

SCREENING SELECTED ISOLATES OF ENDOPHYTIC  
*Fusarium oxysporum* FOR BIOLOGICAL CONTROL OF  
BANANA NEMATODES IN TISSUE CULTURE BANANA

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Screening selected isolates of endophytic *Fusarium oxysporum* for biological control of  
banana nematodes in tissue culture banana

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A thesis submitted in partial fulfillment of the Degree of Master of Science in  
Horticulture in the Jomo Kenyatta University of Agriculture and Technology

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## DECLARATION

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

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## **DEDICATION**

To my loving parents Mr. and Mrs. Waweru, brothers, sisters for their encouragement, moral support, prayers and love. To all my friends who have been an inspiration to me in carrying out this study.

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## ABSTRACT

Studies were carried out to investigate the efficacy of selected isolates of endophytic *Fusarium oxysporum*, against banana nematodes under greenhouse and field conditions. Greenhouse experiments involved the use of two isolates 4MOC321 and 11SR23, tested against *Pratylenchus goodeyi* in two experiments. Isolate V5W2 was used as a positive standard. The field experiments involved the assessment of three isolates V5W2, Emb2.4o and Eny 7.11o from bananas in Uganda, against banana nematodes in both on-farm and on-station trials. Dessert banana cv. Giant Cavendish and cv. Grand Nain were used in all experiments. The on-station trial assessed the response of two month old banana plants, inoculated with one of the three V5W2, Emb2.4o and Eny 7.11o isolates and challenged with mixed nematode species (*P. goodeyi* and *Helicotylenchus multicinctus*) one month later. The on-farm trial assessed the performance of two month old banana plants inoculated with each of the three isolates and planted into a field naturally infested with nematodes (*P. goodeyi*, *H. multicinctus* and *Meloidogyne* spp.). All endophyte isolates significantly suppressed nematode populations and damage to bananas in both greenhouse and field studies. Results from the greenhouse experiment demonstrated a significant reduction of *P. goodeyi* population by >50 % and percentage root necrosis was reduced by >30 % by the endophytes. *Fusarium oxysporum* isolates 4MOC321 and 11SR23 also enhanced plant growth. In the on-station trial, the endophytes suppressed nematode population density by >45 % and reduced percentage root necrosis by >20 %. Nematode damage was also significantly lower in endophyte treated plants compared with control plants in on-farm trial. Isolates Eny7.11o and



V5W2 enhanced plant growth as compared to the control treatments. Banana yields were significantly increased following endophyte treatments. Carry-over effect of endophytes to suckers was also evident. Suckers from endophyte inoculated plants had higher percentage root colonization compared to non-inoculated plants. The study has demonstrated the potential of endophytic *F. oxysporum* to suppress key plant parasitic nematode populations under field conditions and, promote banana plant growth and yield.

## **CHAPTER ONE**

### **1.0 GENERAL INTRODUCTION**

#### **1.1 INTRODUCTION**

Banana is an important crop globally with the world annual production estimated to be 104 million tons with Africa accounting for 32.2 millions tons (Lescot, 2008). The East African region accounts for over 49 % of all bananas produced in Africa and constitute the main staple food for about 50 % of the population (IITA, 2003). In Kenya, the total banana production for 2007 was 1,160,340 tons (MoA, 2007). The key production areas include Kisii, Maragwa, Meru and Embu districts (MoA, 2007). It is the most affordable fruit both in rural and urban households and therefore it has emerged as a major income earner and food item in almost all the provinces (MoA, 2007). Bananas are used for cooking and dessert. The cultivars most commonly grown include Cavendish, Gros Michel, Lacatan Valery, Uganda Green, William's hybrids, Paz, Pisang, Apple, sweet bananas among others.

The total area under banana production has dropped steadily from about 83,687 ha in 2005 to approximately 77,356 ha in 2007 (MoA, 2007). The production is predominantly under the management of small-scale farmers, who own on average 0.3 ha of banana (Smith, 2007). Bananas are grown over a wide range of agro-ecosystems, from sea level up to around 1,800 metres, generally in areas of high rainfall (1000 mm per annum). Production is generally rain-fed, as smallholders tend not to have the resources to implement systematic irrigation.

The continuous decline in banana yields is largely attributed to the infestation by pests (nematodes and weevils) and diseases (panama, black and yellow sigatoka) (Wambugu and Kiome, 2001). On the other hand, the increase in population has equally affected production by reducing the available land for production, poor marketing infrastructure, decline in soil fertility and lack of awareness among farmers of the technologies available to improve yields and control pests and diseases. The combined effect of nematodes (*Pratylenchus* spp., *Radopholus similis*, *Meloidogyne* spp. and *Helicotylenchus* spp.) and banana weevil (*Cosmopolites sordidus*) is considered to be the principal cause of banana decline in Kenya. Due to these constraints, the average banana yield in Kenya has reduced from 16 tons/ha (KARI, 2004) to 12.8 tons/ha (MoA, 2007).

Management of plant parasitic nematodes in banana production systems has mainly relied on chemical, cultural, plant resistance and biological control. In commercial banana production systems, nematode control mainly depend upon application of nematicides, while in subsistence production systems, pesticide use is less common. The use of nematicides has resulted in food safety and environmental concerns due to pesticide residues and reduced effectiveness. Cultural based methods, such as crop rotation is not broad enough to reduce nematode densities below threshold levels due to increased land pressures and polyphagous nature of the nematodes (Sikora *et al.*, 2003). Thus, the search for novel, environmentally friendly alternatives with which to manage plant-parasitic nematode populations has therefore become increasingly important.

The use of clean planting material is essential in overcoming such constraints as nematodes, especially when establishing new fields (Mateille *et al.*, 1994), as infected suckers tend to be the greatest single source of nematode infestation in the field. Tissue culture (TC) banana planting material offers additional benefits to banana production such as uniformity of plants, and permits rapid dissemination. Yields from TC plants are uniform, harvested early and highly marketable (Vuylsteke and Talengera, 1998). However, although sterile, and pest free, they are also free of beneficial microorganisms such as endophytic fungi that are known to help suppress pest densities. Research in Uganda, Kenya, America, Costa Rica and Germany (Dubois *et al.*, 2006; Pocasangre *et al.*, 2007; zum Felde, 2008; Machungo, 2009) has shown that some specific endophytes are better than others in controlling nematodes.

Management of nematodes through the use of beneficial endophytes offers the prospect of an environmentally safe option. Specifically target pests systemically, *in planta*, where they are less exposed to environmental factors (Athman, 2006; Dubois *et al.*, 2006). Beneficial endophytes are microorganisms that colonize plant tissue without causing disease symptoms and may protect the plant against diseases or pests. In some instances, they develop mutualistic relationships with plants, acting as antagonists to pests and diseases (Gold *et al.*, 2003; Sikora *et al.*, 2003). Endophytes can be isolated, re-introduced and re-isolated from the very tissues where pests and diseases are known to attack (Dubois *et al.*, 2006). Introduction of such beneficial organisms into root tissues of TC plants prior to planting would avoid the need for soil applications of biocontrol agents in great quantities, as endophytes are already “on site” and ready to

protect the crop. In addition, there is evidence of transfer of nematode suppression provided by individual fungal endophytes from one banana generation to the next (zum Felde *et al.*, 2006) and this removes the burden of the farmers to apply the endophytes again.

## 1.2 PROBLEM STATEMENT

Banana (*Musa* spp.) is ranked among the most important horticultural crops in Kenya being a source of food and income to millions of rural Kenyans (MoA, 2007). The production has been on the decline for the past two decades due to pests and diseases. Banana nematodes cause yield losses of up to 30-60 % in many countries (Brooks, 2004). Nematode management in bananas is largely reliant on cultural and chemical control. However, in those areas where bananas are grown continuously, crop rotation cannot be practiced, while at the same time, the cost of nematicides is often prohibitive for subsistence production systems and extremely toxic for the environment. Though there is increasing demand for the crop, and the farmers are now growing the high yielding varieties using disease free tissue culture (TC) planting materials. Re-infestation in the field further renders the use of healthy planting materials only a temporary solution to nematode problems in banana (Speijer *et al.*, 1995, 2001). Thus, there is need to develop an effective, sustainable, environmental friendly and easy-use nematode control option which can complement the available management strategies. Use of fungal endophytes is an important alternative for management of banana nematodes and increasing yields, however, their efficacy is not known in Kenya.

### 1.3 JUSTIFICATION

Nematode control requires the development of alternatives to improve integrated pest management strategies that reduce pesticide use and are environmentally safe. The use of mutualistic endophytes such as *Fusarium oxysporum* to biologically enhance TC banana plantlets is considered an important alternative for improving management of banana nematodes and increasing yields (zum Felde *et al.*, 2006). Use of endophytes offers the prospect of an environmentally safe alternative, which can specifically target the pests from within the plant, where they are less exposed to environmental factors (Sikora, 1997). Non-pathogenic *Fusarium* strains isolated from healthy plants are commercially important in that, some of them can induce resistance in host plants, which enhances the plant's ability to defend itself from pathogen and pest attack (Sikora *et al.*, 2007). Introduction of endophytic fungi to TC plants allows for low initial inoculation levels, maintaining low costs and removing the burden for farmers to apply the microbial organism themselves (Dubois *et al.*, 2006).

## **1.4 OBJECTIVE OF THE STUDY**

### **1.4.1 Broad objective**

To evaluate the efficacy of endophytic *F. oxysporum* isolates for biological control of banana nematodes in TC banana plants.

### **1.4.2 Specific objectives**

- i. To determine the efficacy of selected endophytic *F. oxysporum* isolated in Kenya for biological control of banana nematodes under greenhouse conditions.
- ii. To determine the efficacy of three endophytic *F. oxysporum* isolated in Uganda for biological control of banana nematodes under field conditions in Kenya



## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 NEMATODES AS PESTS OF BANANA

Worldwide banana production losses of approximately 20 % are attributed to nematodes annually (Sikora *et al.*, 2003). Nematodes are involved in the destruction of primary roots that disrupts plant anchorage resulting in toppling or uproot of the plants. The most widespread and important are the burrowing nematodes *Radopholus similis*, some species of root lesion nematode *Pratylenchus coffeae* and *Pratylenchus goodeyi*, species of root knot nematode *Meloidogyne javanica*, *Meloidogyne incognita*, the spiral nematode *Helicotylenchus multicinctus* and the semi endoparasites nematodes *Rotylenchulus reniformis* (Stanton, 1994). In addition to these major nematode root parasites, there are numerous other species that are associated with *Musa* spp. throughout the world (Luc *et al.*, 2005).

The geographic distribution of the main banana nematode species generally depends on temperature and elevation (Sikora, 1992; Bridge *et al.*, 1997). The burrowing nematode (*R. similis*) is important in the altitude range of 0-1500 m asl, while *P. goodeyi* and *H. multicinctus* are endemic at higher altitudes (Spejier *et al.*, 1994). The root-knot nematode *Meloidogyne* spp. is ubiquitous sedentary endoparasite of banana, thriving in the warm, moist and friable soils. They feed from stele and are mainly involved in the reduction of the plant's ability to absorb moisture and nutrients. They form conspicuous root galls within which females at various stages of maturation are found.

Depending on mode of parasitism and nematode species, and density, symptoms will differ from the most severe, such as toppling, to the less obvious such as prolonged vegetative cycle. Banana crop losses depend on several factors including nematode species, banana cultivars, climate conditions and soil factors (Sarah, 2000) as well as fungal, virus and bacterial pathogens.

Results from a survey conducted in Kenya by Seshu Reddy (1998) revealed that a complex of banana nematodes (*P. goodeyi*, *P. coffeae*, *R. similis*, *H. multincinctus*, *Meloidogyne* spp.) were common in many banana fields. The nematode species and population varied across the country. At the coast, up to 200 m asl, *H. multincinctus*, *Meloidogyne* spp. were observed in high densities. In the western region, *H. multincinctus* was common, although generally in moderate densities. *Radopholus similis* was observed in central and western regions. In the central and western regions, higher than 1000 m asl, the lesion nematode, *P. goodeyi* was the dominant species and it was also observed on a “Matooke” cultivar grown at Kilifi, coast region. In the central region, in Muranga district, where coffee is being replaced with banana, *P. coffeae* is commonly found. In addition, *Rotylenchus clavicaudatus* (in high numbers in Homa Bay district), *Scutellonema* spp., *Criconema* spp., *Xiphinema* spp., *Hemicycliophora* spp., and a new *Trophurus* sp. were recorded. A similar survey carried out in central Kenya (KARI, 2004) indicates that the most dominant root-parasitic nematodes were the lesion-inducing *Pratylenchus* spp. and *H. multincinctus*. *Pratylenchus goodeyi* has been observed in every banana growing area of East Africa (Gichure and Ondieki, 1977; Bridge *et al.*, 1997). The life cycle of *P. goodeyi* is completed in about four

weeks under optimum conditions. Their distribution is likely to have been increased through the movement of infested cloned planting material. *Pratylenchus goodeyi* 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 (Bridge *et al.*, 1997).

The damage in roots and corms is similar to that caused by *R. similis*, due to the similar feeding activity on the cytoplasm contents of cells in the cortex their migration between and within cells. This causes formation of cavities within the root tissue and results in characteristic, dark purple lesions and necrotic patches. Symptoms are usually confined to the cortex and the stele tissue is generally unaffected. Infected plants become stunted, bunch weight is decreased and the production cycle is increased. Damage leads to a reduction in the size of the root system, the snapping of roots and the toppling of plants. *Helicotylenchus multincinctus* attack and feed on the outer cells of the root cortex and produce small, characteristic necrotic lesions (Gowen *et al.*, 2005). Development of root lesions caused by *H. multincinctus* is slow relative to those produced by *R. similis*. Lesions on primary roots are shallow and superficial, reddish brown to black in colour. The effect of *H. multincinctus* can lead to stunting of plants, lengthening of the vegetative cycle, and reduction of size of the plant and in bunch weight and reduction of the productive life of the plantation. Toppling may also occur in situations where there are heavy infestations.

### **2.1.1 Management of bananas nematodes**

Currently, nematode management for small scale farmers mainly relies on the use of cultural methods such as pared and hot water-treated suckers, mulching, tissue culture-derived planting material and crop rotation (Speijer *et al.*, 1995; Speijer *et al.*, 1999; Sarah, 2000; Sikora *et al.*, 2003). Although cultural control methods contribute to nematode management, both high labour input and material requirements are often limiting factors for adoption of these practices (Gold *et al.*, 2001). Consequently, breeding for resistant cultivars has been suggested as a potential long-term intervention for nematode control, especially on small-scale farms. Chemical nematicides are also commonly used especially in commercial farms (Gowen *et al.*, 2005). Though effective, abundant application of chemical nematicides now creates severe problems in agricultural production, food safety and environmental protection. Furthermore it is often prohibitive for small scale farmer due to high costs. So, a new, effective, environmental friendly and easy-to-use nematicide would be ideal. Such a management option is expected to be developed through biological control research.

### **2.1.2 Biological control of nematodes**

Biological control of plant parasitic nematodes is practiced in the world. A large number of natural enemies, including parasitic and trapping fungi, mycorrhizal fungi, bacteria, viruses, nematodes, insects, mites, invertebrates, are investigated as possible biological control agents against nematodes (Stirling, 1991). Bacteria and fungi are the most abundant organism in the soil and some of them have shown great potential as bio-

control agents for plant parasitic nematodes (Stirling, 1991; Sikora, 1992). To date, most research on biological control has concentrated on plant health promoting rhizobacteria, obligate bacteria parasites and fungal egg pathogens, predacious or trapping fungi, mutualistic endoparasitic fungi and endomycorrhizal fungi. The biological control of PPN by antagonists in general, using rhizobacteria, endophytic bacteria and fungal endophytes has been well documented (Hallmann and Sikora 1994; Niere *et al.*, 1999; Kerry, 2000; Pocasangre, 2000).

A most promising alternative to pesticides is biological enhancement of banana tissue culture plantlets with beneficial fungal endophytes to increase plant resistance to infection to banana nematode (Sikora and Pocasangre, 2004). Mutualistic fungal endophytes have been shown to biologically control parasitic nematodes that attack banana (IITA, 1998). Endophytes are usually not effective because their densities are low under normal field conditions, and the fact that they must compete for food and space with other microorganisms (Sikora and Pocasangre, 2004). Therefore biological enhancement of endophytes in to tissue cultured banana plants, gives the endophytes a head start and a competitive advantage over other microorganisms present. The mechanism of action of antagonistic endophytes is usually related to disruption in juvenile penetration, induced resistance and/or to competition for nutrients that ultimately reduces nematode fecundity (Sikora and Hoffmann, 1993).

## **2.2 ENDOPHYTES AS BIOLOGICAL CONTROL AGENTS**

The term endophyte, with the exception of the endotrophic mycorrhizal fungi was always closely associated with beneficial organisms colonizing the phyllosphere. However, the definition of an endophyte is now broadened by many researchers and can include any organisms that live in plant tissue whether neutral, beneficial or detrimental (Sikora *et al.*, 2007). Beneficial endophytes are microorganisms living within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence (Saikkonen *et al.*, 2004). Typically, these are bacterial and fungal which mostly inhabit healthy and symptomless plants, in various tissues, seeds, roots, stems and leaves. Presence of a mutualistic endophyte acts as a “biological trigger” to activate the stress response system more rapidly and strongly than non-mutualistic plants.

Beneficial endophytes enter plant tissue primarily through the root zone, however, aerial portions of plants, such as flowers, stems, and cotyledons, may also be used for entry (Kobayashi *et al.*, 2000). Inside a plant they may either become localized at the point of entry or spread throughout the plant (Hallmann *et al.*, 1997). These microorganisms can reside within cells, in the intercellular spaces, or in the vascular system. They can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue.

### **2.2.1 Endophytic bacteria**

Bacteria are numerically the most abundant organisms in soil. Endophytic bacteria have been found internally in root tissue, where they persist in most plant species, but do no harm to the plant (Azevedo *et al.*, 2000; Surette *et al.*, 2003). As reported by Kobayashi

(2000), both gram-positive and gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species. The bacteria enter tissues via germinating radicles, secondary roots, stomates, or as a result of foliar damage. Endophytic bacteria have been shown to promote plant growth and to inhibit disease development and nematode pests (Azevedo *et al.*, 2000; Munif *et al.*, 2000; Sturz and Kimpinski, 2004).

Various species of *Pasteuria*, *Pseudomonas* and *Bacillus* have shown great potential for the biological control of nematodes. For example, studies by Rodriguez-romero *et al.*, (2008) showed that *Pseudomonas fluorescens* reduced the number of root knot nematodes. Several bacterial species have also been found to possess activity against root-lesion nematode (*Pratylenchus penetrans*) in soil around the root zone of potatoes. Among them, *Microbacterium esteraromaticum* and *Kocuria varians* have been shown to play a role in root-lesion nematode suppression through the attenuation of host proliferation, without incurring any yield reduction (Munif *et al.*, 2000).

They affect nematodes by a variety of modes; for example parasitizing, producing toxins, antibiotics, or enzymes; interfering with nematode–plant-host recognition; competing for nutrients; inducing systemic resistance of plants; and promoting plant health (Siddiqui and Mahmood, 1999; Compant *et al.*, 2005). They form a network with complex interactions among bacteria, nematodes, plants and the environment to control populations of plant-parasitic nematodes in natural conditions (Kerry, 2000).

### 2.2.2 Endophytic fungi

Endophytic fungi have attracted great attention in the past few decades for two main reasons. First, growing evidence indicates that endophytic fungi occur in all plants, are extremely abundant and are often very diverse (Schulthess and Faeth, 1998; Arnold *et al.*, 2000). Most of these endophytes form internal localized infections in foliage, roots, stems and bark and are horizontally transmitted via spores. A much smaller fraction, mostly found in grasses, form systemic infections in above-ground tissues. Some of these are vertically transmitted via hyphae growing into seeds (Saikkonen *et al.*, 1998). Second, endophytic fungi may produce mycotoxins, or otherwise alter host physiology and morphology. Endophytic mycotoxins are thought to benefit their woody plant hosts as ‘inducible defenses’ against insect herbivores (Carroll, 1991) and their grass hosts as ‘acquired plant defenses’ (Cheplick and Clay, 1988) against both vertebrate and invertebrate herbivores. Endophytic fungi may also alter other physiological, developmental or morphological properties of host plants such that competitive abilities are enhanced, especially in stressful environments (Clay, 1990; Malinowski *et al.*, 1999). This wide array of purported benefits has led to the conclusion that many, if not all, endophytes in host plants are strong plant mutualists (Clay, 1990; Schardl and Phillips, 1997).

The best understood of these are the members of the *Clavicipitaceae* (*Ascomycota*), which are endophytes of some temperate grasses. In these systems, there is usually only one endophytic fungal species per host and this fungus appears highly coevolved with their host. Generally the fungus is transmitted vertically from the mother to the



offspring through seeds (Clay and Schardl, 2002). This transmission pattern is thought to promote beneficial relationships with the host plant (Herre *et al.*, 1999). Nonetheless, in grasses, the net effect of endophytic associations can range from parasitic (e.g. choke disease) to strong mutualistic (Clay and Schardl, 2002). More recently, anti-pathogen protection mediated by endophytic fungi has also been observed in non-gramineous hosts. For example, (Mejia, 2008) isolated fungal endophytes from healthy *Theobroma cacao* (cacao) tissues and screened them *in vitro* for antagonism against major pathogens of cacao. Of the tested endophytic morphospecies, 40 %, 65 %, and 27 % showed *in vitro* antagonism, against *Moniliophthora roreri* (frosty pody rot), *Phytophthora palmivora* (black pody rot) and *Marasmius perniciosus* (witches broom), respectively. In tomato (*Lycopersicon esculentum*), split-root plants treated with *F. oxysporum* strain 162 showed 26-45 % less root knot nematode penetration, 21-36 % less galls and a 22-26 % reduction in the number of eggs in the roots not directly inoculated with the fungus when compared to untreated control plants in repeated tests (Dababat *et al.*, 2007).

Endophytic fungi have also been found to protect bananas from nematodes (Dubois *et al.*, 2004; Athman, 2006; Machungo, 2009). The majority of endophytic fungi isolated from healthy banana tissue belong to the genus *Fusarium*, followed by *Acremonium*, others include soil fungi belonging to the genera *Penicillium*, *Aspergillus*, *Gongronella* and also *Trichoderma* which have biological potential against nematodes (Niere *et al.*, 2002).

Strains of *Fusarium* spp., especially *F. oxysporum* are the most abundant endophytes of banana (*Musa* spp.). Successful inoculation of tissue culture banana plants with fungal endophytes (including *Acremonium* sp., *Fusarium concentricum*, *Fusarium oxysporum*, *Fusarium solani* and *Geotrichum candidum*) was reported by Griesbach (2000), Niere (2001) and Paparu *et al.* (2004). Nematode densities in endophyte-inoculated plants were reduced by 42-79 % and root necrosis by 30-40 % (Niere, 2001). The endophytes have the advantage of targeting the destructive stages of banana nematodes that occur within the plant. Besides acting as antagonists to pests and diseases, fungal endophytes have been reported to increase biomass production in inoculated plants. Griesbach (2000) reported increased banana biomass production of the cultivars Nakyetengu and Nfuuka (AAA-EA) inoculated with the fungal endophyte *F. concentricum* in the field. Various modes of action against pests and diseases have been attributed to endophytes: parasitism, competition, production of secondary metabolites and indirect effects through induced resistance (Dubois *et al.*, 2006). Production of secondary metabolites and induced resistance are considered the main modes of action against banana nematodes (Athman, 2006).

## CHAPTER THREE

### 3.0 GREENHOUSE EVALUATION OF SELECTED KENYAN ENDOPHYTIC *Fusarium oxysporum* ISOLATES FOR CONTROL OF BANANA NEMATODES (*Pratylenchus goodeyi*)

#### ABSTRACT

Greenhouse studies were carried out to evaluate the efficacy of two non-pathogenic *Fusarium oxysporum* isolates (4MOC321 and 11SR23) against *Pratylenchus goodeyi*. The isolates were isolated from East African highland banana at the eastern and coastal region in Kenya as bio-control agents against *Pratylenchus goodeyi*. Isolate V5W2 originating from Uganda was used as a positive standard. Two month old tissue culture-derived plantlets (cv. Giant Cavendish and cv. Grand Nain) were inoculated with endophytes at potting and one month later inoculated with 500 *P. goodeyi*/plant. The experiments were laid out in a completely randomized design with control plants inoculated with nematodes but no endophytes. Plant growth response to endophyte treatment was evaluated over a period of four months. Nematode population, percentage damage and colonization were determined at termination of the experiments. Results demonstrate enhanced growth for plants inoculated with endophytes when compared to the control plants for height, girth of the pseudostem and root weights. The number of dead roots was significantly lower in endophyte inoculated plants and the number of functional roots significantly higher when compared to the control plants. *Pratylenchus goodeyi* population densities in endophyte-inoculated plants compared to non-inoculated plants were reduced >50 %, while percentage root necrosis was reduced by

>30 %. The study demonstrates that the Kenyan endophytic isolates have the potential to enhance growth of tissue cultured banana plants as well as control banana nematode.

### 3.1 INTRODUCTION

Banana nematodes are key pests of bananas worldwide. Although management of these pests has mainly relied on use of clean planting materials, re-infestation in the field remains a critical concern (Speijer *et al.*, 2001). Tissue culture plants are produced axenically, making them pest and disease-free. According to Pereira *et al.* (1999), the axenic nature of the plants production eliminates beneficial microorganisms, which may confer some resistance to nematodes in the field. Thus, the plants are easily attacked by nematodes in the field. Introduction of the beneficial endophytic fungi into tissue culture banana at weaning stage to biologically enhance tissue cultured banana plantlets is considered an important alternative for improving management of banana nematodes (zum-Felde *et al.*, 2006).

The use of microbial agents for biological control of banana nematodes has been investigated *in vitro* in greenhouse trials (Niere, 2001; Athman, 2006; Machungo, 2009). Endophytic *Fusarium oxysporum* have been demonstrated to control *Radopholus similis* (Schuster *et al.*, 1995; Niere, 2001). The endophytes offer a unique opportunity for pest control, as they can be introduced to tissue culture banana plants, are effective at low initial inoculation levels and can achieve control at relatively low costs, compared to other biopesticides (Dubois *et al.*, 2006). *Fusarium oxysporum* endophyte V5W2 has been identified as effective in controlling nematodes (Athman, 2006; Machungo, 2009). Endophytic *F. oxysporum* reduces *R. similis* populations primarily by means of induced resistance (Athman, 2006). They have also been shown to stimulate plant growth (Niere, 2001).

Several greenhouse experiments have been carried out to evaluate the efficacy of non-pathogenic *F. oxysporum* isolates as bio-control agents against banana nematodes (Niere, 2001; Athman, 2006; Machungo, 2009) both in Uganda and Kenya. However, these previous studies have mainly dealt with endophytic isolates that were originally isolated from healthy banana plants in Uganda (Niere, 2001). Recently, endophytic isolates originating from Kenya bananas were isolated and only tested for biological control of banana nematodes *in vitro* (Mwaura, 2008). However, there is need for exploring the potential use of these endophytic fungi isolated from Kenyan bananas as bio-control agent against nematodes under *in vivo* conditions. Thus, the objectives of this study were i) to evaluate the efficacy of selected Kenyan endophytic *F. oxysporum* isolates against *P. goodeyi*, ii) to evaluate the growth response of tissue cultured banana cultivars to endophytic fungi inoculation.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Site description**

All experiments were carried out in a greenhouse at Jomo Kenyatta University of Agriculture and Technology (JKUAT) (Altitude: 1537 m asl, 01° 05' 25.6''S, 037° 00' 45.5''E). Throughout the duration of the experiment, daily temperatures in the greenhouse ranged between 22 and 27°C with natural photoperiod, 12 hrs light and 12 hrs dark.

### 3.2.2 Experimental design and layout

Two non-pathogenic *F. oxysporum* isolates (*4MOC321* and *1ISR23*) isolated from banana in Kenya and one from Uganda (*V5W2*) were evaluated for their efficacy against *P. goodeyi* in greenhouse trials. Two banana cultivars were used; cv. Giant Cavendish (AAA) and cv. Grand Nain (AAA). The experiments were conducted between August 2008 and February 2009. The treatments included; *4MOC321* + nematodes, *1ISR23* + nematodes, *V5W2* + nematodes and control + nematodes laid out in a completely randomized design (CRD) with three replications. The number of plants per treatment was 5, hence 60 plants/cultivar and a total of 120 plants for one trial. The experiment was carried out twice.

### 3.2.3 Fungal isolates

The two Kenya *F. oxysporum* isolates used for this study were originally isolated from the roots and corms of apparently healthy banana plants: *4MOC321* from Meru district (Altitude: 1050 m asl 06° 34S, 037° 46' 23E) and *1ISR23* from Kilifi district (17 m asl 03° 4S 0'' 039° 40' 0''E) in eastern and coast province, respectively (Mwaura, 2008). These isolates were selected for the current study due to their ability to immobilize and cause higher nematode mortality *in vitro* (Mwaura, 2008). The isolate *V5W2* was originally isolated and tested in Uganda (Athman, 2006) and later in pots Kenya (Machungo, 2009) was included for comparison. The isolates were stored on sterile soil tubes at 4 °C.

### **3.2.4 Preparation of fungal inoculum**

Small quantities (0.01 g) of soil containing fungi from the soil tubes were sprinkled on sterile SNA media (1 g  $\text{KH}_2\text{PO}_4$ , 1 g  $\text{KNO}_3$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g  $\text{KCl}$ , 0.2 g glucose, 0.2 g sucrose, 0.6 ml  $\text{NaOH}$  (1M) and 15 g agar  $\text{L}^{-1}$  distilled water) in 90 mm Petri dishes. The Petri dishes were incubated under laboratory conditions (room temperature  $25^\circ\text{C}$ , under natural light conditions) for 14 days to allow the fungus to sporulate. The SNA media was supplemented with 0.05 g chlorotetracycline, 0.1 g penicillin G and 0.2 g streptomycin-sulphate per litre to prevent bacterial contamination. Spore suspensions for inoculating banana plants were produced in 100 ml duran bottle containing 100 ml half strength potato dextrose broth (PDB). Half strength PDB was prepared by dissolving 12 g of PDB per litre of sterile distilled water. The flasks containing PDB were sterilized (autoclaved at  $121^\circ\text{C}$  for 15 minutes) and allowed to cool down. Five mycelial blocks (approximately  $1 \text{ cm}^3$  each) containing spores and mycelium of each fungal isolate were cut from 2-week old cultures on SNA and aseptically transferred to PDB. Five replicate flasks were prepared for each fungal isolate. Five flasks containing non-inoculated broth served as controls. Flasks were incubated under laboratory conditions for 14 days to allow for fungal sporulation. Each day, flasks were manually shaken to disperse spores throughout the media. Fungal spores were harvested by filtering the suspension through a 1-mm diameter sieve to remove mycelial fragments into a 1000 ml sterile beaker. Spore densities for each isolate were estimated using a haemocytometer and the suspensions standardized to provide a final spore concentration of  $1.5 \times 10^6$  spores  $\text{ml}^{-1}$ . The standardization of the



spore suspensions were either diluted with sterilized distilled water or concentrated to achieve the required spore concentration. To concentrate, the spore suspensions were left to settle for a few hours and broth at the top of the solution siphoned off.

### **3.2.5 Inoculation of plants with fungal isolates**

The tissue culture banana plants used for this experiment were obtained from the JKUAT tissue culture laboratory. Two month old tissue culture plants (post-weaning) were removed from trays. Roots were gently rinsed in tap water to remove adhering soil and before cutting back to approximately 2 cm length using sterile scalpel blade to enhance fungal infection. The roots were then immersed in the various spore suspensions for 2 hrs, ensuring that all roots were submerged (Niere, 2001). After inoculation, the plants were potted in 3-L plastic bags (6 x 10 x 10 cm) containing steam sterilized sandy loam forest soil. At the time of potting, 1 ml of each spore suspension was poured around the roots before covering with soil. The plants were watered daily and maintained for one month before inoculation with nematodes.

### **3.2.6 Preparation of the nematode inoculum**

Nematode infected banana roots were obtained from banana plants initially inoculated with *Pratylenchus goodeyi* and maintained in a greenhouse at JKUAT. The roots were washed free of soil with tap water and cut transversally to assess nematode damage. The root segments were further cut into small pieces (1 cm) and macerated in a blender thrice at low speed for 15 seconds with a short interval between. Nematodes were extracted overnight from the macerated roots using the modified Baermann method

(Hooper *et al.*, 2005). The nematode extraction apparatus consisted of a plastic sieve placed in a shallow plastic plate and overlaid with paper towels. After the maceration, the nematode suspension was poured onto the paper towels in the sieve. The plastic plate was filled with tap water to a level that just covers the macerated root and left undisturbed on the laboratory bench for 24 hrs during which time nematodes migrated from the macerated tissues into the water. The nematode suspensions from the plastic plate were then concentrated using a 38  $\mu\text{m}$  and backwashed into sterile 10 ml Bjorn bottles. The nematode suspensions from the Bjorn bottles were concentrated in a 1000 ml beaker and thoroughly mixed in order to have uniform nematode suspension. Using a micro pipette, 1 ml of the nematode suspension was put on a nematode counting slide and the population of living nematodes was determined by species and development stages (female, male and juvenile) using a compound microscope at magnification of 40. The suspension was standardized to provide 500 nematodes  $\text{ml}^{-1}$ .

### **3.2.7 Inoculation of plants with nematodes**

Plants were inoculated with 500 *P. goodeyi* /plant one month after endophyte inoculation. Prior to inoculation, the plants were watered and three holes of 3-5 cm deep were made in the soils at the base of the plants roots using a stick. The three holes were made around the roots at equal distances from each other. The nematode suspension was pipetted into the holes at rate of 0.3 ml per hole after which the holes were covered with soil. Plants were not watered until one day after inoculation to enable the

nematodes to penetrate the roots and avoid washing away the nematodes. After inoculation, plants were maintained in the greenhouse for 12 weeks.

### **3.2.8 Plant growth assessment**

Plant growth parameters (plant height, girth of the pseudostem, number of healthy standing leaves, and the length and width of the youngest leaf) were measured on a fortnight basis for four months. Plant height was measured as the distance from the point where the youngest leaf emerges from the pseudostem to the base of the plant, girth was measured at approximately 2 cm from the base of the plant while the width was measured at the widest point on the youngest leaf. Leaves were considered healthy when more than three quarters of the leaf area was green as opposed to yellow and brown leaves. At the termination of the experiments, plants were removed from the plastic bags and washed free of soil with tap water. For each plant the number of functional and dead roots was recorded and the fresh and dry root and shoot weights were determined. Dry weights were assessed after oven drying at 70 °C for 72 hrs. Root fresh weights were measured after selecting the 3 and 5 roots used for fungus re-isolation and nematode extraction respectively.

### **3.2.9 Determination of fungal colonization**

The ability of the endophytes to colonize tissue culture plants was evaluated at the time of termination. Three plants per treatment per cultivar were randomly selected, uprooted and their roots washed thoroughly with tap water to remove adhering soil. Three healthy roots from each plant were randomly selected and used for fungus re-isolation. Under

aseptic conditions, the roots were surface sterilized for two minutes in 95 % ethanol and flamed for 1 minute. For each root, six pieces of 0.5 cm long were cut from the base, mid and tip portions (two from each portion). Each root piece was plated in 90 mm Petri dishes containing SNA. Petri dishes were incubated in the laboratory for 7-10 days and emerging fungal colonies observed under a light microscope (x40 magnification). *Fusarium oxysporum* colonies were identified based on morphological characteristics, such as the presence of macro conidia (sickle-shaped with attenuated apical cell), short phialides and chlamydospores. Percentage colonization was determined per Petri dish as the number of root pieces with *F. oxysporum* colonies divided by the total number of root pieces plated, multiplied by 100.

### **3.2.10 Assessment of nematode damage and population**

From the three plants selected for fungus re-isolation, five roots were randomly selected and used for assessment of nematode damage according to Speijer and De Waele (1997). Nematode damage was expressed as a percentage of the necrotic root tissue. To estimate the percentage root necrosis, the five roots were cut to approximately 10 cm long segments. The root segments were sliced lengthwise and the percentage of the visible necrotic cortical region determined. One half of the root was used for scoring the percentage of the cortical necrosis. Each root represented a maximum percentage root necrosis of 20 %, with the five roots per plant adding up to a total of 100 % root necrosis. The five root segments were further cut to approximately 1 cm, thoroughly mixed and 5 g sub-sample used for nematode extraction. The sub-samples were

macerated for 15 s at low speed using a kitchen blender prior to extraction using modified Baermann method for 24 hrs (Hooper *et al.*, 2005). The recovered nematode suspensions were then concentrated with 38  $\mu\text{m}$  and backwashed into sterile 10 ml Bjorn bottles. The suspensions were stored at 4 °C until identification and counting could be done. Prior to counting, the suspension was reduced to 5 ml. The recovered nematodes were identified and counted in two, one ml aliquots of the respective suspensions using a compound microscope and the average was calculated. The population of female, male and juvenile of *Pratylenchus goodeyi* was recorded separately.

### **3.3 DATA ANALYSIS**

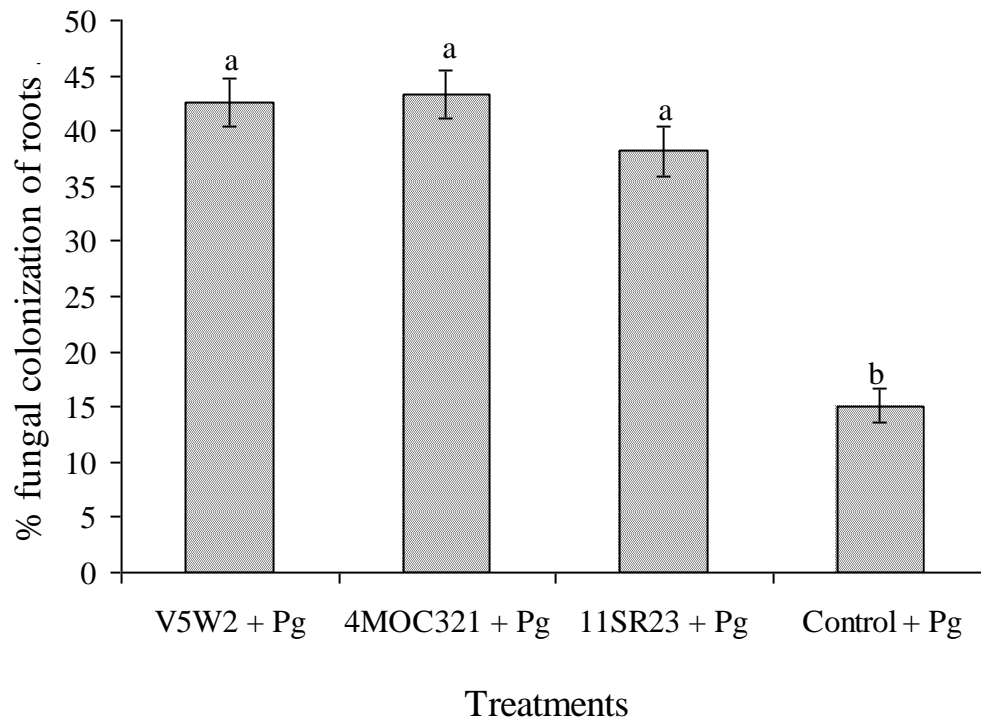
The Levene option of the Hovtest (SAS, 2003) was used to test for normality and variances homogeneity for all factor effects before subjecting to ANOVA. If not normally distributed various transformations were carried out prior to analysis of variance (ANOVA). Nematode counts were calculated per 100 g of root sample after log transformation while percentage nematode damage and colonization data were arcsine-square root-transformed. The generalized linear model (Proc GLM) was used to test for factor effects and their interactions. When significant factor interaction was noted effects of one factor was analysed at each level of the other factor. Means were separated using least significant difference test (LSD) (SAS, 2003). All tests were performed at a significant  $\alpha$  of 0.05.

### **3.4 RESULTS**

No significant differences were observed between the two experiments carried out and therefore the data was pooled together. The two banana cultivars (cv. Grand Nain and cv. Giant Cavendish) reacted similarly to nematode infection and endophyte colonization and plant growth ( $P>0.05$ ). The results did not depend on the cultivar as indicated by an insignificant interaction (treatment\* cultivar,  $P>0.05$ ). However, significant ( $P<0.05$ ) differences were observed between endophyte treated plants and untreated plants for many assessed variables except for the plant growth parameters.

#### **Endophytic Colonization**

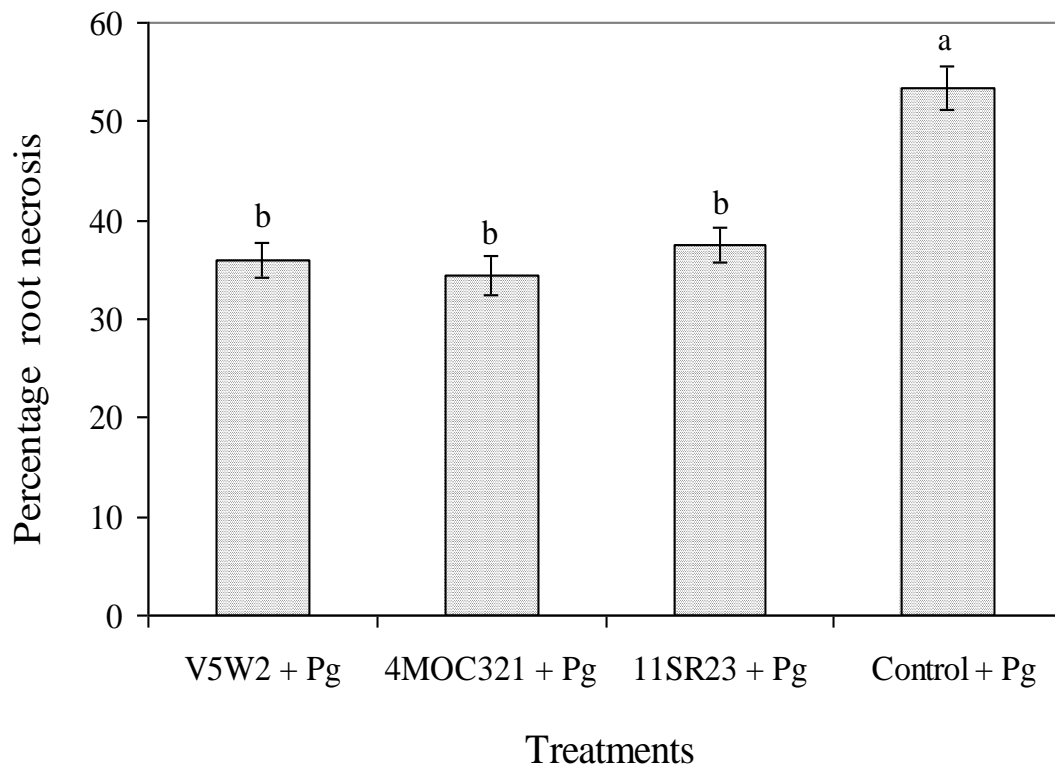
Sixteen weeks after inoculation, percentage colonization of root tissue in endophyte-inoculated plants was between 38.2 % and 43.3 % (Fig. 1), with isolate *4MCO321* having the highest percentage colonization. Tissue colonization was significantly ( $P<0.0001$ ) greater in all endophyte-inoculated plants compared to untreated plants in both experiment but did not vary significantly for all isolates. Though control plants were not inoculated, tissue colonization by *F. oxysporum* was recorded and ranged from 15.1 % to 17.6 %.



**Figure 1:** Percentage root tissue colonization by endophytic *Fusarium oxysporum* isolates in banana plants. Bars and standard errors are means of 3 banana plants replicated three times. Pg- *Pratylenchus goodeyi*.

### **Nematode damage**

Nematode damage to banana roots did not significantly ( $P>0.05$ ) differ between experiment one and two. Percentage root necrosis was significantly ( $P<0.0001$ ) lower in plants inoculated with the fungal isolates compared to the control plants (Fig. 2). Plants treated with isolate *4MOC321* had the least root necrosis of 34.4 % followed by *V5W2* with 35.9 % and 37.4 % for *11SR23* compared with control plants (53.4 %). Root necrosis levels were similar between all three isolates ( $P=0.05$ ).



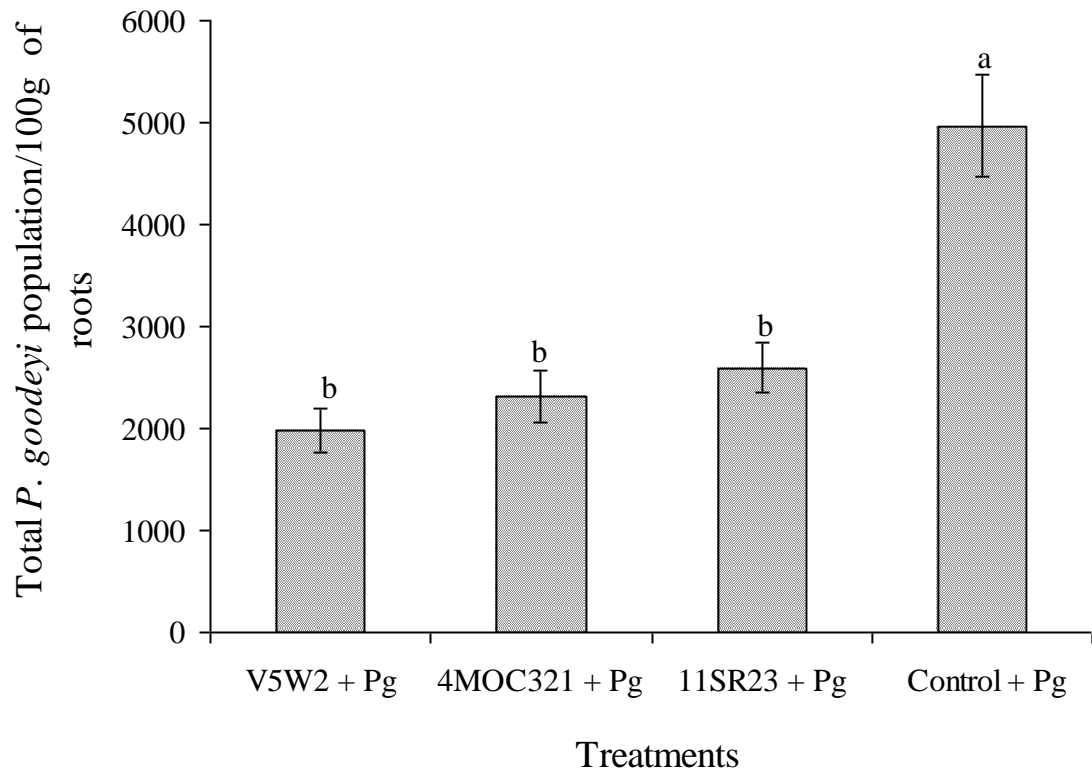
**Figure 2:** The effect of Endophytic *Fusarium oxysporum* isolates on the damage caused by *Pratylenchus goodeyi* in banana roots of 16-week-old banana plants, 12 weeks after inoculation with 500 nematodes. Bars and standard errors are means of 3 banana plants replicated three times. Pg- *Pratylenchus goodeyi*.

#### **Population of *Pratylenchus goodeyi***

No significant differences were observed in nematode counts between experiment one and two ( $P > 0.05$ ). However, the nematode population significantly differed among treatments ( $P < 0.0001$ ) (Fig. 3). The *P. goodeyi* population densities in endophyte-inoculated plants compared to non-inoculated plants were 60 %, 53 % and 47 % lower



than controls for isolate *V5W2*, *4MOC321* and *11SR23*, respectively (Fig. 3), but the differences between isolates were not significant ( $P=0.05$ ).



**Figure 3:** The effect of endophytic *Fusarium oxysporum* isolates on the total *Pratylenchus goodeyi* population density (Males, Females and Juveniles) in roots of 16-week-old banana plants, 12 weeks after inoculation with 500 nematodes. Means and standard errors were calculated from untransformed data. Statistical analysis was performed on log transformed data. Bars and standard errors are means of 3 banana plants replicated three times. *Pg*- *Pratylenchus goodeyi*.

***Pratylenchus goodeyi* (females, males and juveniles)**

The effects of the three endophytic isolates were not sex specific. The population densities of *P. goodeyi* females, males and juveniles on plants treated with the endophytes differed from the control only ( $P < 0.0001$ ) (Table 1). However, inoculation with isolate *V5W2* resulted in relatively fewer *P. goodeyi* females and juveniles compared to the other isolates, while isolate *4MOC321* recorded relatively lower densities of *P. goodeyi* males compared with other isolates. The control plants recorded the greater densities of *P. goodeyi* females, males and juveniles in all the experiments. Endophyte inoculation led to >30 % reduction in the population of *P. goodeyi* females and males, and >55 % reduction in juveniles.

**Table 1:** The effect of endophytic *Fusarium oxysporum* isolates on the densities *Pratylenchus goodeyi* females, males and juveniles in roots of 16-week-old banana plants, 12 weeks after inoculation with 500 nematodes

<b>Population of <i>P. goodeyi</i> (females ,males and juveniles)/100g root</b>			
Treatment	Females	Males	Juveniles
<i>V5W2</i>	565.8 ± 65.89 <sup>b</sup>	239.83 ± 29.24 <sup>b</sup>	1179.6 ± 145.60 <sup>b</sup>
<i>4MOC321</i>	633.3 ± 71.50 <sup>b</sup>	214.81 ± 20.56 <sup>b</sup>	1462.1 ± 186.50 <sup>b</sup>
<i>11SR23</i>	855.6 ± 97.77 <sup>b</sup>	303.75 ± 34.11 <sup>b</sup>	1435.2 ± 136.15 <sup>b</sup>
Control	1256.5 ± 178.28 <sup>a</sup>	437.94 ± 52.49 <sup>a</sup>	3268.6 ± 328.70 <sup>a</sup>
LSD	100.78	315.08	597.32
P value	<0.0001	<0.0001	<0.0001

Data are means of three banana plants replicated three times. Means followed by the same letter within a column (Superscript) are not statistically different at  $P=0.05$ (LSD). Means and standard errors were calculated from untransformed data. Statistical analysis was performed on log transformed data.

### **Plant growth**

Growth parameters (plant height, girth of the pseudostem and number of functional leaves) assessed in trial one and two were not significantly ( $P>0.05$ ) different. Inoculating tissue-cultured banana cv. Giant Cavendish and cv. Grand Nain, with *F. oxysporum* isolates resulted in relatively enhanced growth of 16-week-old plants. Plants inoculated with isolate *V5W2* were relatively taller by 1.5 % with a slight increase in the number of functional leaves by 2.6 % when compared to control treatment. Similarly, inoculation of plants with isolates *V5W2*, *4MOC321* and *1ISR23* led to relative increase in girth of the pseudostem by 8.1 %, 2.7 % and 1.4 %, respectively compared to control plants. However, control plants had relatively wider length and width of the youngest leaf, 13.27 and 5.61cm, respectively compared to the endophyte inoculated plants (Table 2).

**Table 2:** The effect of endophytic *Fusarium oxysporum* isolates on plant height, girth of the pseudostem, number of functional leaves, length and width of the youngest leaf of 16-week-old banana plants), 12 weeks after inoculation with 500 nematodes

Plant Growth Parameters					
Treatment	Height (cm)	Girth (cm)	NOFL	Length (cm)	Width (cm)
<i>V5W2</i>	6.86 ± 0.21	0.80 ± 0.02	5.52 ± 0.11	13.08 ± 0.27	5.54 ± 0.17
<i>4MOC321</i>	6.75 ± 0.21	0.76 ± 0.02	5.33 ± 0.11	12.78 ± 0.26	5.37 ± 0.16
<i>1ISR23</i>	6.45 ± 0.22	0.75 ± 0.02	5.26 ± 0.11	12.81 ± 0.28	5.43 ± 0.17
Control	6.76 ± 0.24	0.74 ± 0.02	5.38 ± 0.11	13.27 ± 0.29	5.61 ± 0.18
LSD	0.61	0.05	0.30	0.76	0.47
P value	0.567	0.191	0.3956	0.5327	0.7682

Data are means ± standard errors of five banana plants replicated thrice. NOFL-Number of functional leaves. Length and width of the youngest leaf.

Shoot and root fresh weights, shoot and root dry weights and number of functional leaves of endophyte-treated plants and control plants were not significantly ( $P > 0.05$ ) different (Table 3). However, isolates *V5W2*, *4MOC321* and *1ISR23* slightly enhanced root fresh weight compared to control treatment though the difference was not significant ( $P = 0.6884$ ) (Table 3). The total number of dead roots significantly ( $P < 0.0001$ ) differed among the treatments with control plants having the most dead roots (5.97), while isolate *1ISR23* had the fewest with (2.61). Isolate *1ISR23*-treated plants had relatively more number of functional roots compared to the other treatments. The fresh shoots, dry shoots and roots were all relatively heavier in control plants.

**Table 3:** The effect of endophytic *Fusarium oxysporum* isolates on fresh root and shoot weights, dry shoot and root weights, total number of functional and dead roots of 16-week-old banana plants, 12 weeks after inoculation with 500 nematodes

Treatment	Shoot fresh Weight(g)	Shoot dry Weight(g)	Root fresh Weight(g)	Root dry Weight(g)	No. functional Roots	Dead Roots
V5W2	94.77 ± 5.7	15.11± 0.6	40.55 ± 1.1	7.87 ± 0.2	12.36 ± 0.5	5.36 ± 0.6 <sup>b</sup>
4MOC321	91.59 ± 3.9	14.87± 0.5	30.50 ± 1.9	7.58 ± 0.5	11.89 ± 0.4	5.42 ± 0.3 <sup>b</sup>
1ISR23	87.55 ± 6.4	14.71± 0.7	29.95 ± 2.0	7.83 ± 0.2	12.56 ± 0.6	2.61 ± 0.4 <sup>a</sup>
Control	95.77± 7.1	15.52± 0.9	29.90± 2.5	7.97 ± 0.3	12.08 ± 0.7	5.97 ± 0.6 <sup>b</sup>
LSD	16.48	1.93	16.03	0.66	1.57	1.22
P value	0.7572	0.853	0.4793	0.6884	0.8421	<0.0001

Data are means and standard errors of three banana plants replicated thrice. Means followed by the same letter within a column (Superscript) are not statistically different at P=0.05(LSD).

### 3.5 DISCUSSION

Non-pathogenic *F. oxysporum* isolates 4MOC321, 11SR23 and V5W2 were all able to successfully colonize tissue cultured dessert banana plantlets when inoculated artificially. Similar results were obtained by Griesbach (2000) and Paparu *et al.* (2004) for various Ugandan strains of *F. oxysporum*. Root tissue colonization varied with endophytic isolate, which was most pronounced with V5W2 although the three assessed isolates provided similar levels of colonization, based on re-isolation at 16 weeks. Colonization of untreated plants by endophytic *F. oxysporum* could be due to possible contamination from irrigation water or from the greenhouse environment, which has been observed and documented previously (Athman, 2006).

Data from the current study demonstrates that growth endophyte-treated plants as compared with control plants appear to be stimulated slightly. Enhanced growth of plants inoculated with fungal endophytes has been reported for tomato (Hallmann and Sikora 1994), maize (Nassar *et al.*, 2005) and banana (Niere, 2001). Niere *et al.* (1999) reported that plant height of some banana cultivars was increased by some endophyte isolates. Higher root fresh weights were also demonstrated in endophyte-treated plants. This concurs with the findings of Pocasangre (2000) who reported plant growth promotion by endophytic *Fusarium* manifested in greater shoot and root weights.

The current study shows that the two Kenyan-derived endophytic isolates 4MOC321 and 11SR23 stimulated the growth of the banana plantlets as effectively as the Ugandan isolate V5W2. The endophytes functioned as growth promoters in healthy tissue

cultured banana plantlets. The mechanism of growth improvement was beyond the scope of this study, but the improved growth of the inoculated plantlets could be attributed to the phytohormone effect produced by endophytic fungi. Previous studies by Nassar *et al.* (2005) showed that numerous *Fusarium* spp. isolates and other endophytic fungi could produce phytohormones, which are capable of stimulating host plant growth.

An absence of significant differences in measured growth parameters between the two cultivars used demonstrates that performance of the endophytes is not cultivar dependent. This can be primarily attributable to the non-host specificity of the endophytes, their growth-promoting properties and the amenability of banana plants to endophyte infection (Pan *et al.*, 1997). This is in contrast to earlier report where significant differences in growth between cv. Giant Cavendish and cv. Grand Nain plants inoculated with endophytic *F. oxysporum* were recorded (Machungo, 2009). Insignificant differences in growth between endophyte inoculated plants and control plants may be due to the short duration within which the experiments were conducted i.e. four months. Similarly, Niere (2001) and Athman (2006) observed limited statistical differences in growth between endophyte-treated and control plants.

In this study, the endophytes tested (*V5W2*, *4MOC321* and *1ISR23*) formed a neutral–beneficial association with the banana plantlet, as the growth of the inoculated plantlets was either similar to or slightly better than that of plantlets without endophytes. Furthermore, the isolates of *F. oxysporum* used for inoculation did not induce wilting

symptoms in the cultivar used during the course of the trials, again demonstrating the non-pathogenic nature of these fungal isolates.

Recent findings have shown *in vitro* antagonism of endophytic isolates originating from Kenya against *P. goodeyi* and *H. multicinctus* in banana plants (Mwaura, 2008). Likewise, results presented in this study clearly show that applications of Kenyan endophytic *F. oxysporum* isolates provide enhanced biological nematode control in a controlled environment. Nematode multiplication was effectively reduced in banana roots of endophyte-inoculated plants. Similar results obtained by Speijer (1993) by simultaneous inoculation of bananas with *F. oxysporum* and *P. goodeyi* resulted in reduced nematode multiplication. Antagonism against *P. goodeyi* did not differ significantly between the Kenyan isolates (*4MOC321* and *11SR23*) and the Ugandan isolate *V5W2*. The possible mechanism of action against plant parasitic nematodes could be attributed to production of secondary metabolites within the plant tissues that are toxic to nematodes (Athman, 2006). Endophyte treatment seems to affect equally the three tested stages of development of *P. goodeyi* (females, males and juveniles). The three isolates did not show variations in their ability to suppress nematode population.

Based on the results from this study, we conclude that the selected Kenyan endophytic *F. oxysporum* isolates *4MOC321* and *11SR23* have the potential to promote plant growth, control plant parasitic nematodes and reduce the nematode induced lesions in the roots. There is however, need to evaluate the efficacy of the Kenyan isolates against banana nematodes under field conditions.



## CHAPTER FOUR

### 4.0 FIELD EVALUATION OF SELECTED UGANDAN ENDOPHYTIC *Fusarium oxysporum* ISOLATES FOR ACTIVITY AGAINST BANANA NEMATODES IN TISSUE CULTURE BANANA PLANTS

#### ABSTRACT

Studies were carried out to evaluate the efficacy of three endophytic *Fusarium oxysporum* isolates (*V5W2*, *Eny 7.11o* and *Emb 2.4o*) against banana nematodes under field conditions. Dessert banana cv. Giant Cavendish and cv. Grand Nain inoculated with the three isolates and challenged with *Pratylenchus goodeyi* and *Helicotylenchus multicinctus* were assessed for a period of twelve months in on-station trial. An additional on-farm trial comprised of banana plants inoculated with the three isolates and planted in a field naturally infested with nematodes was also studied for a period of twelve months. All the three endophytic isolates significantly suppressed *P. goodeyi* and *H. multicinctus* densities and damage in the field. On-station, nematode population densities were reduced by >45 % in endophyte-inoculated plants compared to non-inoculated plants, while percentage root necrosis was reduced by >20 %. Isolate *V5W2* promoted plant growth. Similarly, on-farm, nematode damage and *P. goodeyi* and *H. multicinctus* densities, but not *Meloidogyne* spp. densities, were also significantly lower in endophyte treated plants compared with control plants. The isolate *Eny 7.11o* resulted in enhanced plant growth with treated plants having significantly ( $P < 0.05$ ) higher yields compared to the untreated plants for both trials.

#### 4.1 INTRODUCTION

Bananas (*Musa* spp.) are grown in many districts of Kenya. In the higher western regions of Kenya, the East African highland bananas (*Musa* AAA, ‘Matooke’ and ‘Mbidde’ cultivars) are most common, while in the central and eastern highlands and coastal areas, dessert cultivars are the most popular, especially ‘Cavendish’ and ‘Gros Michel’ (MoA, 2007). Banana production is faced by a number of constraints, although a complex of plant-parasitic nematodes, banana weevil, poor agronomic practices, diseases and poor soil fertility combine to adversely affect yields in Kenya (Nguthi, 1996; Inzaule *et al.*, 2005). Progressive yield decline in plantations of banana is a problem in the small plots of the resource-limited farmers of East, Central and West Africa. Plant-parasitic nematodes cause serious crop losses worldwide and are among the most important agricultural pests (Koenning *et al.*, 1999). Several species of plant parasitic nematodes have been associated with banana in Kenya. The most important are the *P. goodeyi*, *H. multicinctus* and *Meloidogyne* spp. which have a varied distribution (KARI, 2004). Yield losses associated with banana nematodes range between 30-60 % (Brooks, 2004). Roots damaged by nematodes are less able to supply plants with needed water and nutrients. These slow plant growth, lengthen the time to fruiting, reduce bunch weight, and decrease the productive life of the farm. Top-heavy plants may fall over due to the loss of anchoring roots.

The initial key to high productivity lies in the effective management of pests and diseases. The management of nematodes is often more difficult than that of other pests because nematodes attack the underground parts of the plants (Stirling, 1991). Although

chemical nematicides are effective, easy to apply, and show rapid effects, concerns about public health and environmental safety have been raised (Schneider *et al.*, 2003). Furthermore, costs of nematicides are often prohibitive for the small scale farmer. Therefore, efficient approaches to control nematodes that utilize a range of biological options are needed (Kiggundu *et al.*, 2003).

Previous research has demonstrated the potential of endophytes as bio-control agents of banana nematodes (Pocasangre, 2000; Niere, 2001; Athman, 2006; Machungo, 2009) under Greenhouse conditions. The potential of *F. oxysporum* as a bio-control agent of banana nematodes has not been investigated under field conditions in Kenya. Thus, the objective of the current study was to evaluate the efficacy of selected endophytic isolates for biological control of banana nematodes (*Pratylenchus goodeyi* and *Helicotylenchus multicinctus*) in the field (on-station and on-farm trials).

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Site description**

The experiments were carried out on-station at Jomo Kenyatta University Agriculture Technology farm in Juja (Altitude: 1537 m asl 01° 05' 25.6''S, 037° 00' 45.5''E) and on-farm in a key banana production area, Maragwa district (1346 m asl 00° 47' 17.3''S, 037° 08' 18.3''E).

#### **4.2.2 Experimental design and layout**

Three *F. oxysporum* endophytic isolates *V5W2*, *Emb.2.4o* and *Eny 7.11o* were evaluated for their efficacy against banana nematodes under Kenyan environmental conditions using two dessert banana cultivars cv. Giant Cavendish and cv. Grand Nain. The on-station trial comprised of four treatments that included; *V5W2* + nematodes, *Emb.2.4o* + nematodes, *Eny 7.11o* + nematodes and control + nematodes. The on-farm trial treatments included; *V5W2*, *Emb.2.4o*, *Eny 7.11o* and control, plants were transplanted to a field naturally infected with *P. goodeyi*, *H. multicinctus* and *Meloidogyne* spp. nematodes. In the two sites, the treatments were laid out in a split-plot design where the main plot factors were the cultivars and sub-plot factors the fungal inoculations. For on-station, each plot consisted of 12 plants (4 rows and 3 plants per row) while for on-farm, a plot consisted of 16 plants (4 rows and 4 plants per row). The sites were surrounded with a border row of banana plants which acted as guard rows. Treatments were replicated thrice and hence for on-station and on-farm trial there were a total of 36 and 48 plants/cultivar, respectively. Plots were planted using nine month old plants spaced at 3 × 3 m and 5 m gaps between blocks.

#### **4.2.3 Fungal isolates**

Three *F. oxysporum* endophytic isolates *V5W2*, *Emb.2.4o* and *Eny 7.11o* obtained from the International Institute of Tropical Agriculture (IITA) Uganda were evaluated for their efficacy against some banana nematodes in on-station and on-farm trials. The

isolates were selected following demonstration of their ability to cause higher nematodes mortality both *in vitro* and *in vivo* (Athman, 2006; Machungo 2009).

#### **4.2.4 Inoculation of plants with fungal isolates and nematodes**

Inoculation of plants with endophytes and nematodes was carried out according to Machungo (2009). Banana plants were obtained from JKUAT commercial tissue culture laboratory. Tissue culture 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 3-L potting bags (5×9 ×4 cm) where they were allowed to grow for one month before endophyte inoculation. A hole was made in the middle and 2 g maize bran containing the fungal inoculums ( $3.3 \times 10^6$  spores ml<sup>-1</sup>) was mixed in soil around the roots. Maize bran was the solid substrate that was used for fungal sporulation. Plants were inoculated with nematodes at four weeks after endophyte inoculation. Nematode inoculation was done for all plants treated with fungal isolates and control plants. Mixed nematode populations with *Pratylenchus* as the major nematode species were used. Roots for nematode extraction were obtained from plants in nematode infested fields. Species composition of the sample was determined using an identification manual. Each plant was inoculated with 1200 nematodes (95 % *Pratylenchus*, 3 % *Radopholus similis* and 2 % *Helicotylenchus multicinctus*) by digging three holes of approximately 3 cm deep and pour the 2 ml suspension with 1200 nematodes into the holes using a pipette. Plants for on-farm trial were not inoculated with nematodes.

#### **4.2.5 Land preparation**

Pre-trial soil sampling was carried out in the two sites, the number and species of nematodes in the soil determined at the onset of the trials. Nematodes are often distributed unevenly in a field, and therefore samples were collected from several areas within the fields. Samples from the same field were combined to make a composite sample weighing 1-2 kg. The samples were placed in plastic bags and transported to JKUAT laboratory for nematode extraction. The spectrum of nematodes was determined (nematodes present in the sites was a representative of species composition in the region) and plant holes measuring  $1 \times 1$  m were prepared ready for planting.

The on-station site consisted of murrum soil which is unfavourable for banana growth and thus, forest soil was imported from Kieni forest in the Central province of Kenya. The soil mixture for planting comprised of approximately 70 kg of forest soil and 40 kg of goat manure per planting hole. On-farm site, the soil mixture for planting comprised of approximately 70 kg of topsoil and 40 kg of goat manure per planting hole.

#### **4.2.6 Planting**

On-station, nine month old plants inoculated with endophytes ( $3.3 \times 10^6$  spores  $\text{ml}^{-1}$ ) and challenged with nematodes (1200 nematodes/plant) were transplanted in the field mid-May 2007.

On-farm, nine months old Grand Nain (AAA group) and Giant Cavendish (AAA group) plantlets inoculated with endophytes ( $3.3 \times 10^6$  spores  $\text{ml}^{-1}$ ) were transplanted in a field naturally infested with nematodes mid-November 2007. Species of banana nematodes

that were in the site included; 20 % *Pratylenchus goodeyi*, 29 % *Meloidogyne spp.* & 51 % *Helicotylenchus multincinctus* (200nem/100g of soil).

#### **4.2.7 Field maintenance**

The fields were irrigated when necessary depending on weather conditions, during the dry seasons, irrigation was done thrice a week. Plants were weeded manually twice a month and dead leaves removed on a weekly basis. Desuckering was undertaken to maintain optimal banana plant density and manageability, and single suckers were selected when the mother plants were 100 cm high above the ground, with direction of selection (eastern side) uniform to maintain spatial arrangement. Three plants per mat were maintained. Residual banana leaf materials were used as mulch, usually spread within the mats.

At five months after planting, approximately 40 kg of goat manure was added around each plant in both sites. In addition, approximately 70 kg of forest soil was added per plant for the on-station trial.

#### **4.2.8 Assessment of plant growth parameters**

Plant growth parameters of plant height, girth of the pseudostem and number of functional fully opened leaves were recorded on a monthly basis. Plant height was measured as the distance from the point where the youngest leaf emerges from the pseudostem to the base of the plant, girth was measured at the base of the plant, while the leaves were considered healthy when more than three quarters of the leaf area was

green as opposed to yellow leaves. At harvest, the weight of the bunches, number of hands as well the number of fingers from each plant were determined.

#### **4.2.9 Determination of nematode densities and damage**

Root samples were collected at 3, 6, 9 and 12 months after transplanting to assess for nematode damage and population. A hole measuring approximately 5 x 5 x 5 cm was dug 10 cm away from the base of the pseudostem of the mother plant and all the banana roots within the hole collected for each plant. Five functional roots were randomly selected from each sample, cut into 10 cm long pieces and split lengthwise. Procedures for scoring the percentage root necrosis and nematode extraction were carried out as described in section 3.2.10 of Chapter 3. For the nematode extraction a 25 g root subsample was used from each sample. The number of female, male and juvenile of each species of nematode present was identified and recorded.

#### **4.2.10 Determination of fungal colonization**

Endophytic colonization of banana plants roots of the first ratoons was determined. A hole measuring approximately 5 x 5 x 5 cm was dug 10 cm away from the base of the pseudostem of the ratoon plants. Three healthy roots from each plant were randomly selected and used for fungus re-isolation as described in section 3.2.9 of Chapter 3.



### **4.3 DATA ANALYSIS**

The Levene option of the Hovtest (SAS, 2003) was used to test for normality and variances homogeneity for all data. If not normally distributed transformations were carried out prior to analysis of variance (ANOVA). Nematode data calculated per 25 g of root sample were log transformed while percentage nematode damage and colonization data were arcsine-square root-transformed. Plant growth and yield data were subjected to ANOVA. The generalized linear model (Proc GLM) with split-plot design option was used to test for factor effects and their interactions. When significant factor interaction was noted effects of one factor was analysed at each level of the other factor. Means were separated using least significant difference test (LSD) (SAS, 2003). All tests were performed at a significant  $\alpha$  of 0.05. Pearson correlation coefficient was used to assess the relationship between percentage root colonization and percentage root necrosis, nematode population and bunch weights.

## 4.4 RESULTS

### Plant growth

Although there were no significant differences ( $P>0.05$ ) between the endophyte treated plants and the controls, plants treated with *V5W2* and *Eny 7.11o* were relatively taller and thicker with more functional leaves for on-farm trial (Table 4). In the on-station trial, plants treated with *V5W2* were relatively taller and wider compared to controls (Table 5).

**Table 4:** Effect of endophytic isolates on plant height, girth of the pseudostem and number of functional leaves for on-farm trial

Location	Treatment	Height (cm)	Girth (cm)	NOFL
Maragwa	<i>V5W2</i>	134.02 ± 4.18	50.45 ± 1.48	14.64 ± 0.27
Maragwa	<i>Eny 7.11o</i>	134.62 ± 4.02	51.02 ± 1.43	15.04 ± 0.26
Maragwa	<i>Emb 2.4o</i>	129.10 ± 3.99	50.20 ± 1.48	14.74 ± 0.26
Maragwa	Control	130.80 ± 4.23	49.46 ± 1.47	14.42 ± 0.27
LSD		10.56	3.49	0.80
P value		0.685	0.5514	0.5641

Data are means ± standard errors of three replicates obtained from 12 plants.

NOFL- Number of functional leaves.

**Table 5:** Effect of endophytic isolates on plant height, girth of the pseudostem and number of functional leaves for on-station trial

Location	Treatment	Height (cm)	Girth (cm)	NOFL
JKUAT	V5W2	111.20 ± 3.81	41.25 ± 1.24	15.04 ± 0.29
JKUAT	<i>Eny 7.11o</i>	105.72 ± 3.82	39.90 ± 1.24	15.03 ± 0.28
JKUAT	<i>Emb 2.4o</i>	108.21 ± 3.90	39.69 ± 1.27	14.70 ± 0.30
JKUAT	Control	109.49 ± 3.84	40.95 ± 1.28	15.34 ± 0.29
LSD		11.29	4.05	0.74
P value		0.8581	0.9601	0.5977

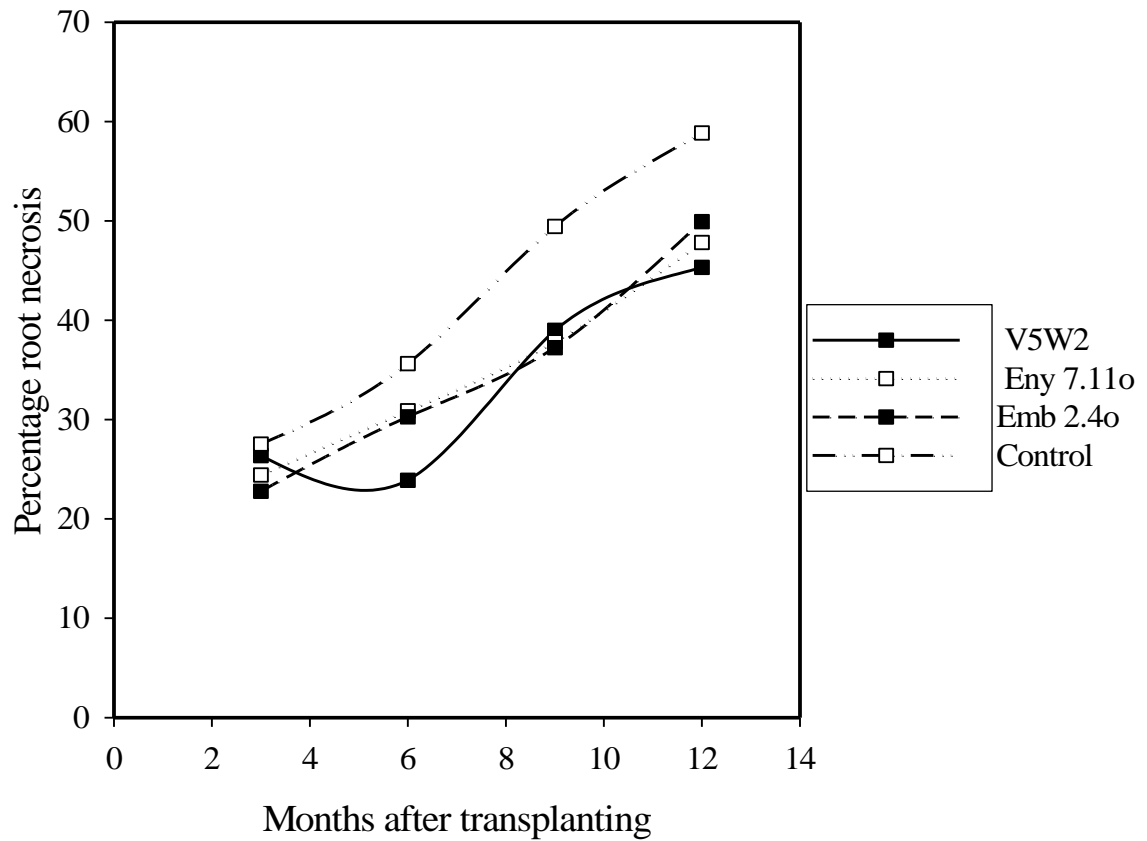
Data are means ± standard errors of three replicates obtained from 9 plants.

NOFL- Number of functional leaves.

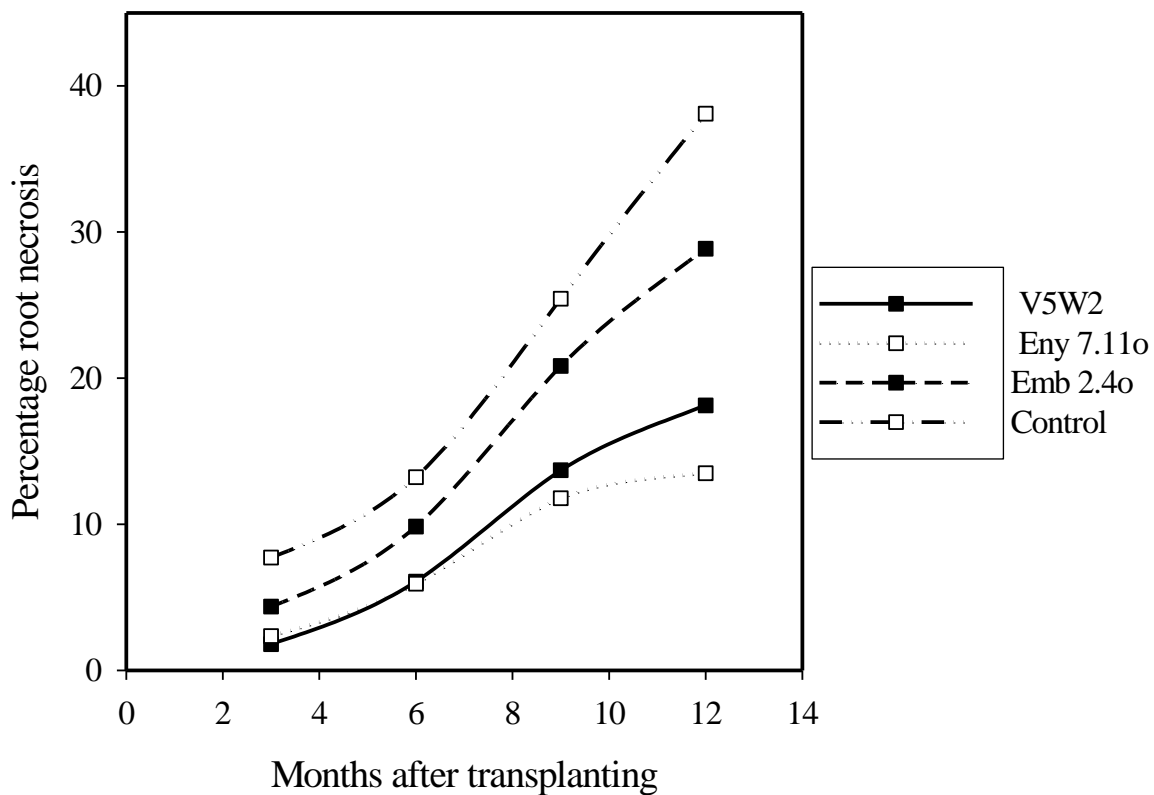
### **Nematode damage**

Inoculating plants with endophytes significantly ( $P < 0.05$ ) reduced the damaged caused by nematodes in banana roots (percentage root necrosis) over time in the on-station (Fig. 4). At three months after transplanting, there were no significance differences ( $P = 0.6097$ ) between plants inoculated with endophyte and non-inoculated in terms of root necrosis. Significant differences were observed at six, nine and twelve months after transplanting in the field, where plants treated with the endophytes had significantly lower necrosis compared to control plants. The percentage reduction in damage in banana roots ranged between 15-33 %, 21-24 % and 15-23 % at six, nine and twelve months, respectively.

On-farm, no significant differences ( $P>0.05$ ) were observed in percentage root necrosis between endophyte-inoculated and non-inoculated plants at three and six months after transplanting. However, the root damage significantly differed ( $P>0.05$ ) at nine and twelve months after transplanting, where plants treated with the fungal isolates had a lower root necrosis compared to the untreated plants (Fig. 5). The percentage reduction in root necrosis was highest with isolate *Eny 7.11o*, followed by *V5W2* and isolate *Emb 2.4o* had the least percentage reduction in damage both at nine and twelve months (Fig. 5).



**Figure 4:** The roots necrosis caused by nematodes on endophytic *Fusarium oxysporum* inoculated plants on cv. Giant Cavendish and cv. Grand Nain (*Musa* spp. AAA) at three, six, nine and twelve months after transplanting (on-station). Data points are means and standard errors of three replicates obtained from 9 banana plants



**Figure 5:** The roots necrosis caused by nematodes on endophytic *Fusarium oxysporum* inoculated on cv. Giant Cavendish and cv. Grand Nain (*Musa* spp. AAA) at three, six, nine and twelve months after transplanting to the field (on-farm). Data points are means and standard errors of three replicates obtained from 12 banana plants

### **Nematode population**

In the on-station, no significant differences ( $P>0.05$ ) were observed in densities of *P. goodeyi* and *H. multincinctus* between endophyte-inoculated and non-inoculated plants at three and six months after transplanting (Table 6). At nine and twelve months, the population of *P. goodeyi* and *H. multincinctus* were significantly ( $P<0.0001$ ) lower following endophyte treatment (Table 6).

Although there were no significant differences ( $P=0.05$ ) on the effects of individual isolates, the total nematode density was lower by 36.6 %, 33.6 % and 15.4 % in plants inoculated with *V5W2*, *Eny 7.11o* and *Emb2.4o* at three months, respectively, compared to un-inoculated plants. At six months nematode density was lower by 29.5 %, 23.1 % and 22.2 % for *V5W2*, *Eny 7.11o* and *Emb2.4o*, respectively (Fig. 6). At nine and twelve months after transplanting, the total nematode density was consistently lower in plants treated with isolates *V5W2*, *Eny 7.11o* and *Emb2.4o* compared to untreated plants (Fig. 6). The total nematode density was lower by 62.1%, 64.3% and 52.1% in plants treated with *V5W2*, *Eny 7.11o* and *Emb2.4o*, respectively, compared to control plants. Nematode population densities in roots varied widely between treatment and among plants within the same treatment.

In the on-farm, the total population of *P. goodeyi*, *H. multincinctus* and *Meloidogyne* spp. of endophyte-treated plants and control plants did not differ ( $P>0.05$ ) at three and six months after transplanting (Table 7). At nine and twelve months after transplanting, the total population of *P. goodeyi* was significantly ( $P<0.05$ ) lower in endophyte-treated plants compared to untreated plants (Table 8). Also, *H. multincinctus* densities were

significantly ( $P=0.0479$ ) suppressed in plants inoculated with the fungal isolates compared with non-inoculated plants at twelve months after transplanting. However, at nine months, endophyte treatment did not significantly ( $P=0.4924$ ) affect *H. multinctus*. The total population of *Meloidogyne* spp. was not affected by the endophyte infection at nine and twelve months (Table 8). Nematode densities were significantly lower in plants inoculated with isolate *Eny 7.11o* and *V5W2*, compared with control plants and plants inoculated with isolate *Emb2.4o* (Table 8).



**Table 6:** Mean population densities of *Pratylenchus goodeyi* and *Helicotylenchus multicinctus*/ 25 g banana root samples of endophyte inoculated and non-inoculated plants three, six, nine and twelve months after transplanting on-station

Treatment	Months after transplanting							
	3		6		9		12	
	<i>P.</i> <i>goodeyi</i>	<i>H.</i> <i>multicinctus</i>	<i>P.</i> <i>goodeyi</i>	<i>H.</i> <i>multicinctus</i>	<i>P.</i> <i>goodeyi</i>	<i>H.</i> <i>multicinctus</i>	<i>P.</i> <i>goodeyi</i>	<i>H.</i> <i>multicinctus</i>
V5W2	223.06 <sup>a</sup>	98.61 <sup>a</sup>	280.28 <sup>a</sup>	80.00 <sup>a</sup>	238.33 <sup>b</sup>	135.28 <sup>b</sup>	1299.7 <sup>b</sup>	640.8 <sup>b</sup>
<i>Eny 7.11o</i>	233.61 <sup>a</sup>	103.06 <sup>a</sup>	298.78 <sup>a</sup>	93.49 <sup>a</sup>	234.72 <sup>b</sup>	117.22 <sup>b</sup>	1322.2 <sup>b</sup>	652.8 <sup>b</sup>
<i>Emb 2.4o</i>	293.06 <sup>a</sup>	136.11 <sup>a</sup>	317.78 <sup>a</sup>	79.72 <sup>a</sup>	303.33 <sup>b</sup>	169.17 <sup>b</sup>	1394.4 <sup>b</sup>	651.4 <sup>b</sup>
Control	345.83 <sup>a</sup>	161.67 <sup>a</sup>	408.06 <sup>a</sup>	102.78 <sup>a</sup>	624.72 <sup>a</sup>	361.11 <sup>a</sup>	2751.6 <sup>a</sup>	1500.1 <sup>a</sup>
LSD	122.41	61.81	164.84	46.84	152.02	106.74	505.9	274.43
P value	0.1696	0.1485	0.4258	0.7106	<.0001	<.0001	<.0001	<.0001

Means calculated from untransformed data. Statistical analysis was performed on log transformed data. Data are means of three replicates obtained from nine plants. Means followed by the same small letter within a column (Superscript) are not significantly different (LSD) at P=0.05.

**Table 7:** Mean population densities of *Meloidogyne* spp., *Pratylenchus goodeyi* and *Helicotylenchus multicinctus*/25 g of banana root of endophyte-inoculated and non-inoculated plants at three and six months after transplanting on-farm

Treatment	Months after transplanting					
	3			6		
	<i>Meloidogyne</i> spp.	<i>P. goodeyi</i>	<i>H. multicinctus</i>	<i>Meloidogyne</i> spp.	<i>P. goodeyi</i>	<i>H. multicinctus</i>
V5W2	325.4 ± 105.9	1.88± 1.4	6.25 ± 3.9	703.8 ± 186.3	75.46± 53.3	27.75 ± 24.3
Eny7.11o	427.5 ± 123.8	23.13 ± 21.9	8.13 ± 6.9	807.3 ± 173.9	36.25± 33.7	38.75 ± 32.4
Emb2.40	440.4 ± 111.1	13.13 ± 12.5	15.63± 9.9	927.5 ± 164.9	87.50 ± 61.6	89.38± 52.9
Control	477.3 ± 144.5	61.25 ± 27.6	42.50 ± 18.9	506.32 ± 194.5	207.5 ± 93.5	137.50 ± 63.4
LSD	343.42	52.46	32	506.32	180.46	129.25
P value	0.8376	0.1343	0.098	0.7563	0.2721	0.3128

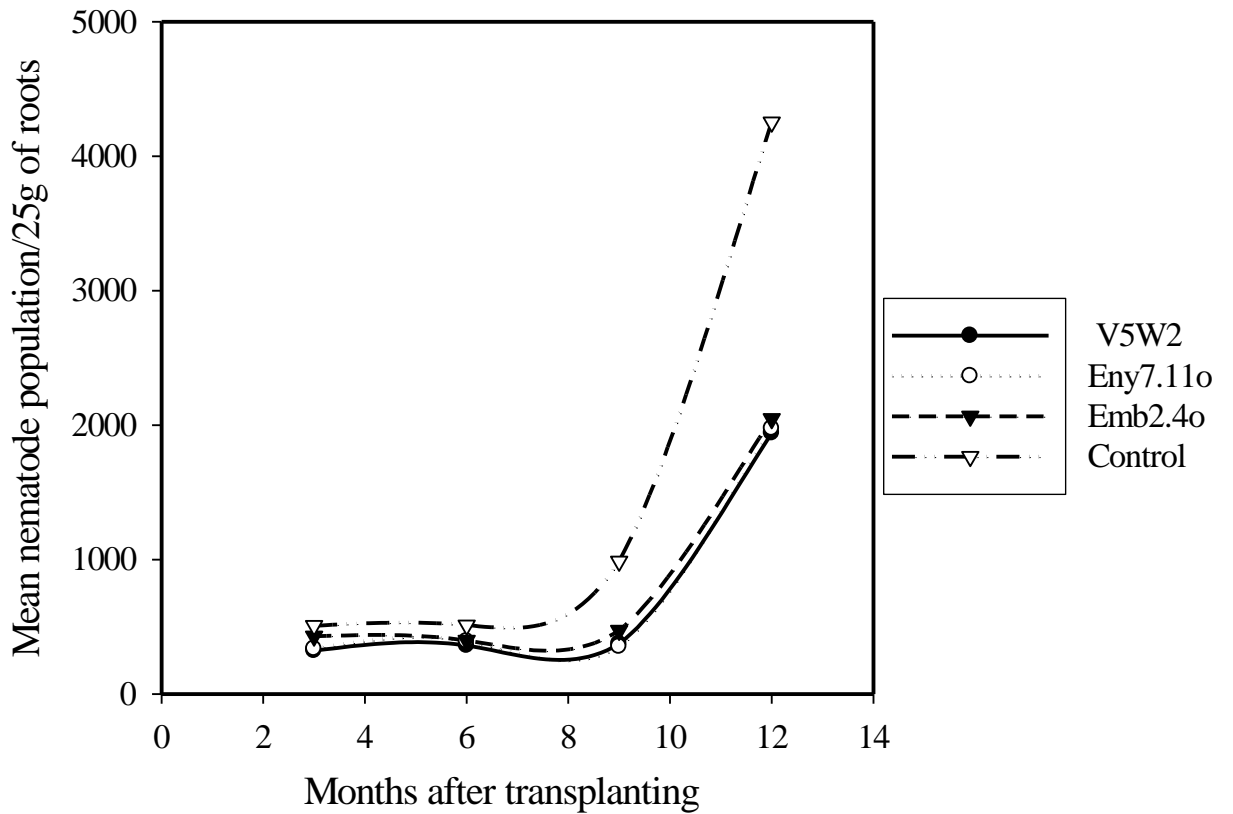
Means were calculated from untransformed data. Statistical analysis was performed on log transformed data.

Data are means of three replicates obtained from twelve plants.

**Table 8:** Mean population densities of *Meloidogyne* spp., *Pratylenchus goodeyi* and *Helicotylenchus Multicinctus*/25 g of banana root samples of endophyte-inoculated and non-inoculated plants at nine and twelve months after transplanting on-farm

Treatment	Months after transplanting					
	9			12		
	<i>Meloidogyne</i> spp.	<i>P. goodeyi</i>	<i>H. multicinctus</i>	<i>Meloidogyne</i> spp.	<i>P. goodeyi</i>	<i>H. multicinctus</i>
V5W2	98.96 ± 85.2 <sup>a</sup>	43.2 ± 12.6 <sup>c</sup>	69.58 ± 22.7 <sup>a</sup>	229 ± 130.7 <sup>a</sup>	276.6 ± 176.7 <sup>b</sup>	105.4 ± 35.0 <sup>b</sup>
Eny7.11o	37.29 ± 23.4 <sup>a</sup>	64.8 ± 25.6 <sup>bc</sup>	47.42 ± 19.3 <sup>a</sup>	164.2 ± 76.8 <sup>a</sup>	133.6 ± 69.8 <sup>b</sup>	82.3 ± 33.7 <sup>b</sup>
Emb2.40	60.21 ± 22.6 <sup>a</sup>	303.7 ± 156.9 <sup>ba</sup>	105.75 ± 48.9 <sup>a</sup>	215.2 ± 58.5 <sup>a</sup>	507.3 ± 178.9 <sup>ba</sup>	248.1 ± 96.4 <sup>ba</sup>
Control	25.21 ± 10.5 <sup>a</sup>	317.5 ± 77.1 <sup>a</sup>	160.88 ± 94.1 <sup>a</sup>	108.8 ± 42.9 <sup>a</sup>	897.9 ± 268.3 <sup>a</sup>	449.2 ± 172.9 <sup>a</sup>
LSD	128.91	248.72	154.75		525.65	286.37
P value	0.6832	0.0436	0.4924	0.737	0.0277	0.0479

Means were calculated from untransformed data. Statistical analysis was performed on log transformed data. Data are means of three replicates obtained from nine plants. Means followed by the same small letter within a column (superscript) are not significantly different (LSD) at P=0.05.



**Figure 6:** Mean parasitic nematodes population present in 25 g of banana roots over time of endophyte-inoculated and non-inoculated banana plants after three, six, nine and twelve months transplanting. Data points representing averages of three replicates obtained from 9 banana plants. Means and standard errors were calculated from untransformed data. Statistical analysis was performed on log transformed data

## Yields

Yields of endophyte treated banana plants significantly ( $P < 0.05$ ) differed from the controls for both cv. Giant Cavendish and cv. Grand Nain in both on-farm and on-station trials. In the on-station trial, mother plants treated with the *V5W2* and *Emb 2.4o* produced heavier bunches compared to the controls (Table 9). Likewise, in the on-farm, *V5W2* and *Eny 7.11o* endophyte treated mother crops produced significantly ( $P < 0.05$ ) heavier bunches compared to untreated mother plants (Table 10). Compared to control plants, endophyte treated plants had higher average bunch weights by a range of 13-20 % and 6-14 % in on-station and on-farm, respectively.

The average number of hands per bunch and average number of fingers per hand were significantly ( $P < 0.05$ ) favoured by endophyte inoculations in both on-farm and on-station trials.

**Table 9:** Effect of endophytic isolates on average bunch weight (kg), average number of hands per bunch and average number of fingers per hand for on-station trial

Location	Treatment	Average bunch weight (Kg)	Average number of hands	Average number of fingers
JKUAT	<i>V5W2</i>	$31.07 \pm 1.04^a$	$9.35 \pm 0.28^a$	$143.88 \pm 5.70^a$
JKUAT	<i>Eny 7.11o</i>	$29.47 \pm 1.45^{ba}$	$9.06 \pm 0.30^a$	$147.41 \pm 6.15^a$
JKUAT	<i>Emb 2.4o</i>	$30.35 \pm 1.34^a$	$9.00 \pm 0.36^a$	$137.06 \pm 5.52^{ba}$
JKUAT	Control	$25.87 \pm 1.30^b$	$8.06 \pm 0.36^b$	$124.82 \pm 4.62^b$
LSD		3.65	0.93	15.61
P value		0.0296	0.0414	0.0275

Data are means  $\pm$  standard errors of three replicates obtained from 9 plants. Means followed by the same small letter within a column (superscript) are not significantly different (LSD) at P=0.05.

**Table 10:** Effect of endophytic isolates on average bunch weight (kg), average number of hands per bunch and average number of fingers per hand for on-farm trial

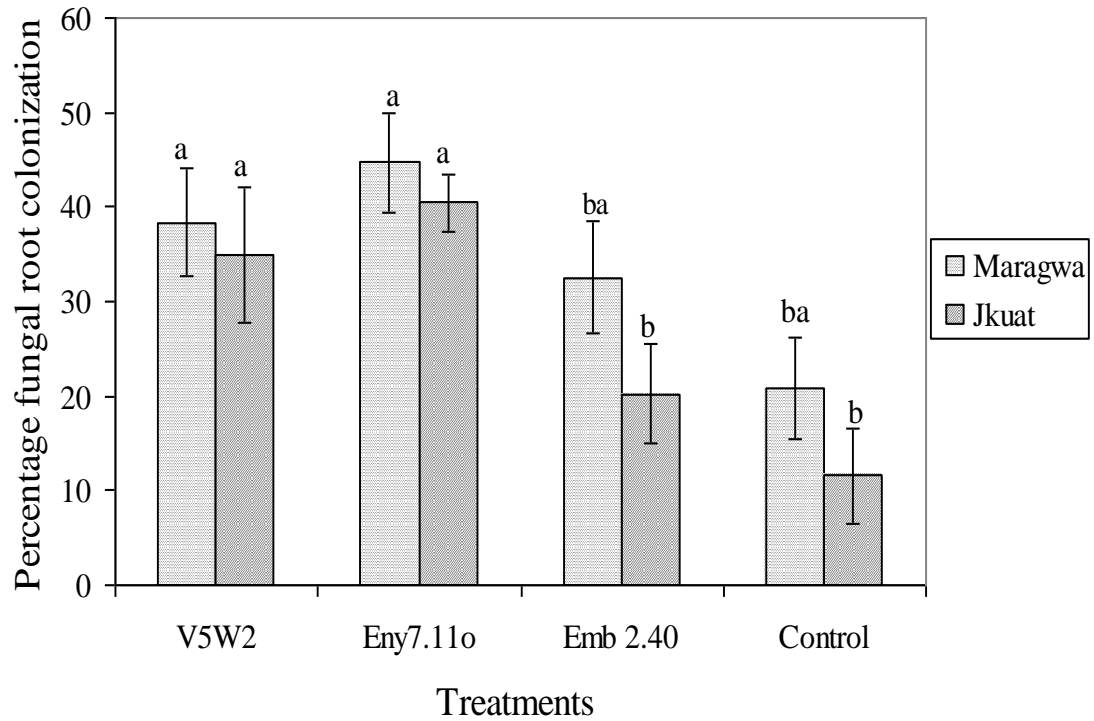
Location	Treatment	Average bunch weight (Kg)	Average number of hands	Average number of fingers
Maragwa	<i>V5W2</i>	28.87 $\pm$ 0.63 <sup>a</sup>	8.87 $\pm$ 0.26 <sup>a</sup>	127.83 $\pm$ 3.59 <sup>a</sup>
Maragwa	<i>Eny 7.11o</i>	29.04 $\pm$ 0.83 <sup>a</sup>	9.09 $\pm$ 0.23 <sup>a</sup>	130.35 $\pm$ 4.32 <sup>a</sup>
Maragwa	<i>Emb 2.4o</i>	26.89 $\pm$ 0.85 <sup>ba</sup>	8.78 $\pm$ 0.23 <sup>a</sup>	124.17 $\pm$ 3.77 <sup>ba</sup>
Maragwa	Control	25.33 $\pm$ 0.83 <sup>b</sup>	7.96 $\pm$ 0.29 <sup>b</sup>	114.87 $\pm$ 4.55 <sup>b</sup>
LSD		2.22	0.71	11.45
P value		0.0030	0.0124	0.0464

Data are means  $\pm$  standard errors of three replicates obtained from 12 plants. Means followed by the same small letter within a column (superscript) are not significantly different (LSD) at P=0.05.

### Endophytic colonization of the first ratoon

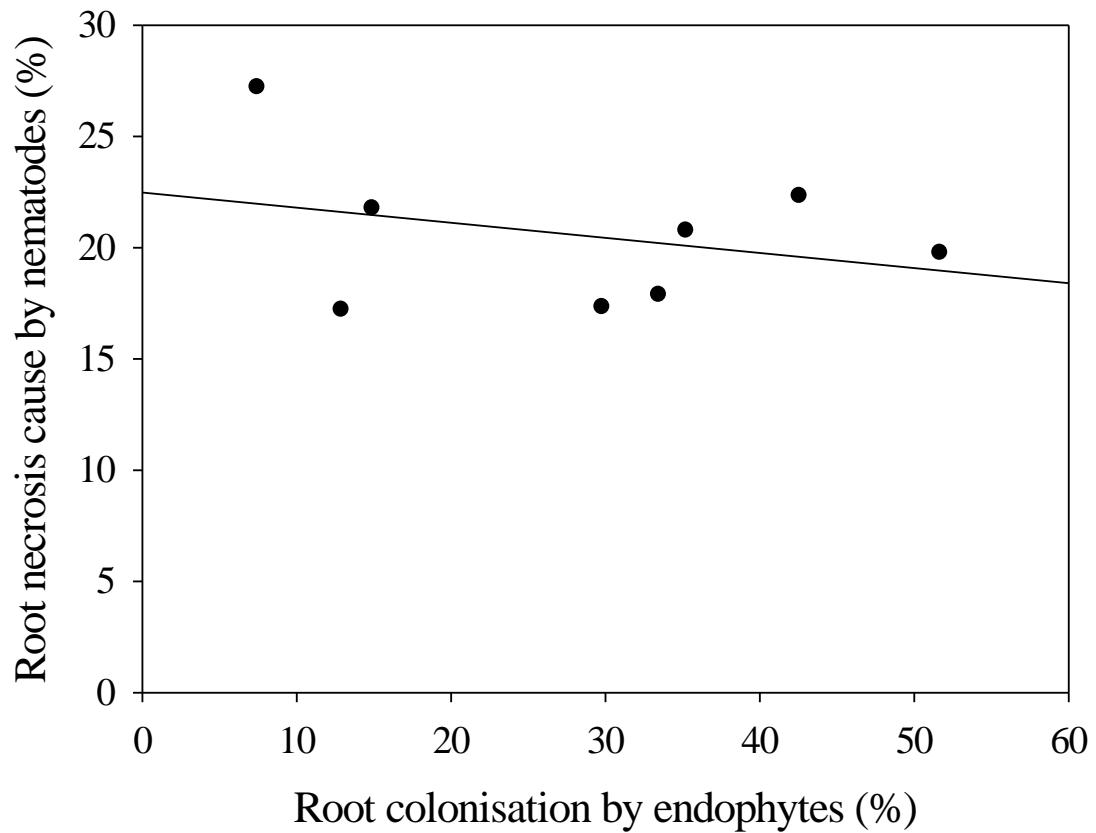
Root colonization was significantly higher in all first ratoons from endophyte-inoculated plants, compared with the ones from non-inoculated plants for on-farm and on-station trials. Similar trends were observed in both trials, whereby root colonization by isolate *Emb 2.4o* did not differ significantly from non-inoculated plants, but colonization by isolate *V5W2* and *Eny7.11o* differed significantly between endophyte-inoculated plants and non-inoculated plants (Fig. 7). Generally, colonization of roots in

non-inoculated plants by naturally occurring isolates of *F. oxysporum* was significantly lower than root colonization by the endophytes in both trials.



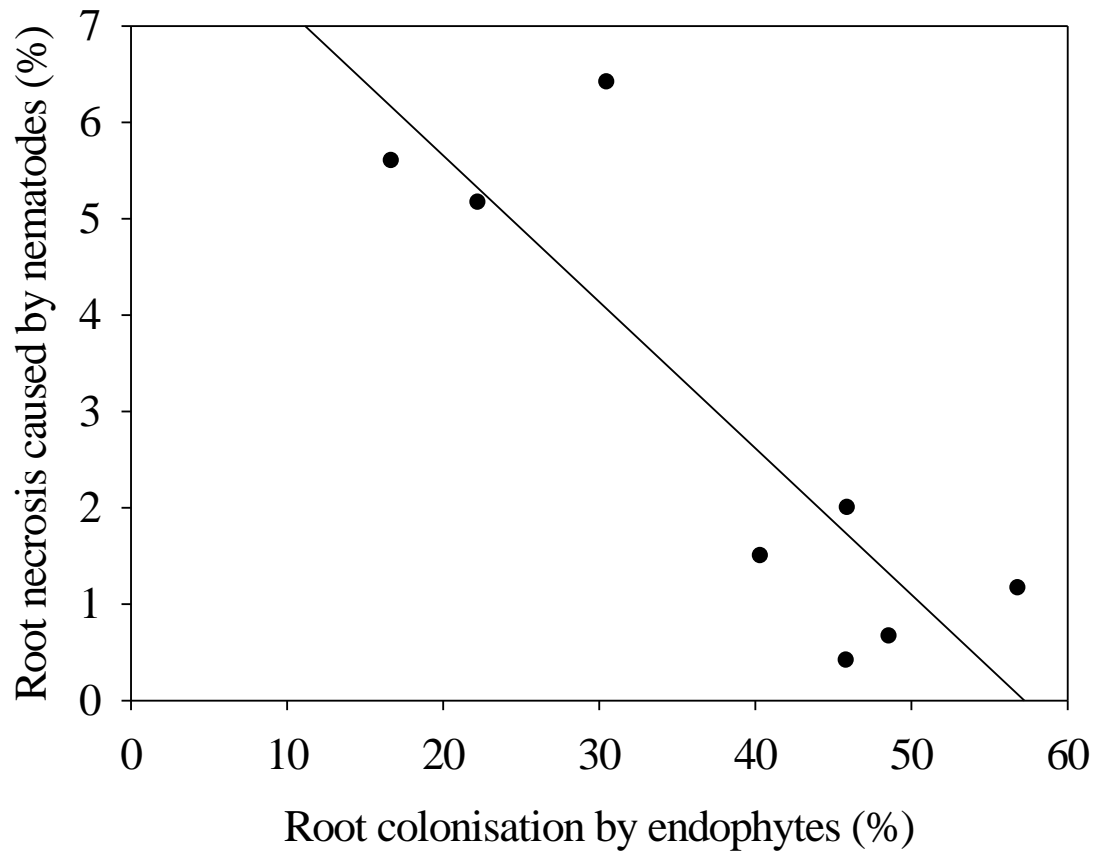
**Figure 7:** Percentage root colonization by endophytic *Fusarium oxysporum* isolates in banana plants (First ratoon). Bars are means and standard errors of three replicates obtained from 12 and 9 suckers for on-farm and on-station trials, respectively.

At both trials, the percentage root colonization by endophytes negatively correlated with percentage root necrosis caused by nematodes. Correlation between nematode population and bunch weights was also negative for both trials.

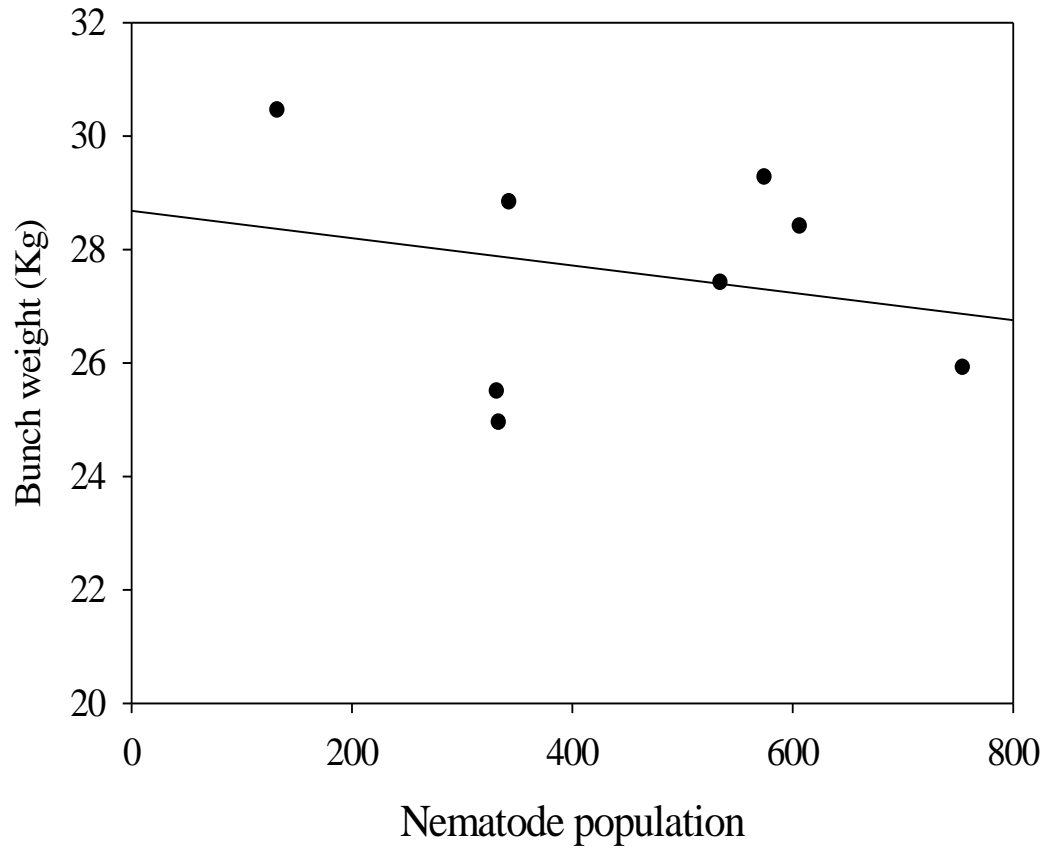


**Figure 8a:** Relationship between percentage root colonization and percentage root necrosis on-station trial

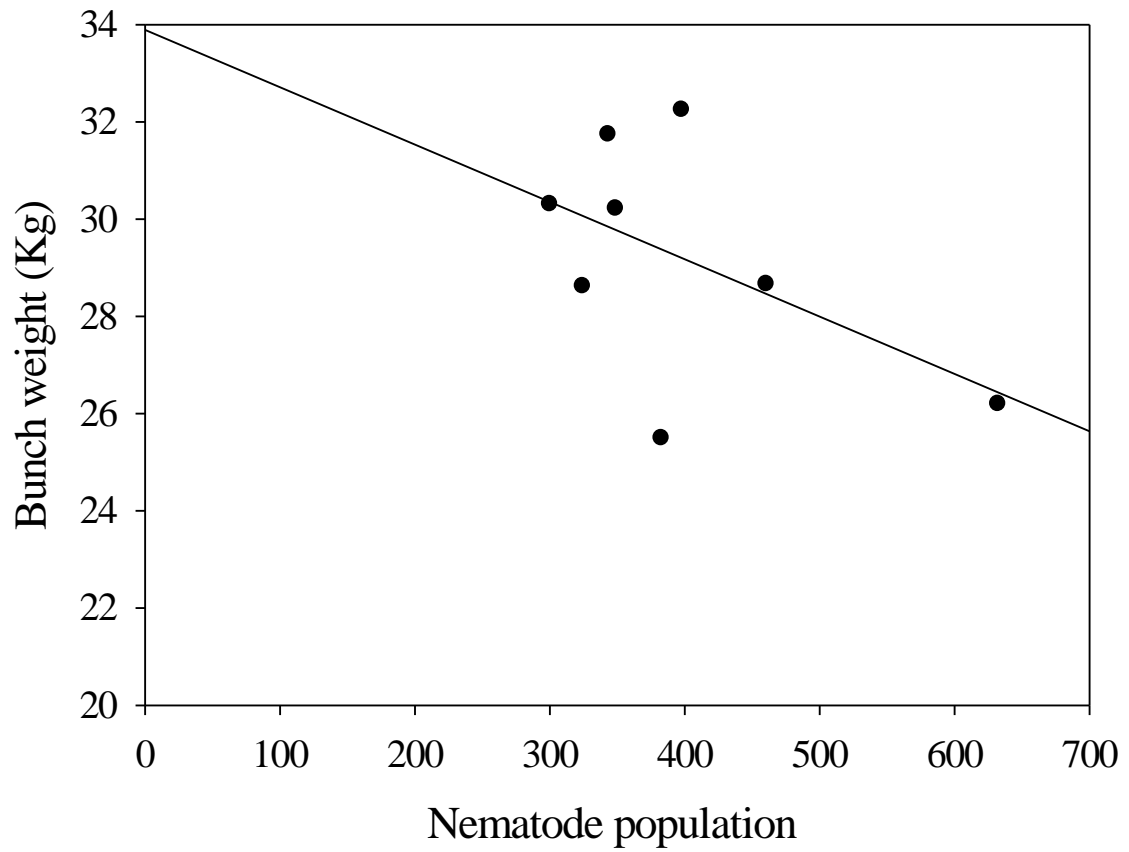




**Figure 8b:** Relationship between percentage root colonization and percentage root necrosis on-farm trial



**Figure 9a:** Relationship between nematode population and bunch weight on- station trial



**Figure 9b:** Relationship between nematode population and bunch weight on-farm trial

#### 4.5 DISCUSSION

Non-pathogenic endophytic *F. oxysporum* isolates (V5W2, *Eny7.11o* and *Emb 2.40*) used to reduce damage caused by *P. goodeyi* and *H. multincinctus* were able to stimulate growth and protect banana plants in the field. A slightly increase in plant height, girth of the pseudostem and number of functional leaves for endophyte inoculated plants were observed. In earlier reports, stimulated growth of *F. oxysporum* endophyte-inoculated plants was observed by Niere (2001) for EAHB while Waller *et al.* (2005) observed similar responses following *Piriformospora indica* Sav. Verma colonization of barley (*Hordeum vulgare*). Increased plant growth for endophyte-inoculated plants challenged with the nematodes observed in the current study could, however, have been the consequences of reduced nematode populations and damage in banana roots.

Little has been documented on effect of endophytes on banana yields, however, effect of endophyte on yields of other crops have been demonstrated. Research carried out by West *et al.* (1988) showed a higher yield in tall fescue plots infected by *Acremonium coenophialium*. Similarly, Yanni *et al.* (1997) demonstrated substantial increases in rice grain yield and N content with a grain yield increase of 46%, grain-content by 53%, straw yield by 15% and straw N-content by 39%, compared to non-inoculated plants. Similar reports on the effect of endophytes on banana yields are lacking.

Nematode density and root damage were lower in endophyte-inoculated plants compared to non-inoculated plants. Reduction in nematode population and subsequent damage could be attributed to the better root colonization by V5W2 and *Eny 7.11o* for

on-station and on-farm trials, respectively. The population of *P. goodeyi* and *H. multicinctus* were significantly suppressed in endophyte treated compared to non-treated plants for both on-farm and on station trial. Higher root colonization led to lower nematode damage as indicated by a negative correlation between the percentage root colonization and percentage root necrosis in both the on-farm and on-station trials (Fig. 8 a, b).

Although endophytic fungi have been shown to protect plants from nematode attack and damage, not all nematode species are affected by endophyte infection. A field study by Niere *et al.* (1999) reported that *H. multicinctus* nematode population was reduced by 75% compared to non-treated plants. Other nematode species present were not affected by the various treatments. In the current study, the population of root knot nematodes (*Meloidogyne* spp.) in the on-farm trial was not significantly affected by the endophyte infection.

Endophytic isolate *Eny 7.11o* and *V5W2* were found to be effective and gave better control compared to *Emb 2.4o* in terms of nematode control and enhancement of plant growth. These results reflect those obtained by Niere (2001), Athman (2006) and Machungo (2009) in pot studies. They reported that the isolate *Eny 7.11o* and *V5W2* performed better than isolate *Emb 2.4o*. The difference in nematode control of endophytic isolates could be attributed to their different abilities to colonize and persist in the plant.

The earliness to bunch harvest of the mother crop of 25 to 30 days of cv. Giant Cavendish and cv. Grand Nain will likely be attributed to the reduction of nematode

infestation in endophyte treated plants compared to control plants. This earliness to maturity might be associated with the low nematode infestation of endophyte treated mother plants compared to untreated plants. Earlier review by Gowen and Quénéhervé (2005) indicate that high nematode infestation results in lengthened vegetative cycle. From the results, application of endophyte led to reduced nematode populations and hence the early maturity due to shortened vegetative cycle.

Compared to the control mother plants, endophyte inoculated plants had heavier bunch weights, more number of fingers and hands. The average total harvest weights were highest in the endophyte treatments i.e. isolate *V5W2* for on-station and *Eny7.11o* for on-farm trial compared to other isolates. The reduction in bunch weights could be associated with higher percentage root necrosis in untreated plants which could be due to high nematode populations in the banana roots. Root damage affects the water and nutrient uptake of the plants which are necessary for fruit formation. Gold and Merrienen (2000) and Brooks (2004) reported that, destruction of the root system due to nematode attack results in reduced water and nutrient uptake and poor anchorage and consequently results in reduced bunch weight and plant toppling.

Results obtained from the studies demonstrate that there is evidence of transmission of endophytic *F. oxysporum* from the mother plant to the succeeding generation (first ratoon). On-station and on-farm trials revealed that the three isolates continued to be retrieved from the first ratoon (*V5W2*, *Eny7.11o* and *Emb2.4o*). In both trials, *F. oxysporum* isolates *Eny 7.11o* and *V5W2* appear superior colonizers than isolate *Emb2.4o*. Similar results were obtained by Paparu *et al.* (2009), whereby isolate *V5W2*

was found to be a better root colonizer than *Emb2.4o*. The increased endophyte colonization by *V5W2* and *Eny 7.11o* may be attributed to the increased plant growth and nematode population reduction that was observed during the studies. Thus, banana bio-enhancement with endophytic isolates *V5W2*, *Eny 7.11o* and *Emb2.4o* can be used for tissue culture banana planted in fields severely affected by nematodes in Kenya.

## CHAPTER FIVE

### 5.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

The beneficial effects of the mutualistic association between plants and endophytes include improved growth of the infected plant. The observed enhanced vigor of endophyte-treated banana plants in this study would have been due, at least in part, to reduced nematode infestations and possibly also to increased phytohormone production. Endophytes promote the growth of plants in various ways, for example through secretion of plant growth regulators; e.g. indole-acetic acid, via phosphate-solubilizing activity (Wakelin *et al.*, 2004b), production of siderophores and by supplying biologically fixed nitrogen.

The population of plant-parasitic nematodes differed greatly according to treatments in the current study. Populations increased in untreated plants compared to endophyte-treated plants. The inoculation of endophytic strains reduced plant-parasitic nematode multiplication and banana root damage. The lowest nematode populations were observed in *V5W2*- and *Eny 7.110*-treated plants.

Nematode population densities in roots varied widely between treatments and among plants. Such variation in nematode populations is not uncommon, especially under field conditions. This variation could be due to aggregated nematode distribution and environmental factors, such as soil heterogeneity, root status, interactions with other organisms, and inter- and intra-specific nematode competition (Bridge and Gowen, 1993; Price and McLaren, 1995; Marin *et al.*, 1998).



Nematode suppression was also observed in endophyte inoculated plants transplanted into nematode infested fields. All endophyte treatments reduced *P. goodeyi* and *H. multincinctus* population twelve months after transplanting to the field. Earlier studies have indicated that the reduction in nematode population is not due to direct antagonistic activity of the fungal isolate on the nematode in the soil system, but is caused by endophyte activity inside the root tissue of plants (Niere *et al.*, 1999).

The fungal application system used for the Greenhouse experiment - dipping tissue culture plants at weaning stage in spore suspensions - can be effectively used in mass propagation of biological enhanced tissue cultured planting material before it is placed in the field. Production of fungal spores is simple, inexpensive, and the weaning process is only slightly altered by the inoculation of the fungal isolates prior to weaning. Furthermore, only small amounts of inoculum are needed (Dubois *et al.*, 2006). Re-introduction of beneficial micro-organisms to sterile tissue cultured plants may substantially improve pest and disease management, including nematodes and as such could contribute to sustainable banana production.

Although endophytic fungi could generally be re-isolated from the plantlets, it was difficult to determine whether the re-isolated fungi were the original ones inoculated. Therefore, to establish actual plant tissue colonization by the introduced fungal endophytes, and their continued presence in particular, there may be need to mark the target strains, or identify them using molecular techniques that will distinguish the re-isolated endophytes from those previously present in the soil.

## 5.1 CONCLUSION

Management of plant parasitic nematodes using endophytic fungi in banana showed great potential for use both under greenhouse and field conditions. However, it is advisable to carry out the study over a long period in order to observe the effect of endophytes on plant growth, nematode control and yields properly. The study demonstrate that the non-pathogenic isolates of *F. oxysporum* are an effective biocontrol agent against *P. goodeyi* and *H. multicinctus* in banana, in addition to *R. similis*, Fusarium wilt and the banana weevil, *Cosmopolites sordidus* on banana under glasshouse and field conditions (Niere *et al.*, 1999; Griesbach, 2000; Pocasangre *et al.*, 2000; Sikora and Pocasangre, 2004; Vu *et al.*, 2006; zum Felde *et al.*, 2006). The best results were achieved using the *V5W2*, *Eny7.11o* and *4MOC321* isolates. Nevertheless, other isolates have shown promising effects. Reduction of nematode infestation and damage following treatment of plantlets with endophytes may result to high banana production. Thus, would be beneficial for commercial application of the technique.

Endophyte-treated banana plants suppressed the nematode population well below the economic threshold level of 10,000 nematodes per 100 g roots both in the greenhouse and fields. This finding could have practical applications in the integrated management of banana nematodes. By applying endophytes, the promotion of labor-intensive practice of cultural practices such as paring and hot water treatment of banana corms before planting (to reduce the banana parasitic nematode load) would be less critical.

In addition, inoculation of endophytes into tissue cultured banana plants allows for low initial inoculation levels, maintaining low costs and removing the burden for farmers to

apply the microbial organism themselves (Griesbach, 2000; Dubois *et al.*, 2006). The effectiveness of endophytic fungi for banana nematode management is of practical significance since, unlike chemical methods, the use of endophytes has neither human nor environmental hazards as are associated with chemical nematicides.

## **5.2 RECOMMENDATION**

Further research needs to be conducted on:

- (1) Genetic characterization of the Kenyan isolates used in this study.
- (2) Evaluate the efficacy of the promising Kenyan isolates against banana nematodes under field conditions

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## APPENDICES

Appendix 1: Summary of ANOVA table for plant height (Greenhouse )

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	37.72795	12.57598	0.67	0.5676
Variety	1	5.834077	5.834077	0.31	0.5759
Treatment*Cultivar	3	27.29777	9.099256	0.49	0.6905

Appendix 2: Summary of ANOVA table for girth of the pseudostem

(Greenhouse)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	0.688424	0.229475	1.58	0.1913
Cultivar	1	0.382893	0.382893	2.64	0.1042
Treatment*Cultivar	3	0.016243	0.005414	0.04	0.9903

Appendix 3: Summary of ANOVA table for number of functional leaves

(Greenhouse)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	13.95048	4.65016	0.99	0.3959
Cultivar	1	11.59631	11.59631	2.47	0.1161
Treatment*Cultivar	3	2.642788	0.880929	0.19	0.9047

Appendix 4: Summary of ANOVA table for length of the youngest leaf

(Greenhouse)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	63.95192	21.31731	0.73	0.5325
Cultivar	1	13.51477	13.51477	0.46	0.4956
Treatment*Cultivar	3	120.5379	40.1793	1.38	0.2469

Appendix 5: Summary of ANOVA table for width of the youngest leaf

(Greenhouse)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	12.93469	4.311562	0.38	0.7683
Cultivar	1	0.82616	0.82616	0.07	0.7876
Treatment*Cultivar	3	37.38125	12.46042	1.09	0.3501

Appendix 6: Summary of ANOVA table for percentage root colonization

(Greenhouse)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	5	109946.4	21989.27	55.89	<0.0001
Variety	2	1101.824	550.9118	1.4	0.2473
Treatment*Variety	10	3987.083	398.7083	1.01	0.4303

Appendix 7: Summary of ANOVA table for total nematode population

(Greenhouse)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	17.1523	5.717432	13.59	<.0001
Cultivar	1	0.012952	0.012952	0.03	0.861
Treatment*Cultivar	3	0.198065	0.066022	0.16	0.925

Appendix 8: Summary of ANOVA table for percentage root damage

(Greenhouse)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	8422.601	2807.534	19.62	<.0001
Cultivar	1	189.796	189.796	1.33	0.2515
Treatment*Cultivar	3	102.4478	34.14926	0.24	0.8693

Appendix 9: Summary of ANOVA table for plant height (On-station)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	4	7124.30926	1781.07731	0.57	0.6850
Cultivar	1	12916.875	12916.875	4.13	0.0424
Treatment*Cultivar	4	1332.99074	333.24769	0.11	0.9803

Appendix 10: Summary of ANOVA table for girth of the pseudostem (On-station)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	4	1038.262963	259.565741	0.76	0.5514
Cultivar	1	385.208333	385.208333	1.13	0.2885
Treatment*Cultivar	4	92.185185	23.046296	0.07	0.9917

Appendix 11: Summary of ANOVA table for number of functional leaves (On-station)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	4	53.86111111	13.46527778	0.74	0.5641
Cultivar	1	48.13333333	48.13333333	2.65	0.1039
Treatment*Cultivar	4	79.1537037	19.78842593	1.09	0.3605

Appendix 12: Summary of ANOVA table for percentage root colonization (On-station)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	9552.965767	3184.322	6.55	0.0006
Cultivar	1	453.087674	453.0877	0.93	0.3380
Treatment*Cultivar	3	1118.444337	372.8148	0.77	0.5169

Appendix 13: Summary of ANOVA table for percentage root necrosis (On-station)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	4	5490.61566	1372.65391	10.38	<.0001
Time	3	38138.35948	12712.78649	96.16	<.0001
Cultivar	1	73.92679	73.92679	0.56	0.4551
Replicate	2	125.5961	62.79805	0.48	0.6223
Treatment*Cultivar	4	19.12086	4.78022	0.04	0.9975
Treatment*Time	12	1855.25162	154.6043	1.17	0.3041
Treatment*Time*Cultivar	15	2180.00872	145.33391	1.1	0.3558

Appendix 14: Summary of ANOVA table for total nematode population (On-station)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	16.1109651	5.3703217	12.82	<.0001
Cultivar	1	1.0092824	1.0092824	2.41	0.1218
Time	3	187.4010851	62.4670284	149.14	<.0001
Replicate	2	1.9511555	0.9755777	2.33	0.0994
Cultivar*Treatment	3	0.9331784	0.3110595	0.74	0.5275
Time*Treatment	9	4.9593911	0.5510435	1.32	0.2289
Time*Cultivar*Treatment	12	9.1693944	0.7641162	1.82	0.0447

Appendix 15: Summary of ANOVA table for plant height (On-farm)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	4	5766.7197	1441.68	0.33	0.8581
Cultivar	1	58893.55227	58893.55	13.47	0.0003
Treatment*Cultivar	4	15702.00455	3925.501	0.9	0.4646

Appendix 16: Summary of ANOVA table for girth of the pseudostem (On-farm)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	4	352.457576	88.11439	0.16	0.9601
Cultivar	1	80.018939	80.01894	0.14	0.7063
Treatment*Cultivar	4	1535.5	383.875	0.68	0.6047

Appendix 17: Summary of ANOVA table for number of functional leaves (On-farm)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	4	52.08636364	13.02159	0.69	0.5977
Cultivar	1	0.825	0.825	0.04	0.8342
Treatment*Cultivar	4	10.42878788	2.607197	0.14	0.968

Appendix 18: Summary of ANOVA table for percentage root colonization (On-farm)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	7407.540178	2469.18	3.67	0.0152
Cultivar	1	1363.471705	1363.472	2.03	0.1579
Treatment*Cultivar	3	3238.048806	1079.35	1.61	0.1938

Appendix 19: Summary of ANOVA table for percentage root necrosis (On-farm)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	9842.46	3280.82	13.05	<.0001
Time	3	24485.2	8161.74	32.45	<.0001
Cultivar	1	121.022	121.022	0.48	0.4883
Replicate	2	1998.47	999.237	3.97	0.0197
Cultivar*Treatment	3	1294.18	431.393	1.72	0.1635
Time*Treatment	9	3200.02	355.557	1.41	0.1804
Time*Cultivar*Treatment	12	3128.96	260.747	1.04	0.4142

Appendix 20: Summary of ANOVA table for total nematode population (On-farm)

Source of variation	DF	SS	MS	F value	Pr>F
Treatment	3	19.9454	6.64848	4.09	0.0073
Cultivar	1	0.0688	0.0688	0.04	0.8372
Time	3	74.3574	24.7858	15.24	<.0001
Replicate	2	3.04557	1.52279	0.94	0.3934
Cultivar*Treatment	3	4.71013	1.57004	0.97	0.4096
Time*Treatment	9	3.64202	0.40467	0.25	0.9867
Time*Cultivar*Treatment	12	23.5099	1.95916	1.2	0.2797