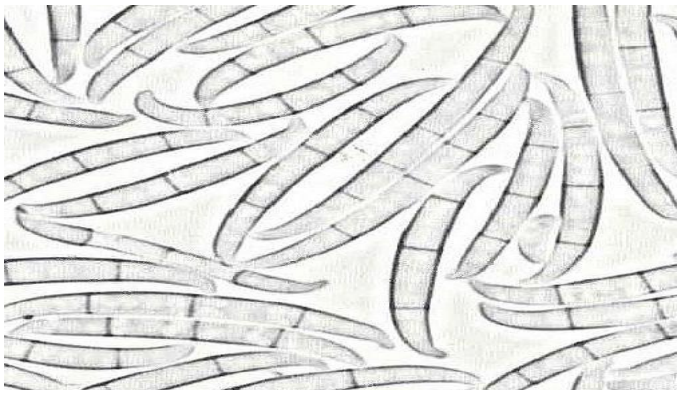

Plate 7. Macroconidia used to identify *Fusarium oxysporum* emerging from cultured root pieces.

Percentage colonization was determined as the number of *F. oxysporum* forming colonies divided by the total number of pieces plated multiplied by 100 per Petri dish as shown in the equation below.

% Colonisation per dish = (Number of *F. oxysporum* forming colonies per dish ÷6) ×100

6.2.4 Data analysis

All data were first checked for normality and variance homogeneity using the Lervene option of the Hovetest prior to analysis (SAS institute 2002). Percentage root colonization data was arcsine-square root transformed prior to analysis. Factor effects

and factor interaction effects were determined using the Generalised Linear Model procedure of the SAS programme (SAS Institute, 1999) and means compared using the Tukey test at $\alpha = 5\%$. With significant factor interaction analysis for the effects on one factor was performed at each level of the other factor.

6.3 RESULTS

6.3.1 Effect of different post flask weaning techniques on root colonization

Percentage root colonization was significantly higher in endophyte inoculated plants when compared to the control treatment for potted plants and for nutrient solution plants (P<.0001) (Table 10). However, the endophyte inoculation technique had no effect on percentage root colonization (P=0.4458). For the inoculation techniques, neither was there any cultivar effect in percentage root colonization (P=0.6274). Though there were no significant differences in percentage root colonization between the two inoculations techniques, marginally higher rates of root colonization were observed on potted plants. Percentage root colonization for Giant Cavendish potted plants was 55.6%, 53.7% and 57.6% for endophytic isolates *V5W2, Eny7.110* and *Emb2.4o,* respectively, and for nutrient solution plants 47.9%, 48.3 % and 55.5%, respectively (Table 10). For Grand naine plants percentage root colonization for potted plants for the three endophytic isolates ranged between 50% to 53.8% while for plants in nutrient solution before inoculation ranged from 45% to 52.4%. The three endophytic *F. oxysporum* isolates tested colonized tissue culture banana plant roots similarly.

Table 10. Effect of two post flask weaning techniques, plants potted before inoculation and plants with roots suspended in nutrient solution before inoculation, on root colonization of tissue culture banana roots (Musa AAA) var Giant Cavendish and Grand naine by three endophytic *Fusarium oxysporum* isolates.

	Percentage root colonization			
Treatments	**Giant Cavendish**		**Grand naine**	
	Potted	**Nutrient Solution**	**Potted**	**Nutrient Solution**
V5W2	55.6 ± 3.2aA	47.9 ±1.9 aA	53.8 ± 2.7aA	52.4 ± 3.1aA
Eny 7.11o	53.7 ±3.3aA	48.3 ± 3.9aA	50.9 ± 3.1aA	46.9 ± 2.0aA
Emb2.4o	57.6 ±2.8aA	55.5 ± 3.5aA	53.1 ± 2.6aA	45.0 ± 4.0aA
Maize bran Control	21.5 ±3.4bA	25.8 ± 3.3bA	29.2 ± 3.9bA	29.0 ± 3.3bA
Complete control	18.8 ±3.4bA	25.0 ± 4.1bA	17.2 ± 4.1bA	24.0 ± 2.9bA

In columns means followed by different small case letters are significantly different between treatments within an inoculation technique at $P<0.05$. In rows means followed by different Capital letters are significantly different between inoculation techniques for each treatment at $P<0.05$. Values represent the mean ± standard error of three replicates (n=36).

6.3.2 Effect of different inoculum delivery methods on root colonization

Within each inoculation method there were significant differences in percentage root colonization between the endophyte inoculated plants and the control treatment ($P<.0001$). Percentage root colonization for endophytes inoculated ranged between 59.1% to 79.8% across inoculation methods and endophyte isolates for the tow banana varieties (Table 11). However, the endophyte inoculation method had no effect on percentage root colonization ($P=0.4458$). There was also no effect of banana cultivar ($P=0.2140$). Though there were no significant differences in percentage root colonization among the three inoculation methods, marginally higher rates of root colonization were recorded using solid substrate, for the isolate *Eny7.11o* (79.8%) and *V5W2* (77.5%) for Grand naine plants (Table 11). Percentage root colonization for solid substrate method ranged between 59.1% to 79.8%, 59.7% to 74.9% for dipping method and 66.0% to 70.8% for the drenching method for the three endophytic isolates across the two banana varieties. (Table 11). The three endophytic *F. oxysporum* isolates showed no significant variations in their ability to colonize tissue culture among the inoculation methods for the two banana varieties.

Table 11. Effect of three inoculum delivery methods of three endophytic *Fusarium oxysporum* isolates on root colonization of tissue culture banana roots (Musa AAA) var. Giant Cavendish and Grand naine

Treatments		**Percentage root colonization**	
Giant Cavendish	**Solid Substrate method**	**Dipping method**	**Drenching Method**
V5W2	66.7± 4.3aA	59.7± 6.4aA	70.8± 4.8aA
Eny 7.11o	68.6 ± 4.3aA	69.3± 5.6aA	66.0 ± 3.8 aA
Emb2.4o	59.1± 4.9aA	62.6 ± 6.01aA	69.5 ± 3.3 aA
Control	34.3± 5.4 bA	21.1± 6.6 bA	24.4 ± 6.2bA
Grand Naine	**Solid Substrate method**	**Dipping method**	**Drenching Method**
V5W2	77.5±3.6 aA	70.5 ± 4.1 aA	70.5 ± 4.1 aA
Eny 7.11o	79.8±4.1 aA	74.9 ± 3.9 aA	70.0 ± 4.3 aA
Emb2.4o	76.3±3.9 aA	69.8 ± 3.9 aA	68.2 ± 6.0 aA
Control	24.0±5.1 bA	24.1 ± 5.0 bA	26.6 ± 5.0 bA

In columns means followed by different small case letters are significantly different between treatments within an inoculation method at $P<0.05$. In rows means followed by different Capital letters are significantly different between inoculation method for each treatment at $P<0.05$. Values represent the mean ± standard error of three replicates, n=24.

Although percentage root colonization was not affected by inoculation method pooled data indicated that use of solid substrate leads to marginally higher percentage root colonization (71.3%), than drenching (69.2%) and dipping (67.8%) (P=0.4040) (Fig 10).

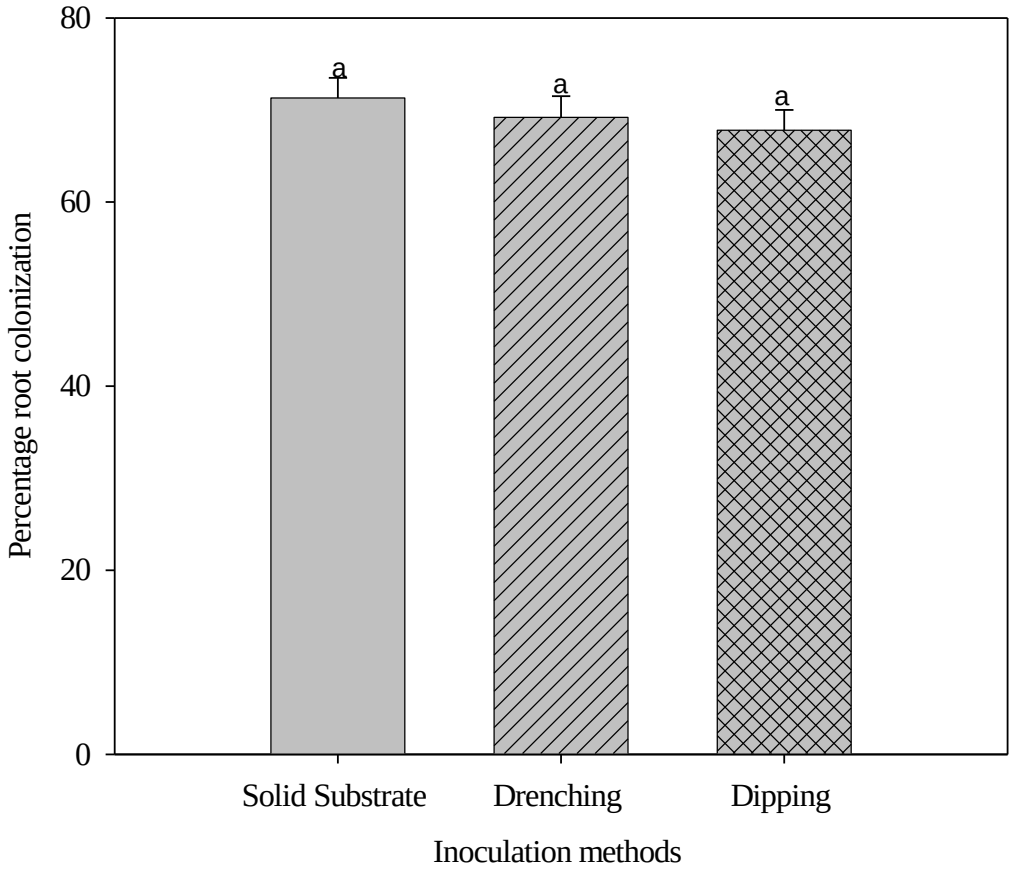

Figure 10. Effect of different inoculation methods on percentage root colonization of 8 weeks old tissue culture banana plants by endophytic *Fusarium oxysporum*

Bars with different letters are significantly different at P=0.05 according to Turkey's Studentized range test. Values represent mean of two replicates n=72

6.4 DISCUSSION

In selecting the fungal isolates most suitable as biological control agents, their biological activity against the pest as well as the capability to colonize plant tissue play an outstanding role. The isolates used in the current experiments have already demonstrated their potential for biological control of banana nematodes under screen house conditions (Chapter 3). Results presented demonstrate the ability of the three studied endophytic *F. oxysporum* isolates to successfully colonize tissue culture banana plantlets. In both experiments conducted percentage root colonization was predictably higher in endophyte inoculated plants, compared to the control treatments, although controls also had relatively high colonisation, given that they had not been inoculated. Similar results were obtained by Griesbach (1999), Niere (2001), Paparu *et al.* (2004) and Athman (2006). The three endophytic isolates studied also did not vary in their colonization of plant roots in the current study, although this did vary in earlier studies (Chapter 2, 3). Originally the endophytes were isolated from different plant parts of different banana cultivars. For example, isolate *V5W2* originated from roots, *Eny7.11o* from rhizomes of banana cv. Enyeru (*Musa* AAA-EA) and *Emb2.4o* from rhizome of banana cv. Embiire (*Musa* AAA-EA) (Athman, 2006). This implies that plant endophytic *F. oxysporum* isolates may not be cultivar specific, and thus a single isolate may be suitable for use with a range of banana cultivars, irrespective of the original host.

The main aim of suspending plant roots in nutrient solution prior to endophyte infection is to facilitate enhanced root development. The process is however expensive and labour intensive and probably cumbersome to apply on a commercial scale. In the current experiment using nutrient solution did not significantly affect percentage root colonization of endophytes compared with potted plants, which also had relatively higher colonization rates. This indicates that the use of nutrient solution prior to endophyte inoculation may not necessarily be important. Thus tissue culture banana plants can be directly inoculated with endophytes after weaning.

Regarding inoculation method and the results of the current study demonstrate that under screen house conditions *F. oxysporum* endophytes can similarly be inoculated using solid substrate, through dipping of roots in spore suspension or drenching planting media with spore suspension. Use of the solid substrate, while not leading to significant differences in root colonization was relatively more effective than dipping and drenching, which may indicate that root tissue colonization is affected by the period of root exposure to fungal endophyte inoculum. For the solid substrate inoculation method the period of root exposure to endophyte inoculum is longer owing to the fact the inoculum remains in contact with roots following its application on the root during the planting. Additionally the fungal inoculum is contained in a medium through which growth can continue before the fungus is able to penetrate the roots. Paparu *et al.* (2004) observed higher root colonization rates of tissue culture banana plants inoculated with

endophytic *F. oxysporum* isolates using solid substrate when compared to root and corm dip methods. Future investigations on methods of inoculating tissue culture bananas with endophytes should focus on how to increase the duration of exposure of plants roots to fungal inoculum. There is also need to further evaluate the applicability and how cost effective each method would be to a commercial tissue culture banana producer.

The type of banana cultivar had no significant effect on root colonization rates in either experiment in the current study. The two banana cultivars used Giant Cavendish (*Musa* AAA) and Grand Naine (*Musa* AAA) both belong to the Cavendish subgroup, thus their pattern of endophyte colonization may be similar. Therefore there is need to evaluate colonization of banana cultivars that belong to different subgroups. The results also contrast with findings from previous studies. Griesbach (1999) for instance, reported a significant effect of banana cultivar on colonization rates, where rates of root colonization in cv. Bogoya (*Musa* AAA) were twice as high as that of cv. Valery (*Musa* AAA). The interactions between fungal endophytes and different banana cultivars is important in designing protocols for biological enhancement of tissue culture banana plants with endophytes and thus need to be studied in details.

It was not possible to confirm with certainty that re-isolated fungi were the original isolates inoculated into the plants. Given that controls also had colonisation of approximately 30%, it is likely that contamination occurred, and also contributed to overall colonisation of endophyte treated plants. A system should be put in place to

identify reisolated fungi and compare them with original isolates. Tracking of individual isolates once inoculated into tissue culture banana plants may also be necessary to determine the mode of action of endophytic *F. oxysporum*. There is also need to determine persistence of these endophytic *F. oxysporum* isolates under field conditions and their ability to move from the mother plant to colonize subsequent suckers.

CHAPTER 7

7.0 CONCLUSION AND RECCOMENDATIONS

Endophytic micro-organisms are potential bio-control agents of banana nematodes and can be utilized to protect tissue culture banana plants before they are transferred to the field. The overall goal of the current study was to evaluate the efficacy of selected endophytic *Fusarium oxysporum* isolates obtained from Uganda for control of banana nematodes in Kenya under screen house conditions. The isolates had been previously tested in Uganda for their *in vitro* and *in vivo* activity against the burrowing nematode *R. similis.* This study focused specifically on efficacy of these isolates against the lesion nematode *P. goodeyi.* The specific objectives were to evaluate the effect of the endophytic isolates on growth of tissue cultured banana plants, to screen the isolates for control of nematodes in tissue culture banana plants and determine the effect of different inoculation methods of tissue colonization of tissue culture banana plants by the endophytic isolates.

The study on the effect of endophytes on growth of tissue culture banana plants revealed that endophytes may have potential to enhance growth of tissue culture banana plants. Though the difference in growth between endophyte inoculated plants and control treatment plants was not significant relatively higher measurements for most growth parameters considered were recorded inoculated plants when compared to the control treatment. The effect of endophyte on growth of tissue culture plants from this study was

found not to be cultivar dependent. The duration of time in which the experiments were conducted was probably not adequate to determine the exact effect of endophytes on growth of tissue culture bananas thus there is need to conduct the experiments for relatively longer period of times and probably under field conditions.

All the three endophytic *F. oxysporum* isolates evaluated in this study demonstrated ability to reduce nematode damage and population density of *P. goodeyi* in inoculated plants. Reduction in nematode damage quantified as percentage root necrosis was further evidenced by significantly lower numbers of dead roots and higher numbers of functional roots in endophyte inoculated plants when compared to the control treatments. The three endophyte isolates had a significant effect on all stages of the lesion nematode *P. goodeyi*. Nematode control was not dependent on the host genotype. Biological control was related to reduced development of juveniles as evidenced by the high number of females, but low juvenile densities in roots of plants inoculated with endophytes. Mechanisms leading to nematode control were not investigated in this study. Elucidation of different mechanisms of action of endophytes against nematodes is essential for designing control programmes that would maximally utilize the nematode controlling potential of endophytes. Results presented are based on screen house trials thus there is need to evaluate the efficacy of this isolates for control of nematodes under field conditions where there is a wide diversity of nematode species attacking the crop.

The effect of different inoculation methods on root colonization of tissue culture banana plants was also investigated in this study. Results presented demonstrate the ability of endophytic *F. oxysporum* isolates to colonize tissue culture banana plants. The different inoculation techniques and methods evaluated did not have a significant effect on root colonization. From the study it was concluded that it may not be necessary to place plants in nutrient solution prior to endophyte inoculation, a process which is expensive and labour intensive particularly for a commercial scale. The study also demonstrated that the use of solid substrate for inoculating tissue culture banana plantlets with endophytes could be the most ideal method due to duration of exposure of plant roots to fungal inoculum. However further research into the use of the three methods is needed to evaluate the most applicable method for commercial scale. It is also important to determine the threshold colonization rates that are capable of eliciting effective and durable plant defense responses in plants. The possibility of maternal transmission of introduced endophytes to suckers need to be investigated to minimize the need for additional or frequent applications for the technique to be self sustaining.

The use of fungal endophytes for management of banana nematodes is a strategy likely to contribute enormously to banana production in Kenya. Biological enhancement of tissue culture banana plants with endophytes before they are transferred to farmers is a targeted, environmentally safe and economically feasible approach for management of banana nematodes at the subsistence farm level. Their incorporation in banana

management systems can provide protection to bananas against nematodes throughout the first growth cycle. However, use of endophytes for control of nematodes should not be seen as a complete alternative to nematicides since they are unlikely to achieve the levels of control provided by nematicides. Their use must be maximized in an integrated nematode management programme.

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APPENDICES

Appendix 1. Nutrient composition of Polyfeed fertilizer (Haifa Chemicals, Haifa Bay, Israel) used to enhance root development of tissue culture plants prior to endophyte inoculation

Nutrient	**Quantity (g/kg)**
Nitrogen	0.19
Phosphorus	0.19
Potassium	0.19
Iron	1
Manganese	0.5
Boron	0.2
Zinc	0.15
Copper	0.11
Molybdenum	0.07

Appendix 2. ANOVA Results Tables

ANOVA Results Tablesof the effect of endophyte treatments, variety and their interaction on height of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	96.85896	65.61965	0.78	0.5049
Experiment	1	13620.07735	13620.07735	161.96	<.0001
Variety	1	789.29018	789.29018	9.39	0.0022
Replicate	2	1991.24638	995.62319	11.84	<.0001
Variety*Treatment	3	19.98465	6.66155	0.08	0.9713

Appendix 3. ANOVA results of the effect of endophyte treatments, variety and their interaction on girth of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	12.7476788	4.2492263	1.01	0.3886
Experiment	1	252.5833178	252.5833178	59.86	<.0001
Variety	1	38.2969093	38.2969093	9.08	0.0026
Replicate	2	26.9832363	13.4916181	3.20	0.0411
Variety*Treatment	3	5.2985031	1.7661677	0.42	0.7397

Appendix 4. ANOVA results of the effect of endophyte treatments, variety and their interaction on Number of functional leaves (NOFL) of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	4.9490195	1.6496732	0.54	0.6529
Experiment	1	616.4439175	616.4439175	202.92	<.0001
Variety	1	99.3948714	99.3948714	32.72	<.0001
Replicate	2	88.5083994	44.2541997	14.57	<.0001
Variety*Treatment	3	11.4719287	3.8239762	1.26	0.2869

Appendix 5. ANOVA results of the effect of endophyte treatments, variety and their interaction on lengh of the youngest leaf of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	103.78231	34.59410	0.39	0.7603
Experiment	1	12099.13403	12099.13403	136.35	<.0001
Variety	1	974.97632	974.97632	10.99	0.0009
Replicate	2	2663.45396	1331.72698	15.01	<.0001
Variety*Treatment	3	62.57512	20.85837	0.24	0.8720

Appendix 6. ANOVA results of the effect of endophyte treatments, variety and their interaction width of the youngest leaf of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	112.420010	37.473337	0.88	0.4515
Experiment	1	3898.352035	3898.352035	91.38	<.0001
Variety	1	98.066850	98.066850	2.30	0.1296
Replicate	2	482.683267	241.341633	5.66	0.0035
Variety*Treatment	3	51.804408	17.268136	0.40	0.7496

Appendix 7. ANOVA results of the effect of endophyte treatments, variety and their interaction on shoot fresh weight of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	19890.21034	6630.07011	2.26	0.0847
Experiment	1	818.50132	818.50132	0.28	0.5984
Variety	1	2012.71422	2012.71422	0.69	0.4092
Replicate	2	10587.55489	5293.77744	1.80	0.1689
Variety*Treatment	3	3412.97499	1137.65833	0.39	0.7622

Appendix 8. ANOVA results of the effect of endophyte treatments, variety and their interaction on shoot dry weight of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	362.9732465	120.9910822	0.87	0.4601
Experiment	1	558.5722898	558.5722898	4.00	0.0475
Variety	1	26.0779043	26.0779043	0.19	0.6663
Replicate	2	802.6109369	401.3054684	2.88	0.0600
Variety*Treatment	3	775.2825773	258.4275258	1.85	0.1410

Appendix 9. ANOVA results of the effect of endophyte treatments, variety and their interaction on root fresh weight of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	509.82956	169.94319	0.29	0.8335
Experiment	1	10042.90826	10042.90826	17.06	<.0001
Variety	1	41.64221	41.64221	0.07	0.7907
Replicate	2	21.48171	10.74086	0.02	0.9819
Variety*Treatment	3	620.00523	206.66841	0.35	0.7884

Appendix 10. ANOVA results of the effect of endophyte treatments, variety and their interaction on root dry weight of tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	3	14.3408563	4.7802854	0.38	0.7657
Experiment	1	328.0073284	328.0073284	26.25	<.0001
Variety	1	0.0033014	0.0033014	0.00	0.9871
Replicate	2	0.8182198	0.4091099	0.03	0.9678
Variety*Treatment	3	7.1339929	2.3779976	0.19	0.9028

Appendix 11. ANOVA results of the effect of endophyte treatments, variety and their interaction on percentage root necrosis on tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	40168.27778	10042.06944	39.59	<.0001
Experiment	1	9374.45000	9374.45000	36.96	<.0001
Variety	1	893.33889	893.33889	3.52	0.0623
Replicate	2	3406.43333	1703.21667	6.71	0.0016
Variety*Treatment	4	254.74444	63.68611	0.25	0.9087

Appendix 12. ANOVA results of the effect of endophyte treatments, variety and their interaction on number of functional roots in tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	537.8220076	134.4555019	2.80	0.0276
Experiment	1	630.2907006	630.2907006	13.14	0.0004
Variety	1	0.0973879	0.0973879	0.00	0.9641
Replicate	2	204.5914765	102.2957382	2.13	0.1219
Variety *Treatment	4	449.8789539	112.4697385	2.34	0.0570

Appendix 13. ANOVA results of the effect of endophyte treatments, variety and their interaction on number of dead roots in tissue cultured bananas

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	385.9273646	96.4818411	9.77	<.0001
Experiment	1	1.9572764	1.9572764	0.20	0.6567
Variety	1	46.7647127	46.7647127	4.74	0.0310
Replicate	2	35.4082004	17.7041002	1.79	0.1697
Variety*Treatment	4	42.5984922	10.6496231	1.08	0.3689

Appendix 14. ANOVA results of the effect of endophyte treatments, variety and their interaction on population density of female nematodes in tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	155074.7000	38768.6750	19.82	<.0001
Experiment	1	76921.3389	76921.3389	39.32	<.0001
Variety	1	6.0500	6.0500	0.00	0.9557
Replicate	2	9215.2111	4607.6056	2.36	0.0980
Variety*Treatment	4	1691.3667	422.8417	0.22	0.9292

Appendix 15. ANOVA results of the effect of endophyte treatments, variety and their interaction on population density of nematode juveniles in tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	17357.38889	4339.34722	10.14	<.0001
Experiment	1	23621.35556	23621.35556	55.22	<.0001
Variety	1	278.75556	278.75556	0.65	0.4207
Replicate	2	2592.74444	1296.37222	3.03	0.0510
Variety*Treatment	4	430.07778	107.51944	0.25	0.9085

Appendix 16. ANOVA results of the effect of endophyte treatments, variety and their interaction on population density of male nematodes in tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	1125.922222	281.480556	6.11	0.0001
Experiment	1	1032.005556	1032.005556	22.39	<.0001
Variety	1	42.050000	42.050000	0.91	0.3409
Replicate	2	46.900000	23.450000	0.51	0.6022
Variety*Treatment	4	100.144444	25.036111	0.54	0.7043

Appendix 17. ANOVA results of the effect of endophyte treatments, variety and their interaction on total population density of nematodes in tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Treatment	4	301916.6333	75479.1583	18.13	<.0001
Experiment	1	220780.0889	220780.0889	53.04	<.0001
Variety	1	752.3556	752.3556	0.18	0.6713
Replicate	2	21350.1000	10675.0500	2.56	0.0800
Variety*Treatment	4	1775.8111	443.9528	0.11	0.9801

Appendix 18. ANOVA results of the effect of post flask weaning techniques, endophyte treatments, variety and their interaction percentage root colonization in tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Inoculation technique	1	233.9822	233.9822	0.82	0.3667
Treatment	4	100307.0659	25076.7665	87.49	<.0001
Variety	1	62.9802	62.9802	0.22	0.6394
Replicate	2	1790.0563	895.0281	3.12	0.0449
Variety*Treatment	4	2506.9998	626.7499	2.19	0.069

Appendix 19. ANOVA results of the effect different inoculation methods, endophyte treatments, variety and their interaction percentage root colonization in tissue cultured bananas.

Source	DF	SS	Mean Square	F Value	Pr > F
Inoculation method	2	243.1066	121.5533	0.29	0.7503
Treatment	3	122022.9427	40674.3142	96.21	<.0001
Variety	1	676.7210	676.7210	1.60	0.2065
Replicate	1	230.4589	230.4589	0.55	0.4607
Variety*Treatment	3	7248.4102	2416.1367	5.71	0.0008