Host-endophyte-pest interactions of endophytic *Fusarium oxysporum* antagonistic to *Radopholus similis* in banana (*Musa* spp.)

By

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Dedication

To my family

Declaration

I declare that the thesis which I hereby submit at the University of Pretoria for the award of the degree PhD (Plant Pathology) is my work and has not been submitted by me for a degree to any other university or institution of higher education.

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Shahasi Yusuf Athman

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General introduction

Bananas (*Musa* spp.) are among the most important tropical fruits in Sub-saharan Africa, providing the bulk of dietary carbohydrates and daily calorie intake for millions of people. Highland cooking banana (*Musa* spp. group AAA-EA) is the most important staple food crop in the Lake Victoria basin (INIBAP, 1986). Uganda is one of the largest banana producing and consuming countries in the world, and a secondary center of diversity of the highland bananas of the *Musa* AAA group. The primary center of diversity for AAA banana group members is the Malaysian region from where the plants were introduced to the East African Highlands (Simmonds, 1987). The dominant cultivars in Uganda belong to the East African highland cooking bananas, which comprise over 76% of banana production in the country (Karamura, 1993). Banana production is primarily undertaken by semi-subsistent, small-scale households, and most bananas are locally consumed as a starch staple after cooking.

In recent years, there have been marked changes in the location and intensity of banana production in Uganda. Highland banana production in the country has declined sharply. Karamura (1993) reported a production decline of more than 25% from more than 8 tons/ha in the 1970s to less than 6 tons/ha in the 1990s. The major constraints to banana production in the region are mainly pests and diseases (Gold *et al*., 1993). This has led to the replacement of highland cooking bananas in some traditional growing areas in Central Uganda with more pest and disease tolerant brewing and dessert bananas (Gold, *et al*., 1999). A complex of plant-parasitic nematodes affect banana production: the burrowing nematode *Radopholus similis* (Cobb) Thorne, the spiral nematode *Helicotylenchus multicinctus* (Cobb) Golden, the lesion nematodes *Pratylenchus goodeyi* (Sher and Allen) and *Pratylenchus coffeae* (Goodeyi), and the root-knot nematodes *Meloidogyne* spp. (Goeldi). These nematodes, together with the banana weevil *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae), are the primary banana pest constraints in East Africa (Gold *et al*., 1993; Karamura, 1993; Speijer *et al*., 1999a).

Control measures against *R. similis* include the use of (uninfested) planting material such as pared and hot water treated suckers and tissue culture plants (Stanton, 1999; Speijer *et al*., 1999b). Clean planting material may provide adequate control in the first crop cycle, but the problem of re-infestation of plants in the field is a major disdavantage (Speijer *et al*., 1995; Speijer *et al*., 2001). Host plant resistance would offer a safe and long-term intervention

strategy against *R. similis*. Resistance to *R. similis* is, however, yet to be identified in East African highland cooking banana cultivars. Thus, research is currently focusing on several alternative ways, including the use of microbial antagonists such as endophytes, for controlling banana pests and diseases (Sikora and Schuster, 1999; Pocasangre, 2000; Niere, 2001).

Endophytes are microorganisms that spend some time in their life cycle living symptomlessly within plant tissues (Petrini, 1991). Although tissue culture plants may provide healthy, nematode-free planting material, the effects may offer only a temporary solution to nematode problems in banana, necessitating the need for affordable, sustainable and environmentally friendly management strategies that complement the benefits of clean planting material. Due to the sterile conditions under which tissue culture plants are produced, the plants loose naturally beneficial microorganisms such as endophytes (Pereira *et al*., 1999). The artificial introduction of endophytic fungi in these sterile plants at the hardening phase may offer protection against pests and diseases to the young plants in the early growth stages and extend the life of planting material (Sikora and Schuster, 1999). This strategy would form part of an integrated pest management approach consisting of biological control and clean planting material.

Research on the use of endophytes for the biological control of the main banana pests in Uganda was initiated at the International Institute of Tropical Agriculture (IITA) in the late 90s. This led to the isolation of hundreds of endophytic fungal isolates from apparently healthy banana plants growing in nematode and banana weevil infested plantations (Griesbach, 2000; Niere, 2001). These isolates are being preserved at the IITA laboratory in Namulonge, Uganda. Although various fungal endophytes were isolated, *Fusarium* spp. and especially *Fusarium oxysporum* Schlecht.: Fries were the dominant endophytic taxa. Research aimed at identifying fungal isolates with nematode-controlling activities has since focused on *F. oxysporum* isolates against the most economically important nematode species *R. similis*. Preliminary results have shown that some fungal isolates tested against *R. similis* possessed *in vitro* nematicidal activity and also led to a reduction in nematode populations in tissue culture plants under screen house conditions (Niere, 2001).

Problem statement

Preliminary results involving *R. similis* control with endophytic *F. oxysporum* isolates have been very promising (Niere, 2001). The isolates tested have shown the potential to kill *R. similis in vitro* and also reduce *R. similis* populations in endophyte-treated tissue culture plants. Despite these exciting results, information on the interactions between the host plant, the endophyte and the nematode, and the mechanisms involved in nematode suppression, remains limited. Little is known on the effects of the endophyte on host preferences, invasion and root penetration, and population build-up of *R. similis* in banana plants. The role of induced resistance by endophytes against *R. similis* has also been postulated but not confirmed. Knowledge of these interactions is essential in elucidating the mechanisms involved in nematode suppression. Understanding how endophytes control *R. similis* in banana plants would further be useful in designing appropriate nematode control strategies and also in maximizing benefits of endophyte inoculation in plants.

Research objectives

The main objective of this research was to identify endophytic *Fusarium* spp. isolates for use as biological control agents against the major banana nematode *R. similis* and to study the interactions and mechanisms of control involved.

Specific objectives:

- To screen endophytic *Fusarium* spp. for *R. similis* suppression both *in vitro* and *in vivo* in tissue culture banana plants.
- To determine the effect of endophytic *F. oxysporum* isolates on the *R. similis*-banana plant association.
- To determine mechanisms involved in *R. similis* control by endophytic *F. oxysporum* isolates.

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Chapter 1

Review of the role of endophytes in biological control of plant-parasitic nematodes with special reference to the banana nematode, *Radopholus similis* (Cobb) Thorne

1. Introduction

Plant-parasitic nematodes cause significant damage and losses to most agricultural crops in the tropics and sub-tropics (Luc *et al*., 2005). The need to control and manage nematode populations to acceptable levels and reduce losses remains a big concern for nematologists. Increased scientific interest in biological control of plant parasitic nematodes is mainly a response to growing public concerns over the use of agrochemicals. The need to reduce dependence on chemical control using nematicides and the increased pressure to use pest control measures that do not pollute or degrade the environment has provided the impetus for more research geared towards the search for and exploitation of potential biological control agents of plant parasitic nematodes (Cook, 1988; Gerhardson, 2002). Stirling (1991) defined biological control as 'the reduction of nematode populations through the action of living organisms other than the nematode-resistant host plant, and which occurs naturally, or through the manipulation of the environment or the introduction of antagonists'.

Nematodes have long been known to have numerous antagonists (Kerry, 1987). Several organisms have been described and exploited for the management of plant parasitic nematodes in agricultural crops. A large number of organisms including fungi, bacteria, viruses, predatory nematodes, insects and mites have been found to parasitize on vermiform stages of nematodes or females and eggs of root-knot nematodes and cyst nematodes (Stirling, 1991). To date, most research on biological control of plant-parasitic nematodes has concentrated on nematophagous egg parasitic fungi and the nematode predatory fungi and antagonistic bacteria. In this respect, several reviews have been published (Jatala, 1986; Stirling 1991; Sikora, 1992; Siddiqui and Mahmood, 1995; 1996; Akhtar and Malik, 2000; Kerry, 2000).

More recently the use of endophytic micro-organisms resident within plant tissues for protection of plants against pests and diseases has been exploited. The most studied is the grass-endophyte associations in which endophytic fungi associated with grasses have been shown to protect grasses against pests and diseases. Most grass endophytes are members of the Ascomycete family Clavicipitaceae (Clay, 1991). Biological control with endophytes has mostly emphasized resident or mutualistic fungi of grasses, which render hosts unpalatable to herbivores and insects (Clay, 1988; 1989). Detrimental effects of grass endophytes on fungal pathogens have also been demonstrated. For example, isolates of *Acremonium lolii* Link ex

Fries, and *A. coenophialum* Morgan-Jones & W. Gams showed antibiosis against a range of fungal plant pathogens in culture (White and Cole, 1985). Research on grass endophytes has clearly demonstrated the nature and extent of protection afforded to the host plants by the interactions, with mutualistic associations between grasses and endophytic fungi benefiting the host plants in most circumstances (Clay, 1990). In mutualistic associations, endophyte-infected plants are protected from attack by some species of insects, nematodes and fungi while, in return, the endophyte is provided with shelter and nutrition by the host plant (Latch, 1993; Saikkonen *et al*., 1998; Schardl *et al*., 2004).

Although most reports on host plant infection by endophytes concern grass endophytes, symptomless infections of other plants by endophytic fungi belonging to diverse taxonomic groups have been known for many years (Carroll, 1988). The presence of endophytes has been demonstrated in many plants, including important crops such as banana (Brown *et al*., 1998; Pereira *et al*., 1999; Cao *et al*., 2004a,b; Cao *et al*., 2005), maize (*Zea mays* L.)(Fisher *et al*., 1992) rice (*Oryza sativa* L.)(Fisher and Petrini, 1992), and tomato (*Lycopersicon esculentum* L.) (Hallman and Sikora, 1994c; Cao *et al*., 2004a). Some of the principal groups of root colonizing plant beneficial fungi, which have developed symbiotic relationships with the host plants, belong to *Fusarium* and *Trichoderma* spp. (Haas and Defago, 2005). Endophytic bacterial species have been found in many plant species (McInroy and Kloepper, 1995; Hallman *et al*., 1997b; Li *et al*., 2002). Several studies have shown that the interaction between plants and endophytic bacteria may be beneficial through plant growth promotion and biological control against plant pathogens (Lalande *et al*., 1989; Chen *et al*., 1995; Hallman *et al*., 1998). Chen *et al.* (1995) demonstrated reduction of root rot caused by *Rhizoctonia solani* Kühn and vascular wilt caused by *Fusarium oxysporum* f. sp. v*asinfectum* W.C. Synder & H.N. Hans in cotton (*Gossypium hirsutum* L.) plants by endophytic bacteria. M'piga *et al*. (1997) demonstrated that an endophytic isolate of *Pseudomonas fluorescens* Migula increased resistance to *F. oxysporum* f. sp. *radicis-lycopersici* Garvis & Shoemaker in tomato plants. Intensity of colonization by the pathogen was markedly reduced together with accumulation of electron dense material in the epidermal and outer cortex layers, and callose cell wall appositions in bacterized plants.

In this review, the role of endophytic microorganisms in the management of plant-parasitic nematodes in agricultural crops is discussed. Since limited information is available on the use of endophytes for control of the banana nematode *Radopholus similis* (Cobb) Thorne, the

review will focus on existing literature on the interactions between endophytes and plant-parasitic nematodes in grasses and other crops, highlight the implications of infection of plants by endophytes, and discuss the beneficial effects of endophytic microorganisms in the management of plant-parasitic nematodes. Finally, banana production in Uganda, production constraints and nematode control options, including the potential of endophytes, are discussed.

2. Why endophytes?

Several definitions for endophytism have been proposed (Carroll, 1988; Clay, 1990). For the purpose of this review, the term endophyte will refer to fungi or bacteria, which for all or part of their life cycle, invade and live inside tissues of living plants without causing any disease symptoms in or any apparent injury to the host plant (Petrini, 1991; Wilson, 1995). Epiphytes are bacteria and fungi that colonize plant surfaces. In contrast to epiphytes, endophytes are contained entirely within plant tissues, are asymptomatic and may be described as mutualistic (Clay, 1990). Bacteria associated with roots and the rhizospheres of many plant species known to benefit the plants through growth promotion and biological protection from pests and diseases (Rammamorthy *et al*., 2001) are referred to as plant growth promoting rhizobacteria (PGPR). Most PGPR belong to the genus *Bacillus* and *Pseudomonas* and are capable of proliferating not only around the plants roots but also inside (Van Peer *et al*., 1990; Pleban *et al*., 1995; Hallman *et al*., 1997b; Shishido *et al*., 1999). Similarly, fungi associated with roots and rhizospheres of the plants are referred to as plant growth promoting fungi (PGPF). Some of the important PGPF belong to the genus *Trichoderma* and *Gliocladium* (Altomare *et al*., 1999) and the arbuscular mycorrhizal fungi (AMF), which form symbiotic associations with plant roots and are also capable of colonizing the roots of their hosts (Gera Hol and Cook, 2005).

The use of endophytes for the control of plant-parasitic nematodes is a relatively new approach. Since endophytes spend most of their life cycle inside plant tissues they are less exposed to the external environmental factors and thus do not entirely depend on the environment for their multiplication and survival (Siddiqui and Shaukat, 2003a). Endophytes occupy a similar niche as the pests and are thus in close contact with the pests which makes them an edge over other biological control agents (Hallman *et al*., 1996b; 1997b). Inside the plant tissue, the host plant provides a relatively uniform and protected environment enabling

the endophytes to avoid microbial competition and extreme environmental conditions such as fluctuations in temperature and moisture (Ramamoorthy *et al*., 2001). They are easy to culture *in vitro* and can be applied as seed treatments or on transplants, reducing the inoculum levels required (Sikora, 1992; Musson *et al*., 1995; Sikora and Schuster, 1999). Another advantage is that once developed, farmers will not need to apply the control product themselves as this may be done by public/private organizations engaged in commercial tissue culture production (Dubois *et al*., 2006). Inspite of these advantages over other biological control agents, the potential of bacterial and fungal endophytes in pest and disease management in crops remains largely unexplored.

3. Colonization of plants by endophytes

The processes of colonization of plant tissue by endophytes are complex and include host recognition, spore germination, penetration and colonization. Endophytes penetrate their host plants through natural openings or wounds or actively using hydrolytic cellulases and pectinases (Hallman *et al*., 1997b; Rutherford *et al*., 2002), forming inconspicuous infections within healthy plant tissues for all or part of their life cycles (Siegel *et al*., 1987). Plant wounding induced by biotic factors such as plant-parasitic nematodes also constitute a major factor for entry of bacterial endophytes (Hallmann *et al*., 1998).

For many years, endophytic microorganisms colonizing plants have been thought to be weakly virulent pathogens residing latently within plant tissues (Sinclair and Cerkauskas, 1996). The distinction between endophytic and latent infections is not very clear. In latent infections, the host plant does not show any symptoms, with the infection persisting latently until symptoms are prompted to appear by environmental or nutritional stress conditions. The state of the host plant and the pathogen may also provide signals for symptom expression. Since the production of disease symptoms is dependent upon the interaction between the host, parasite and environment over time, endophytic colonization is considered not to cause any disease (Sinclair and Cerkauskas, 1996).

To detect endophytic colonization of plants, several methods for *in-situ* detection of fungal endophytes in plant tissues have been developed. A simple method involves microscopic examination of differentially stained samples of endophyte-infected plants (Saha *et al*., 1988). This method is, however, time consuming and less reliable since histological staining is not

endophyte-specific (Hahn *et al*., 2003). Other methods for *in situ* detection of endophytes have been developed. Some examples include the use of monoclonal antibodies (Hiatt *et al*., 1997; Hiatt *et al*., 1999), tissue print immunoblotting (Gwinn *et al*., 1991), tissue print immunoassays (Hahn *et al*., 2003), electron microscopy (Sardi *et al*., 1992) and autoradiography (You *et al*., 1995).

4. Methods for isolation of endophytes from plants

The growth of fungal hyphae outwardly from internal tissues of surface-sterilized plant tissues is considered the main evidence for endophytism (Petrini, 1986; Petrini, 1991). Several surface sterilization methods can be used depending on the plant tissue (Petrini 1991; Hallman *et al*., 1996a; Cao *et al*., 2005). The most common procedure involves surface sterilization of plant tissue using various disinfectants. Some of the disinfectants used include various concentrations of sodium hypochlorite (Fisher *et al*., 1992, Hallman *et al*., 1997b), ethanol (Fisher *et al*., 1992; Dong *et al*., 1994) and hydrogen peroxide (McInroy and Kloepper, 1995). Surface sterilization using ethanol involves dipping the plant tissue into ethanol and flaming the surface. This method is advantageous in that it is simple, fast and allows for processing of a large number of samples. Concentrations of the disinfectants and the length of sterilization times differ according to the plant species, age and the plant part. Afterwards, the samples are rinsed in sterile water and dried on sterile tissue paper. The surface sterilized samples are then placed on microbiological media and pure cultures are made followed by identification. Complete surface sterilization is often difficult to achieve and more advanced techniques for isolation of endophytes from plants have been developed. For example, techniques such as vacuum and pressure extraction of plant sap (Hallman *et al*., 1997a) and centrifugation of intercellular fluid of plant tissue (Dong *et al*., 1994) have been used successfully to isolate endophytic bacteria from plants. These two methods have been shown to bypass the problems associated with surface sterilization techniques.

5. Endophytic fungi and nematode control

The first report of nematode antagonism by fungal endophytes was described on tall fescue plants (*Festuca arundinacea* Schreb.) infected by *Pratylenchus scribneri* Steiner. West *et al*., (1988) compared nematode populations of migratory endoparasitic *P. scribneri* present in the soil surrounding endophyte free roots to those in roots of tall fescue plants infected with *A.*

coenophialium. Their results showed a reduction in nematode populations both in pots and field experiments and higher yield in tall fescue plots infected by *A. coenophialium*.
The effects of endophyte infection of tall fescue by root knot nematodes (*Meloidogyne* spp.) are probably best studied. Endophytic fungi adversely affected *Meloidogyne marylandi* Jepson and Golden, a root-knot nematode commonly associated with pasture grasses. A reduction in *M. marylandi* and *M. graminis* Sledge & Golden populations in tall fescue roots infected by the endophytic fungus *A. coenophialium* has been reported (Kimmons *et al*., 1989; Kimmons *et al*., 1990; Elmi *et al*., 2000). Juvenile emergence from eggs, the number of egg masses per pot and the number of eggs per egg mass were found to be lower than in endophyte-free tall fescue plants. Similarly, Ball *et al*. (1997) demonstrated higher numbers of *M. marylandi* in roots of perennial ryegrass plants free of the endophytic fungus *Neotyphodium lolii*. *Pratylenchus scribneri* penetrated roots of *N. coenophialum*-infected and endophyte-free tall fescue plants equally well but reproduction of the nematodes was hindered in the presence of the endophyte (Kimmons *et al*., 1990).

Endophyte effects on nematodes in other crops have also been demonstrated. For example, Hallman and Sikora (1994a, 1994b, 1994c, 1996c) demostrated that endophytic *F. oxysporum* isolated from tomato roots had detrimental effects on *Meloidogyne incognita* (Kofoid and White). Colonization of tomato roots by the endophyte resulted in 60% reduction of *M. incognita* infection.

The response to fungal endophytes by plant parasitic nematodes depends on the particular way in which the nematode species feeds (Cook and Lewis, 2001). Ectoparasitic nematodes remain in the soil or on the root surfaces feeding on the outer cells. Migratory endoparasites penetrate roots and feed on internal cells. Sedentary endoparasitic nematodes form specialised feeding cells in the plant tissue and remain embedded in the tissue. Due to protection by surrounding plant tissue, they are difficult to control by soil and rhizosphere microorganisms. Therefore, endophytic microorganisms colonizing plant root tissues may be better able to manage sedentary endoparasitic nematodes due to the fact that both occupy the same ecological niche and are in close contact (Hallman *et al*., 1996b; 1997b; Siddiqui and Shaukat, 2003a).

The length of time that the nematode spends feeding on plant cells may influence the chances of contact between the nematode and the endophyte or its by-products. The more intimate the

relationship between the nematode and plant, the more sensitive the nematode species are to fungal metabolites (Hallman and Sikora, 1996c). Selectivity for trophic groups has also been demonstrated by fungal endophytes. *Fusarium oxysporum* isolate 162 inactivated nematode species in similar trophic groups (Hallman and Sikora, 1996c). Between 60 to 100% of plant-parasitic nematodes were inactivated within 24 hrs of exposure, whereas the mobility of mycophagous and bacteriophagous nematodes was not altered (Hallman and Sikora, 1996c). The nematode stage may also influence the sensitivity to fungal metabolites and juvenile stages may be more sensitive than more advanced stages.

Although endophytic fungi have been shown to protect plants from nematode attack and damage, not all nematode species are affected by endophyte infection. Results obtained range from reduced root and soil nematode populations to no effect on nematode populations and increased levels of nematode infestation (Kimmons *et al*., 1990; Cook *et al*., 1991; Sikora *et al*., 2003). Populations of plant parasitic nematodes in three grassland sites on perennial ryegrass infected or not infected by the endophytic fungus *A. lolii* were studied over a 2-year period. In two of the three sites, nematodes were not reduced, but rather increased in the endophyte-infected plants, while no effect on nematode populations was observed at the third site (Cook *et al*., 1991).

6. Endophytic bacteria and nematode control

Information on the potential use of endophytic bacteria for nematode control is scarce. Hallman *et al*., (1995) provided evidence that endophytic bacteria may contribute to the control of *M. incognita* in tomato. Some endophytic bacterial isolates from cotton also resulted in a significant reduction of root galling by *M. incognita* (Hallman *et al*., 1998). Production of 2,4-diacetylpholoroglucinol, a bacterial secondary metabolite from an endophytic *P. fluorescens* isolate in culture, reduced egg hatching and resulted in substantial mortality of *Meloidogyne javanica* (Treub) Chitwoodi juveniles. Under glasshouse conditions, application of this isolate reduced root-knot development in tomato plants compared to untreated plants (Siddiqui and Shaukat, 2003b).

7. Mechanisms of action of endophytes against nematodes

Although endophytes have long been known to protect plants from both biotic and abiotic stresses, little is known on how they suppress pests and diseases. A thorough understanding of the mechanisms of action by endophytes, though, is needed to maximize the use, efficiency and consistency of biological control. Various mechanisms of action by fungal endophytes have been suggested (Clay, 1987). These include production of nematicidal metabolites (Hallmann and Sikora, 1994a, b; Cook and Lewis, 2001; Siddiqui and Ehteshamul-Haque, 2001; Li *et al*., 2002), changes in the host plant physiology (West *et al*., 1988; 1993; 1994; Assuero *et al*., 2000; Elmi *et al*., 2000) and the induction of general plant defense responses (Kimmons *et al*., 1990; Fuchs *et al*., 1999; Schulz *et al*., 1999, Siddiqui and Shaukat, 2003b). Direct parasitism of nematodes by endophytes could not be demonstrated (Vu *et al*., 2004) and may thus not represent an important effect of endophytes on nematodes.

7.1 Production of nematicidal secondary metabolites

The production of toxic compounds (antibiosis) is an important mechanism of action of beneficial endophytic microorganisms against plant parasitic nematodes. Grass endophytes, mainly those belonging to *Neotyphodium* spp., produce a wide range of metabolites both in culture and in plants (Cook and Lewis, 2001). The production of alkaloids toxic to both insects and herbivores by grass endophytes has been documented (Breen, 1994). These toxins have been isolated successfully from both plants and pure cultures of grass endophytes. Infection of tall fescue plants by *N. coenophialum* resulted in both qualitative and quantitative differences in the production of volatile compounds between endophyte-infected and endophyte-free plants (Yue *et al*., 2001). Other examples of production of toxic metabolites by endophytic fungi have also been reported. Ma *et al*. (2004) reported production of rhizoctonic acid, momomethylsulochrin, ergosterol and 3β,5α,6β-trihydroxyergosta-7,22-diene by an endophytic isolate of *Rhizoctonia* sp. in Bermuda grass *Cynodon dactylon* (L.), which were toxic to the bacterium *Helocibacter pylori* Warren & Marshall.

Despite strong evidence for the production of toxic metabolites by grass endophytes, information regarding nematicidal compounds from non-grass fungal endophytes is scarce. Köpcke *et al*. (2002a; b) reported the production of pregaliellalactone and other related lactones by non-graminaceous endophytes, which had nematicidal activity. Production of 3-

hydroxypropionic acid (3-HPA) in culture by endophytic fungi isolated from above-ground plant organs has been demonstrated (Schwarz *et al*., 2004). This compound showed selective nematicidal activity against *M. incognita* and *Caenorhabditis elegans* (Maupas). Dead juveniles of *M. incognita* incubated for 12 hours in purified 3-HPA appeared stiff, turgid and straight. Culture filtrates of an endophytic *F. oxysporum* isolated from tomato roots reduced mobility of *M. incognita* within 10 minutes of exposure (Hallman and Sikora, 1994a; b). Ninety-eight percent of juveniles were inactivated after 60 minutes exposure to the culture filtrates, demonstrating the production of toxic metabolites in culture by this isolate.

Bacterial endophytes have also been shown to produce metabolites toxic to plant-parasitic nematodes. Li *et al*. (2002) reports production of toxic metabolites in culture filtrates by an endophytic *Burkholderia ambifaria* sp. novel (Coenye *et al*., 2001) isolate from corn roots. Culture filtrates inhibited egg hatch and mobility of second-stage juveniles of *M. incognita*. Results from purified culture filtrates showed that a <3kDa fraction was responsible for inhibition of *M. incognita*. *Burkholderia cepacia* Palleroni & Holmes has also been shown to be antagonistic to *M. incognita* (Meyer *et al*., 2000). An endophytic isolate of *Pseudomonas aeruginosa* (Schroeter) Migula produced toxic compounds *in vitro,* which resulted in substantial mortality of *M. javanica* juveniles (Siddiqui and Ehteshamul-Haque, 2001). Production of 2,4-diacetylpholoroglucinol, by an endophytic *Pseudomonas fluorescens* Migula isolate in culture reduced egg hatch and resulted in substantial mortality of *M. javanica* juveniles (Siddiqui and Shaukat, 2003b).

The ability of endophyte-infected plants to produce biologically active compounds may depend on the location and concentration of the endophyte in the plant. Distribution of these compounds in the plant may also vary depending on the compound itself and the season (Cook and Lewis, 2001). Toxins produced in endophyte-infected plants may be translocated elsewhere (Watson *et al*., 1993) and exuded into the surrounding soil, affecting nematode populations. For example, smaller populations of root lesion nematodes in the roots and rhizosphere of endophyte-infected grasses were associated with translocation of lolitrem and peramines from the stems and leaves to the roots (Kimmons *et al*., 1990).

Although toxic metabolites produced by most endophytes in culture may show antagonistic activity against nematodes *in vitro,* the role of these compounds in nematode reduction in the plant can only be shown if they are present in detectable concentrations in the plant tissues

and rhizosphere of plants that are infected by the endophyte, compared to endophyte-free plants (Lopez-Llorca and Olivares-Bernabeu, 1998; Kerry, 2000). Great uncertainty exists between the products of endophytes in culture compared with in nature. Production of bioactive compounds by endophytes may facilitate domination of a biological niche within the host plant or provide protection to the plant (Tan and Zhou, 2001). Although microbial endophytes may be a source of novel natural products that could be used as alternatives to chemical treatments in agriculture, very few plant species have been explored for the identification of endophytes and, subsequently, their associated products (Tan and Zhou, 2001; Strobel and Daisy, 2003).

Both the type and quantity of secondary toxic metabolites produced in endophyte-infected plants might depend on the fungal genotype (Cook and Lewis, 2001). For example, tall fescue endophytes grown *in vitro* differed in the production of ergot alkaloids (Bacon, 1988). Hill *et al*. (1990) also found that different isolates of *A. coenophialum* from tall fescue plants differed in the amounts and types of ergopeptine alkaloids produced. The host plant may also affect production and concentration of secondary metabolites. For example, concentrations of lolitrem B, ergovaline and peramine were found to vary among tall fescue cultivars infected with the same isolate of *A. lolli,* suggesting that phenotypic differences in tall fescue plants may have contributed to differences in endophyte-grass interactions (Hill *et al*., 1990). The results obtained suggest that the production of toxic compounds in the host plant by fungal endophytes may require some kind of plant-endophyte recognition. Additionally, the host-endophyte symbiosis is affected by the environmental conditions under which the host is growing, especially temperature. Breen (1994) suggested that the compatibility of an endophyte and its host may be important in determining production of toxic metabolites by the endophyte. It is, thus, important to determine compatible host-endophyte-genotype combinations in order to maximize the benefits of the association (Hill *et al*., 1990; Breen, 1994; Siddiqui and Shaukat, 2003a).

7.2 Changes in host plant physiology

Endophyte-infected grasses have improved physiological responses to adverse environmental conditions. Probably the most documented feature of abiotic stress tolerance in endophyte-infected grasses is tolerance to, and recovery from, drought stress. Reduced nematode parasitism in endophyte infected tall fescue plants has been associated with enhanced root

growth and osmotic adjustment in growing points of the plant, thereby reducing the effects of drought on the host plant (Elmi *et al*., 2000). Endophyte-infected tall fescue plants were shown to be more drought tolerant than their endophyte-free counterparts (West *et al*., 1988; West *et al*., 1993; West, 1994). Morse *et al*. (2002) demonstrated that infection by *N. coenophialum* was beneficial to the growth of Arizona fescue (*Festuca arizonica* Vasey) under low water availability. Endophyte infection of grasses has been demonstrated to have positive effects on the plant's growth during water stress. For example, endophyte infected tall fescue plants had a higher net growth rate during water deficit than endophyte-free plants (Assuero *et al*., 2000). Under water stress, the tall fescue endophyte was associated with increased cell wall elasticity, which in turn conferred the drought tolerance response in the endophyte-infected grass (White *et al*., 1992).

While most studies have shown that endophyte-infected grasses are more drought-tolerant than endophyte-free counterparts, this may not always be true. For example, Cheplick (2004) demonstrated that endophyte-free perennial ryegrass under drought conditions had more tillers, greater leaf area and a higher total mass than grasses infected by the endophytic fungus *N. lolii*. After drought recovery, endophyte-free plants showed an equal or greater allocation to tiller bases than endophyte-infected grasses. Similarly, Ahlholm *et al*. (2000) demonstrated that endophyte-infected *Festuca pratensis* Huds. Syn. plants had less tillers and lower biomass production in low nutrient and water conditions than endophyte-free plants. Other results with *N. coenophialum* in *F. arizonica* showed that, under water and nutrient stress conditions in the field, the endophytes decreased growth and seed production in the host (Faeth and Sullivan, 2003).

Endophyte infection may improve the growth, size and quality of the host plants. Cook *et al.* (1991) demonstrated that endophyte-infected ryegrass grew much better than uninfected plants. The enhanced plant growth could lead to increased nematode populations compared to uninfected ryegrass, as a consequence of better growth of endophyte-infected plants. Enhanced growth of endophyte-infected tall fescue and perennial ryegrass compared to endophyte-free plants has been demonstrated. Malinowski and Belesky (2000) and Clay and Schardl (2002) showed that endophyte infected plants of *Lolium multiforum* Lam had more vegetative tillers and allocated more biomass to roots and seeds than endophyte free plants.

Endophytes have also been shown to influence photosynthesis rates in host plants. For example, tall fescue plants infected by *N. coenophialum* photosynthesized faster and flowered earlier than uninfected plants (Newman *et al*., 2003). Also, endophyte-infected tall fescue plants exhibited higher survival and flowering frequency (Hill *et al*., 1991). Such attributes of endophyte infection confer an ecological advantage to the endophyte-infected plants, enabling their survival and dominance over endophyte-free plants (West *et al*., 1988; Vila-Aiub *et al*., 2005).

Despite strong evidence for improved host physiology and tolerance to adverse abiotic stresses by endophyte-infected grasses, the mechanisms underlying this phenomena is still unknown. Research focused on this aspect is still needed and may help in selection of endophyte isolates that improve the plant's tolerances to both biotic and abiotic stresses. Apart from evidence gathered from research with grass endophytes, little to nothing is known about the influence of non-grass endophytes on their hosts' physiological responses.

7.3 Induced plant defense responses

Induction of systemic resistance by non-pathogenic microorganisms against pests and diseases is a well-documented phenomenon (Rammamorthy *et al*., 2001; Compant, 2005a). For example, a non-pathogenic *F. oxysporum* isolate (Fo47) induced resistance in tomato plants to *F. oxysporum* f. sp. *lycopersici* Jarvis et Shoem. when inoculated prior to infection with the pathogen (Fuchs *et al*., 1999). The endophyte *Acremonium kiliense* Grütz. induced resistance to *F. oxysporum* and *Clavibacter michiganense* pv *michiganense* (Smith), leading to delayed wilt symptoms, reduction of disease severity, and diminished loss of plant biomass in tomato plants (Bargmann and Schonbeck, 1992).

Induced systemic resistance (ISR) is defined as the resistance in plants induced by localized infection or treatment with microbial components or their products, or chemical compounds (Kuc, 2000; Ramamoorthy *et al*., 2001). ISR is different from systemic acquired resistance (SAR). SAR develops in plants in response to both biotic (pathogen attack) and abiotic factors (chemicals) and depends on the accumulation of salicylic acid (Van Loon *et al*., 1998). The onset of SAR is characterized by expression of genes for pathogenesis-related proteins (PR proteins) such as PR-1, PR-2, chitinases and peroxidases (M'Piga *et al*., 1997; Ramamoorthy

et al., 2001; Jeun *et al*., 2004). ISR, on the other hand is dependent on the jasmonic acid and phenylpropanoid pathways (Pieterse *et al*., 1998; Van Loon *et al*., 1998; Ramamoorthy *et al*., 2001). ISR leads to synthesis of plant defense products including peroxidases, polyphenol oxidases and phenylalanine ammonia-lyases (PAL). Polyphenol oxidases catalyse the formation of lignin through polymerization of phenols while PAL are involved in synthesis of phytoalexins and of phenolic compounds. It has not been demonstrated that endophytes induce SAR, which involves the hypersensitive reaction (Ramamoorthy *et al*., 2001).

Plant growth-promoting rhizobacteria (PGPR) are one of the most widely studied inducers of resistance in host plants against pest and pathogen attack (Ramamoorthy *et al*., 2001). Some PGPR may enter the interior of plant roots and establish endophytic associations (Kloepper *et al*., 1999). The ability of PGPR to establish endophytic relationships with their host plants indicate the possibility that naturally occurring endophytes may be able to induce resistance responses similar to those induced by PGPR. Endophytes may be suitable inducers of plant defense mechanisms, because by colonizing internal plant tissues they establish intimate relationships over a long period of time with their host plants (Siddiqui and Shaukat, 2003b). Induction of plant defense responses may occur through enhancement of the physical and mechanical strength of the plant cell wall, as well as biochemical changes in the host reaction, which leads to the synthesis of defense-related chemicals against the pest or pathogen (Ramamoorthy *et al*., 2001).

7.3.1 Induced physical and mechanical defense responses

Few studies have been conducted on the host physical changes induced by endophyte infection. Nevertheless, there are a few indications that plants undergo structural changes due to infection by endophytes. Kimmons *et al*. (1990) noted a correlation between the thickness of the wall of the root endodermis and the presence of *P. scribneri* in tall fescue roots. Induced structural changes in the roots, mainly thickening of the endodermal cell walls, were also associated with endophyte infection in tall fescue, which in turn led to reduced *M. marylandi* populations. Thickening of the endodermis might reduce the ability of *M. marylandi* to penetrate the stele, induce giant cells and reproduce (Kimmons *et al*., 1990; Gwinn and Bernard, 1993).

7.3.2 Induced biochemical defense responses

Induction of biochemical changes by PGPR is well documented. Accumulation of phenolic compounds, which occurs after challenge inoculation with the pathogens in plants, may be one of the mechanisms through which fungal endophytes become beneficial to the host plant (Biggs, 1992). For example, the endophytic bacterium *Burkholderia phytofirmans* Sessitsch *et al.* induced phenolic compound accumulation and strengthening of the cell walls in the exodermis in grapevines (*Vitis vinifera* L.) (Compant *et al*., 2005b). Schulz *et al*. (1999) demonstrated a higher phenolic metabolite concentration in roots of larch (*Larix larcina* (Duroi) K.Koch) and barley (*Holdeum vulgare* L.) plants infected by endophytic *Cryptosporiopsis* sp. and *Fusarium* sp. than in endophyte-free plants. Increased chitinase activity observed in endophyte-infected clones of tall fescue was associated with reduced nematode effects (Roberts *et al*., 1992). Although the authors could not directly link increased chitinase activity and nematode response, they concluded that, since chitinase is a PR-protein (Van Loon *et al*., 1998), it may have some effect on the resistance mechanism of the plants leading to reduced nematode populations. Chitinases indirectly release oligosaccharide signal molecules capable of activating a variety of plant defenses against pests and diseases (Ryan, 1988).

7.3.3 How to detect ISR

The evidence for ISR is mainly obtained by separation of the biological control agent from the infection courts and the subsequent decrease of disease upon challenge inoculation with the pathogen. The split-root technique provides a convenient method to assess ISR as it gives spatial separation of the inducing agent and the pathogen for the duration of the experiment (Van Loon, 1997). Since split-root experiments prevent direct interaction between the biological control agent and the pest/pathogen, the observed pest or disease reduction is usually attributed to increased defense responses due to colonization of the roots by the biological control agent (Fravel *et al*., 2003). For example, Duijff *et al*. (1998) used split root experiments to demonstrate that induced resistance was a mechanism of control by endophytic *F. oxysporum* in the suppression of fusarium wilt of tomato. Similarly, Reitz *et al*. (2000), using split-root experiments, showed that soil treatments on one half of the root system with lipopolysacharides (LPS) of the rhizobacteria *Rhizobium etli* sp. novel L. Segovia**,** Young and Martinez-Romero resulted in a reduction of root penetration by the cyst

nematode, *Globodera pallida* (Stone) on the untreated half of the split root system on potato. Their results showed that LPS of *R. etli* act as the inducing agent of systemic resistance against *G. pallida* in potato roots. Although the antagonistic bacterium used was isolated from the rhizosphere, these results lead to the assumption that endophytic bacteria would induce similar responses in other plants. Spatial separation of the pathogen or pest and the inducing agent can also be achieved when the pest is applied on the roots and the inducing agent in the foliage, or by inoculating the pest and inducing agent on different leaves of the same plant. For example, Bargabus *et al*. (2004) demonstrated a reduction in Cercospora leaf spot symptoms by inoculating the biological control agent *Bacillus pumilus* Meyer and Gottheil on different leaves of sugar beet (*Beta vulgaris* L.) than the pathogen.

7.3.4 Benefits of ISR

Biological pest and disease management by endophytes mediated through induction of resistance is advantageous because sustained pest and disease suppression can be observed, even when the population of the inducing bacteria or fungus declines over time. Additionally, activation of plant defense mechanisms may be maintained for prolonged periods of time and may be effective against multiple pathogens (Ramamoorthy *et al*., 2001). Non-specificity of ISR has been demonstrated, although the effectiveness of ISR against different pathogens may vary (Kúc, 2000). If ISR is the main mechanism of action by endophytes against nematodes, then the need for complete colonization of the plant root system is unnecessary. Threshold colonization rates capable of stimulating the plant to defend itself would need to be determined (Hallman, 2001).

8. Banana production in Uganda

The National Agricultural Research Organization (NARO) reported that bananas occupied the largest cultivated area among staple food crops in Uganda (NARO, 2001) with more than 75% of all farmers growing the crop (Gold *et al*., 1993). *Per capita* annual consumption of bananas in Uganda is the highest in the world, estimated at around 250 kg / year / person (INIBAP, 2000; NARO, 2001). Bananas are consumed as fruit, prepared by cooking, roasting or drying, or fermented for the production of alcoholic beverages as well as for non-alcoholic banana juice (Karamura, 1993).

Bananas are primarily grown as a subsistence crop for both rural and urban consumption with surplus production sold in local markets (Karamura, 1993; Mugisha and Ngambeki 1994; Karamura *et al*., 1996). Most banana production takes place on small subsistence farms (plots of less than 0.5 ha) with low input farming methods (Gold *et al*., 1998; Karamura, 1999). The life span of banana plantations depends on agro-ecological conditions and management practices, and may range from as low as 4 years in Central Uganda to over 30 years in Western Uganda (Speijer *et al*., 1999a).

Most of the banana varieties grown in Uganda are endemic to the East African highlands. The clones are triploid hybrids belonging to the East African highland bananas (*Musa* AAA-EA). NARO (2001) also reports that an estimated 85% of bananas grown in the country are East African highland bananas. These endemic banana varieties (AAA-EA genomic group) consist of two types based on their use: cooking bananas (*matooke*) and beer bananas (*mbidde*). They are classified by morphological characteristics into five clone sets: Musakala, Nakabululu, Nakitembe, Nfuuka and Mbidde (Karamura and Karamura, 1994; Gold *et al*., 1998). The non-endemic bananas grown in Uganda have their origins in Southeast Asia and include exotic beer and sweet bananas (AB, ABB and AAA genomic groups), and roasting bananas or plantains (AAB genomic group). Banana production is characterized by wide cultivar diversity with farmers growing 10 to 15 different banana cultivars in stands of less than 200 banana mats (Karamura, 1993; Karamura *et al*., 1996; Gold *et al*., 1998).

Geographic shifts of banana production towards new growing areas (Southwestern Uganda) and the abandonment of the crop in traditional areas (Central Uganda) have occurred in recent years. In the traditional banana growing areas of Central Uganda, production of endemic East African highland bananas has stimulated an increase in the production of non-endemic bananas and other food crops (e.g. maize, sweet potato, cassava). Combinations of pest and disease pressure, declining soil fertility, and socio-economic constraints (reduced labor availability and management of the plants) have been cited as the causes for this trend (Gold *et al*., 2000). The major pest constraints to banana production are the banana weevil *Cosmopolites sordidus* (Germar) and a complex of plant-parasitic nematodes.

9. Nematode problems of banana

A complex of plant-parasitic nematodes comprising *Radopholus similis* (Cobb) Thorne, *H. multicinctus* (Cobb) Golden and *Pratylenchus goodeyi* (Sher and Allen) have been identified as major nematode species affecting banana production in Uganda (Gold *et al*., 1993, Karamura, 1993, Speijer *et al*., 1999a). Geographic distribution of the main nematode species is governed by temperature and elevation. *Radopholus similis* and *H. multicinctus* are the predominant species between 1000-1350 m above sea level (Kashaija *et al*., 1994) while at higher elevations and cooler temperatures *P. goodeyi* is the predominant species (Speijer *et al*., 1994). Mixed populations occur at lower elevations. Cultivar, cropping systems, farm management and sources of planting material influence abundance of individual nematode species, while species composition varies among sites and between farms within sites (Kashaija *et al*., 1994). All three nematode species are migratory endoparasites with their life cycles being completed in the root tissues. Among these nematode species, the burrowing nematode *R. similis* is considered to be most important economically in Uganda (Kashaija *et al*., 1994) and worldwide (Sarah *et al*., 1996).

10. Biology of *Radopholus similis*

Radopholus similis is a migratory endoparasitic nematode completing its life cycle in 20-25 days in root and corm tissue under optimal temperature conditions of 30ºC. Embryonic development occurs within 4-10 days and the subsequent juvenile stages are completed in 10-15 days. They attack almost all banana cultivars as well as abáca (*Musa textilis* L.) and other seeded *Musa* spp. (Gowen and Quénéhervé, 2005). Distribution of *R. similis* is governed by preferences for temperature fluctuations between 24ºC and 32ºC with optimum reproduction occurring around 30ºC (Sarah *et al.,* 1996). Being primarily endoparasitic, *R. similis* is hardly found in the soil.

Penetration of nematodes in banana roots occurs mainly at the root apex though invasion can occur at any portion along the root length (Sarah *et al*., 1996). After root penetration, the nematode occupies an intercellular position in the cortical parenchyma and migrates in and between cells in the root cortex feeding on cell cytoplasm. This results in collapsed cell walls, cavities and tunnels. On the corm, lesions begin to develop where infested roots are attached and then spread outwards. Necrosis can extend to the whole cortex of the corm and the roots

but the root stele is rarely damaged. *Radopholus similis* females lay eggs inside roots and all development occurs within the root. All juvenile stages and females of *R. similis* are infective and cause damage to the roots while males lack a developed stylet and are not plant-parasitic (Speijer and De Waele, 1997). Though primary, secondary as well as tertiary roots can be affected, *R. similis* were found to occur in higher numbers in primary roots than in lateral roots (Kashaija, 1996). Spatial distribution of nematodes in banana root parts revealed the highest *R. similis* numbers in the cortical parenchyma and less in the epidermis and vascular cylinder (Araya and De Waele, 2001).

11. Damage and economic importance of *Radopholus similis*

Damage due to *R. similis* infection occurs because of its migration and feeding activity inside the plant roots. Symptoms of nematode damage can be identified early in longitudinal sections of roots as reddish brown necrotic patches usually confined to the cortex region and extending from the root surface to the centre. On corms, necrosis appears as reddish brown discoloration, usually beginning where roots leave the corm (Speijer and De Waele, 1997). Nematode feeding destroys root and corm tissue, reducing water and mineral uptake, resulting in a reduction of plant growth and development. This leads to a severe reduction of bunch weight and a significant increase in time period between two successive harvests. Due to weakening of the root systems, plants can easily be blown over by strong winds, a condition referred to as toppling (Sarah *et al.,* 1996; Jones, 2000; Gowen and Quénéhervé, 2005).

Nematodes and banana weevils frequently occur together on the same plant (Speijer *et al*., 1994). On young suckers, nematode and banana weevil damage are highly associated, which aggravates the loss of banana plants (Speijer *et al*., 1993). Due to the association between banana nematodes and weevils on banana plants, it's often very difficult to estimate the actual yield losses caused by nematodes alone (Speijer *et al*., 1993). Damage caused by weevils and nematodes is often confused leading to underestimation of nematode damage with most of the damage on the corm attributed to the weevil (Gold *et al.,* 1993).

Radopholus similis can cause yield losses of up to 30-50% per cycle in on-station trials (Speijer *et al*., 1999b; Speijer and Kajumba, 2000). The East African highland banana cv. Mbwazirume infected by both *R. similis* and *P. goodeyi* had bunch weight reductions of up to 30% with the percentage of toppled plants being four times higher when both nematodes were

present compared to when either of the two occurred alone (Talwana *et al*., 2003). Nematode-induced losses are a result of an increase in the number of dead roots, root necrosis, reduced number of standing leaves, reduced flower production, increased plant toppling and reduction in bunch weight (Speijer *et al*., 1999b; Speijer and Kajumba, 2000; Talwana *et al*., 2003).

12. Management of *R. similis* in banana

Radopholus similis control has mainly relied on the use of chemical nematicides in commercial plantations and although these nematicides are very effective, the feasibility for use is beyond the economic means of most small-scale farmers in developing countries such as Uganda (Gowen, 1991). Conventional methods of establishing plantations using nematode-infested suckers as planting material are the main avenues for introducing these pests to new fields (O'Bannon, 1977; Sarah, 1989). Dissemination of the banana nematodes through infested planting material continues to occur unabated as farmers continue to use infested suckers. Most farmers in Uganda perceive control strategies against banana pests (nematodes, weevils and diseases) to be too costly, labour consuming and/or ineffective (Gold *et al*., 1993). Nevertheless, several nematode management options are available.

12.1 Cultural control

The most important measure to control nematodes in banana stands is the use of healthy planting material (Sarah, 2000). Using pest and disease-free planting material reduces the spread of nematodes to new fields (Speijer *et al*., 1995). Clean planting material can be obtained in several ways. Corm paring, which involves removal of nematode infested roots and corm tissue, can reduce initial infestation (Speijer *et al*., 1995; Gold *et al*., 1998). Additional hot water treatment of corm-pared suckers by dipping in hot water at a temperature of 53ºC for 20 min rids the plants of nematodes (Speijer *et al*., 1995; Gold *et al*., 1998) leading to crop yield improvements of about 30% in the first crop cycle (Speijer *et al*., 1999a). Though these methods have proven effective and feasible, they only offer temporary control as re-infestation of the plants readily occurs in the fields (Speijer *et al*., 1995; Speijer *et al*., 2001). Acceptance of the hot water treatment technology at the farmer level is also constrained by problems of transportation of the suckers and hot water tank (Speijer *et al*., 1999b).

Mulching can also mitigate the effects of nematodes on banana plants. Obiefuna (1991) demonstrated that mulching with crop residues effectively decreased *R. similis* populations and increased yield in plantain. Mulching reduced the population densities and damaging effects of *R. similis* on cv. Mbwazirume (*Musa* spp. AAA-EA). Banana mats in mulched plots had lower soil temperatures than unmulched plants, which may have slowed down *R. similis* reproduction (Talwana *et al*., 2003). In contrast, McIntyre *et al*. (2001) obtained higher nematode numbers and root necrosis in mulched banana plots. Mulching may reduce the impacts of nematodes through increased soil nutrient availability, increased soil porosity and increased surface rooting and may also has beneficial effects on plant growth (Speijer *et al*., 1999a; McIntyre *et al*., 2000).

Use of legume intercrops was found to have no effect on nematode and the banana weevil populations and damage. Though intercrops may not reduce the effects of nematodes they may be incorporated in banana farming systems to increase land use efficiency and provide food security (McIntyre *et al*., 2001). Use of intercrops is mainly hampered by the ability of *R. similis* to reproduce in many plant species as well as weeds (O'Bannon, 1977).

12.2 Host plant resistance

Strong resistance to *R. similis* has been identified in Pisang jari buaya clones (*Musa* spp. AA group) (Pinochet and Rowe, 1979) and in the banana cultivars Yangambi KM5 (AAA), Gros Michel (AAA) (Fogain and Gowen, 1997; Sarah *et al*., 1997) and Kunnan (AB) (Collingborn *et al*., 2000). Studies to investigate the resistance mechanisms in Yangambi KM5 revealed greater amounts of preformed phenolic compounds (Valette *et al*., 1998) and while cultivar Kunnan was found to possess high amounts of condensed tannins (Collingborn *et al*., 2000). Resistance may offer a long-term intervention against nematodes for resource poor farmers in Africa. However, resistance to nematodes has not been identified in highland cooking banana cultivars. Banana improvement by means of conventional plant breeding has proved extremely difficult due to the genetic complexity of the crop and the long period required to evaluate crossings for resistance to different nematode collections (Stover and Buddenhagen, 1986; Tripathi, 2003). Additionally, most banana varieties are triploid genotypes that are almost or fully sterile, which further complicates the situation.

12.3 Transgenic banana

Due to difficulties involved in conventional breeding methods, there has been increased research interest in the potential of genetic engineering to tackle pressing biotic problems in banana production. Banana plants may be genetically transformed to express resistance genes for protein inhibitors such as cystatins (Tripathi, 2003). Cystatins are naturally occurring proteinase inhibitors used by plants as a defense against insects and pests (Atkinson *et al*., 1995). They impair digestion of dietary protein by nematodes and suppress nematode multiplication (Atkinson *et al*., 2004). Cystatins are effective against a wide range of nematodes and can protect banana from different combinations of pest species that occur in plantations. Atkinson *et al.* (2004) successfully transformed Grand Naine plants (Cavendish subgroup-AAA) with a rice cystatin. Transformed plants achieved a reduction of 70% in *R. similis* population densities. The acceptance and adoption of transgenic banana in developing countries such as Uganda may be undermined by lack of biosafety rules and regulations. Although developing transgenic banana may offer a long term control option against banana pests and diseases, it may take a long time before such cultivars are freely available to farmers (De Waele, 2000).

12.4 Tissue culture banana plants

Use of tissue culture banana plants is one means to provide clean planting material (Mateille *et al*., 1994; Sarah, 2000). Tissue culture plants are produced axenically, making them pest- and disease-free. Additional advantages are the rapid multiplication, higher yields and uniformity (Robinson, 1996). Advocacy for the use of tissue culture plants is on the increase, though adoption by farmers in Uganda remains limited, especially due to the low availability and relatively high cost. Loss of beneficial microorganism such as endophytes through the axenic production of tissue culture plants may probably make them more vulnerable to nematode attack in the field (Pereira *et al*., 1999). Research has shown that tissue culture plants are more susceptible to nematode and disease attack in the field than plants derived from suckers (De Waele *et al*., 1997; Stanton, 1999; Viaene *et al*., 2003; Blomme *et al*., 2004). Tissue culture plants infested with *R. similis* had more nematodes and higher root damage than plants derived from suckers (Viaene *et al*., 2003). Although tissue culture plants may offer temporary solutions to nematode problems in banana, there is a need to develop affordable, sustainable and environmentally friendly management strategies that complement

the benefits of clean planting material offered by tissue culture. The artificial introduction of beneficial microorganisms such as endophytic fungi in these sterile plants at the hardening phase may offer protection to the young plants in the early growth stages and extend the life of planting material (Sikora and Schuster, 1999).

12.5 Biological control

Biological control of plant-parasitic nematodes may offer an alternative to nematicides due to environmental and health concerns associated with the chemicals. However, biological control should not be used in isolation but should form part of an integrated nematode management approach. Several biological control agents have been used to reduce the effects of *R. similis* in banana.

12.5.1 Rhizosphere bacteria and Actinomycetes

Four isolates of rhizosphere fluorescent bacteria (three *P. fluorescens* and a *P. putida* Trevisan Migula isolate) showed *in vitro* repellent effects to *R. similis* and resulted in lower nematode invasion and development in greenhouse plants (Aalten *et al*., 1998). Application of *Streptomyces costaricanus* sp. nov. Esnard**,** Potter and Zuckerman resulted in improved plant growth and lower *R. similis* populations (Esnard *et al*., 1998). Combining these cultures with wheat mash further improved results. Pre-inoculation of banana plantlets using a commercial water dispersible *Paecilomysces lilacinus* (Thom) Samson product (strain 251) resulted in decreased *R. similis* activity in the field, with a positive correlation between rates of application and degree of *R. similis* control (Mendoza *et al*., 2004)

12.5.2 Mycorrhizae

The use of arbuscular mycorrhizal fungi (AMF) to control nematodes in banana in pot experiments has also been demonstrated. AMF have also been shown to promote plant growth. Declerck *et al*. (1995) reported a significant increase in growth of tissue culture banana plantlets inoculated with AMF. Inoculation of plantain tissue culture plants with the AMF *Glomus mosseae* (Nicolson & Gerdemann) Gerd during the weaning phase significantly improved plant growth and reduced *R. similis* populations compared to non-mycorrhized

plants. Similar results were reported by Elsen *et al*. (2003) and Fogain and Njifenjou (2003). Elsen *et al*. (2003) demonstrated that mycorrhization of different banana genotypes differing in root morphology resulted in better plant growth even in the presence of nematodes.

12.5.3 *Endophytes*

Endophytes offer a novel and environmentally friendly means of nematode management in banana. Since *R. similis* is an endoparasite completing its life cycle in banana root and corm tissues, the prospects for using endophytes, which occupy the same niche, are promising. Additionally, application of endophytes in vegetatively propagated crops such as banana may ensure continued pest and disease suppression in later ratoon crops if the endophytes are transmitted to the sucker plants (Ramamoorthy *et al*., 2001). Banana is a perennial herb and the introduction of endophytes into tissue culture plantlets at an early stage of growth is feasible. Since tissue culture plants contain no microorganisms, introduced endophytes should easily establish in the plant tissues (Cao *et al*., 2005). Many effective endophytes have been found in other crops for the management of nematodes, which makes biological control of banana nematodes using fungal endophytes a promising option.

Evidence for the presence of fungal endophytes in banana plants has been reported. Endophytic fungi have been isolated from healthy banana plants growing in nematode and banana weevil infested plantations in Uganda (Schuster *et al*., 1995; Griesbach, 1999; Niere 2001), Central America (Pocasangre, 2000) and South Africa (Athman *et al*., unpublished). The main fungi isolated as endophytes from roots and corms of banana plants mainly belonged to the genus *Fusarium*. *Fusarium oxysporum* was the predominant species in Uganda, Central America and South Africa.

Artificial inoculation of 1-month-old tissue culture banana plants has successfully been achieved through root and corm dip. Tissue culture plants are deflasked and dipped in the fungal spore suspension for variable time periods allowing spore attachment and the subsequent endophytic colonization of the roots and corm tissues (Griesbach, 2000; Niere, 2001; Paparu *et al*., 2004). Fungal isolates inoculated into banana plants are able to colonize the plants roots, which has led to enhanced plant growth and lower nematode infection (Griesbach, 2000; Sikora *et al*., 2000; Niere, 2001). Inoculation of endophytes into tissue

culture plants may serve to biologically protect the plants at a crucial stage before they are transplanted to the field. The treatment of tissue culture plants provides the advantage of lower costs due to the low levels of inoculum required (Sikora *et al*., 2003). Application of the endophyte pre-grown on a solid substrate such as maize bran also offers an alternative method for artificially introducing the endophyte in tissue culture plants by mixing the fungal inoculum with the potting soil at transplanting time (Paparu, 2005).

Various isolates of endophytic *F. oxysporum* have been shown to produce nematostatic and nematicidal compounds against *R. similis* in culture filtrates. Pocasangre (2000) and Niere (2001) demonstrated inactivation of *R. similis* motile stages in culture filtrates of endophytic *Fusarium* spp. isolates from banana and related this effect to possible toxic metabolites in the culture filtrates. Although these toxins were not identified, it was concluded that the fungal isolates tested produced secondary metabolites in culture, which resulted in inactivation and mortality of *R. similis*. *In vitro* tests for parasitism of four endophytic *F. oxysporum* isolates in the absence of banana plants demonstrated a lack of direct parasitism on *R. similis* although nematode activity decreased significantly (Vu *et al*., 2004).

Positive effects of endophyte infection of banana plants in screen house experiments have been demonstrated. Pocasangre (2000), working on fungal endophytes isolated from banana plants in Central America, found a lower number of *R. similis* in banana roots. A reduction in penetration of nematodes in root tissue of up to 74%, and increased shoot and root weights in endophyte-inoculated banana plants was also noted. Speijer (1993) reported decreased penetration and development of *P. goodeyi* in banana plants colonized by a non-pathogenic isolate of *F. oxysporum*.

Plant growth promotion effects of banana plants manifested in higher root and shoot weights in endophyte-treated plants than in non endophyte-plants, although this was not consistent for all isolates tested (Pocasangre, 2000; Niere, 2001). Griesbach (2000) reported increased banana biomass production in field plants of the cultivars Nakyetengu and Nfuuka (AAA-EA) inoculated with the fungal endophyte *Fusarium concentricum* Nirenberg & O'Donnell. Reduced nematode populations in endophyte-treated plants may depend on the fungal isolate and banana clone (Niere *et al*., 2004). The endophytic *F. oxysporum* isolate V5W2 has been earmarked for its biocontrol potential against the banana nematode *R. similis*. *In planta* screening experiments using tissue culture plants have shown this isolate to reduce *R. similis*

reproduction by between 22.9 and 60.6% in the banana cultivars Enyeru and Kibuzi (AAA-EA), respectively, compared to control plants (Dubois *et al*., 2004).

Colonization and persistence of introduced endophytic fungal isolates in tissue culture banana plants has been extensively studied through re-isolation from infected roots and corm tissues of banana plants after surface sterilization (Paparu, 2005). The identity of re-isolated fungal isolates was further confirmed with vegetative compatibility tests. No differences were observed among endophyte isolates or banana cultivars, indicating a lack of interaction between isolate and cultivar (Paparu, 2005). One month after inoculation of plants with the fungal isolates, corms were colonized to a higher extent compared to roots. However, colonization rates declined over time and there was substantial decline in percentage colonization after field planting of the plants. Decline in the colonization rates of corm tissue was more pronounced than in roots up to 33 weeks after plants had been inoculated with the endophytes. Different methods of introducing fungal endophytes resulted in differences in percentage colonization (Paparu, 2005). The use of a solid substrate medium for introducing endophytes into banana roots gave the best results, followed by root and corm dip of plants with a bigger root biomass in a conidial suspension. To enhance formation of a bigger root biomass, plants were first grown in a nutrient solution for 1 month before being inoculated with the endophyte. The lowest colonization rates were observed in plants that were inoculated with the conidial suspensions after being removed straight from rooting medium.

13. Conclusions

Endophytes have important implications for their host plants, in providing protection against biotic and abiotic stresses. Presently, little is known of the potential benefits of endophytic microorganisms. Endophytic microorganisms are potentially attractive biocontrol agents and could be utilized to protect tissue culture plants before they are transplanted to the field. This would expose the plant to microorganisms that could provide protection through systemically induced plant responses. While the use of endophytic microorganisms for nematode and pest control in crops is relatively new and unexplored, it is an environmentally friendly alternative to pesticides at a time that the latter become increasingly restricted. Information on the diversity of endophytes and mutualistic interactions with crops other than grasses remains scanty, and more research is needed to understand their full potential as biocontrol agents.

Culture filtrates of endophytic fungi have shown *in vitro* activity against nematodes, suggesting that these fungi produce secondary metabolites that are nematicidal. However, only a limited number of research projects have focused on isolation and identification of such compounds and their effects. Additionally, production of these compounds in culture (*in vitro*) does not necessarily reflect what would happen in the plant. Their effects on nematodes in the plants may only be proven if the compounds can be extracted from endophyte-infected plants. Screening isolates and banana cultivars for the production of toxins is necessary to determine the role of antibiosis in nematode suppression in the plant.

Although research on the use of endophytes remains recent, there is strong evidence that they could provide a source of novel nematicidal compounds capable of protecting plants from pest and disease attack. Little is known about the interactions between the host plant, endophyte and the pest. Understanding the host-endophyte-pest interactions is essential for the successful development of biological control as it would help in selection of the most suitable candidate isolates and also help in designing control programmes that maximally utilize their nematode controlling potentials. Elucidation of different mechanisms of action would also facilitate combination of isolates, thus providing a broader spectrum of control.

Decline in colonization of banana plants by introduced fungal endophytes with continued pest suppression provides strong evidence for the presence of other mechanisms of action acting through the host plant. Continued nematode population reduction, even when endophyte colonization rates fall substantially, is an indication that additional mechanisms are involved, especially induced resistance mechanisms. It is, therefore, important to determine threshold colonization rates that are capable of eliciting effective and durable plant defense responses in plants.

The use of endophytes for inducing systemic resistance may be more beneficial to vegetatively propagated crops such as banana. As a perennial crop banana remains in the field for relatively long periods and is thus continuously challenged by pest and disease attack. Since the frequency of re-isolation of the endophyte isolates on banana plants decreases over time, long-term protection in banana may not be high. How long the plant remains in the induced state is also unknown. Earlier studies so far suggest that introduced endophytes can provide protection throughout the first growth cycle (Niere *et al*., unpublished). Although initial introduction of endophytes in tissue culture plants may provide

protection during this vulnerable stage, what happens at later stages of the plants growth is unknown. The possibility of maternal transmission of introduced endophytes to suckers needs to be investigated. This would minimize the need for additional or frequent applications as the introduced endophytes become self-perpetuating and sustaining. The effect and necessity for booster applications following initial inoculation, especially when plants are in the field, needs to be investigated.

Finally, while endophytes may be successfully used in plant-parasitic nematode management programmes, they are unlikely to achieve the levels of control provided by pesticides and, therefore, are unlikely to replace pesticides (Kerry, 1990; Van Loon *et al*., 1998). Their performance is inconsistent under screen house conditions (Niere, 2001). Their use must, therefore, be maximized in an integrated nematode management programme that include cultural practices and field management (Sikora *et al*., 2003). Additionally, combinations of biocontrol agents with complementary or synergistic mechanisms of action may be used to further reduce pest and disease pressure. Biological control of plant-parasitic nematodes using endophytes may be an additional option for environmentally friendly and benign nematode management.

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Chapter 2

In vitro screening of endophytic *Fusarium* isolates against the banana burrowing nematode, *Radopholus similis* (Cobb) Thorne

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Abstract

Radopholus similis is one of the key pests of banana in the East African Highlands and in the world. Although control of this pest has mainly relied on the use of clean planting material, re-infestation of plants in the field remains a critical concern. Alternative management options such as the use of fungal endophytes may be used to provide protection and extend plant life. In this study, a total of 35 endophytic *Fusarium* isolates were screened for the production of secondary metabolites antagonistic to *R. similis* in culture. Undiluted and diluted culture filtrates were tested against motile stages of *R. similis* and eggs. Tests were conducted using culture filtrates of the fungal isolates grown in banana corm broth. All isolates tested demonstrated some level of *in vitro* antagonistic activity on the mobility of *R. similis* mixed stages (males, females and juveniles). The percentage of immobilized nematodes increased with increase in the length of exposure time to culture filtrates. After 24 hrs exposure, up to 100% of nematodes were immobilized compared to 26.5% in control treatments. Mortality of mixed stages of *R. similis* exposed to culture filtrates for 24 hrs, followed by rinsing with sterile distilled water, demonstrated that the effects of culture filtrate treatment were irreversible, as nematodes did not recover and were considered dead. Nematode mortality rates after 24-hr exposure periods ranged from 76.4% to 100.0%. The effects of culture filtrates on *R. similis* motile stages increased with increasing culture filtrate concentration. Culture filtrates also demonstrated inhibitory effects on hatching of *R. similis* eggs. *Radopholus similis* males were more sensitive to culture filtrate treatment than females. The results obtained demonstrate the potential for using endophytic *Fusarium* isolates as biological control agents against *R. similis* and/or toxic derivatives as potential nematicides against the nematode.

Introduction

The banana burrowing nematode, *Radopholus similis* (Cobb) Thorne, is one of the major constraints to banana production in the world (Sarah, 1989). *Radopholus similis* feeds inside the plant roots, and the damage it causes leads to necrosis and impaired water and nutrient uptake. Root necrosis, in turn, reduces the plant's anchorage in the soil and plants can easily be toppled during strong winds. Yield losses caused by *R. similis* can be as much as 50% (Speijer and Kajumba, 2000). Chemical control of the nematode is not a feasible option to small-scale farmers due to their high cost and lack of availability. Management of *R. similis,* thus, relies on cultural methods, such as the use of healthy, clean planting material obtained from pared and hot water-treated suckers, and tissue culture plants (Speijer *et al*., 1995; Speijer *et al*., 1999). These methods offer temporary control of nematodes only, as reinfestation in the fields occurs readily (Stanton, 1999; Speijer *et al*., 2001).

Biological control of *R. similis* using fungal endophytes offers a novel and promising nematode management option. In the past, fungal endophytes have successfully been used for nematode control in crops such as tomato (Hallman and Sikora, 1994). It was also previously demonstrated that fungal endophytes from healthy banana roots were able to kill nematodes *in vitro* and to protect tissue culture banana plants against nematodes in the screen house (Pocasangre, 2000; Niere, 2001).

Fungal endophytes protect plants against nematodes in several ways. The main method by which nematodes are inhibited *in vitro* is through antibiosis. Antibiosis is the antagonism resulting from the production of secondary metabolites by one microorganism that are toxic to another microorganism (Alabouvette and Lemanceau, 1999). Production of nematode-antagonistic metabolites by fungi and bacteria in culture is a well-documented phenomenon (Anke and Sterner, 1997; Sharon *et al*., 2001), and has been reported for *Paecilomyces lilacinus* (Thom) Samson (Cayrol *et al*., 1989), *Fusarium equiseti* (Corda) Sacc. (Nitao *et al*., 2001), various rhizosphere fungi (Alam *et al*., 1973), *Fusarium solani* (Mart) Sacc. (Mani and Sethi, 1984; Mani *et al*., 1986; Zareen *et al*., 2001), *Penicillium* and *Aspergillus* spp. (Dahiya and Singh, 1985; Molina and Davide, 1986; Ansari *et al*., 2002), *Pseudomonas striata* (Chester) Migula (Ansari *et al*., 2002), *Pseudomonas aeruginosa* (Schroeter) Migula (Ali *et al*., 2002), *Trichoderma virens* Miller, Giddens & Foster (Meyer *et al*., 2000), *Trichoderma*

harzianum Rifai (Ansari *et al*., 2002) and *Chaetomium globosum* Kunze ex Steud. (Nitao *et al*., 2002). As a whole, antagonistic activity by fungi against the root-knot nematodes *Meloidogyne* spp. and the cyst nematodes *Heterodera* spp. has received the greatest attention.

Various non-endophytic *Fusarium* isolates have been shown to produce filtrates toxic to plant-parasitic nematodes *in vitro* (Ciancio, 1995; Anke and Sterner, 1997; Nitao *et al*., 2001, Meyer *et al*., 2004; Mennan *et al*., 2005). Hallman and Sikora (1996) demonstrated production of toxins by an endophytic isolate of *Fusarium oxysporum* Schlecht.: Fries from tomato plants against *Meloidogyne incognita* (Kofoid & White). Similarly, various endophytic *Fusarium* isolates obtained from banana plants have been shown to produce secondary metabolites in culture that immobilized and caused mortality of *R. similis* (Pocasangre, 2000; Niere, 2001; Dubois *et al*., 2004; Niere *et al*., 2004).

The use of fungal culture filtrates to demonstrate toxicity against plant-parasitic nematodes can help to rapidly identify strains with potential for biological control (Nitao *et al*., 1999). The objectives of the current study were, therefore, to screen various endophytic *Fusarium* isolates for production of metabolites antagonistic to *R. similis* motile stages (males, females and juveniles), to determine the effects of fungal culture filtrates on hatchability of *R. similis* eggs, and to determine the effects of different culture filtrate concentrations on motile stages of *R. similis*. These tests could be used for developing a rapid bioassay screening procedure with which different fungal isolates could be discerned.

Materials and methods

Endophytic fungal isolates

Thirty-five endophytic fungal isolates belonging to various *Fusarium* spp. were obtained from the fungal culture collection at the International Institute of Tropical Agriculture (IITA) laboratory in Namulonge, Uganda. These isolates are preserved at 4°C in soil tubes at IITA. Nine of the isolates were previously isolated from the healthy roots and corms of banana plants growing in nematode-infested plantations in Uganda (Schuster *et al*., 1995; Griesbach, 2000) and have been tested both for *in vitro* and *in vivo* activity against *R. similis* (Table 1).

The results of the initial *in vitro* screening, however, were not quantified (Niere, 2001). The other 26 isolates were selected from a collection of *Fusarium* spp. that were originally obtained from mature and healthy banana plants of the cultivar Kibuzi (*Musa* spp., AAA-EA) in 2001 in Uganda (Table 2). Selection of these isolates was based on the developmental stage of the plant and the plant part from which the isolates were obtained. Isolates were, thus, collected either from a flowering plant or a sucker attached to the flowering plant, from roots, or from the outer corm tissues. These isolates had not been screened against *R. similis* before.

Radopholus similis *cultures*

Pure sterile cultures of *R. similis* were prepared and are maintained on carrot disks at the IITA laboratory in Namulonge, Uganda according to the method described by Speijer and De Waele (1997). Nematode-infested banana roots were obtained from banana fields, cut into 1-cm-long pieces and macerated in a Waring blender (Waring, Connecticut, USA) with tap water for 15 s. The macerated suspension was collected in a modified Baermann dish and nematodes extracted overnight. Nematodes that collected at the bottom of the dish were transferred into a beaker, concentrated in a 28-µm sieve, and transferred to a counting slide. Individual *R. similis* females were handpicked with a sterile sucking tube from the slide under a dissecting microscope and transferred into a 25 ml sterile glass test tube. The sucking tube was obtained by burning the tips of two Pasteur pipettes pressed against each other. The pipettes were pulled apart after melting, resulting into a tiny opening that was used to suck nematodes from the suspension. The nematode-containing suspension was made up to 2 ml, and the nematodes surface-sterilized by adding 1 ml of a 6000 ppm streptomycin sulphate solution to the suspension for 4 hrs. The streptomycin sulphate solution was siphoned from above the settled nematodes, and the nematodes rinsed with sterile distilled water (SDW) (autoclaved at 121°C for 15 min). The rinsing step was repeated with a 30 min interval between the two rinses.

Fresh carrots were surface-sterilized by dipping them in 96% ethanol, followed by flaming. The outer peel was removed with a flame-sterilized carrot peeler and the process repeated twice. After surface sterilization, the carrots were cut with a sterile scalpel into disks *ca.* 0.5 cm thick and placed into 30-mm-diameter sterile glass Petri dishes. Approximately 25 gravid females were inoculated on the cortex of each carrot disk and the Petri dishes sealed with

parafilm. The Petri dishes were maintained in an incubator at 27°C. Three to 4 weeks later, nematodes were harvested by rinsing the Petri dishes with SDW under laminar airflow and the suspension collected in a sterile beaker. The nematodes were then either sub-cultured onto new carrot disks to replenish the nematode inoculum bank, or used for experiments.

Culturing of fungal isolates and preparation of culture filtrates

Fungal isolates preserved in soil tubes were cultured on synthetic nutrient agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar in 1000 ml SDW) in 55-mm-diameter Petri dishes for 1 week. The isolates were then transferred to liquid banana corm broth (BCB). BCB was used in an attempt to simulate the growth conditions under which the endophytes naturally occur. The broth was prepared as follows: Corms were obtained from approximately 50 cm high suckers of the cultivar Enyeru (syn. Nabusa, *Musa* spp. AAA-EA) in Namulonge, Uganda. The corms were pared to remove roots and adhering soil, and cut into pieces of *ca.* 1 cm^3 in the laboratory. Five hundred grams of corm tissue was boiled in 1 L of tap water until soft. The mixture was then passed through cheesecloth to remove corm pieces and the resultant solution topped up to 1 L with SDW. Aliquots of 100 ml were dispensed into 250-ml Erlenmeyer flasks and sterilized at 121°C for 15 min. The medium was allowed to cool and then aseptically inoculated with 7-day-old agar disks of the different fungal isolates grown on SNA. Inoculated flasks were incubated for 14 days at laboratory conditions of ± 25°C with a natural photoperiod of 12 hrs light and 12 hrs darkness. Control flasks were not inoculated with fungal cultures. The flasks were manually shaken each day to disperse the fungal spores in the medium.

Pure fungal culture filtrates were obtained by centrifuging the fungal cultures in BCB at 6000 rpm for 10 min (Meyer *et al*., 2000). The resultant supernatant was aseptically transferred to sterile 25 ml screw cap glass bottles and filtered through 0.22 µm millipore filters. The pH of the fungal filtrates was measured and the average pH calculated. The pH of the control treatment was also measured and adjusted to equilibrate the average pH of the fungal culture filtrates. An additional control treatment with unadjusted pH was included. Culture filtrates were kept in the fridge at 4°C overnight.

Nematicidal activity of undiluted culture filtrates

One milliliter of 2-wk-old undiluted culture filtrates of each of the nine *F. oxysporum* isolates was transferred to 30-mm-diameter sterile glass Petri dishes. The filtrates were inoculated with approximately 100 sterile mixed stages (females, males and juveniles) of *R. similis* kept in 100 µl SDW. Control Petri dishes had pure BCB inoculated with the mixed nematode culture. Two control treatments were included in the study: one with unadjusted pH and another in which the pH was adjusted to the average pH of the culture filtrates. Three replicate Petri dishes were used for each fungal isolate and for the controls, and the experiment was repeated three times. During the course of each experiment, Petri dishes were left completely randomized and undisturbed on laboratory benches.

For the bioassays with the nine prescreened *F. oxysporum* isolates, the effect of culture filtrates on nematode activity (paralysis) was assessed by recording the number of active and inactive nematodes after exposure times of 3, 6 and 24 hrs. Reversibility of toxic effects of culture filtrates and mortality of nematodes exposed to the culture filtrates was determined after an exposure period of 24 hrs. To assess mortality, the nematodes were first rinsed through a 28-µm sieve with SDW and transferred back into the Petri dishes. The Petri dishes were then incubated under laboratory conditions for a further 24 hrs. Nematodes that remained paralysed after probing with a fine needle were considered dead (Cayrol *et al*., 1989).

Separate bioassays were conducted for the 26 *Fusarium* isolates obtained from roots and corms of banana plants from Masaka district, Uganda, since all isolates could not be handled in one bioassay. The bioassay was performed at an exposure time of 6 hrs, based on results obtained with the nine prescreened *F. oxysporum* isolates. A reference isolate *V5W2*, previously selected as the best of the nine prescreened *F. oxysporum* isolates, was included for comparison. Four replicate Petri dishes were used for each fungal isolate and for the controls, and the experiments repeated three and six times for corm and root isolates, respectively.

Nematicidal activity of different culture filtrate concentrations

Two *F. oxysporum* isolates, *V5W2* and *Eny1.31i*, were selected to test the effect of culture filtrate concentrations on the paralysis and mortality of mixed stages of *R. similis*. Fungal isolates were grown in BCB, and culture filtrates prepared as described above. Six concentrations, 2.5, 5, 10, 25, 50 and 100% of the filtrate were prepared by diluting the culture filtrates with SDW. One milliliter of the diluted culture filtrates of each test isolate was transferred into 30-mm-diameter sterile glass Petri dishes. Control Petri dishes received uninoculated broth, with the pH of the control treatment adjusted to equal the average pH of the test isolates. Approximately 100 *R. similis* (mixed stages) in 100 µl sterile distilled water were transferred to the filtrates. The treatments were replicated four times, and the experiment was repeated once. The activity (mobility) of nematodes immersed in diluted culture filtrates was determined by counting the number of active and inactive nematodes after an exposure time of 6 hrs. Reversibility of the toxic effects of culture filtrates and mortality of nematodes was determined as described above.

Effect of fungal culture filtrates on hatching of R. similis *eggs*

R. similis egg hatch was determined following treatment with the nine pre-screened *F. oxysporum* isolates. Single eggs of *R. similis* were obtained from 3- to 4-week-old carrot disk nematode cultures. The carrot disk cultures were macerated in a Waring blender, and both motile stages and eggs of *R. similis* collected overnight in a modified Baermann dish (Hooper *et al*., 2005). The suspension was concentrated in a 28-µm sieve and transferred to counting-slides, from where the eggs were hand-picked under a stereomicroscope and transferred to sterile test tubes. The eggs were surface sterilized in a 6000-ppm streptomycin sulphate solution for 1 hour (Speijer and De Waele, 1997) and washed twice with SDW, with a 30 min interval. Undiluted fungal culture filtrates (1 ml) were transferred into 30-mm-diameter glass Petri dishes, and approximately 50 surface-sterilized eggs were added. The eggs were incubated in the fungal filtrate for 48 hrs and then transferred to SDW before incubation for 15 days. The number of juveniles that emerged was recorded at 3-day intervals. Three repeat bioassays were conducted. Two controls were used for comparison: one in SDW, and the other in uninoculated BCB. Three replicate Petri dishes were used for each fungal isolate and the control treatments, and the experiments were repeated three times. In the first bioassay,

the control treatment was SDW only. All bioassays were conducted on laboratory benches in a completely randomized design.

Data analysis

Separate counts for mobile and immobilized males, females and juveniles of *R. similis* were made for all bioassays. From the counts of inactive/active and dead/living nematodes, percentage paralysis or mortality were calculated, respectively, and the means for each treatment calculated. Prior to analysis, all nematode data were tested for normality and homogeneity of variances using Shapiro-Wilkinson, Levene Welch and Kolmogorov-Smirnov tests. Normal probability plots, box plots and stem leaf plots were additionally used to confirm normality of data. If not normally distributed, various transformations were tested until the most suitable transformation was obtained. Percentage paralysis and mortality were arcsine sqrt-transformed to normalize data and homogenize variances.

For all bioassays using motile stages of *R. similis*, one-way ANOVA was conducted to investigate differences among treatment means. If different, means were separated using Tukey's studentized range test (SAS Institute, 1989). Two-way ANOVA was used to demonstrate interaction effects between treatments and exposure time, treatments and sex, and sex and exposure time. When the sex of the nematodes was included as a factor, juveniles were omitted from the analysis, as they represented less than 5% of the total nematodes.

Percentage egg-hatching was calculated as the number of hatched juveniles, divided by number of eggs originally placed in a Petri dish, and multiplied by 100. Before analysis, percentage egg-hatching was arcsine sqrt-transformed to normalize data. One-way ANOVA was conducted to demonstrate variability among treatment means. If different, means were separated using Tukey's studentized range test. From the bioassays with different culture filtrate concentrations, the LC_{50} values for isolates *V5W2* and *Eny1.31i* were calculated using the Calcusyn 1.1 (Biosoft, Cambridge, UK) software package (Chou & Hayball, 1996).

Results

In vitro screening of Fusarium oxysporum *isolates*

Culture filtrates of the nine *F. oxysporum* fungal isolates (Table 1) paralysed all stages of *R. similis in vitro*. Significant differences (P<0.0001) were observed between the control treatments and some of the endophyte treatments in each of the three bioassays (Table 3). Isolate *Eny7.11o* paralyzed nematodes significantly in all but one (bioassay 2, after 24 hrs exposure) of the treatments, while isolates *Eny1.3li* and *1113W3* reduced activity after 6 of the 9 exposure time periods in the three bioassays. The percentage of paralyzed nematodes differed between the three independent bioassays (P=0.0006), indicating that the fungal isolates' effects varied between repeat experiments.

The effect of fungal culture filtrates on nematode paralysis was influenced by the length of exposure of the nematodes to the culture filtrates (Table 3). Bioassays differed significantly (P<0.0001) in the percentage of nematodes paralyzed at each exposure time. The percentage of paralysed nematodes increased with an increase in the duration of exposure to the culture filtrate (Table 3). At 3 hrs exposure time, the percentage nematode paralysis by filtrates of some of the fungal isolates tested (e.g. *Eny7.11o*, *III3W3 V1W7* and *V2W2*) differed from the controls in Bioassays 1 and 3. After 6 hrs of exposure, most of the fungal isolates differed from the control treatments and often from each other, while few differences were observed among isolates after 24 hrs of exposure. Thus, fungal isolates could be best differentiated from each other after 6 hrs of exposure to culture filtrates.

Nematicidal effects on *R. similis* were observed for all the undiluted culture filtrates after exposure for 24 hrs (Table 4). Dead nematodes appeared straight (uncurved) with elongated bodies, while live nematodes retained the normal sigmoid shape and exhibited some slight movement. In all three bioassays conducted with the nine *F. oxysporum* isolates, the percentage of dead nematodes was significantly higher for all fungal treatments compared to the controls (P<0.0001, Table 4). Nematode mortality rates in culture filtrates ranged from 76.4 to 100.0%, with a strong interaction effect observed between treatments and bioassays (P<0.0001; Table 4).

Culture filtrates from the nine *F. oxysporum* isolates exhibited selectivity for *R. similis* sexes. *Radopholus similis* males were more sensitive to culture filtrates than females after 3 and 6 hrs of exposure (Table 5). No differences, however, were observed between males and female *R. similis* response to culture filtrates after 24 hrs of exposure.

In vitro screening of Fusarium *spp. from banana plants*

The percentage nematodes paralyzed in the culture filtrates of different *Fusarium* spp. obtained from banana corms in Masaka differed among the three bioassays ($P<0.0001$), with a strong interaction between treatments and bioassays ($P<0.0001$). This means that the percentage paralyzed nematodes in culture filtrates of the same isolate differed across repeat bioassays. In bioassay 1, the percentage of nematodes paralyzed in the control treatments was significantly lower than that in culture filtrates of isolates *M86* and *M127*. Culture filtrates of these two isolates also resulted in significantly higher nematode paralysis compared to all other isolates with an exception of isolates *M*66 (Table 6). In bioassay 2, all fungal isolates except, isolates *M72* and *M120* caused significantly higher levels of nematode paralysis compared to the controls, while in bioassay 3, culture filtrates of all tested isolates with an exception of isolates *M119*, *M120 and M127* paralyzed significantly more nematodes in comparison to the controls (Table 6). The percentage of paralyzed nematodes did not differ significantly ($P=0.05$) between the two controls in all three bioassays, although the percentage of nematodes paralyzed in the pH-adjusted control (Control 2) was numerically lower than in the control with unadjusted pH in 2 out of the 3 bioassays (Table 6).

The culture filtrates of the different *Fusarium* isolates from corms caused variable levels of nematode mortality after 6 hrs of exposure (Table 6). The interaction between treatments and bioassays was significant ($P<0.0001$). In bioassay 1, isolates *M86* and *M66* resulted in a significantly higher nematode mortality compared to the controls, while in bioassay 2, isolate *M66* caused significantly more death to nematodes than the control treatments. In bioassay, isolates *M121* and *M127* caused significantly higher mortality than the controls. The control treatment with the adjusted pH (control 2) had a lower nematode mortality than the control with unadjusted pH in bioassays 1 and 2, although the difference was not significant ($P>0.05$). In all three bioassays, nematodes recovered from the immobile state after rinsing

with sterile distilled water (Table 6). Nematode recovery rates ranged from 10.8% to 61.9%, 2.7% to 71.3% and 20.2% to 56.6% in bioassay 1, 2 and 3, respectively.

The percentage nematodes immobilized in culture filtrates of *Fusarium* isolates obtained from banana roots in Masaka differed across repeat bioassays (P<0.0001). A strong interaction effect between treatments and bioassays (P<0.0001) was observed. In all the six bioassays conducted with these isolates, the percentage nematodes immobilized by some *Fusarium* culture filtrates were significantly higher than the control treatments (Table 7). In bioassay 1, for example, culture filtrates from all tested isolates with an exception of isolates *M321*, *M360* and *M388* caused significantly higher nematode immobilization than the controls. The percentage of immobilized nematodes did not significantly differ between the two control treatments. Culture filtrates from several isolates (e.g. *M*387, *M*343, *M*291, *M*325, *M*419 and *M*365) resulted in nematode immobilization rates similar to that of the reference isolate *V5W2* (Table 7) in some of the bioassays in which isolate *V5W2* was tested.

Percentage nematode mortality in culture filtrates of some *Fusarium* isolates obtained from banana roots in Masaka was significantly higher than the nematode mortalities obtained in the controls (Table 8). The percentage nematodes killed in the fungal culture filtrates also differed across repeat bioassays (P<0.0001) with a strong interaction between treatments and bioassays (P<0.0001). No significant differences were observed between the control treatments in the percentage of nematodes killed in bioassays 1, 3, 4 and 6. In these bioassays, however, the control with adjusted pH (control 2) tended to cause lower nematode mortality than the control with unadjusted pH. Culture filtrates of isolates *M*290, *M*304, *M*291, *M*325, *M*304, *M*329 and *M*379 resulted in nematode mortalities similar to those of the reference isolate, *V5W2*.

Effect of different culture filtrate concentrations on Radopholus similis *motile stages*

The percentage nematodes immobilized by culture filtrates of both isolates *V5W2* and *Eny1.31i* increased with an increase in filtrate concentration (Table 9). Differences in the percentage of immobilized nematodes as the culture filtrate concentration increased from 2.5% to 100% were observed for both isolates. This difference was significant when the concentration of the filtrate was reduced from 100 to 50% for isolate *Eny1.31i* (P=0.0001),

but not for isolate *V5W2* (*P*=0.1682) in bioassay 1. For the repeat bioassay, the reduction was significant for both isolates (*P*<0.0001). No significant differences, however, were observed when the filtrate concentration was reduced from 50 to 25%, or among filtrate concentrations of 2.5, 5% and 10% (Table 9).

Percentage mortality of nematodes differed significantly among the different culture filtrate concentrations for both isolates *V5W2* and *Eny1.31i* (*P*<0.0001), and increased as the filtrate concentration increased (Table 9). Significant differences in the percentage of dead nematodes were evident only between the 50% and 100% filtrate concentrations. Once a dilution rate of 25% or less was obtained, the percentage of nematodes killed in culture filtrates were not significantly reduced any more (Table 9). The LC_{50} values for isolate *V5W2* was 21.9 ± 2.5% and 20.4 ± 6.4% in bioassay 1 and 2, respectively, while the LC_{50} for isolate *Eny1.31i* was 18.5 ± 5.2 and 9.6 ± 1.7% in bioassays 1 and 2, respectively.

Effect of fungal culture filtrates on Radopholus similis *egg hatch*

Hatching of *R. similis* eggs was significantly reduced by fungal culture filtrates and the culture filtrate control when compared to the treatment with sterile distilled water (P<0.0001) (Fig. 1). The number of eggs that hatched increased with the increase in incubation days. In bioassay 1, percentage egg hatch in SDW was >70.3% (Fig. 1A) while in the culture filtrates, egg hatch was <15% after 15 days of incubation. In bioassays 2 (Fig. 1B) and 3 (Fig. 1C), percentage egg hatch in SDW was lower than in bioassay 1 (30.3% and 40.2%, respectively), but still significantly lower than the culture filtrate treatments. Eggs appeared normal in shape in both the control and culture filtrate treatments.

Discussion

In this study, culture filtrates of *Fusarium* endophytes from banana roots and corms resulted in the *in vitro* paralysis and mortality of motile stages of *R. similis*. Endophytic *F. oxysporum* isolates that resulted in consistently good paralysis and mortality of *R. similis* included *V5W2*, *V4W5*, *Eny1.31i* and *Eny7.11o*. Similarly, several *Fusarium* isolates (*M*66, *M*86, *M*127 and *M*387) from roots and corms of banana plants in Masaka caused consistently high paralysis and mortality. Despite the great variation that occurred within treatments and the absence sometimes of significant differences among isolates, results obtained in this study may be considered when selecting candidate isolates for *in planta* studies in the greenhouse and the field. Nitao *et al.* (1999) have previously used in vitro screening using fungal filtrates to provide a simple and rapid method of identifying potential biological control agents. In the current study, culture filtrates from various *Fusarium* spp. isolates have demonstrated the potential for using the isolates as possible biological control agents of *R.similis*.

All isolates of *F. oxysporum* studied by Niere (2001) reduced the mobility of nematodes in the current study. This demonstrated the ability of the isolates to retain *in vitro* nematicidal activity even after long periods of storage. Culture filtrates of *Fusarium* isolates from roots and corms of plants from Masaka demonstrated the capacity to immobilize motile *R. similis* stages after 6 hrs of exposure. Similarly, isolates *M*66 (*Fusarium chlamydosporium* Wollenw. & Reinking), *M*86 (*F. solani*) and *M*127 (an unidentified *Fusarium* sp.) from Masaka district exhibited good *in vitro* inhibition of *R. similis* motile stages. These species should, therefore, be considered as potential biological control agents against *R. similis* in future studies.

Different levels of nematode immobilization and mortality were obtained for the same *Fusarium* isolates despite efforts to maintain the same experimental conditions for repeat bioassays. Several factors may have contributed to these inconsistencies, such as the type of culture medium (Cayrol *et al*., 1989), the age of the fungal culture filtrates (Mankau, 1969), the culture filtrate concentration and the nematode incubation period (Mani and Sethi, 1984; Meyer *et al*., 2000; Ali *et al*., 2002). Cayrol *et al*. (1989) reported that the culture medium and cultural conditions, such as aeration and pH, affected production of inhibitory compounds by some microorganisms. The culture medium used for the current study was made from banana

corms, and the age of the banana suckers and the length of boiling might have influenced results. However, the pH of fungal filtrates on nematode inhibition in this study seems negligible, as no significant differences were found between the control with unadjusted pH and the one with pH adjusted to the average pH of the culture filtrates except in a few bioassays. The metabolites produced in the culture filtrates by the various *Fusarium* isolates were, therefore, primarily responsible for immobilizing and killing *R. similis* motile stages. Cayrol *et al*. (1989) also reported that, although toxin production by fungi may be influenced by pH, the toxins act in a wide range of pH values and are independent of the culture filtrate pH.

Little information is available on the nematode-inhibiting components of fungal culture filtrates and on specific phytotoxins produced by the fungi. The fact that the culture filtrates in this study were free of spores and mycelia, however, strongly implies that the compounds in the filtrates were toxic to *R. similis*. It has been demonstrated before that *Fusarium* spp. can produce toxic secondary metabolites such as zearalanone, fumonisins, tricothecenes and fusaric acid (Vey *et al*., 2001). Ciancio (1995) further demonstrated that *Fusarium* mycotoxins, such as T2-toxin, monilliformin, verrucarin A, cytochalasin B and Enniatin B, caused significant mortality of *Meloidogyne javanica* (Treub) Chitwoodi juveniles in the laboratory. Similarly, culture filtrates of non-endophytic strains of *Penicilliun oxalicum* Currie & Thom, *Penicillium anatolicum* Stolk and *Aspergillus niger* Van Tieghem caused mortality to *R. similis* after 48 hrs immersion (Molina and Davide, 1986).

Fungal culture filtrates demonstrated an increase in *in vitro* activity as the length of exposure time increased. A mortality rate of up to 100.0% was obtained when nematodes were exposed to *F. oxysporum* culture filtrates for 24 hrs. When exposed to culture filtrates for shorter periods, nematodes underwent partial paralysis that was reversible after rinsing off the filtrates with SDW. This was demonstrated in the bioassays conducted with the 26 *Fusarium* spp., whereby a certain percentage of nematodes exposed to culture filtrates for 6 hrs recovered from the immobile state after the culture filtrates were rinsed out, indicating that the toxin activity was neurotropic. The toxic effects of the culture filtrates were not reversible after nematodes had been exposed to it for 24 hrs, indicating that these nematodes could be considered dead. Reversibility of toxic effects has also been demonstrated with culture filtrates of *P. lilacinus* against different nematode species (Cayrol *et al*., 1989) when exposed

for less than 48 hours. The fact that male *R. similis* was immobilized and killed more rapidly than the females is of little significance, as male nematodes are not infective on banana (Speijer and De Waele, 1997).

The use of diluted culture filtrates was intended to mimic the natural conditions encountered by *R. similis* in the banana plants. As expected, the percentage immobilized nematodes decreased with a reduction in culture filtrate concentration. This is in agreement with findings by others regarding the effects of fungal filtrate concentrations on the immobilization of nematodes (Mankau, 1969; Mani and Sethi, 1984; Zareen *et al*., 2001). The low rates of nematode immobilization at low culture filtrate concentration might be due to the dilution of the toxic compounds. If the fungal isolates produce the toxins inside the plant, that concentration would probably be lower than in the bioassays conducted with undiluted culture filtrates. Thus, the levels of nematode control in the plants due to toxins can be expected to be lower than in the laboratory. Production of toxins in the plant may, however, act as an additional form of armory that would help the plant guard itself from invasion by the nematode. Production of toxic metabolites in the plant by *Fusarium* endophytes may inhibit not only nematode mobility, but also host searching and the infection processes (Sikora *et al*., 2003). While the production of toxic metabolites may be the main mechanism of action in the laboratory, the value of nematicidal compounds in the plant, and the mechanism whereby endophytes protect banana plants against *R. similis*, should be further investigated. It is important to recognize that isolates not showing *in vitro* activity against nematodes may be able to induce systemic resistance in the plant.

Culture filtrates of endophytic *F. oxysporum* isolates substantially inhibited egg hatch of *R. similis*. Inhibition of egg hatch of nematodes by fungal culture filtrates has been demonstrated for root-knot nematodes before. Ansari *et al*. (2002) demonstrated inihibitory effects of *P. striata*, *T. harzianum* and *T. viride* culture filtrates on egg hatch of *M. javanica* after 7 and 14 days of incubation. Reduction of egg hatch of *M. incognita* by microbial extracts of *P. oxalicum*, *P. anatolicum* and *A. niger* was also reported by Molina and Davide (1986). In contrast to these studies, some fungal filtrates have been shown to stimulate nematode egg hatch (Meyer *et al*., 2004). As *R. similis* eggs are reported to hatch within 4 to 10 days (Gowen *et al*., 2005), the eggs that did not hatch after 15 days of incubation in the current study were considered dead. The mechanism whereby fungal culture filtrates inhibited egg

hatch of *R. similis* was not investigated in this study, but may be due to toxic effects on eggshell permeability that inhibits or suppresses larval development (Bone *et al*., 1987; Samaliev *et al*., 2000). A low molecular weight compound, flavipin, was isolated from *C. globosum* which inhibited both egg hatch and juvenile mobility of *M. incognita* and *Heterodera glycines* Ichinohe (Nitao *et al*., 2002).

From the current study it was evident that *Fusarium* endophytes may be a rich source of bionematicides that can be harnessed for the management of *R. similis* and possibly other nematode species. Purification and identification of such compounds may also be useful in identifying genes responsible for their production, and the potential generation of transgenic banana plants expressing such genes. Using this technology, Atkinson *et al*. (2004) have produced transgenic Grande Naine banana plants, modified with a proteinase inhibitor gene, that reduced damage caused by *R. similis* with 70%.

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Table 1: The identity and origin of nine endophytic *Fusarium oxysporum* isolates obtained from banana (*Musa* spp.) plants in Uganda that were screened against the motile stages and eggs of *Radopholus similis* in the laboratory.

Isolate	Species	Cultivar	Plant part	*In-vitro* activity*
Eny1.31i	*F. oxysporum*	Enyeru (AAA-EA)	Corm	nt
Eny7.11o	*F. oxysporum*	Enyeru (AAA-EA)	Corm	nt
III3W3	*F. oxysporum*	Unknown AAA-EA	Root	++
III4W1	*F. oxysporum*	Unknown AAA-EA	Root	++
V5W2	*F. oxysporum*	Unknown AAA-EA	Root	++
V4W5	*F. oxysporum*	Unknown AAA-EA	Root	+
V2W2	*F. oxysporum*	Unknown AAA-EA	Root	(+)
V1W7	*F. oxysporum*	Unknown AAA-EA	Root	+
Emb2.4o	*F. oxysporum*	Embiire (AAA-EA)	Corm	nt

**In vitro* inactivation of *R. similis* with *F. oxysporum* culture filtrates, as cited in Niere (2001), with ++ constantly good inactivation; + good inactivation; (+) moderate/varying inactivation; nt, not tested.

Table 2: The identity and origin of 26 endophytic Fusarium isolates obtained from banana (Musa spp.) plants in Masaka District, Uganda that were screened against the motile stages of *Radopholus similis* in the laboratory.

Isolate	Species	Cultivar	Plant No	Stage of plant	Plant part
M-66	*F. chlamydosporium*	Kibuzi (AAA-EA)	2	Sucker	Outer corm
M-72	*F. subglutinans*	Kibuzi (AAA-EA)	2	Sucker	Outer corm
M-86	*F. solani*	Kibuzi (AAA-EA)	3	Sucker	Outer corm
M-87	*F. oxysporum*	Kibuzi (AAA-EA)	3	Sucker	Outer corm
M-110	*F. anthophilum*	Kibuzi (AAA-EA)	3	Flowering	Outer corm
M-119	*F. solani*	Kibuzi (AAA-EA)	1	Sucker	Outer corm
M-120	*Fusarium* sp.	Kibuzi (AAA-EA)	1	Sucker	Outer corm
M-121	*Fusarium* sp.	Kibuzi (AAA-EA)	1	Sucker	Outer corm
M-122	*Fusarium* sp.	Kibuzi (AAA-EA)	1	Sucker	Outer corm
M-127	*Fusarium* sp.	Kibuzi (AAA-EA)	5	Sucker	Outer corm
M-290	*F. solani*	Kibuzi (AAA-EA)	1	Sucker	Root
M-291	*F. oxysporum*	Kibuzi (AAA-EA)	1	Sucker	Root
M-304	*F. oxysporum*	Kibuzi (AAA-EA)	2	Sucker	Root
M-321	*F. anthophilum*	Kibuzi (AAA-EA)	3	Sucker	Root
M-325	*F. oxysporum*	Kibuzi (AAA-EA)	3	Sucker	Root
M-337	*F. oxysporum*	Kibuzi (AAA-EA)	4	Sucker	Root
M-343	*Fusarium* sp.	Kibuzi (AAA-EA)	4	Sucker	Root
M-360	*Fusarium* sp.	Kibuzi (AAA-EA)	5	Sucker	Root
M-365	*F. oxysporum*	Kibuzi (AAA-EA)	5	Sucker	Root
M-379	*F. oxysporum*	Kibuzi (AAA-EA)	1	Flowering	Root
M-385	*F. oxysporum*	Kibuzi (AAA-EA)	3	Flowering	Root
M-387	*F. oxysporum*	Kibuzi (AAA-EA)	2	Flowering	Root
M-388	*F. anthophilum*	Kibuzi (AAA-EA)	2	Flowering	Root
M-419	*F. oxysporum*	Kibuzi (AAA-EA)	4	Flowering	Root
M-428	*Fusarium* sp.	Kibuzi (AAA-EA)	4	Flowering	Root
M-459	*F. oxysporum*	Kibuzi (AAA-EA)	5	Flowering	Root

Table 3: Percentage paralysis (mean ± S.E.) of *Radopholus similis* mixed stages (males, females and juveniles) in culture filtrates of nine endophytic *Fusarium oxysporum* isolates after exposure times of 3, 6 and 24 hrs.

Treatment	Exposure time in culture filtrate								
	Bioassay 1			Bioassay 2			Bioassay 3		
	3 hrs	6 hrs	24 hrs	3 hrs	6 hrs	24 hrs	3 hrs	6 hrs	24 hrs
Control 1	33.8 ± 4.9^{bc}	62.4 ± 0.5^{cd}	93.0 ± 0.7^{b}	34.0 ± 0.9^{bc}	38.0 ± 0.7^{b}	98.4 ± 0.9^{ab}	13.8 ± 3.1^{cd}	18.1 ± 3.5^{d}	20.8 ± 3.9^{c}
Control 2	33.4 ± 2.9^{c}	43.3 ± 1.1^{e}	21.1 ± 2.9^{c}	27.6 ± 3.2^{bc}	30.8 ± 3.3^{b}	29.9 ± 1.1^{d}	18.7 ± 4.9^{d}	25.9 ± 3.9^{d}	32.5 ± 4.9^{c}
Emb2.4o	29.8 ± 3.2^{c}	63.6 ± 7.1^{c}	96.5 ± 1.2^{ab}	40.3 ± 7.2^{bc}	72.3 ± 4.9^{a}	99.3 ± 0.7^{a}	18.7 ± 2.8^{d}	46.5 ± 7.4^{cd}	83.6 ± 1.6^{ab}
Eny1.31i	37.1 ± 6.1^{bc}	80.8 ± 4.4^{ab}	100.0 ± 0.0^{a}	42.6 ± 2.5^{bc}	83.0 ± 3.7^{a}	100.0 ± 0.0^{a}	63.5 ± 3.7^{a}	77.7 ± 1.7^{a}	88.2 ± 4.4^{ab}
Eny7.11o	68.4 ± 4.9^{a}	82.9 ± 2.9^{ab}	100.0 ± 0.0^{a}	71.6 ± 9.1^{a}	71.3 ± 7.1^{a}	100.0 ± 0.0^{a}	50.3 ± 1.1^{ab}	66.8 ± 5.0^{abc}	91.1 ± 2.7^{ab}
1113W3	67.4 ± 2.7^{a}	86.1 ± 1.5^{a}	99.1 ± 0.5^{ab}	39.4 ± 4.4^{bc}	36.3 ± 4.9^{b}	73.5 ± 4.7^{c}	43.1 ± 8.8^{ab}	72.8 ± 5.9^{ab}	91.8 ± 2.6^{ab}
1114W1	55.3 ± 4.7^{ab}	84.1 ± 1.7^{a}	100.0 ± 0.0^{a}	53.1 ± 6.9^{ab}	72.0 ± 3.6^{a}	100.0 ± 0.0^{a}	33.2 ± 3.6b^{cd}	51.1 ± 4.7^{c}	90.0 ± 2.2^{ab}
V1W7	66.5 ± 4.6^{a}	60.6 ± 2.3^{cd}	94.6 ± 2.7^{ab}	50.5 ± 6.5^{ab}	39.8 ± 5 .7^{b}	90.1 ± 2.0^{b}	47.1 ± 1.9^{ab}	63.9 ± 0.7^{abc}	93.6 ± 4.4^{a}
V2W2	28.6 ± 5.0^{c}	48.5 ± 1.8^{de}	98.1 ± 0.5^{ab}	72.5 ± 5.2^{a}	39.5 ± 7.3^{b}	100.0 ± 0.0^{a}	16.2 ± 1.9^{d}	58.8 ± 2.3^{abc}	100.0 ± 0.0^{a}
V4W5	31.8 ± 3.2^{c}	90.6 ± 0.2^{a}	97.9 ± 0.4^{ab}	49.1 ± 4.0^{ab}	72.6 ± 4.4^{a}	100.0 ± 0.0^{a}	41.2 ± 6.4^{bc}	52.8 ± 2.5^{bc}	72.4 ± 8.8^{b}
V5W2	27.2 ± 3.6^{c}	68.8 ± 2.0^{bc}	99.2 ± 0.8^{ab}	17.7 ± 3.5^{c}	76.6 ± 2.8^{a}	100.0 ± 0.0^{a}	14.5 ± 1.4^{d}	54.8 ± 1.4^{bc}	88.4 ± 2.3^{ab}
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

In control 1, the pH was unadjusted (5.89, 6.31 and 6.46), and in control 2 to the average pH of the culture filtrates (9.16, 7.37 and 7.68) in bioassay 1, 2 and 3, respectively. In each column, the means followed by the same letter (superscript) are not significantly different at $P>0.05$ according to Tukey's studentized range test. n=3.

Table 4: Percentage mortality (mean ± S.E.) of *Radopholus similis* mixed stages (males, females and juveniles) in culture filtrates of nine endophytic *Fusarium oxysporum* isolates after an exposure time of 24 hrs, n=3.

Treatment	Bioassay		
	1	2	3
Control 1	79.1 ± 3.6 [b]	41.2 ± 2.7 [b]	47.7 ± 12.3 [b]
Control 2	14.8 ± 1.2 [c]	14.2 ± 2.1 [c]	36.9 ± 4.8 [b]
Emb2.4o	97.7 ± 0.6 [a]	97.4 ± 1.5 [a]	96.1 ± 1.1 [a]
Eny1.31i	99.0 ± 0.5 [a]	99.1 ± 0.9 [a]	77.6 ± 5.1 [a]
Eny7.11o	99.5 ± 0.4 [a]	100.0 ± 0.0 [a]	84.2 ± 3.0 [a]
1113W3	100.0 ± 0.0 [a]	76.4 ± 1.1 [a]	88.2 ± 2.6 [a]
1114W1	98.6 ± 0.7 [a]	98.5 ± 0.8 [a]	84.8 ± 4.8 [a]
V1W7	94.2 ± 0.5 [a]	95.3 ± 1.3 [a]	97.5 ± 0.4 [a]
V2W2	97.9 ± 1.3 [a]	96.1 ± 1.6 [a]	100.0 ± 0.0 [a]
V4W5	99.6 ± 0.4 [a]	100.0 ± 0.0 [a]	89.9 ± 2.5 [a]
V5W2	99.2 ± 0.8 [a]	100.0 ± 0.0 [a]	99.0 ± 1.0 [a]
P-value	<0.0001	<0.0001	<0.0001

In control 1 the pH was unadjusted (5.89, 6.31 and 6.46), and in control 2 the pH was adjusted to the average pH of the culture filtrates (9.16, 7.37 and 7.68) in bioassay 1, 2 and 3, respectively. Mortality rates were determined after rinsing out nematodes from the culture filtrates and incubating them in sterile distilled water for 24 hrs. In each column, the means followed by the same letter (superscript) are not significantly different at $P>0.05$ according to Tukey's studentized range test.

Table 5: Effect of exposure time to culture filtrates of endophytic *Fusarium oxysporum* isolates on *Radopholus similis* males and females, indicated by the percentage paralysis.

	R. similis paralysis (%)								
	Bioassay 1			Bioassay 2			Bioassay 3		
Exposure time (hrs)	F	M	*P*-value	F	M	*P*-value	F	M	*P*-value
3	44.5[a]	64.3[b]	<0.0001	36.0[a]	58.8[b]	<0.0001	27.6[a]	51.7[b]	<0.0001
6	34.7[a]	81.2[b]	<0.0001	50.1[a]	63.8[b]	<0.0001	47.9[a]	75.7[b]	<0.0001
24	82.3[a]	86.9[a]	0.0011	88.5[a]	93.2[a]	0.0700	76.6[a]	85.4[a]	<0.0001

F= female *R. similis*, M=male *R. similis*. In each row per bioassay, means followed by the same letter (superscript) are not significantly different at $P>0.05$ according to Tukey's studentized range test. n=3.

Table 6: Percentage paralysis and mortality of mixed stages of *Radopholus similis* (males, females and juveniles) following treatment with culture filtrates of nine endophytic Fusarium isolates obtained from corms of banana plants in Masaka district, for 6 hrs.

	Bioassay 1		Bioassay 2		Bioassay 3	
Treatment	% Paralysis	% Mortality	% Paralysis	% Mortality	% Paralysis	% Mortality
Control 1	39.5 ± 3.1^{bc}	40.4 ± 2.2^{b}	19.8 ± 3.3^{d}	30.9 ± 3.8^{bc}	38.2 ± 4.2^{gf}	40.4 ± 3.2^{ef}
Control 2	31.6 ± 3.4^{c}	17.6 ± 3.7^{b}	15.1 ± 2.7^{d}	14.2 ± 2.5^{d}	33.7 ± 1.6^{g}	53.1 ± 5.3^{cde}
M-66	61.9 ± 4.2^{ab}	43.7 ± 2.8^{ab}	75.3 ± 6.9^{a}	52.1 ± 5.2^{a}	67.4 ± 3.8^{cde}	29.2 ± 2.8^{f}
M-72	33.3 ± 1.5^{c}	18.3 ± 1.0^{b}	34.9 ± 5.6^{bcd}	13.9 ± 1.2^{d}	73.2 ± 6.4^{bcd}	41.8 ± 6.2^{def}
M-86	87.7 ± 5.2^{a}	33.4 ± 9.9^{ab}	70.5 ± 1.9^{a}	34.5 ± 2.8^{bc}	90.2 ± 2.9^{a}	61.1 ± 5.7^{bcd}
M-87	36.7 ± 2.4^{bc}	25.1 ± 3.7^{ab}	38.1 ± 4.6^{bc}	17.9 ± 3.2^{cd}	89.8 ± 0.9^{ab}	57.1 ± 1.2^{cde}
M-110	38.9 ± 3.0^{bc}	31.3 ± 6.6^{ab}	41.6 ± 4.5^{b}	18.2 ± 5.4^{cd}	58.8 ± 4.5^{de}	46.9 ± 1.6^{def}
M-119	45.5 ± 2.6^{bc}	38.5 ± 12.3^{ab}	39.3 ± 2.0^{bc}	38.2 ± 4.3^{ab}	53.8 ± 1.1^{ef}	72.4 ± 3.8^{bc}
M-120	45.3 ± 6.9^{bc}	26.5 ± 7.3^{ab}	34.9 ± 4.0^{bcd}	23.1 ± 1.5^{bcd}	37.9 ± 2.1^{gf}	46.7 ± 4.8^{def}
M-121	47.8 ± 6.9^{bc}	38.6 ± 5.4^{ab}	67.3 ± 2.6^{a}	19.3 ± 2.8^{cd}	80.3 ± 1.6^{abc}	80.4 ± 3.6^{ab}
M-127	83.3 ± 2.3^{a}	56.4 ± 12.7^{a}	69.3 ± 3.8^{a}	32.2 ± 2.0^{bc}	53.2 ± 5.1^{ef}	95.7 ± 0.9^{a}
V5W2	nt	nt	nt	nt	87.3 ± 1.4^{ab}	69.5 ± 5.2^{bc}
P-value	<0.0001	0.0159	<0.0001	<0.0001	<0.0001	<0.0001

In control 1 the pH was unadjusted (5.78, 5.91 and 5.71) and in control 2, the pH was adjusted to the average pH of the culture filtrates (7.84, 7.49 and 7.65) in bioassay 1, 2 and 3 respectively. Mortality rates were determined after rinsing out nematodes from the culture filtrates and incubating them in sterile distilled water for 24 hrs. In each column, the means followed by the same letter (superscript) are not significantly different at P>0.05 according to Tukey's studentized range test. nt, not tested. n=4.

Table 7: Percentage paralysis of mixed stages of *Radopholus similis* (males, females and juveniles) following treatment with culture filtrates of seventeen endophytic Fusarium spp. isolates obtained from roots sin Masaka for 6 hrs.

	Bioassay number*					
Treatment	1	2	3	4	5	6
Control 1	22.9 ± 5.1^{bc}	20.4 ± 1.2^{c}	55.8 ± 3.8^{def}	21.2 ± 3.5^{g}	44.1 ± 0.9^{fg}	55.1 ± 3.0^{c}
Control 2	19.4 ± 2.9^{c}	25.1 ± 3.7^{c}	34.4 ± 2.1^{f}	31.6 ± 0.6^{fg}	37.2 ± 4.4^{g}	25.6 ± 2.3^{d}
M122	89.9 ± 3.9^{a}	24.2 ± 1.6^{c}	36.1 ± 1.3^{f}	nt	nt	nt
M290	80.8 ± 2.0^{a}	61.2 ± 4.0^{ab}	61.5 ± 3.4^{de}	nt	nt	nt
M304	97.7 ± 1.1^{a}	35.8 ± 2.0^{c}	68.1 ± 2.7^{cde}	64.8 ± 2.3^{abc}	81.9 ± 0.6^{bcd}	62.4 ± 3.6b^{c}
M321	39.3 ± 5.6^{b}	66.6 ± 4.2^{a}	53.1 ± 9.8^{def}	nt	nt	nt
M343	91.9 ± 3.5^{a}	59.8 ± 7.4^{ab}	73.8 ± 1.7^{bcd}	nt	nt	nt
M360	33.1 ± 2.4^{b}	41.5 ± 8.6^{bc}	93.1 ± 0.8^{ab}	nt	nt	nt
M379	92.3 ± 4.3^{a}	66.7 ± 4.6^{a}	50.5 ± 1.2^{ef}	32.5 ± 3.4^{fg}	67.8 ± 5.6^{e}	50.3 ± 2.1^{c}
M387	87.2 ± 4.1^{a}	65.9 ± 2.1^{a}	89.7 ± 1.7^{abc}	70.4 ± 3.1^{ab}	77.3 ± 2.1^{cde}	90.7 ± 3.0^{a}
M388	22.8 ± 4.6^{bc}	57.1 ± 3.7^{ab}	86.3 ± 2.0^{abc}	nt	nt	nt
M428	91.4 ± 2.8^{a}	34.3 ± 2.8^{c}	73.7 ± 7.2^{bcd}	nt	nt	nt
M459	94.5 ± 1.3^{a}	60.5 ± 1.9^{ab}	70.7 ± 3.0^{cde}	35.1 ± 6.2^{efg}	76.4 ± 2.4^{de}	52.5 ± 5.0^{c}
M291	nt	nt	nt	53.8 ± 2.7^{bcd}	99.4 ± 0.3^{a}	53.7 ± 3.9^{c}
M325	nt	nt	nt	28.5 ± 6.2^{fg}	90.9 ± 1.5^{abc}	57.3 ± 2.9^{c}
M329	nt	nt	nt	73.6 ± 1.3^{a}	51.1 ± 3.8^{f}	66.1 ± 4.8^{bc}
M365	nt	nt	nt	29.2 ± 1.0^{fg}	99.0 ± 0.7^{a}	79.8 ± 3.6^{ab}
M385	nt	nt	nt	30.9 ± 4.1^{fg}	91.3 ± 1.2^{ab}	50.7 ± 4.8^{c}
M419	nt	nt	nt	38.5 ± 2.9^{def}	87.7 ± 4.0^{abcd}	77.7 ± 7.2^{ab}
V5W2	nt	nt	97.3 ± 1.4^{a}	50.5 ± 1.8^{cde}	92.9 ± 1.1^{ab}	64.1 ± 1.2^{bc}
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

* Bioassays 1 – 6 are repeat bioassays. In control 1 the pH was unadjusted (5.83, 5.62, 6.54, 6.02, 6.01 and 5.71) and in control 2, the pH was adjusted to the average pH of the culture filtrates (7.71, 8.58, 7.06, 8.52, 7.86 and 7.65) in bioassay 1, 2, 3, 4, 5 and 6, respectively. In columns, means followed by the same letter are not significantly different at $P>0.05$ according to Tukey's Studentized Range Test. nt, not tested. n=4.

Table 8: Percentage mortality of mixed stages of *Radopholus similis* (males, females and juveniles) following treatment with culture filtrates of seventeen endophytic Fusarium spp. isolates obtained from roots sin Masaka for 6 hrs.

	Bioassay number*					
Treatment	1	2	3	4	5	6
Control 1	46.1 ± 5.1^{b}	80.8 ± 3.5^{a}	15.3 ± 3.3^{c}	26.9 ± 3.6^{b}	18.7 ± 2.4^{f}	80.6 ± 2.8^{a}
Control 2	41.5 ± 5.6^{b}	9.2 ± 2.4^{h}	19.8 ± 3.3^{c}	25.6 ± 2.5^{b}	40.6 ± 1.4^{de}	74.8 ± 2.1^{a}
M122	81.8 ± 1.2^{a}	8.9 ± 1.2^{h}	24.2 ± 2.3^{c}	nt	nt	nt
M290	88.8 ± 3.4^{a}	33.1 ± 4.4^{defg}	80.8 ± 14.6^{ab}	nt	nt	nt
M304	46.5 ± 4.6^{b}	76.1 ± 1.4^{ab}	59.5 ± 2.1^{b}	87.6 ± 1.9^{a}	50.7 ± 1.4^{a}	12.9 ± 2.4^{cd}
M321	38.9 ± 2.7^{b}	42.9 ± 1.2^{de}	30.1 ± 7.1^{c}	nt	nt	nt
M343	30.4 ± 4.3^{b}	17.8 ± 1.7^{gh}	10.8 ± 1.8^{c}	nt	nt	nt
M360	31.6 ± 4.8^{b}	63.2 ± 5.7^{bc}	33.1 ± 1.5^{c}	nt	nt	nt
M379	51.1 ± 3.4^{b}	48.8 ± 4.7^{cd}	70.1 ± 2.3^{b}	27.2 ± 4.9^{b}	48.6 ± 1.9^{a}	31.6 ± 6.4^{bc}
M387	44.3 ± 4.7^{b}	32.3 ± 3.5^{efg}	8.1 ± 1.5^{c}	28.4 ± 1.6^{b}	52.7 ± 3.3^{a}	98.7 ± 0.7^{d}
M388	28.1 ± 5.6^{b}	38.9 ± 2.6^{def}	10.9 ± 2.4^{c}	nt	nt	nt
M428	41.8 ± 6.0^{b}	23.5 ± 3.2^{fgh}	8.7 ± 2.3^{c}	nt	nt	nt
M459	42.1 ± 6.7^{b}	35.4 ± 2.7^{def}	12.2 ± 1.5^{c}	26.4 ± 5.7^{b}	37.9 ± 3.5^{cd}	88.7 ± 2.0^{a}
M291	Nt	nt	nt	85.4 ± 3.4^{a}	47.6 ± 6.9^{a}	5.0 ± 3.1^{d}
M325	Nt	nt	nt	40.2 ± 15.4^{b}	45.5 ± 3.4^{ab}	90.5 ± 0.7^{a}
M329	Nt	nt	nt	74.5 ± 4.6^{a}	53.4 ± 4.9^{a}	24.3 ± 4.6^{bc}
M365	Nt	nt	nt	21.9 ± 4.5^{b}	39.0 ± 4.9^{cb}	88.9 ± 2.6^{a}
M385	Nt	nt	nt	20.1 ± 3.8^{b}	28.9 ± 5.6^{ef}	86.5 ± 2.5^{a}
M419	Nt	nt	nt	27.0 ± 0.8^{b}	42.2 ± 3.2^{cd}	35.5 ± 8.8^{b}
V5W2	Nt	nt	96.2 ± 1.7^{a}	27.7 ± 4.4^{b}	62.1 ± 2.9^{ab}	19.1 ± 1.1^{cd}
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

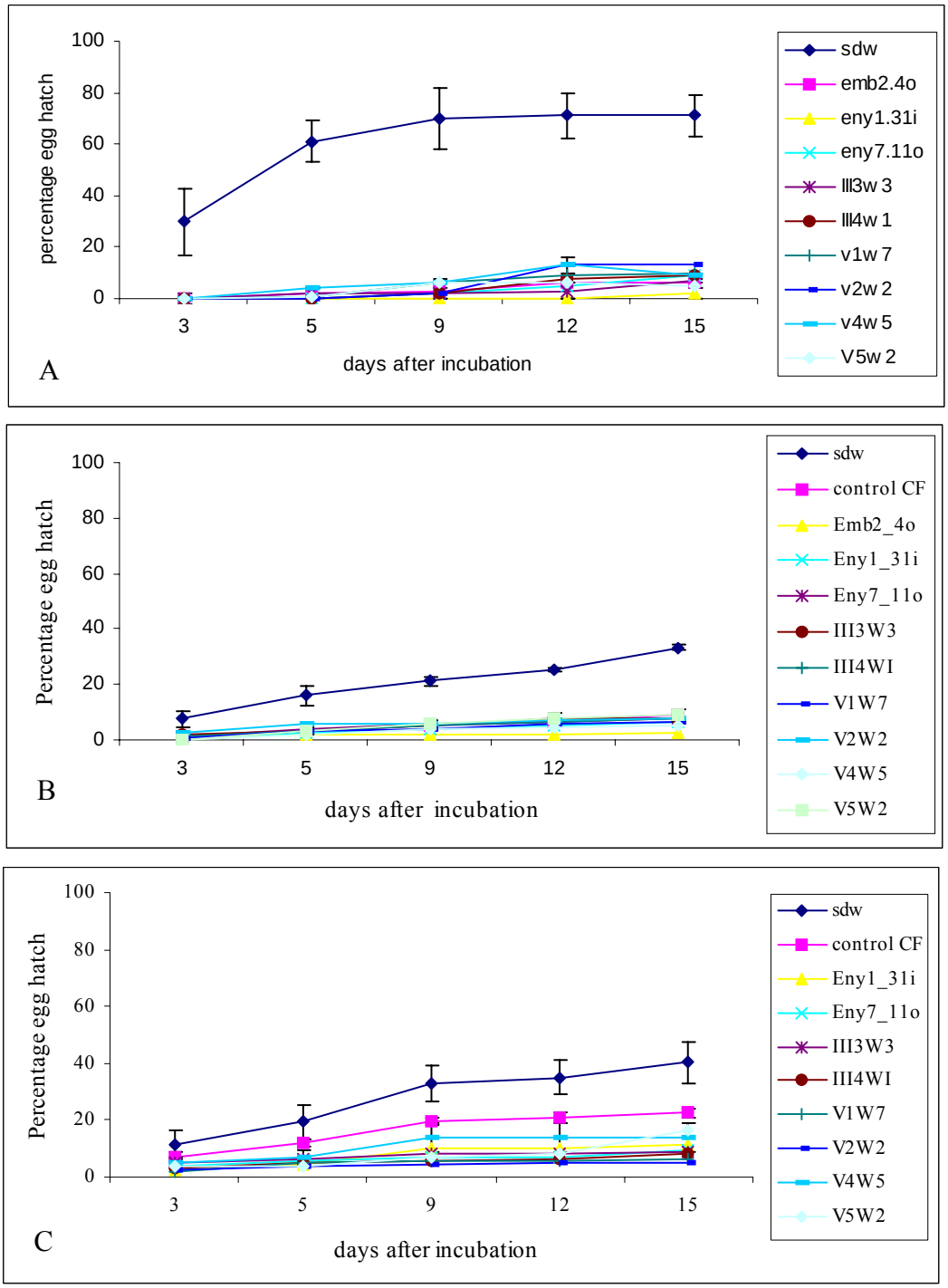
* Bioassays 1 – 6 are repeat bioassays. In control 1, the pH was unadjusted to 5.83, 5.62, 6.54, 6.02, 6.01 and 5.71 and in control 2, the pH was adjusted to the average pH of the culture filtrates (7.71, 8.58, 7.06, 8.52, 7.86 and 7.65) in bioassay 1, 2, 3, 4, 5 and 6, respectively. In each column, means followed by the same letter (superscript) are not significantly different at *P*=0.05 according to Tukey's studentized range test. nt, not tested. n=4.

Table 9: Percentage paralysis and mortality of mixed stages of *Radopholus similis* in different culture filtrate concentrations of two endophytic *Fusarium oxysporum* isolates after 6 hrs exposure.

Isolate		% paralyzed *R. similis*		% mortality of *R. similis*	
V5W2	CF concentration (%)	Bioassay 1	Bioassay 2	Bioassay 1	Bioassay 2
	100	75.1 ± 9.3^{a}	81.4 ± 0.9^{a}	95.7 ± 0.1^{a}	95.8 ±2.1^{a}
	50	63.9 ± 2.5^{a}	67.1 ± 4.2^{b}	68.0 ± 4.4^{b}	52.3 ±3.1^{b}
	25	57.9 ± 4.5^{a}	61.8 ± 2.1^{b}	19.3 ± 4.2^{c}	20.9 ±1.4^{c}
	10	46.8 ± 10.0^{a}	49.4 ± 1.6^{c}	13.1 ± 3.8^{c}	23.9 ±2.5^{c}
	5	52.6 ± 5.4^{a}	41.8 ± 1.1^{c}	22.6 ± 5.6^{c}	21.1 ±2.5^{c}
	2.5	51.5 ± 9.3^{a}	39.4 ± 1.1^{c}	22.9 ± 3.3^{c}	23.2 ±1.4^{c}
	P-value	0.1682	<0.0001	<0.0001	<0.0001
	LC50	-	-	21.9 ± 2.5	20.4 ± 6.4
Eny1.31i	CF concentration (%)	Bioassay 1	Bioassay 2	Bioassay 1	Bioassay 2
	100	89.7 ± 0.7^{a}	95.2 ± 1.4^{a}	96.8 ± 1.1^{a}	98.5 ± 1.4^{a}
	50	53.4 ± 4.5^{b}	80.7 ± 2.8^{b}	75.7 ± 5.7^{b}	77.9 ± 4.2^{b}
	25	57.4 ± 6.3^{b}	70.2 ± 1.5^{b}	27.4 ± 3.4^{c}	33.2 ± 1.2^{c}
	10	43.5 ± 1.5^{b}	55.2 ± 2.3^{c}	17.7 ± 1.1^{c}	37.7 ± 1.9^{c}
	5	42.9 ± 6.4^{b}	50.3 ± 2.1^{c}	13.6 ± 2.7^{c}	34.7 ± 4.9^{c}
	2.5	43.4 ± 6.3^{b}	51.6 ± 3.1^{c}	23.1 ± 3.3^{c}	36.0 ± 3.9^{c}
	P-value	<0.0001	<0.0001	<0.0001	<0.0001
	LC50	-	-	18.4 ± 5.2	9.6 ± 1.7
Control 1	-	49.5 ± 6.1^{b}	46.2 ± 1.1^{c}	19.5 ± 3.6^{c}	36.8 ±1.3^{c}
Control 2	-	40.3 ± 3.1^{b}	20.8 ± 2.9^{c}	22.1 ± 4.9^{c}	64.4 ±3.9^{b}

CF= culture filtrate. In control 1, the pH was unadjusted to 5.29 and 5.22, and in control 2, the pH was adjusted to the average pH of the test fungal filtrates (8.21 and 8.68) in bioassay 1 and 2, respectively. Means followed by the same letter (superscript) for each isolate in each bioassay are not significantly different at P=0.05 according to Tukey's studentized range test. LC50 values were calculated using the Calcusyn 1.1 software for dose-effect relationships. n=4.

Figure 1: Percentage hatch of *Radopholus similis* eggs in culture filtrates of nine endophytic Fusarium oxysporum isolates after 3, 5, 9, 12 and 15 days incubation, sdw = sterile distilled water control, Control CF=Un-inoculated broth. A, B and C are repeat bioassays. Errors bars represent standard errors of the mean, n=3.



Chapter 3

In vivo screening of endophytic *Fusarium oxysporum* isolates for activity against *Radopholus similis* in tissue culture banana plants

Abstract

The effects of nine endophytic *Fusarium oxysporum* isolates on *Radopholus similis* were tested in tissue culture plants of the East African highland banana cultivar Enyeru (*Musa* spp. AAA-EA). A series of nine screen house experiments were conducted to determine the effects of the fungal isolates on *R. similis* reproduction rate, nematode damage and plant growth and to determine *in vivo* consistency in the biological control activity of the fungal isolates. Tissue culture plants at the weaning stage were inoculated with fungal isolates by immersing plant roots in a spore suspension for 2 hrs and acclimatized in a humidity chamber for 1 month. Plants were transferred to loamy soil in 3-liter plastic bags in the screen house. Eight weeks after endophyte inoculation, plants were inoculated with 500 *R. similis* mixed stages and harvested 8 weeks later. Endophyte treatments did not significantly affect *R. similis* female, juvenile or male densities, although in most experiments there was a trend for endophyte-treated plants to have less nematodes than untreated plants. The reduction in nematode populations in endophyte-treated plants ranged between 1.08% to 59.34%. When the damage caused by the nematodes was presented as a percentage of necrotic root tissue, it was not reduced by endophyte treatment. Although endophyte treatment did not significantly enhance plant growth, endophyte-treated plants tended to have higher fresh root and shoot weights, as well as higher dry shoot weights than the untreated plants. Plant height, the number of standing leaves, and the length and width of the youngest leaf were also not significantly affected by endophyte treatment. The performance of introduced fungal isolates was inconsistent and varied between repeat experiments.

Introduction

The use of nematode-infested suckers as planting material is the main avenue for introducing these pests to new banana fields (O'Bannon, 1977; Sarah, 1989). Due to the unrestricted movement of nematode-infested suckers, the spread of *Radopholus similis* Cobb (Thorne) and other nematode species continues. The main control measure of *R. similis* to small-scale farmers, therefore, involves the use of clean planting material such as pared or hot water treated suckers (Speijer *et al*., 1999) and tissue culture (TC) banana plants (Mateille *et al*., 1994). TC plants offer additional benefits to banana production such as rapid multiplication rates, higher yields and uniformity of plants (Robinson, 1996). However, the use of TC plants by most small-scale farmers is limited by availability and cost. A number of studies have also shown that TC plants are more susceptible to nematode and disease attack in the field than plants derived from suckers (De Waele *et al*., 1997; Stanton, 1999; Viaene *et al*., 2003; Blomme *et al*., 2004). Re-infestation of clean planting material in the field further renders the use of healthy planting material only a temporary solution to nematode problems in banana (Speijer *et al*., 1995; Speijer *et al*., 2001).

A nematode management strategy that can complement the benefits of TC banana plants is important to develop. The artificial introduction of beneficial microorganisms, such as endophytic fungi, may protect TC plants against pests and diseases in their early growth stages (Sikora and Schuster, 1999; Sikora *et al*., 2000b). The low levels of fungal inoculum required to treat TC plants with endophytes further makes it a viable option for nematode management in banana.

Biological control of *R. similis* in TC banana plants using endophytic *Fusarium* isolates has been demonstrated in the laboratory and screen house. Pocasangre (2000) reported lower *R. similis* populations in TC banana plants treated with unidentified fungal endophytes that were originally isolated from Cavendish banana plants in Central America, than in non-endophyte treated plants. Similarly, Niere (2001) demonstrated that some endophytic *Fusarium oxysporum* Schlecht.: Fries isolates suppressed *R. similis* densities in banana roots to varying degrees in Uganda. He further reported that the efficiency of nematode suppression depended on the banana cultivar colonized, and that some endophytes stimulated plant growth. With his work, Niere (2001) provided the background and foundation for the current study. The four

experiments he conducted, however, included plants of different ages and inoculated with different nematode densities. Replications per treatment were few, ranging from five to nine, and due to limitations in availability of TC plants, experiments were not repeated. It was, therefore, difficult to derive conclusive evidence as to how effective the fungal isolates were in controlling *R. similis*.

Various endophytic *Fusarium* isolates produce secondary metabolites antagonistic to motile stages and eggs of *R. similis* (Chapter 2), suggesting that these fungal isolates may control *R. similis* in the plant. The objective of the current study was to screen the nine *F. oxysporum* isolates studied by Niere (2001) to (i) determine the effect of endophyte treatment on *R. similis* reproduction and damage in TC banana plants (ii) assess the effect of endophyte infection on the plant's growth and performance and (iii) evaluate consistency in the performance of fungal isolates in repeat screen house experiments.

Materials and methods

Site description

All experiments were carried out in a screen house at the International Institute of Tropical Agriculture (IITA) Research Station in Namulonge, Uganda, approximately 30 km northeast of Kampala. The site is situated at approximately 1,150 m above sea level, 32º 34'N latitude, with a mean annual rainfall of 1,255 mm and an average daily temperature of 22ºC.

Tissue culture plants

The East African highland cooking banana cultivar Enyeru (*Musa* spp. AAA-EA) was selected for all *in vivo* screening experiments, based on its popularity (Karamura *et al*., 1993) and its susceptibility to *R. similis* (Speijer and Ssango, 1999). Plants were micropropagated from sword suckers in tissue culture using standard shoot-tip culture protocols for banana as outlined by Vuylsteke (1998). Cultures were initiated from explant materials collected from the IITA germplasm collection in Namulonge, Uganda. The initiation medium was based on the Murashige and Skoog (1962) mineral salt mixture (MS) with some modifications as

suggested by Vuylsteke (1998) (Table 1). The initiation medium was amended with 5 g/l benzyl amine.

Plant multiplication started 8 weeks after initiation by sub-culturing the emerging buds every 4 weeks to modified MS medium in 250-ml sterile glass jars until a sufficient number of plants were obtained for rooting. The multiplication stage took between 12 and 16 weeks, after which root growth was induced. Shoots were transferred to rooting medium for root initiation, each shoot being rooted singly in a 25 x 150 mm culture test tube. The rooting medium was amended with 0.1 mg/l naphthalene acetic acid as rooting hormone. Plants at the initiation, multiplication and rooting stages were placed in incubators at 27°C with a photoperiod of 14 hrs light and 10 hrs darkness. Four weeks after rooting, plants were removed from the test tubes and were ready for weaning and inoculation with endophytes.

Fungal isolates

The nine non-pathogenic *F. oxysporum* isolates selected for this study were originally isolated from the roots and corms of apparently healthy East African highland cooking banana plants in Uganda by Schuster *et al*. (1995) (Table 1). The isolates were selected because of their ability to immobilize and kill nematodes *in vitro* (Chapter 2; Niere, 2001). Seven out of the nine isolates had previously been studied for their effect on *R. similis* by Niere (2001). All nine isolates are maintained in soil tubes at 4 ºC.

Preparation of fungal inoculum

Fungal isolates preserved in soil tubes at 4ºC were re-grown on synthetic nutrient agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar/Ldistilled water) in 65-mm diameter Petri dishes under laboratory conditions (± 25°C and a natural photoperiod of 12 hrs light and 12 hrs darkness) for 7 days. The SNA medium was supplemented with 10 mg chlortetracycline, 100 mg penicillin G and 50 mg streptomycin-sulphate per liter to prevent bacterial contamination. Spore suspensions for inoculating banana plants were produced in 500-ml Erlenmeyer flasks containing 200 ml of half strength potato dextrose broth (PDB) (Sigma-Aldrich). Half strength PDB was prepared by dissolving 12 g of PDB per liter of sterile distilled water

(SDW). The flasks containing PDB were sterilized (autoclaved at 121 ºC for 15 min) and allowed to cool down.

Mycelial blocks of each fungal isolate were cut from 1-week-old cultures on SNA and aseptically transferred to the PDB under laminar airflow. Two replicate flasks were prepared for each fungal isolate. Two flasks containing non-inoculated broth served as controls. Flasks were incubated under laboratory conditions for 1 week to allow for fungal sporulation. Each day, flasks were manually shaken to disperse spores throughout the medium. Fungal spores were harvested by filtering the suspension through a 1-mm-diameter sieve to remove mycelial fragments. Spore densities were then estimated using a heamocytometer and the suspensions standardized to provide a final spore concentration of 1.5 to 1.75 x 10^6 spores / ml. The spore suspensions were either diluted with SDW or concentrated to achieve the required spore concentration. To concentrate, the spore suspensions were left to settle for a few hours and some broth at the top of the solution siphoned off.

Radopholus similis *cultures*

Pure nematode cultures of *R. similis* were multiplied on carrot disks in 30-mm-diameter glass Petri dishes according to the procedure of Speijer and De Waele (1997). Nematode infested banana plant roots were obtained from the IITA fields, cut into small pieces and macerated in a Waring blender (Waring, Connecticut, USA) at low speed for 15 s. Nematodes were extracted overnight from the macerated roots using the modified Baermann method (Hooper *et al*., 2005). Individual *R. similis* females were handpicked from the nematode suspension obtained and surface sterilized with a 600-ppm streptomycin sulphate solution. Fresh carrots were surface sterilized by dipping in absolute ethanol followed by flaming, cut into discs of ca. 0.5 cm diameter and placed in sterile 30-mm diameter glass Petri dishes. The sterilized females were inoculated on the cortex regions of the sterile carrot discs and the Petri dishes sealed with parafilm. The carrot discs were incubated at 27 ºC for 3 - 4 weeks after which nematodes were harvested from the Petri dishes for experiments.

Inoculation of plants with fungal isolates

When ready for weaning (4 weeks after plants were transferred to rooting medium), TC plants were removed from test tubes and selected for uniformity in size. Roots were rinsed in tap water to remove adhering medium and cut back to ca. 2 cm long to mimic natural wounding and enhance fungal infection. The roots were then immersed in the various spore suspensions or sterile PDB (controls) for 2 hrs, ensuring that all roots were submerged (Niere, 2001). Flasks were shaken occasionally to re-suspend spores in the solution. After inoculation, the plants were potted in 150-ml plastic pots containing steam-sterilized loamy forest soil. The different treatments (fungal isolates or control) plants were placed on separate plastic trays and transferred to a humidity chamber (1.92 m high x 1.59 m long x 1.23 m wide) for 1 month. The humidity chamber was constructed from a wooden frame covered with a clear polythene sheet. The humidity chamber was misted regularly by spraying the interior with tap water to maintain a high humidity level. After 1 month in the humidity chamber, plants were transplanted into 3-liter plastic bags containing steam-sterilized loamy forest soil, and transferred to the screen house.

Inoculation of plants with nematodes

Plants were inoculated with nematodes 8 weeks after endophyte inoculation. Pure cultures of *R. similis* were obtained from carrot disk cultures and suspensions of the nematodes in SDW collected in a beaker. The nematode suspension density was estimated using a light microscope (magnification x 100) and standardized to provide 500 female and juvenile nematodes/2 ml SDW. Males were not taken into account when preparing nematode inoculum because they are not infective (Speijer and De Waele, 1997). To inoculate plants with nematodes, three holes of ca. 3-5 cm deep were made in the soil at the base of the plant around the roots using a 0.5 cm-diameter stick. Care was taken not to damage the plants roots when drilling the holes. The three holes were made around the roots at equal distances from each other. The nematode suspension was pipetted into the holes at a rate of ca. 0.6 ml per hole after which the holes were covered with soil. Plants were not watered until 24 hrs after inoculation to enable the nematodes to enter the roots, and to avoid washing away the nematodes. After nematode inoculation, plants were maintained in the screen house for 8 weeks and watered daily. This time would permit for at least two nematode generations.

Experimental design and layout

Experiments to determine the effect of endophytic *F. oxysporum* isolates on *R. similis* reproduction and damage to TC banana plants, and their effects on plant growth and performance were conducted between May 2002 and January 2003, with an overlap in experimental timeframes between sequential experiments. All experiments were conducted in a completely randomized design under similar screen house conditions. The number of plants per treatment ranged from 14 to 25, depending on the availability of TC plants, and the experiments were repeated nine times. The duration of each experiment was 16 weeks, from the time of weaning to termination of the experiment.

Assessment of plant growth parameters

Plant growth parameters (plant height, number of healthy standing leaves and the length and width of the youngest leaf) were measured on a monthly basis for the duration of each experiment. Plant height was measured as the distance from the point where the youngest leaf emerges from the pseudostem to the base of the plant while the width of the youngest leaf 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 or brown and dry leaves.

Assessment of nematode reproduction and damage

At termination of an experiment (8 weeks after nematode inoculation), plants were uprooted from the plastic bags and washed free of soil with tap water. For each plant, the number of dead and functional roots was recorded. The fresh roots and shoots were weighed, and the dry shoot weight determined after drying shoots in an oven at 70ºC for 48 hrs. Nematode damage was expressed as a percentage of the necrotic root tissue, as described by Speijer and Gold (1996) (Fig. 1). To estimate the percentage root necrosis, five functional roots were randomly selected from each plant and cut to ca. 10-cm-long segments. The root segments were sliced lengthwise and the percentage of visible necrotic cortical region determined. 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 used for estimating nematode damage were further cut into 0.5-cm-long pieces with a knife, thoroughly mixed, and a 5-g sub-sample taken for nematode extraction. Nematode extraction was performed according to the modified Baermann funnel method (Hooper *et al*., 2005). The nematode extraction apparatus consisted of a sieve of ca. 2 cm deep and 15 cm in diameter made out of a plastic ring, the base of which was covered with plastic netting with ca. 1 mm diameter openings. The sieve was placed in a shallow plastic plate of 20 cm in diameter and overlaid with tissue paper. The root samples were macerated in a Waring Blender at low speed for 15 s and the suspension poured onto the tissue paper in the sieve. The plastic plate was filled with tap water to a level that just covered the macerated root pieces. Nematodes were extracted overnight, during which time they migrated from the macerated root tissue into the water. The nematode suspension in the plastic plate was then rinsed into 100-ml glass sample bottles and stored at 4ºC until counting could be done, usually within a week. Prior to counting, the nematode suspension was reduced to 25 ml. From each 25-ml sample, the nematode population density was determined in three 2-ml aliquots, and the average calculated. Female, male and juvenile *R. similis* counts were recorded separately.

Determination of fungal colonization

Endophytic colonization of banana plant roots was determined for experiments 2, 7 and 9. After harvest, three roots were randomly obtained from each plant and surface-sterilized by dipping in 100% ethanol, followed by flaming. The sterilized roots were cut into 0.25-cm long segments under sterile conditions and plated on SNA medium in 65-mm-diameter Petri dishes. From each root, 6 segments were plated per Petri dish. Petri dishes were incubated for 7 days in the laboratory. Fungal colonies growing from the root pieces were identified under a light microscope (magnification x 400). *Fusarium oxysporum* colonies were identified based on the presence of short phialides, the shape of macroconidia and the presence of chlamydospores (Nelson *et al*., 1983). Percentage colonization was determined per Petri dish as the number of *F. oxysporum* colonies divided by the total number of pieces plated, multiplied by 100.

Data analysis

Prior to statistical analysis, all data were tested for normality and homogeneity of variances using Shapiro-Wilkinson, Levene Welch and Kolmogorov-Smirnov tests. Normal probability plots, box plots and stem leaf plots were additionally used to confirm normality of data and equality of variances. If not normally distributed, various transformations were tested until the most suitable transformation was obtained. Plant growth data was square root transformed. For plant growth parameters measured on a monthly basis, the mixed model procedure was used to test for the effects of treatments (fungal isolates or control) with time as a regression factor. Experiments and treatments were treated as random factors. Percentage root necrosis and colonization were arcsine-sqrt-transformed prior to analysis. Nematode counts were calculated per 100 g of root sample and then sqrt (x +0.5) -transformed prior to analysis. One-way ANOVA was used to determine differences among experiments. When differences were observed between experiments, data from each experiment was analyzed separately. Within each experiment, a one-way ANOVA was conducted to demonstrate variability among treatments and, if different, means were separated using Tukey's studentized range test (SAS Institute, 1989).

Results

Significant differences (P<0.05) were observed among experiments for all the assessed variables. Consequently, data from each experiment was analyzed separately.

Radopholus similis reproduction

The population density of *R. similis* females signficantly differed among the nine experiments (P<0.0001) The interaction between experiments and treatments was, however, not significant (P=0.1053). Within experiments, the population density of female *R. similis* was not significantly different between the endophyte treatments and the control treatment (P=0.0985 – 0.9141) (Table 3). Some endophytic isolates resulted in lower *R. similis* female densities in treated compared to untreated plants. For example, plants treated with the fungal isolate *Eny1.31i* had lower *R. similis* female densities in seven out of eight experiments where this isolate was tested, *V5W2*-treated plants had lower female *R. similis* densities in four out of seven and *Eny7.11o*-treated plants had fewer females in five out of seven experiments. In some experiments, endophyte-treated plants had higher female *R. similis* densities than in the control plants. For example, in experiment 8, plants treated with fungal isolates *III4W1*, *V4W5*, *III3W3* and *Emb2.4o* had higher female *R. similis* densities compared to the control treatment. The coefficient of variation for *R. similis* females in the nine experiments varied from 40.2% to 71.7%.

Endophyte treatment did not significantly affect the densities of *R. similis* males (P=0.0893 – 0.7244) (Table 4), juveniles (P=0.0575 – 0.7752) (Table 5) or the total population density (females + males + juveniles) (P=0.0749 – 0.7966) (Table 6) in all experiments. However, the densities of *R. similis* males, juveniles and the total nematode density differed significantly among experiments (P<0.0001) indicating that the isolates resulted in different nematode densities in each experiment. A significant interaction in the numbers of *R. similis* juveniles (P=0.0079) and the total nematode densities (P=0.0140) between treatments and experiments indicated that the fungal isolates performed differently across experiments. No interaction was observed between the density of *R. similis* males and experiments (P=0.4295). A trend in which endophyte-infected plants had lower numbers of *R. similis* males, juveniles and also total nematode densities was observed in most experiments. For instance, the total nematode

densities in experiments 3, 5 and 7 were consistently lower in most endophyte treatments compared to the controls (Table 7). In some experiments, such as experiment 4, the number of male, juvenile and total nematodes was consistently higher in endophyte-treated than untreated plants. The high coefficients of variation (ranging from 40.6 to 67.2%) show that the variation within an experiment was high and may be the reason for the lack of statistical significance.

The total nematode densities were consistently lower in plants treated with fungal isolates *V5W2*, *Eny1.31i* and *Eny7.11o* in comparison to the controls (Table 7). In all the experiments in which isolate *V5W2* was tested, total *R. similis* population densities were lower, with the reduction ranging from 4% to 38%. Plants treated with isolate *Eny1.31i* had lower *R. similis* population densities ranging from 18% to 59% while isolate *Eny7.11o* resulted in a reduction ranging between 7% and 45% in comparison with the controls. A different trend in which endophyte-treated plants supported more nematodes than the controls was observed in experiments 2 and 4, and in one instance each of experiments 5 and 6. In these experiments, the total *R. similis* population densities in plants treated with isolates *III3W3*, *V4W5*, *III4W1*, *V1W7*, *V2W2* and *Emb2.4o* were higher compared to the control plants (3.5% to 97.2% increase). Particularly, in experiment 4, the numbers of *R. similis* in endophyte-treated plants were higher than in the control plants except for the plants treated with isolates *Eny7.11o* and *V5W2* which had lower nematode densities (Table 7). Despite the observed trend, in all the experiments, the reduction in nematode densities in endophyte-treated plants was not significantly different from the controls ($P<0.05$).

Radopholus similis damage

Nematode damage to banana roots was not significantly affected by endophyte treatment in any of the experiment ($P=0.0568 – 0.7011$) other than experiment 3 ($P=0.0175$) (Table 8). In experiment 3, plants treated with isolates *III4W1* had a significantly higher percentage root necrosis than control plants. A non-significant reduction in nematode damage by most of the fungal isolates was observed in experiments 1 and 6. The high variation in experiments (coefficient of variation 10.7% to 63.7%) may be responsible for the lack of a statistical significance of the observed data.

Plant growth and performance

Plant fresh root weights differed significantly among experiments (P<0.0001), and a significant interaction (P=0.0006) between experiments and treatments indicated that the effects of treatments on plant fresh root weight varied across experiments. Inoculation of plants with fungal isolates resulted in variable effects on plant fresh root weights across experiments. Within each experiment, no significant differences were observed between endophyte treatments and the control treatment, except for experiment 3 (P=0.0008) (Table 9). In this experiment, plants treated with isolate *V5W2* had a significantly higher fresh root weight than the control treatment and that of the treatment with isolate *V2W2*, but it did not differ significantly from the other fungal isolates. Plants treated with isolates *V5W2* further had a higher fresh root weights for 5 out of 7 experiments.

A significant difference for fresh shoot weights was found among experiments (P<0.0001), and the interaction between treatments and experiments was not significant (P=0.0503). Within each experiment, fresh shoot weight data demonstrated a lack of significant difference between the endophyte treatments and the controls, except for experiments 3 (P=0.0156) and 8 (P=0.0170) (Table 10). In these experiments, plants treated with fungal isolates *Eny7.11o* and *Eny1.31i*, respectively, resulted in significantly higher shoot weights compared to control plants. Although differences in fresh shoot weights did not reach statistical significance in most of the experiments, a general trend was observed for plants treated with the different fungal isolates to have higher shoot weights than the control plants. For example, isolates *V5W2* and *Eny7.11o* resulted in higher fresh shoot weights in all the experiments in which these isolates were tested, compared to the control plants.

The dry shoot weights differed significantly between experiments (P<0.0001), with a non-significant interaction between treatments and experiments (P=0.4090). Within each experiment, dry shoot weights did not differ between endophyte treatments and the control treatment (P=0.1305 – 0.9420), except for experiments 3 (P=0.0004) and 8 (P=0.0105). In experiment 3, plants treated with fungal isolates *III4W1* and *Emb2.4o* had significantly higher dry shoot weights, while in experiment 8, plants treated with fungal isolate *Eny1.31i* had a significantly higher dry shoot weight compared to the control plants (Table 11). In most of the experiments apart from experiment 5, although not significant, a trend occurred in which

plants treated with the various fungal isolates had higher dry shoot weights compared to the control was observed (Table 11). Isolates *Eny7.110* and *III3W3* resulted in an increase of dry shoot weight in six of the seven experiments.

Plant height of endophyte-treated and untreated plants (data not shown) differed among repeat experiments ($P<0.0001$). Within each experiment, however, no differences were observed in plant height between the endophyte treatments and control treatment ($P=0.1525$). The number of standing leaves, length and width of the youngest leaf were also not influenced by endophyte treatment (data not shown).

The total number of roots per plant in endophyte-treated and untreated plants (data not shown) also differed among repeat experiments ($P<0.0001$). A significant interaction between experiments and treatments was observed ($P=0.0070$), indicating that the effect of treatments on the total number of roots per plant varied across experiments. Within each experiment no differences were observed in the total number of roots per plant between the control and endophyte treatments, except for experiments 3 ($P=0.0044$) and 5 ($P=0.0186$). In experiment 3, plants treated with isolates *III4W1*, *V5W2* and *V4W5* had significantly more roots than all the other endophyte-treated plants and the control. In experiment 5, control plants had significantly more roots than the endophyte-treated plants, except for plants treated with isolate *Eny1.31i*, which had significantly less roots than control plants.

The number of functional roots per plant differed among experiments ($P<0.0001$) (Table 12), with a significant interaction between experiments and treatments ($P=0.0046$). This indicates that the effect of treatments on the number of functional roots differed across experiments. Within each experiment, no differences in the number of functional roots between endophyte treatments and the control treatment were observed, except for experiments 5 ($P=0.0065$), 6 ($P=0.0339$) and 9 ($P=0.0400$). Plants treated with isolates *Eny1.31i* and *Emb2.4o* had significantly fewer roots compared to the controls in experiments 5 and 6, respectively. In experiment 9, plants treated with *Eny1.31i* had more functional roots than the control plants. The percentage of dead roots varied across experiments ($P<0.0001$) with a non-significant interaction between treatments and experiments ($P=0.1640$). Within each experiment, differences in the percentage of dead roots between the endophyte treatment and the control treatment were not significant (data not shown).

Endophytic colonization

Percentage colonization of roots treated with the various fungal isolates ranged from 44.9 to 68.8% in experiment 2, 22.6 to 65.3% in experiment 7, and 17.5 to 22.4% in experiment 9 (Table 13). Endophytic *F. oxysporum* was also re-isolated from control plants. Colonization of roots of control plants by *F. oxysporum* was 45.2%, 36.0% and 16.6% in experiments 2, 7 and 9, respectively. Percentage colonization of plant roots of endophyte-treated plants was not statistically different from the control plants in experiments 2 (P=0.1171), 7 (P=0.4467) and 9 (P=0.8519). Other fungi were also frequently isolated from endophyte-treated and control roots. The most commonly isolated fungal genera were *Penicillium* spp. and other sterile fungi (data not shown). The levels of colonization of plants by fungal isolates varied across the experiments. Percentage colonization by *F. oxysporum* was higher in experiment 2 and 7 than in experiment 9. For example, percent colonization by isolate *V5W2* was 58.4%, 34.6% and 22.4% in experiments 2, 7 and 9, respectively.

Discussion

Artificial inoculation of TC plants with fungal endophytes may offer a cost effective nematode management strategy due to the low levels of fungal inoculum required through targeted application on the plants roots (Sikora *et al*., 2000a). In the current study, however, treatment of TC banana plants with non-pathogenic *F. oxysporum* endophyte isolates did not significantly reduce *R. similis* densities, nematode damage to banana roots, or enhance plant growth in the screen house. The high variation observed in each experiment were mostly responsible for the lack of significant differences, as a general reduction in nematode densities and root damage, and enhancement in plant growth properties, was observed for endophyte-treated plants compared to control treatments. In a separate investigation, Pocasangre (2000) demonstrated that inoculation of endophytes into TC Cavendish banana plants gave protection against *R. similis*.

While the use of TC plants provided relatively uniform plants, the results obtained depicted large unexplained variations in both nematode densities, root damage and plant growth data. The high coefficient of variation was observed both within and among experiments. Under similar screen house conditions, Niere (2001) also found no significant differences between endophyte-treated and control plants, despite endophyte-treated plants having 40% to 50% less nematodes compared to control plants. The substantial variation among repeated experiments could be due to differences in environmental conditions in the screen house, the vigor of the nematode inoculum, small sample size, and root colonization by endophytes. The nine experiments in the current study were conducted over a period of 9 months, and the variation in weather conditions may have influenced the outcome of each experiment. In this time, the air temperature in the screen house where the experiments were conducted varied between 19.1 ºC to 32.8 ºC, while the soil temperature in the plastic bags varied between 20.4 ºC to 26.3 ºC. Since the soil temperature was inside the range for *R. similis* reproduction (Sarah *et al.,* 1996), the air temperature in the screen house may have resulted in the observed variation. Viaene *et al*. (2003) also observed variation in root weights and *R. similis* densities in banana genotypes in a series of experiments conducted under similar glasshouse conditions. A lack in endophytic colonization of banana roots and differences in nematode vigor may have also accounted for the observed lack in significant differences, as successful colonization by the fungal endophytes was not demonstrated for all experiments, and virulence in

nematodes under the different environmental conditions was not investigated. Similar variation in the effects of endophytes on nematode population densities between experiments, however, has been reported several times before (Cook *et al*., 1991; Niere, 2001; Sikora *et al*., 2003).

In the three experiments where fungal re-isolation was conducted, it was demonstrated that plants were colonized by endophytic *F. oxysporum* although colonization of endophyte-treated plants was not different from that of control plants. Whether the re-isolated fungi were actually the ones originally inoculated has not been determined. Re-isolation of endophytic *F. oxysporum* from uninoculated control plants was also demonstrated, with no differences in colonization rates between endophyte-treated and control plants. Possible contamination of control plants by endophytic *F. oxysporum* may have come from the soil, irrigation water or from the screen house environment. The extent to which these contaminants may have influenced the results is however unknown and may be responsible for the lack of significant differences observed between the endophyte treatments and control treatment. The problem of contaminants may be overcome by conducting future experiments in controlled environments like growth cabinets or using sterile irrigation water. The ability to establish and monitor colonization of plants by the introduced fungal endophytes is an important aspect of biological control (Gullino *et al*., 1995; Kerry, 2000). Although it was possible to recover fungal isolates to varying degrees, it is not possible to confirm with certainty that the re-isolated fungi were the original isolates that were inoculated, due to lack of adequate marking techniques. Since control plants were also colonized, a system should be put in place to identify the reisolated fungi and compare them to the original isolates. Biochemical markers and DNA fingerprinting can be used to trace individual strains after release into the soil (Gullino *et al*., 1995) and may also be useful to trace endophytic *F. oxysporum* strains. Use of mutants developed on selective medium may also be used for endophyte tracking purposes. For example, benomyl-resistant mutants obtained by UV treatment (Yamaguchi *et al*., 1998) may be used to track inoculated isolates over time. The fungal isolates can also be genetically transformed with fluorescent proteins (Bao *et al*., 2000). The feasibility however, for using genetically transformed isolates under Ugandan conditions may be hindered by biosafety rules surrounding genetically modified organisms. Thus, the use of fungicide-resistant mutants offers a more feasible option in Uganda.

Endophyte treatment of banana plants seems to affect all stages *R. similis* (females, males and juveniles). The reduction in the female and male *R. similis* densities may be due to endophyte effects on the post-embryonic development of the juvenile stages into adults. Probably, the endophytes affected the feeding activity of the nematodes, thereby reducing the numbers of juveniles that developed to maturity. Niere (2001) also showed that endophyte-treated plants had lower densities of *R. similis* females that subsequently resulted in lower total *R. similis* densities. The lower juvenile densities observed in the current study may be due to an inhibition of egg hatching inside the plant. Although this may be difficult to demonstrate inside the plant, inhibition of the hatching of *R. similis* eggs by culture filtrates of these isolates was demonstrated earlier (Chapter 2).

Plant growth stimulation appeared to be the parameter least affected following inoculation of TC banana plants with non-pathogenic *F. oxysporum* endophytes. According to Fallas *et al*. (1995), shoot and root weight reductions are useful indicators for nematode pathogenicity and may be used to assess growth promotional effects of fungal (endophyte) inoculation. In the current study, a higher fresh root and shoot weight was generally demonstrated with endophyte-treated plants, but plant height, the number of standing leaves and the length and width of the youngest leaf were not influenced by endophyte treatment. Plant growth promotion by some endophytic *Fusarium* isolates that manifested in bigger root and shoot weights of banana plants were previously reported by Pocasangre (2000) and Niere (2001). Niere (2001) also reported that the height of banana plants of different cultivars was not influenced by endophyte treatment.

Damage due to nematode infection was assessed as a percentage necrotic root tissue and the number of dead roots (Gold *et al*., 1994; Speijer and Gold, 1996). Root necrosis was not significantly affected by endophyte treatment in any of the experiments although a general trend was observed for roots of endophyte-treated plants to have less nematode damage than those of control plants. Assessment of nematode damage based on visual ratings is subjective however, and varies naturally with the individuals scoring for the damage. For consistency of nematode damage data, one individual scored for necrosis in all the experiments. There were large variations in nematode damage between replications in a given treatment, which may be responsible for the observed lack of differences. Additionally, variation within a replication was observed where from the same plant, some roots had no nematode damage while others

had high levels of nematode damage. Niere (2001) also reported a high variation in nematode damage, which resulted in a lack of statistical significance.

Inoculation of TC banana plants with some endophytic isolates resulted in substantially lower *R. similis* population densities, despite these not being statistically significant. From these results, three isolates, *V5W2*, *Eny1.31i* and *Eny7.11o* offer potential for biological control of *R. similis* in TC banana plants. In previous experiments with endophytes in banana, Niere (2001) reported similar trends for *R. similis* densities in plants of the banana cv. Enyeru inoculated with the fungal isolates *V1W7*, *Eny1.31i* and *Eny7.11o*. These three isolates will, therefore, be used for studies on detailed plant-endophyte interactions and mechanism of endophyte-control of nematodes.

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

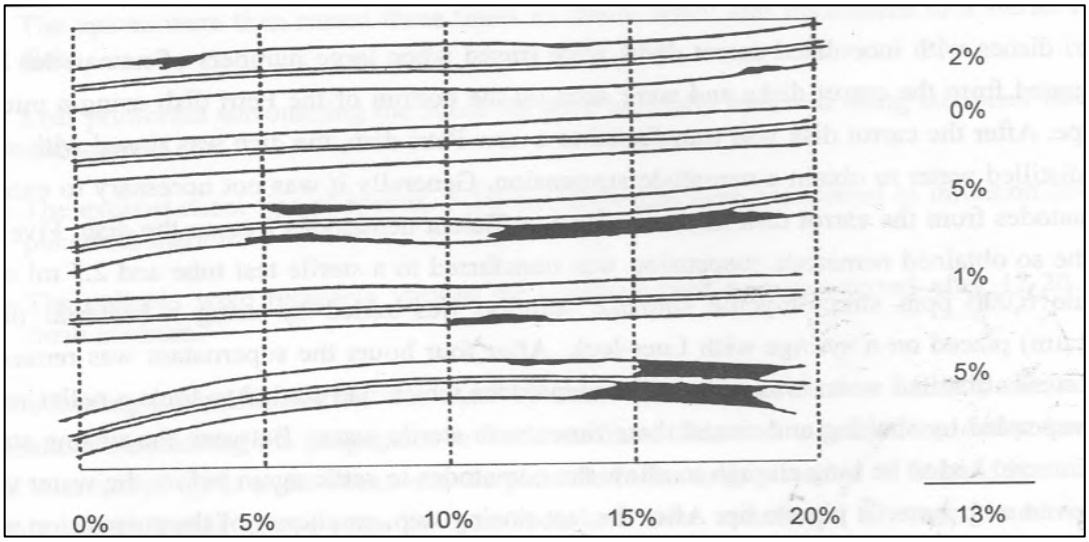

Table 1: Composition of Murashige and Skoog (1962) mineral salt (MS) mixture, for growing tissue culture banana plants *in vitro*.

Major salts	Quantity (mg / l)	Minor salts*	Quantity (mg / l)
KNO_3	1900	$MnSO_4.4H_2O$	22.3
NH_4NO_3	1650	H_3BO_3	6.2
$CaCl_2.2H_2O$	440	$ZnSO_4.4H_2O$	8.6
$MgSO_4.7H_2O$	370	KCl	0.83
KH_2PO_4	170	$Na_2MoO_4.2H_2O$	0.25
$FeSO_4.7H_2O$	27.8	$CuSO_4.5H_2O$	0.025
$Na_2EDTA.2H_20$	37.3	$CoCl_2.6H_2O$	0.025

*Modified by Vuylsteke (1998) by replacing $MnSO_4.4H_2O$ (22.3 mg/l) with $MnSO_4.4H_2O$ (16.9 mg/l).

Table 2: The identity and origin of endophytic non-pathogenic *Fusarium oxysporum* isolates used in *in vivo* screening experiments against *Radopholus similis* in tissue culture banana plants

Fungal isolate	Cultivar	Plant part	Number of times tests repeated*	*In vivo* activity**
Eny1.31i	Enyeru (AAA-EA)	Rhizome	8	Reduced
Eny7.11o	Enyeru (AAA-EA)	Rhizome	7	Reduced
III3W3	Unknown AAA-EA	Root	7	nt
III4W1	Unknown AAA-EA	Root	8	Reduced
V5W2	Unknown AAA-EA	Root	7	Reduced
V4W5	Unknown AAA-EA	Root	7	Reduced
V2W2	Unknown AAA-EA	Root	7	Reduced
V1W7	Unknown AAA-EA	Root	6	Reduced
Emb2.4o	Embiire (AAA-EA)	Rhizome	5	nt

* The number of times each fungal isolate was tested out of the total of nine experiments conducted during this study

**Effect of fungal isolate on *R. similis* population density in TC banana plants of different cultivars as reported by Niere (2001). Reduction in *R. similis* densities was not significantly different from control plants. nt= not tested.

Table 3: The effect of endophytic *Fusarium oxysporum* isolates on the density of *Radopholus similis* females in roots of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8 weeks after inoculation with 500 nematodes.

Treatment	*Numbers of *Radopholus similis* females (x 100) / 100g roots								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	3.6 ± 0.6	6.2 ±	22.4 ± 3.8	9.4 ± 2.1	30.4 ± 8.6	31.5 ± 6.8	57.3 ± 10.7	62.2 ± 13.7	133.9 ± 17.9
Eny.131i	2.6 ± 0.7	5.8 ±	34.2 ± 2.2	7.6 ± 2.8	17.4 ± 4.9	nt	34.4 ± 6.7	56.6 ± 15.1	83.7 ± 9.5
V5W2	2.6 ± 0.4	7.6 ±	23.0 ± 7.9	9.9 ± 4.2	nt	19.4 ± 4.3	39.0 ± 5.1	nt	127.0 ± 20.7
Env7.11o	4.1 ± 0.9	6.5 ±	18.2 ± 6.2	8.0 ± 1.4	19.8 ± 8.9	nt	35.0 ± 4.5	59.9 ± 8.2	nt
III4W1	3.4 ± 0.8	12.3 ±	37.3 ± 9.4	24.6 ± 7.3	30.4 ± 6.9	nt	50.7 ± 15.9	73.6 ± 18.2	114.7 ± 18.5
V4W5	4.2 ± 0.7	8.5 ±	23.0 ± 10.0	11.5 ± 3.2	33.6 ± 10.0	nt	44.2 ± 6.8	73.0 ± 25.8	nt
III3W3	3.9 ± 0.7	18.4 ±	21.6 ± 2.9	11.2 ± 2.6	14.3 ± 2.5	nt	52.3 ± 9.2	174.7 ± 65.2	nt
V1W7	nt	9.2 ±	26.0 ± 10.5	9.5 ± 2.4	nt	31.4 ± 3.8	47.2 ± 10.3	nt	nt
V2W2	nt	13.1 ±	31.8 ± 14.9	15.1 ± 5.8	20.0 ± 5.8	-	50.8 ± 10.5	68.9 ± 19.9	nt
Emb2.4o	nt	nt	23.3 ± 9.7	15.6 ± 5.7	-	47.5 ± 12.1	53.3 ± 13.7	71.9 ± 24.5	nt
P-value	0.3096	0.6183	0.9141	0.4305	0.2830	0.0985	0.8050	0.418	0.2432
CV	52.9	65.2	71.7	55.9	62.8	46.7	40.2	51.6	37.7

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt(x +0.5) transformed data. Each column represents one experiment; nt=not tested.

Table 4: The effect of endophytic *Fusarium oxysporum* isolates on the density of *Radopholus similis* males in roots of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8 weeks after inoculation with 500 nematodes.

Treatment	*Numbers of *Radopholus similis* males (x100) / 100 g root								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	3.0 ± 0.7	5.1 ± 1.2	17.8 ± 3.2	5.3 ± 1.0	17.8 ± 2.9	38.2 ± 8.5	71.9 ± 17.3	65.2 ± 11.3	76.6 ± 98.5
Eny.131i	3.3 ± 0.8	4.3 ± 1.1	11.7 ± 2.8	7.2 ± 2.2	19.9 ± 5.2	nt	35.0 ± 9.3	48.9 ± 11.0	50.9 ± 63.1
V5W2	1.8 ± 0.5	3.4 ± 0.8	15.9 ± 4.7	5.2 ± 1.6	nt	21.6 ± 5.4	45.5 ± 5.7	nt	73.8 ± 124.8
Eny7.11o	2.9 ± 0.6	3.8 ± 0.7	10.1 ± 1.5	7.5 ± 2.4	9.6 ± 2.5	nt	41.3 ± 7.1	48.8 ± 10.1	nt
III4W1	3.3 ± 1.0	8.6 ± 2.2	16.2 ± 4.3	12.5 ± 3.6	19.9 ± 3.9	nt	54.5 ± 15.6	68.6 ± 21.5	77.3 ± 138.0
V4W5	3.0 ± 0.4	5.0 ± 0.9	8.8 ± 2.2	5.3 ± 1.8	24.8 ± 7.9	nt	47.5 ± 11.1	64.5 ± 19.8	nt
III3W3	2.6 ± 0.6	6.2 ± 1.8	14.0 ± 3.1	9.4 ± 1.9	13.0 ± 2.4	nt	53.9 ± 9.5	134.8 ± 47.2	nt
V1W7	nt	4.8 ± 1.1	12.2 ± 4.3	8.3 ± 3.4	nt	35.2 ± 3.8	41.7 ± 5.5	nt	nt
V2W2	nt	4.6 ± 1.1	10.8 ± 3.3	10.6 ± 3.2	13.9 ± 3.8	nt	44.7 ± 11.1	66.1 ± 23.9	nt
Emb2.4o	nt	nt	9.5 ± 2.9	12.8 ± 4.8	nt	44.2 ± 9.1	60.3 ± 18.3	77.8 ± 27.0	nt
P-value	0.5737	0.6210	0.1868	0.7244	0.1351	0.0893	0.7201	0.6934	0.3474
CV	67.2	52.6	56.9	61.2	56.4	43.2	46.0	57.3	40.6

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt (x +0.5) transformed data. Each column represents one experiment; nt= not tested.

Table 5: The effect of endophytic *Fusarium oxysporum* isolates on the density of *Radopholus similis* juveniles in roots of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8 weeks after inoculation with 500 nematodes.

	*Numbers of *Radopholus similis* juveniles (x100)/ 100 g root								
Treatment	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	47.1 ± 8.3	118.0 ± 23.5	277.4 ± 49.6	62.8 ± 13.1	131.3 ± 23.6	187.4 ± 30.1	294.9 ± 48.6	541.3 ± 76.9	238.9 ± 36.4
Eny.131i	29.3 ± 6.6	72.4 ± 17.6	156.6 ± 49.0	81.8 ± 29.0	111.5 ± 30.6	nt	192.6 ± 53.8	427.1 ± 91.0	135.6 ± 14.8
V5W2	30.7 ± 6.0	111.9 ± 28.5	176.4 ± 36.5	40.9 ± 8.8	nt	118.0 ± 21.9	208.4 ± 21.3	nt	199.1 ± 23.3
Eny7.11o	39.6 ± 6.8	77.1 ± 20.2	143.3 ± 28.4	58.0 ± 10.0	96.9 ± 22.7	nt	241.1 ± 36.2	511.0 ± 80.1	nt
III4W1	38.7 ± 8.1	135.2 ± 35.1	155.8 ± 27.7	100.0 ± 25.9	126.6 ± 17.2	nt	227.9 ± 57.9	519.3 ± 124.6	229.3 ± 36.2
V4W5	36.9 ± 5.5	120.2 ± 30.5	130.5 ± 34.4	83.2 ± 24.7	130.8 ± 21.5	nt	232.2 ± 36.1	452.6 ± 119.2	nt
III3W3	44.8 ± 1.4	123.1 ± 21.0	148.0 ± 30.3	102.6 ± 20.3	105.7 ± 15.7	nt	209.3 ± 32.6	1009.2 ± 339.6	nt
V1W7	nt	110.4 ± 29.1	159.7 ± 35.5	122.3 ± 34.1	nt	172.3 ± 17.2	236.9 ± 26.0	nt	nt
V2W2	nt	94.0 ± 19.8	121.9 ± 21.0	95.3 ± 22.3	105.1 ± 19.9	-	234.1 ± 47.2	491.7 ± 136.8	nt
Emb2.4o	nt	nt	135.1 ± 30.7	116.3 ± 42.2	nt	198.8 ± 37.4	284.1 ± 64.0	477.8 ± 91.8	nt
P value	0.5063	0.7660	0.4144	0.3427	0.7383	0.1757	0.7752	0.5403	0.0575
CV	44.6	45.9	48.6	44.5	41.3	35.2	36.9	43.3	35.3

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt (x +0.5) transformed data. Each column represents one experiment. nt, not tested

Table 6: The effect of endophytic *Fusarium oxysporum* isolates on the total *Radopholus similis* population density (males + female + juveniles) in roots of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8 weeks after inoculation with 500 nematodes.

Treatment	*Total *Radopholus similis* population density (x100) /100 g root								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	53.7 ± 8.9	129.3 ± 5.3	317.8 ± 52.8	76.7 ± 14.7	179.6 ± 32.7	257.1 ± 43.8	424.2 ± 72.8	668.8 ± 98.0	449.5 ± 51.6
Eny.131i	35.4 ± 7.5	82.2 ± 18.6	202.5 ± 70.4	96.7 ± 33.8	153.9 ± 42.0	nt	262.1 ± 68.4	532.7 ± 115.2	270.3 ± 27.1
V5W2	35.2 ± 6.5	123.0 ± 29.6	215.4 ± 44.3	56.0 ± 14.3	nt	157.1 ± 29.3	293.1 ± 30.1	nt	400.0 ± 52.6
Eny7.11o	46.7 ± 7.7	87.5 ± 20.7	172.7 ± 30.9	73.6 ± 12.4	126.4 ± 32.3	nt	317.5 ± 45.9	619.8 ± 92.9	nt
III4W1	45.4 ± 9.1	156.1 ± 39.7	209.4 ± 35.9	137.2 ± 35.2	177.0 ± 26.7	nt	333.2 ± 87.7	661.6 ± 161.3	421.3 ± 62.8
V4W5	44.2 ± 6.3	133.8 ± 31.2	136.4 ± 42.6	100.1 ± 29.3	189.2 ± 36.6	nt	324.0 ± 47.0	590.3 ± 162.5	nt
III3W3	51.5 ± 15.1	147.8 ± 30.0	183.6 ± 38.2	131.0 ± 23.4	133.1 ± 19.8	nt	315.6 ± 46.9	1318.8 ± 450.1	nt
V1W7	nt	124.5 ± 30.3	198.1 ± 46.6	140.1 ± 38.9	nt	239.1 ± 21.5	325.8 ± 36.2	nt	nt
V2W2	nt	111.8 ± 22.7	164.7 ± 36.6	121.1 ± 30.3	139.2 ± 27.8	-	329.7 ± 65.6	626.8 ± 178.2	nt
Emb2.4o	nt	nt	167.9 ± 40.2	144.7 ± 52.3	nt	290.5 ± 56.1	397.8 ± 92.5	627.7 ± 139.5	nt
P-value	0.5151	0.7668	0.3218	0.4349	0.5400	0.1135	0.7966	0.5425	0.0749
CV	41.8	44.5	48.5	44.6	42.8	35.8	36.6	44.1	33.1

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt (x +0.5) transformed data. Each column represents one experiment; nt= not tested.

Table 7: Percentage change in total *Radopholus similis* populations in roots of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA) inoculated with various endophytic *Fusarium oxysporum* isolates in comparison with control plants, 8 weeks after inoculation with 500 nematodes.

Treatment	Percentage change in total* *Radopholus similis* populations/ 100 g root								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Eny.131i	-34.14	-26.87	-36.23	26.07	-18.71	nt	-59.34	-20.35	-39.87
V5W2	-11.00	-4.88	-32.18	-26.94	nt	-38.91	-30.91	nt	-11.00
Eny7.11o	-10.93	-32.29	-45.62	-4.03	-29.61	nt	-25.16	-7.33	nt
III3W3	-4.23	14.30	-42.18	56.95	-25.89	nt	-25.60	97.17	-6.26
V4W5	-17.79	3.52	-48.87	30.46	5.34	nt	-23.61	-11.75	nt
III4W1	-15.50	20.77	-34.07	78.85	-1.47	nt	-21.44	-1.08	nt
V1W7	nt	-3.74	-37.63	82.59	nt	-7.03	-23.18	nt	nt
V2W2	nt	-13.54	-48.14	57.81	-22.48	nt	-22.28	-6.29	nt
Emb2.4o	nt		-47.13	88.58	nt	13.00	-6.23	-6.16	nt

*Total of females + males + juveniles. Negative change indicates a reduction in total *R. similis* populations while a positive change indicates an increase in *R. similis* population compared to the control treatment.

Table 8: The effect of endophytic *Fusarium oxysporum* isolates on the damage caused by *Radopholus similis* in banana root tissue (mean ± S.E.) in 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8-weeks after inoculation with 500 nematodes.

Treatment	Percentage root necrosis								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	14.9 ± 3.5	6.1 ± 1.4	15.8 ± 1.0^{b}	11.5 ± 2.0	22.3 ± 1.6^{ab}	32.2 ± 2.9	21.3 ± 2.0	38.1 ± 5.3	32.5 ± 3.4
Eny.131i	6.7 ± 2.3	10.5 ± 1.7	16.6 ± 0.7^{ab}	16.8 ± 9.2	19.2 ± 3.7^{ab}	nt	24.4 ± 2.2	31.4 ± 3.9	37.2 ± 2.3
V5W2	8.5 ± 2.6	7.0 ± 2.2	19.7 ± 0.6^{ab}	8.8 ± 1.8	nt	25.2 ± 3.0	22.1 ± 2.5	nt	35.5 ± 3.0
III4W1	9.2 ± 3.7	6.6 ± 1.7	20.1 ± 0.7^{a}	13.3 ± 2.3	24.3 ± 2.5^{ab}	nt	23.2 ± 3.0	27.5 ± 5.9	36.2 ± 2.4
V4W5	11.9 ± 3.5	8.3 ± 1.7	17.4 ± 1.3^{ab}	11.4 ± 2.3	27.1 ± 2.6^{a}	nt	20.9 ± 2.8	36.1 ± 5.2	nt
Eny7.11o	9.7 ± 3.4	12.7 ± 2.4	17.3 ± 0.8^{ab}	17.8 ± 3.9	16.0 ± 2.0^{b}	nt	28.7 ± 3.3	35.4 ± 3.2	nt
III3W3	14.9 ± 2.6	4.9 ± 1.0	17.1 ± 0.7^{ab}	11.9 ± 9.8	16.1 ± 2.2^{b}	nt	28.3 ± 3.1	40.4 ± 6.6	nt
V1W7	nt	12.7 ± 2.9	18.5 ± 1.0^{ab}	11.6 ± 2.2	nt	23.0 ± 3.3	26.4 ± 1.7	nt	nt
V2W2	nt	6.3 ± 1.4	16.5 ± 0.7^{ab}	12.1 ± 2.2	18.3 ± 2.2^{ab}	nt	19.7 ± 4.0	31.4 ± 3.9	nt
Emb2.4o	nt	nt	16.7 ± 0.8^{ab}	8.8 ± 1.4	nt	21.6 ± 2.3	26.0 ± 2.6	nt	nt
P-value	0.2667	0.0568	0.0175	0.7011	0.0052	0.1049	0.1463	0.5295	0.4482
CV	63.7	40.2	10.7	37.0	25.0	26.1	24.2	26.6	22.1

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt (x +0.5) transformed data. Each column represents one experiment; nt= not tested. In columns, means followed by the same small letter (superscript) are not statistically different at P=0.05 according to Tukey's studentized range test.

Table 9: The effect of endophytic *Fusarium oxysporum* isolates on fresh root weight of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8-weeks after inoculation with 500 nematodes.

Treatment	*Fresh root weight (g)								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	56.4 ± 3.6	45.2 ± 3.9	29.7 ± 3.2^{bc}	20.1 ± 3.4	31.8 ± 2.8	27.7 ± 2.1	28.0 ± 3.3	11.9 ± 2.9	19.4 ± 1.8
Eny.131i	61.3 ± 2.8	42.8 ± 2.8	31.7 ± 3.2^{abc}	14.8 ± 2.7	24.6 ± 4.5	nt	25.7 ± 2.2	13.9 ± 2.0	20.6 ± 1.2
V5W2	56.4 ± 3.6	47.9 ± 4.4	47.9 ± 3.0^{a}	24.3 ± 4.0	nt	25.3 ± 2.7	29.5 ± 1.8	nt	20.3 ± 1.4
III4W1	56.5 ± 2.3	51.3 ± 6.3	46.4 ± 3.4^{ab}	27.6 ± 3.1	30.3 ± 2.1	nt	24.9 ± 2.5	10.5 ± 2.5	21.0 ± 1.4
Eny7.11o	61.2 ± 4.1	40.8 ± 3.0	40.3 ± 4.6^{abc}	24.5 ± 4.5	28.0 ± 2.4	nt	31.8 ± 2.8	15.5 ± 2.5	nt
V4W5	57.7 ± 3.1	55.5 ± 5.6	36.0 ± 3.8^{abc}	14.6 ± 2.3	26.6 ± 2.7	nt	27.2 ± 2.1	11.5 ± 2.4	nt
III3W3	53.8 ± 3.3	50.1 ± 3.1	36.6 ± 3.1^{abc}	19.1 ± 3.2	24.9 ± 2.2	nt	23.7 ± 2.2	7.6 ± 1.8	nt
V1W7	nt	34.8 ± 4.7	43.3 ± 3.9^{abc}	15.1 ± 5.6	nt	25.2 ± 2.5	28.3 ± 2.5	nt	nt
V2W2	nt	42.4 ± 3.5	27.8 ± 3.8^{c}	19.7 ± 3.0	30.1 ± 2.5	nt	25.6 ± 3.3	13.3 ± 2.0	nt
Emb2.4o	nt	nt	37.0 ± 3.3^{abc}	24.2 ± 4.1	nt	20.8 ± 1.8	25.0 ± 2.1	16.9 ± 1.7	nt
P-value	0.6199	0.0861	0.0008	0.0899	0.2989	0.2373	0.6152	0.0830	0.7601
CV	13.0	19.1	22.3	31.3	23.9	20.3	21.1	31.8	20.0

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt (x +0.5) transformed data. Each column represents one experiment. nt, not tested. In columns, means followed by the same small letter (superscript) are not statistically different at P=0.05 according to Tukey's studentized range test.

Table 10: The effect of endophytic *Fusarium oxysporum* isolates on fresh shoot weight of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8-weeks after inoculation with 500 nematodes.

Treatment	*Fresh shoot weight (g)								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	84.4 ± 4.3	95.5 ± 7.0	82.7 ± 6.1[b]	67.5 ± 5.5	54.8 ± 2.7	49.2 ± 2.9	64.1 ± 4.4	26.3 ± 4.1[b]	53.5 ± 2.9
Eny.131i	96.7 ± 3.6	103.7 ± 6.1	106.8 ± 5.9[ab]	52.1 ± 6.2	45.4 ± 5.9	nt	63.8 ± 3.7	44.7 ± 4.1[a]	53.1 ± 2.3
V5W2	95.4 ± 3.1	104.7 ± 6.2	117.1 ± 3.2[ab]	70.3 ± 4.8	nt	52.5 ± 3.5	66.6 ± 1.7	nt	59.4 ± 2.5
III4W1	94.0 ± 2.4	98.7 ± 5.8	117.8 ± 5.3[ab]	71.8 ± 4.4	52.7 ± 2.4	nt	58.5 ± 3.6	33.7 ± 2.3[ab]	59.9 ± 1.9
Eny7.11o	94.0 ± 3.5	100.1 ± 2.8	170.3 ± 56.1[a]	67.8 ± 6.1	57.4 ± 3.3	nt	66.4 ± 4.5	37.9 ± 3.6[ab]	nt
V4W5	95.2 ± 3.2	101.4 ± 6.2	113.7 ± 6.2[ab]	50.2 ± 6.4	53.4 ± 3.7	nt	66.5 ± 3.3	35.6 ± 4.2[ab]	nt
III3W3	87.4 ± 3.1	105.4 ± 3.9	105.4 ± 4.3[ab]	69.3 ± 8.1	52.7 ± 2.4	nt	60.3 ± 3.0	28.0 ± 3.7[ab]	nt
V1W7	nt	89.2 ± 8.0	109.9 ± 8.7[ab]	55.0 ± 9.4	nt	56.1 ± 2.8	65.1 ± 2.6	nt	nt
V2W2	nt	94.1 ± 4.9	94.3 ± 8.8[b]	66.3 ± 4.7	54.2 ± 3.2	nt	56.0 ± 5.2	39.8 ± 3.5[ab]	nt
Emb2.4o	nt	nt	108.5 ± 4.3[ab]	64.2 ± 6.5	nt	49.7 ± 2.9	60.9 ± 4.2	41.2 ± 2.8[ab]	nt
P-value	0.0594	0.6670	0.0156	0.1695	0.2509	0.3884	0.4345	0.0170	0.1004
CV	8.1	13.5	20.6	18.8	14.5	12.6	13.3	17.7	11.8

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt(x +0.5) transformed data. Each column represents one experiment; nt= not tested. In columns, means followed by the same small letter (superscript) are not statistically different at P=0.05 according to Tukey's studentized range test.

Table 11: The effect of endophytic *Fusarium oxysporum* isolates on dry shoot weights of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8-weeks after inoculation with 500 nematodes.

Treatment	*Dry shoot weight (g)								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	22.8 ± 2.3	12.9 ± 1.9	12.7 ± 0.8^{b}	8.8 ± 0.7	6.6 ± 0.2	6.0 ± 0.3	14.3 ± 0.6	3.2 ± 0.4^{b}	6.6 ± 0.3
Eny.131i	21.4 ± 2.4	14.6 ± 2.1	15.6 ± 1.1^{ab}	6.7 ± 0.7	5.7 ± 0.7	nt	14.4 ± 0.5	5.2 ± 0.3^{a}	6.5 ± 0.3
V5W2	22.7 ± 2.6	12.6 ± 1.4	17.4 ± 0.7^{ab}	9.1 ± 0.5	nt	6.6 ± 0.4	14.4 ± 0.3	nt	7.1 ± 0.3
III4W1	22.5 ± 2.4	14.0 ± 1.7	18.7 ± 1.3^{a}	9.4 ± 0.5	6.5 ± 0.2	nt	13.8 ± 0.6	3.9 ± 0.3^{ab}	7.3 ± 0.3
V4W5	25.6 ± 2.4	16.7 ± 1.9	14.3 ± 1.2^{ab}	6.4 ± 0.8	6.2 ± 0.4	nt	14.8 ± 0.6	4.5 ± 0.4^{ab}	nt
Eny7.11o	23.0 ± 2.8	11.7 ± 0.6	16.1 ± 0.9^{ab}	8.9 ± 0.8	6.8 ± 0.3	nt	14.8 ± 0.4	4.5 ± 0.4^{ab}	nt
III3W3	23.1 ± 2.4	14.9 ± 1.7	15.5 ± 0.9^{ab}	8.9 ± 1.0	6.3 ± 0.3	nt	14.4 ± 0.4	3.3 ± 0.4^{ab}	nt
V1W7	nt	11.1 ± 1.5	15.7 ± 0.7^{ab}	7.3 ± 1.2	nt	6.7 ± 0.3	14.6 ± 0.4	nt	nt
V2W2	nt	10.5 ± 0.9	13.5 ± 1.1^{b}	8.6 ± 0.7	6.4 ± 0.3	nt	13.8 ± 0.8	4.9 ± 0.4^{ab}	nt
Emb2.4o	nt	nt	19.5 ± 1.9^{a}	8.4 ± 0.9	nt	6.1 ± 0.3	13.8 ± 0.4	4.9 ± 0.2^{ab}	nt
P-value	0.9420	0.1305	0.0004	0.1580	0.4129	0.3524	0.8623	0.0105	0.2183
CV	23.2	21.3	14.3	17.9	12.7	10.5	7.8	15.1	12.2

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt(x +0.5) transformed data. Each column represents one experiment; nt= not tested. In columns, means followed by the same small letter (superscript) are not statistically different at P=0.05 according to Tukey's studentized range test.

Table 12: The effect of endophytic *Fusarium oxysporum* isolates on the number of functional roots of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA), 8-weeks after inoculation with 500 nematodes.

Treatment	*Number of functional roots								
	Experiment No.								
	1	2	3	4	5	6	7	8	9
Control	18.4 ± 0.9	15.2 ± 1.3	14.3 ± 0.9^{ab}	9.0 ± 1.1	10.9 ± 0.8^{a}	10.1 ± 0.6^{a}	10.5 ± 1.0	5.8 ± 1.2	9.6 ± 0.8^{b}
Eny.131i	19.2 ± 0.8	14.4 ±1.2	14.3 ± 0.7^{ab}	5.8 ± 0.8	6.3 ± 1.2^{b}	nt	11.6 ± 0.8	8.3 ± 1.0	13.0 ± 1.2^{a}
V5W2	18.4 ± 0.8	16.8 ± 1.2	17.8 ± 0.7^{a}	7.9 ± 1.1	nt	8.1 ± 0.9^{ab}	11.1 ± 0.7	nt	10.8 ± 0.6^{ab}
III4W1	18.1 ± 0.6	16.7 ± 0.7	18.5 ± 0.7^{a}	9.4 ± 0.9	10.5 ± 0.7^{a}	nt	11.3 ± 1.1	6.6 ± 0.9	11.0 ± 0.6^{ab}
V4W5	18.4 ± 0.4	18.5 ± 0.7	16.5 ± 0.8^{ab}	7.8 ± 1.0	11.0 ± 1.0^{a}	nt	12.2 ± 0.9	6.3 ± 1.1	nt
Eny7.11o	18.4 ± 0.7	17.5 ± 1.3	15.7 ± 0.7^{ab}	9.3 ± 1.3	9.4 ± 0.8^{ab}	nt	11.5 ± 1.4	7.1 ± 0.7	nt
III3W3	18.0 ± 0.9	17.0 ± 0.8	15.2 ± 0.9^{ab}	7.6 ± 1.2	9.0 ± 0.9^{ab}	nt	10.3 ± 0.7	6.1 ± 0.9	nt
V1W7	nt	14.4 ±1.7	16.5 ± 0.9^{ab}	5.0 ± 0.8	nt	8.1 ± 0.7^{ab}	11.1 ± 0.9	nt	nt
V2W2	nt	14.7 ± 0.8	13.6 ± 1.0^{b}	8.1 ± 0.4	9.8 ± 0.7^{a}	nt	10.5 ± 1.1	8.0 ± 1.2	nt
Emb2.4o	nt	nt	14.6 ± 0.9^{ab}	8.7 ± 1.1	nt	6.7 ± 0.7^{b}	10.3 ± 0.7	6.9 ± 0.4	nt
P-value	0.9526	0.1816	0.0016	0.1497	0.0065	0.0339	0.9475	0.5846	0.0400
CV	9.6	15.9	12.4	24.4	24.7	20.7	20.8	25.0	20.2

* Mean ± S.E

Means and standard errors were calculated from untransformed data. Statistical analysis was performed on sqrt(x +0.5) transformed data. Each column represents one experiment; nt=not tested. In columns, means followed by the same small letter (superscript) are not statistically different at P=0.05 according to Tukey's studentized range test.

Table 13: Percentage colonization of 16-week-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA) by various endophytic *Fusarium oxysporum* isolates inoculated at the weaning stage.

Fungal isolate	*Colonization of roots (%)		
	Experiment No.		
	2	7	9
Control	45.2 ± 8.3	36.0 ± 12.7	16.6 ± 4.3
Eny.131i	68.8 ± 5.2	48.0 ± 20.3	17.5 ± 4.9
V5W2	58.4 ± 4.7	34.6 ± 15.1	22.4 ± 5.7
III4W1	44.9 ± 9.3	25.3 ± 12.0	17.5 ± 5.3
V4W5	50.3 ± 6.0	24.0 ± 10.8	nt
Eny7.11o	63.3 ± 6.1	65.3 ± 8.3	nt
III3W3	47.4 ± 8.9	36.0 ± 13.1	nt
V1W7	56.9 ± 4.2	38.6 ± 7.7	nt
V2W2	49.1 ± 4.0	53.3 ± 15.3	nt
Emb2.4o	nt	22.6 ± 11.6	nt
P-value	0.1171	0.4467	0.8519

* Mean ± S.E
nt=not tested

Chapter 4

Effect of endophytic *Fusarium oxysporum* isolates on host preference, attraction, root penetration and reproduction of *Radopholus similis* in tissue culture banana plants

Abstract

The effects of three endophytic *Fusarium oxysporum* isolates, *V5W2*, *Eny1.31i* and *Eny7.11o*, on host preference and attraction, root penetration and reproduction of *Radopholus similis* in tissue culture banana plants *cv*. Enyeru (*Musa* spp., AAA-EA) were evaluated under laboratory and screen house conditions. Treatment of tissue culture banana plants with the three isolates did not alter host preferences and attraction of *R. similis*, as similar numbers of nematodes migrated towards plants and root segments from both endophyte-inoculated and uninoculated plants. The number of *R. similis* that penetrated the roots of endophyte-inoculated and un-inoculated plants was not influenced by the three endophytic fungal isolates, either in the laboratory or in the screen house. *Radopholus similis* reproduction was, however, highly influenced by endophyte infection. Differences in *R. similis* reproduction rates between endophyte-inoculated and un-inoculated plants were observed at 50 and 75 days after nematode inoculation. No differences were observed 25 days after inoculation. Endophyte-un-inoculated plants supported higher nematode densities than endophyte-inoculated plants, and the nematode reproduction rates in control plants were 10, 4 and 3 times higher than in plants treated with isolates *V5W2*, *Eny7.11o* and *Eny1.31i* at 75 days after inoculation, respectively. Isolate *V5W2* resulted in the highest reduction in *R. similis* reproduction for all nematode stages (females, males and juveniles). The results of this study imply that the early processes of banana root infection by *R. similis* are not affected by endophytes and that the main endophyte effects are post-infectional and occur only during the nematode reproduction phase.

Introduction

Radopholus similis (Cobb) Thorne is a migratory endoparasite that completes its life cycle in the roots and rhizomes of banana plants (*Musa* spp.) in 20 to 25 days (Gowen and Quénéhervé, 2005). Only female and juvenile stages are infective, as males have degenerate stylets. *Radopholus similis* penetrates banana roots at the root apex mainly, but penetration can also occur at any position along the root (Sarah *et al*., 1996). After root penetration, the nematodes occupy the intercellular spaces of the cortical parenchyma. Nematodes may migrate both within and between cells in the root cortex to feed on cell cytoplasm. This results in collapsed cell walls, cavities and tunnels in the root (Sarah *et al*., 1996). Symptoms of nematode damage become visible as reddish brown necrotic patches that are confined to the cortex region. In the rhizome, necrosis appears as a reddish-brown discoloration that begins where roots attach to the rhizome (Speijer and De Waele, 1997). Nematode feeding destroys root and rhizome tissue, reduces water and mineral uptake, and results in a reduction of plant growth and development. This leads to a severe reduction of bunch weight and a significant increase in time between successive harvests (Sarah *et al.,* 1996; Gowen *et al*., 2005).

During their initial life stages nematodes migrate through the rhizosphere to the plant roots (Kaplan and Keen, 1980). Host recognition involves signals from plant roots that influence egg hatch, attraction towards roots and root penetration (Zhao *et al*., 2000). Plant-parasitic nematodes are attracted to plant roots by a variety of factors, which may operate over considerable distances (Prot, 1980; Prot and Van Gundy, 1980; Zuckerman and Jansson, 1984; Spiegel *et al*., 2001; Luc *et al*., 2005). The major factors of nematode attraction to plant roots are chemostatic factors and secretions emanating from the host plant, while minor factors include thermal, vibratory and tactile stimuli (Prot, 1980; Zuckerman and Jansson, 1984; Perry, 1996). Following attraction, nematodes penetrate the host plant cells by using their stylets where they feed and reproduce.

Differences in the response of the host plant to nematode infection have been used to classify plants as resistant, tolerant, intolerant, host or non-host. Resistant plants allow for nematode penetration but not reproduction. Tolerant plants suffer little damage even when heavily infested, while susceptible plants suffer heavy damage under light nematode infestations. Plants are further classified as hosts or non-hosts depending on whether nematode

reproduction occurs or not. Plants that allow high nematode reproduction are classified as good hosts, while those that allow for low nematode reproduction are classified as non-hosts (Luc *et al*., 2005).

Antagonistic microorganisms can reduce early root infection by nematodes. Oostendorp and Sikora (1989; 1990) reported a reduction in egg hatch and early root infection of sugar beet (*Beta Vulgaris* L.) by the sugar beet nematode *Heterodera schachtii* Schmidt after seed treatment with antagonistic rhizobacteria. However, the application of rhizobacteria to the root surface of sugar beet seedlings did not alter migration of *H. schachtii* second stage juveniles. The authors concluded that bacterial alteration of root exudates might have influenced nematode hatch, attraction and root penetration behavior.

Endophytic *Fusarium oxysporum* Schlecht.: Fries reduced *R. similis* populations in tissue culture banana plants (Chapter 3; Pocasangre, 2000; Niere, 2001; Gold and Dubois, 2005). The mechanism through which nematode populations were reduced, however, is unknown. The objectives of this study, therefore, were to determine the effect of endophytic *F. oxysporum* isolates on (i) host preferences and relative attractiveness of endophyte-inoculated plants to *R. similis* (ii) root penetration by *R. similis* and (iii) reproduction of *R. similis* in tissue culture banana plants.

Materials and methods

Site description

Experiments were carried out in the laboratory or screen house at the International Institute of Tropical Agriculture (IITA) Research Station in Namulonge-Uganda, approximately 30 km Northeast of Kampala, Uganda. The screen house air temperatures ranged from 19.1 to 32.8ºC, while the soil temperature in the polythene bags ranged from 20.4 to 26.3ºC. The site is situated at 1150 m above sea level, 32º 34'N latitude, with a mean annual rainfall of 1255 mm and an average temperature of 22ºC.

Fungal isolates and nematode cultures

Three *F. oxysporum* isolates (*V5W2*, *Eny1.31i* and *Eny7.11o*) were included in all experiments. These isolates were obtained from healthy East African highland cooking banana plants roots and rhizomes in Uganda (Schuster *et al*., 1995) and are preserved in soil tubes (Niere, 2001). The isolates were selected on their ability to consistently reduce nematode population build-up in banana plants in *in vivo* screening experiments (Chapter 3). Pure *R. similis* cultures maintained on carrot disks were used as the source of nematode inoculum (Chapter 1; Speijer and De Waele, 1997).

Tissue culture plants

Tissue culture banana plants of the cultivar Enyeru (*Musa* spp. AAA-EA) were used in this study. The plants were micropropagated from sword suckers using standard shoot-tip culture protocols for banana (Vuylsteke, 1998). When ready for weaning, plants were transferred to an aquaculture system comprised of 250-ml lidded plastic pots filled with 200 ml of nutrient solution to allow for root development prior to inoculation with the endophytes (Fig. 1). The nutrient solution was prepared by dissolving a commercial fertilizer in sterilized (autoclaved at 121ºC for 15 min) tap water. In the root penetration experiments, plants were fertilized with Multifeed Classic (Gouws and Scheepers Ltd., Witfield, South Africa) at a rate of 2.5 g/L (Table 1). For the *R. similis* host preference, attraction and reproduction experiments, plants were grown in 1 g/L of Poly-Feed (Haifa Chemicals, Haifa Bay, Israel) (Table 1). In each pot, a plant was supported by the lid with the roots submerged in the nutrient solution. Strips of sterile sponge were used to stabilize the plants through a hole made in the middle of the lids. To simulate dark soil conditions and to discourage growth of algae on the roots, the pots were wrapped with a brown paper bag. The plastic pots then were transferred to a humidity chamber (Chapter 3) for a 1-month acclimatization period, during which the nutrient solution was renewed weekly.

Inoculation of plants with fungal isolates

The fungal isolates were pre-grown on synthetic nutrient agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar/L distilled water) in 65-mm-diameter Petri dishes at ± 25°C and 12 hrs light, 12 hrs darkness photoperiod (laboratory conditions) for 1 week. The SNA medium was supplemented with 10 mg chlortetracycline, 100 mg penicillin G and 50 mg streptomycin-sulphate/L to prevent bacterial contamination. Half strength potato dextrose broth (PDB) (Sigma-Aldrich, MO, USA) was prepared by dissolving 12 g of PDB in 1 L of distilled water. One hundred-ml aliquots of PDB were dispensed into 250-ml Erlenmeyer flasks and sterilized. After cooling, flasks were inoculated with 4 to 5 disks of agar of each fungal isolate. Uninoculated PDB was used as the control treatment. Duplicate flasks were prepared for each fungal isolate and the control. Inoculated flasks were incubated in the laboratory for 7 days to allow for fungal growth and sporulation. Fungal spore suspensions were filtered through a 1-mm-diameter sieve to remove mycelial fragments. The spore suspensions were then adjusted to provide a final spore count of 1.5×10^6 spores/ml.

For the inoculation of banana tissue culture plants with endophytic *F. oxysporum* isolates, 1-month-old plants were removed from the nutrient solution and the roots cut back to 10 cm in length. Plants were selected for uniformity according to size and subdivided among the different treatments. To inoculate the plants with the different fungal isolates, the root systems were dipped in the different fungal spore suspensions for 2 hrs. Control plants were dipped in sterile un-inoculated PDB for the same duration of time. After fungal inoculation, plants were transplanted in steam-pasteurized heavy loamy soil either into 3-L polythene bags for the root penetration and reproduction experiments, or into 250-ml plastic pots for the host preferences and attraction experiments.

To determine colonization of plant roots by the fungal isolates, three healthy primary roots were randomly selected from each plant at harvest and surface sterilized in 75% ethanol for 1 min, followed by sterilization in 2% NaOCl for 30 s. Root pieces were blotted dry on sterile tissue paper and cut into ca. 0.25 cm-long segments. Six segments per root were randomly selected and placed on SNA in 65-mm-diameter Petri dishes. The plates were incubated in the laboratory for 7 days under laboratory conditions. Fungal colonies growing from the root

pieces were identified under a light microscope (magnification x 400) as *F. oxysporum* based on the presence of microconidia carried in false heads on short phialides, the shape of macroconidia and the presence of chlamydospores (Nelson *et al*., 1983). The number of root pieces with *F. oxysporum* colonies were recorded and the percentage recovery of the fungus calculated.

Host preference and attraction experiments

Two experiments were conducted to investigate host preference and relative attractiveness of endophyte-inoculated and un-inoculated (control) banana plants to *R. similis*. Each experiment was repeated once. These included a detached root bioassay where root segments from endophyte-inoculated and uninoculated banana plants were paired in a Petri dish, and an intact plant bioassay using endophyte-inoculated and un-inoculated banana plants paired in a polyvinyl chloride (PVC) tube apparatus. Both experiments had seven treatments that involved combinations of the three endophyte-inoculated *V5W2* (E1), *Eny1.31i* (E2) and *Eny7.11o* (E3) and uninoculated (C) banana plants in the following pairs: C-C, C-E1, C-E2, C-E3, E1-E1, E2-E2 and E3-E3. The detached root bioassay and the intact plant bioassay were replicated four and five times, respectively.

Detached root bioassay for host preference and attraction

Endophyte-inoculated and uninoculated plants were uprooted 1 month after being transplanted into the 250-ml pots and washed free of soil. In the laboratory, healthy roots of the same age and size were selected from the plants and cut into 1-cm-long segments. For each replication, root segments were obtained from the same plant. Root segments from endophyte-inoculated and uninoculated plants were paired and placed on opposite sides in 90-mm-diameter Petri dishes filled with a 50-mm thick layer of moistened sterile sand (Fig. 2). The bottom of the Petri dish was divided into two equal sections, A and B, and two root segments from either an endophyte-inoculated or uninoculated plant placed in each of the sections. The distance between the root segments was 70 mm, and each root segment was placed 10 mm away from the wall of the Petri dish. Petri dishes were covered and left to stand for 12 hrs. Approximately 500 *R. similis* mixed stages (females, males and juveniles) in 0.3 ml water were inoculated in the middle of the Petri dish equal distances from the root

segments using a micropipette. After 24 hrs, nematodes were extracted from the sand and from root segments on either side of the inoculation point by means of the modified Baermann technique (Hooper *et al*., 2005). Extraction from the sand was carried out for 48 hrs by spreading the sand on the tissue paper in the modified Baermann funnel apparatus. Nematodes were extracted for 24 hrs from the root segments following maceration in a Waring blender (Waring, Connecticut, USA) at low speed for 15 s.

Intact plant bioassay for host preference and attraction

A PVC tube apparatus was designed to determine whether *R. similis* is differentially attracted to endophyte-inoculated or uninoculated (control) banana plant roots (Prot, 1979). The PVC apparatus consisted of a 22.5–cm-long and 5-cm-diameter PVC tube divided into seven sections, each 2.5 cm long (Fig. 3). The sections were filled with moistened sterile heavy loam soil and joined together with adhesive tape. The use of PVC pipes not only reduced the amount of soil from which nematodes had to be extracted, but also restricted nematode movement from the point of inoculation to either of the two direction.

Endophyte-inoculated and uninoculated plants transplanted into 250-ml plastic pots were grown until they were 8 weeks old. An endophyte-inoculated plant was then paired with an uninoculated plant on opposite sides of the PVC apparatus. The bottom parts of the pots were first removed to expose the roots, and the pots were then fixed to the ends of the PVC tubes in section number 1 and number 7 (Fig 3). Treatments were paired in such a way that the first plant which is either endophyte-inoculated (E1, E2 or E3) or uninoculated (C) in each treatment pair was placed in PVC section number 1 and the last in section number 7. A 3-mm-diameter opening was drilled at the top of the middle of each PVC section to facilitate watering and nematode inoculation.

One week after transplanting the banana plants into the PVC apparatus, 2000 mixed stages of *R. similis* were pipetted into the soil in the middle section (section 4) that was an equal distance from both plants. The tubes were maintained horizontally at laboratory conditions. The PVC sections were separated 5 days after introduction of the nematodes by cutting through the adhesive tape using a knife. The number of nematodes in each section was determined by extracting the nematodes from the soil over 48 hrs using the modified

Baermann funnel method (Hooper *et al*., 2005). Nematode extraction from the plant's roots in sections number 1 and 7 was conducted for 24 hrs after maceration in a Waring blender for 15 s. Nematode suspensions were concentrated on a 28-µm sieve prior to counting.

Root penetration experiments

To study the effect of endophytes on root penetration by *R. similis*, two experiments were conducted on 8-week-old banana plants inoculated with three different fungal endophytes (*V5W2*, *Eny1.31i* and *Eny7.11o*). The first experiment involved the *in vitro* root penetration of endophyte-inoculated and un-inoculated plants by nematodes in the laboratory. The second experiment was an *in vivo* root penetration experiment in the screen house, conducted as a time course study. Nematode inoculation was performed on the whole root system and plants were sampled to determine the number of nematodes that had penetrated into the roots 3, 6 10 and 14 days after inoculation. Six plants were randomly harvested after each time interval. Each experiment was repeated twice.

In vitro *root penetration experiments*

Endophyte-inoculated and un-inoculated banana plants were uprooted from their polythene bags and their roots washed free of soil. These plants were then transplanted to rectangular plastic trays (90 x 143 x 230 mm) that were filled with *ca*. 500 cm^3 moistened sterile sand (Fig 4). A hole was cut on the side of the trays, and a slit made from the rim to the hole to facilitate placement of the plant. A strip of sponge was used to stabilise the plants and avoid damage to their pseudostems. Three 65-mm-diameter Petri dishes in which two V-shaped cuts were made on the opposite sides were placed inside the trays and filled with moistened sterile sand. Three healthy primary roots of the same size and age were randomly selected from the transplanted plants, and each one of roots placed inside one of the three Petri dishes.

A nematode suspension containing a total of 50 *R. similis* mixed stages in *ca*. 0.1 ml of water was pipetted directly on the root segments in each Petri dishes 24 hrs after transfer of the plants. The roots were then covered with sterile sand. The plastic trays were covered with a lid to prevent dehydration of the plants and root segments. Inoculated root segments were excised after 24 hrs and stained with acid fuschin to observe nematodes that had penetrated

the roots (Byrd *et al*., 1983). After staining, root segments were flattened between two microscope slides and the number of nematodes visible in the root tissue was recorded. There were five plants for each treatment, with three roots selected per plant, each representing a single replication (i.e. a total of 15 replications per treatment).

In vivo root penetration experiments

Eight-week-old endophyte-inoculated and un-inoculated banana plants grown in 3-L polythene bags were inoculated with a nematode inoculum containing 500 mixed stages of *R. similis* per 2 ml water as follows. Three holes were made at the base of the plants around the roots. To each hole, *ca*. 0.6 ml of the nematode suspension was pipetted, and the hole covered with soil. Six plants from each treatment were then uprooted 3, 6, 10 and 14 days after nematode inoculation. Nematodes were extracted from 5 g of each root samples by first macerating them in a Waring blender at low speed for 15 s, and subsequently collecting the nematodes for 24 hrs using a modified Baermann technique (Hooper *et al*., 2005). Nematode suspensions were transferred into sample bottles and kept in the fridge at 4ºC until counted. Prior to counting, nematode suspensions were concentrated on a 28-µm sieve.

Radopholus similis reproduction experiments

A single-root inoculation technique developed for screening banana plants for resistance to *R. similis* (De Schutter *et al*., 2001) was adopted to study the effects of endophytes on *R. similis* reproduction. One-month-old endophyte-inoculated and un-inoculated plants were grown for a further 2 months in 3-L plastic polythene bags after acclimatization in the humidity chamber. The 3-month-old plants were then uprooted and their roots separated. The plants were placed into 24 x 54 cm plastic buckets filled three quarters with sterile sawdust (Fig. 5). Three equally developed primary roots were selected from each plant and planted in 150-ml plastic pots filled with moistened sterile sand. After transplanting, the roots not planted in the pots were covered with sterile soil. A nematode suspension containing 50 *R. similis* in approximately 0.3 ml of sterile water was pipetted directly onto each of the root segments planted inside the plastic pots. The inoculated roots were then also covered with moistened sterile sand. Following inoculation, the banana plants were maintained in the screen house (20.4 to 26.3ºC) and watered two times per week.

Nematodes were extracted from inoculated banana roots 25, 50 and 75 days after nematode inoculation. At each harvest date, five plants were randomly selected and the plastic pots excavated from the sawdust. The root segments inside the pots were removed and washed with tap water. The percentage root necrosis was determined by slicing each root segment lengthwise and estimating the percentage necrotic area (Speijer and De Waele, 1997). The sliced root segment was then cut into 0.5-cm pieces and macerated in a Waring blender at low speed for 15 s. Nematodes were extracted from the macerated root tissue over 24 hrs using the modified Baermann funnel method (Hooper *et al*., 2005). All *R. similis* stages (females, males, juveniles) were counted. Nematode reproduction rates (RR) were calculated by dividing the final population density (*Pf*) with the initial population density (*Pi*). Reisolation of fungal endophytes was conducted on the root segments adjacent to nematode inoculated root segment as described above.

Data analysis

Nematode data in each experiment were tested for normality and homogeneity of variances using the Shapiro-Wilkinson, Levene-Welch and Kolmogorov tests. Normal probability plots, box plots and stem leaf plots were additionally used to confirm normality of data. If not normally distributed with equal variances across treatments, various transformations were tested until the most suitable transformation was obtained.

For the host preference and nematode attraction bioassays using detached roots in Petri dishes, statistical analysis was performed on untransformed data. One-way ANOVA was used to demonstrate differences in the total number of nematodes in each treatment pair. If different, treatment means were separated using Tukey's Studentized range test. Within each treatment pair, differences between treatments were evaluated using a paired t-test.

For the bioassay using intact plants in the PVC tube apparatus and for the *R. similis* reproduction experiments, nematode counts were square root (sqrt) (x+0.5)-transformed prior to analysis. Within each treatment, differences between the proportion of nematodes attracted to either endophyte-inoculated or uninoculated plants were evaluated using a paired t-test. For the reproduction experiments, a two-way ANOVA was conducted to demonstrate variability among treatments (control and fungal isolates) and days after nematode inoculation. Prior to

analysis, the nematode RR was sqrt-transformed x+0.5 to normalise data. Percentage root necrosis and colonization were arcsine sqrt-transformed x+0.5 prior to analysis. If different, means were separated using Tukey's studentized range test (SAS Institute, 1989).

Results

Host preference and attraction experiments

Detached root bioassay for host preference and attraction

Similar numbers of nematodes were attracted to banana roots, whether they were inoculated or uninoculated with *F. oxysporum* endophytes (Fig. 6 and 7) Comparison between the total numbers of *R. similis* that migrated towards opposite sections of the Petri dish containing either an endophyte-inoculated or un-inoculated root segment revealed no significant differences between any of the treatments pair (Table 2). When two endophyte-uninoculated root segments (treatment pair C-C) were paired in opposite sides of a Petri dish, the number of nematodes attracted to either root segment did not differ significantly (Table 2). Likewise, when an endophyte-uninoculated root segment was paired with an endophyte-inoculated root segment (treatment pairs C-E1, C-E2 and C-E3), the number of nematodes that migrated towards either root segment did not differ significantly. When two endophyte-inoculated root segments were paired (treatment combinations E1-E1, E2-E2 and E3-E3), no differences were observed in the number of *R. similis* that migrated towards either root segment (Table 2). The only exception was observed in experiment 2, when two root segments infected with isolate *Eny1.31i* (E2-E2) and isolate *Eny7.11o* (E3-E3) were paired, where significant differences in the number of *R. similis* that migrated to either root segment were observed (Table 2).

Intact plant bioassay for host preference and attraction

THE EXPERIMENTS USED TO DETERMINE NEMATODE ATTRACTION SHOWED THAT THE NUMBER OF NEMATODES (EXPRESSED AS A PERCENTAGE OF THE TOTAL NEMATODES RECOVERED PER TREATMENT) THAT MIGRATED FROM THE POINT OF INOCULATION IN PVC TUBES DID NOT DIFFER BETWEEN THE TREATMENT PAIRS. IN EXPERIMENT 1 (*P*=0.1544), THE PROPORTION OF NEMATODES THAT MIGRATED RANGED FROM 10.2 ± 3.5% IN TREATMENT PAIR E3-E3 (TWO *ENY7.11O*-INFECTED PLANTS PAIRED) TO 35.9 ± 3.5% IN C-C (WHEN TWO CONTROL PLANTS WERE PAIRED). IN BIOASSAY 2 (*P*=0.3776) THE PROPORTION OF NEMATODES THAT MIGRATED FROM THE POINT OF INOCULATION RANGED FROM 40.4 ± 1.6 WHEN TWO *ENY7.11O*-TREATED PLANTS WERE PAIRED TO 57.7 ± 10.1% WHEN A CONTROL PLANT WAS PAIRED WITH A PLANT TREATED WITH *ENY.11O*. PAIRED T-TESTS DEMONSTRATED THAT THE PROPORTION OF

NEMATODES THAT MIGRATED TO OPPOSITE SIDES OF THE INOCULATION POINT TOWARDS EITHER AN ENDOPHYTE-INOCULATED PLANT OR UNINOCULATED PLANT DID NOT DIFFER AMONG THE TREATMENT PAIRS IN BOTH BIOASSAYS EXCEPT IN THE TREATMENT WERE TWO *ENY7.11O*-INFECTED PLANTS WERE PAIRED (TABLE 3).

In both bioassays, the total number of nematodes present in each section of the PVC tube differed significantly between the sections ($P<0.0001$) and was influenced by the distance from the nematode inoculation point. In all treatment pairs, most nematodes were found in the middle section (section 4), the point of nematode inoculation (Fig. 8 A and B), with less nematodes in sections further from the point of nematode inoculation. The number of nematodes recorded in sections at the same distance from the point of nematode inoculation was not different within a given treatment ($P<0.05$).

Root penetration experiments

In vitro experiments

No significant difference was found in the number of nematodes that penetrated root segments of endophyte-inoculated and uninoculated plants 24 hrs after inoculation with *R. similis* ($P=0.6064$, 0.2528 and 0.8752 for experiment 1, 2 and 3 respectively) (Fig. 9). Nematodes were observed in the cortex region of the root segments only and not the stele.

In vivo experiments

No significant differences in the penetration of banana roots by *R. similis* were observed between the endophyte-inoculated and uninoculated banana plants planted in polythene bags (Table 4). There were also no significant differences in the number of nematodes that penetrated the roots 3, 6, 10 and 14 days after nematode inoculation for any of the treatments.

Radopholus similis reproduction

Endophyte treatment significantly reduced the number of *R. similis* females, males, juveniles and the total nematode density at 75 days after nematode inoculation (DAI) in the first

experiment, but not the second one (Table 5). In the former, the percentage reduction in *R. similis* females was 65.0%, 67.4% and 35.5% after 75 days, whilst juvenile numbers compared to the control were lower by 55.2%, 77.4% and 90.8% in plants infected with *Eny1.31i*, *Eny7.11o* and *V5W2*, respectively. The reduction in the number of *R. similis* males was 63.4%, 83.6% and 92.8% and the reduction in the total nematode density was 65.7%, 76.5% and 90.2% in plants treated with the fungal isolates *Eny1.31i*, *Eny7.11o* and *V5W2* respectively compared to control plants (Table 5). In the second experiment, only isolate *V5W2* resulted in less females, males, juveniles and the total nematode density although this did not differ significantly from the control. Isolates *Eny1.31i* and *Eny7.11o*-treated plants had more nematode than the controls 75 DAI (Table 5).

No differences were observed in the number of females, juveniles, males and the total nematode density between endophyte-inoculated and un-inoculated plants at 25 and 50 DAI, (Table 5). Although not significantly different, *R. similis* female numbers were lower by 71.1, 40.5 and 23.0% in plants infected with the fungal isolates *Eny1.31i*, *Eny7.11o* and *V5W2* 50 DAI, respectively, compared to the un-inoculated plants. Similarly, the number of *R. similis* juveniles was lower by 50.7%, 51.7% and 18.6% in plants treated with the fungal isolates *Eny1.31i*, *Eny7.11o* and *V5W2*, respectively (Table 5). The most important reduction in the second experiment, although not significant, was achieved with *Eny1.31i* that reduced nematode numbers by 62.9% after 25 DAI, and after 50 days, juvenile numbers by 78.5%, 24.3% and 43.4%, and males were reduced by 74.2, 15.2 and 37.7% in plants treated with isolates *Eny1.31i*, *Eny7.11o* and *V5W2* respectively.

The DAI affected the female, male, juvenile and total *R. similis* density significantly influenced ($P<0.0001$) in both endophyte-inoculated and uninoculated plants. No differences were observed in the nematode numbers at 50 and 75 DAI in both endophyte-treated plants while the number of nematodes in both endophyte-inoculated and un-inoculated plants at 25 DAI were significantly lower than those at 50 and 75 DAI ($P<0.0001$).

Treatment with endophytes did not significantly reduce the damage caused by nematodes to banana roots (percentage root necrosis) ($P=0.5593$), but the damage was reduced significant over time ($P=0.0239$) (Table 6). At 25 and 75 DAI, plants had similar levels of damage, whether they were inoculated with endophytes or not. The only significant differences in

percentage root necrosis were observed in experiment 1 50 DAI where plants treated with *V5W2* and *Eny1.31i* had significantly more necrosis than control plants, but not in experiment 2 (Table 6).

Nematode reproduction rate (RR) was significantly reduced by endophyte treatment after 75 days in experiment 1, but not experiment 2 (Table 6). At 25 and 50 DAI, no significant differences were observed in the RR between endophyte-inoculated and un-inoculated plants. For both endophyte-inoculated and un-inoculated plants, the nematode RR increased with an increase in DAI, except for *V5W2*-treated plants where the RR was higher at 50 DAI than at 75 DAI (Table 7).

Endophytic colonization of banana roots

In the *in vivo* root penetration experiments, the percentage colonization of plants treated with isolates *Eny1.31i* (16.9 ± 6.8%), *Eny7.11o* (15.5 ± 5.9%) and *V5W2* (20.5 ± 5.5%) was higher than in the control plants (3.6 ± 2.9%). Root colonization increased with increase in the days after nematode inoculation (DAI). In experiments 1 and 3, colonization was significantly influenced by treatment (P=0.0003) but not by DAI (P=0.6210). Lowest root colonization was recorded in endophyte-un-inoculated plants compared to colonization rates in roots inoculated with the various fungal isolates.

At all harvest dates in the first *R. similis* reproduction experiment, percentage colonization of roots by endophytic *F. oxysporum* did not differ between endophyte-inoculated and un-inoculated plants (Table 6). Significant differences, however, were found between un-inoculated and endophyte-inoculated plants 25 (P=0.0002) and 75 (P=0.0004) DAI. Although *F. oxysporum* was re-isolated from control plants 50 DAI, no isolations were made 25 and 50 DAI in either experiment. Still, the difference between the control and endophyte treatments was not significant, even where control yielded 0% re-isolation, and the treated plants 61% re-isolation.

Discussion

This study has demonstrated that endophyte-inoculated roots of tissue culture banana plants did not influence host preferences, attraction and penetration by *R. similis*. This is evident from the lack of repulsion or attraction of *R. similis* in the host preference and attraction experiments. Similar penetration rates into plant roots indicate that the initial processes of host recognition and root penetration, and invasion are not affected by the endophytes tested. The results suggest that, once in the field, roots of plants infected with the endophytic *F. oxysporum* isolates used in this study will still be infected by *R. similis*. However, endophytes inhibit reproduction of the nematode over several generations.

The results obtained in the current study are in contrast to those obtained with other fungal endophytes. Bernard and Gwinn (1991) reported that more nematodes (*Pratylenchus scribneri* Steiner) migrated towards endophyte-free tall fescue (*Festuca arundinacea* Schreb) root segments when both endophyte-infected and endophyte-free root segments were paired in a petri dish. Evidence that biological control agents other than endophytes affect early root infection by nematodes was demonstrated by Oostendorp and Sikora (1989; 1990). These authors reported a reduction in egg hatch and early root infection of sugar beet by the sugar beet nematode, *H. schachtii* after seed treatment with antagonistic rhizobacteria. Treatment of sugar beet seedlings with the rhizobacteria did not alter migration of *H. schachtii* second stage juveniles. These examples, however, involve different crops and nematode species and thus the response in banana may be different.

The results obtained in this study are in contrast to results obtained by Pocasangre (2000) who reported reduction in root penetration by *R. similis* in Cavendish banana plants treated with endophytic isolates of *Fusarium* spp. The contradiction in results may be due to the fact that the banana cultivars, *R. similis* populations and endophytic isolates in the two studies were different and experiments were conducted under different environmental conditions. In grasses, the presence of morphological barriers such as thickening of the endodermis in tall fescue (*Festuca arundinacea* Shreb.) infected by the endophyte *Neotyphodium coenophialium* ([Morgan-Jones and Gams] Glenn, Bacon, and Hanlin) was associated with reduced root penetration of root-knot nematodes *Meloidogyne marylandi* Jepson and Golden (Gwinn and Bernard, 1993) but not of the migratory nematode *Pratylenchus scribneri* Steiner. Reduced

root penetration subsequently led to reduced growth and reproduction of *M. marylandi* in the endophyte-infected grasses. In the current study, penetration into roots of endophyte-treated as well as untreated plants may be an indication that the endophytes did not induce morphological and structural changes that limit nematode penetration.

The complete life cycle of *R. similis* takes 20-25 days a t a temperature range of 24-32ºC (Loos, 1962). In the current study, nematodes reproduced both in endophyte-inoculated and un-inoculated plants. The nematode reproduction rates were, however, lower in endophyte-inoculated plants compared to uninoculated plants. Endophyte-inoculation did not have an effect on the first *R. similis* generation; reduction in nematode populations was observed at the second and third generation only. It is probable that, at 25 DAI, the nematodes that were found in the roots were those that survived after initial inoculation. The lower reproduction rates of *R. similis* in endophyte-treated plants during the second and third generations may probably be due to inhibition of nematode migration inside the plant roots, interference with the feeding processes or reduction in nematode reproduction capacity (Oostendorp and Sikora, 1989; 1990; Jones and Bernard, 1997). These conclusions, however, require further investigation. Another implication of these results is that endophytes may protect banana plants against *R. similis* for multiple nematode generations. Banana is a perennial crop and protection for several seasons by endophytes would greatly benefit the plant (Sikora *et al*., 2003). Hunt *et al*. (2005) defined resistant plants as those that allow for nematode penetration but restrict nematode reproduction. Based on this definition, endophyte infection of tissue culture banana plants restricted nematode reproduction and thus conferred some degree of resistance to the host plant. The lack of differences in *R. similis* reproduction between endophyte-inoculated and uninoculated plants in one of the experiments may have been due to differences in the vigour of both the plants and nematode inoculum used. Large variation in the data may also be responsible for the observed lack of differences. Nevertheless, endophyte-inoculated plants supported less nematodes than uninoculated plants.

Endophytic colonization was higher in the endophyte-inoculated plants than in the non-inoculated plants, but not significantly different. The true identity of the re-isolated endophytic *F. oxysporum* strains, however, is unknown, since endophytic *F. oxysporum* strains have also been re-isolated from the control treatment. The endophytic *F. oxysporum* from non-inoculated plants could have come from the soil, water or screen house

environment. Before it is possible to deduce whether the inoculated *F. oxysporum* indeed contributed to the reduction in reproduction rates, and also to the non-significance of endophyte treatment in host preference, attraction and penetration, it would be necessary to conduct these experiments under greenhouse conditions that prevent the re-introduction of endophytes into control plants. Colonization of control plants by *F. oxysporum*, however, is a common occurrence in screen house studies, and has been reported before by Niere (2001) and Paparu (2005). Niere (2001) reported colonization of uninoculated plants of up to 40%, while Paparu (2005) reported up to 15% colonization of uninoculated plants.

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Table 1. Nutrient composition of Multifeed Classic (Gouws and Scheepers Ltd., Witfield, South Africa) and Polyfeed fertilizers (Haifa Chemicals, Haifa Bay, Israel) used to enhance root development of tissue culture plants prior to fungal inoculation.

Ingredient	Multifeed Classic (g/kg)	Polyfeed (g/kg)
Nitrogen	190	0.19
Phosphorus	82	0.19
Potassium	158	0.19
Iron	0.75	1
Manganese	0.3	0.5
Boron	1	0.2
Zinc	0.35	0.15
Copper	0.075	0.11
Molybdenum	0.07	0.07
Magnesium	0.9	0

Table 2. Paired *t*-tests for the differences in the total number of *Radopholus similis* present in the sand, roots and in both sand and roots, which migrated towards root segments obtained from endophyte-inoculated or un-inoculated plants paired in a Petri dish.

		Bioassay 1						Bioassay 2					
		Sand		Root		Sand + Root		Sand		Root		Sand + Root	
Treatment pair *	Difference**	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|
C-C	A-B	2.37	0.0986	0.28	0.7999	2.23	0.1119	1.29	0.2861	-0.34	0.7587	1.09	0.3538
C-E1	A-B	0.64	0.5684	-2.71	0.0729	0.45	0.6860	0.41	0.7116	1.36	0.2671	0.65	0.5641
C-E2	A-B	0.63	0.5723	-0.90	0.4339	0.35	0.7492	-0.49	0.6749	-0.8	0.4798	-1.31	0.2809
C-E3	A-B	1.28	0.2918	-0.42	0.7016	0.92	0.4236	1.43	0.2474	-0.72	0.5239	1.26	0.2982
E1-E1	A-B	1.62	0.2033	1.68	0.1920	2.11	0.1253	1.36	0.2666	-0.61	0.5848	1.16	0.3315
E2-E2	A-B	-1.77	0.1749	0.11	0.9173	-2.06	0.1312	-4.01	0.0279	-0.78	0.4917	-3.46	0.0407
E3-E3	A-B	-0.50	0.6512	0.28	0.7987	-0.42	0.7017	-3.35	0.044	0.73	0.5158	-2.97	0.0589

*C= control, E1=endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*. E3=endophyte isolate *Eny7.11o*, root segments from either control or endophyte-infected plants were paired in a Petri dish as indicated above.

**The Petri dishes were divided into two sections, A and B, with the first and second treatment in each treatment pair placed in section A and B respectively (Fig. 2).

Table 3: Paired *t*-tests for the differences between the proportion of nematodes that migrated towards an endophyte-inoculated or un-inoculated plant in the intact plant host preference and attraction bioassays conducted using PVC tube apparatus.

		Bioassay 1		Bioassay 2	
Treatment pair*	Difference**	T Value	P>|t|	T Value	P>|t|
C-C	A-B	2.17	0.0958	-0.59	0.5877
C-E1	A-B	0.76	0.4879	-1.40	0.2344
C-E2	A-B	-1.05	0.3517	-1.70	0.1644
C-E3	A-B	0.26	0.8042	0.34	0.7507
E1-E1	A-B	-0.09	0.9305	0.91	0.4321
E2-E2	A-B	-2.49	0.0673	-0.37	0.7234
E3-E3	A-B	4.54	0.0105	2.79	0.0493

*C= control, E1= endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*. E3= endophyte isolate *Eny7.11o*.

**Control and endophyte-infected plants were paired in a polyvinyl chloride (PVC) tube divided into 7 sections (Fig. 3), with the first and second treatment in each treatment pair placed on section 1 and 7, respectively. Nematodes were inoculated at the middle section (4) of the tube equidistant from the two plants. A and B represents sections on either side of the nematode inoculation point and correspond to the plant (treatment) on that side of the inoculation point.

Table 4. Average number of *Radopholus similis* in 5 g banana root samples of endophyte-inoculated and un-inoculated plants 3, 6, 10 and 14 days after nematode inoculation in in vivo root penetration experiments in the screen house.

Treatment	Days after nematode inoculation				*P*-Value[2]
	3	6	10	14	
Experiment 1					
Control	13.5 ± 0.5	19.2 ± 0.6	15.6 ± 1.9	14.8 ± 5.7	0.1028
V5W2	9.2 ± 4.6	6.6 ± 2.1	13.6 ± 0.6	8.3 ± 5.9	0.7772
Eny1.31i	5.8 ± 1.1	8.8 ± 2.8	7.6 ± 3.4	13.1 ± 3.7	0.8447
Eny7.11o	6.1 ± 2.4	4.8 ± 1.4	7.6 ± 3.4	13.1 ± 3.7	0.3514
P-value[1]	0.6771	0.6340	0.1301	0.7300	
Experiment 2					
Control	4.1 ± 1.1	5.2 ± 2.2	3.4 ± 1.3	1.8 ± 0.3	0.5584
V5W2	1.7 ± 0.4	4.2 ± 1.1	6.0 ± 1.6	3.9 ± 0.8	0.2099
Eny1.31i	1.0 ± 0.5	10.9 ± 9.5	4.0 ± 0.6	3.8 ± 1.1	0.5859
Eny7.11o	2.8 ± 0.9	1.5 ± 0.4	5.9 ± 3.3	3.3 ± 0.8	0.3147
P-value[1]	0.1179	0.7025	0.6780	0.1909	
Experiment 3					
Control	6.0 ± 1.4	4.2 ± 1.3	5.5 ± 1.4	13.7 ± 5.4	0.1531
V5W2	2.8 ± 0.6	5.5 ± 1.4	6.2 ± 2.9	26.5 ± 11.9	0.0693
Eny1.31i	5.3 ± 3.1	3.3 ± 0.5	5.5 ± 1.7	4.6 ± 1.8	0.6586
Eny7.11o	12.1 ± 6.7	2.2 ± 0.4	4.5 ± 2.4	4.8 ± 0.6	0.4473
P-value[1]	0.5981	0.2858	0.7552	0.0643	

P-Value[1] is the *P*-value for treatments along columns in each experiment. *P*-Value[2] is the *P*-value for days after inoculation along rows within each treatment.

Table 5. Effect of endophyte treatment of tissue culture banana plants cv. Enyeru (*Musa* spp. AAA-EA) with fungal isolates *V5W2*, *Eny1.31i* and *Eny7.11o* on the reproduction of *Radopholus similis* 25, 50 and 75 days after inoculation of individual primary roots with 50 *Radopholus similis* females.

Treatment	Females			Juveniles			Males			Total*		
	Days after nematode inoculation			Days after nematode inoculation			Days after nematode inoculation			Days after nematode inoculation		
	25	50	75	25	50	75	25	50	75	25	50	75
Experiment 1												
Control	1.1 ±0.3^{a}	115.4±44.7^{a}	270.0±55.1^{a}	1.0±0.3^{a}	143.3±53.7^{a}	581.8±110.9^{a}	0.1 ±0.1^{a}	80.4±33.7^{a}	268.5±47.8^{a}	2.0 ±0.6^{b}	339.2 ±130.6^{a}	1120.6 ±196.7^{a}
Eny1.31i	7.5±3.3^{a}	33.4 ±11.3^{a}	94.5±47.1^{b}	4.3±1.9^{a}	70.6±28.7^{a}	190.5±98.7^{b}	2.1 ±0.8^{a}	36.0±13.2^{a}	98.5±56.5^{b}	12.8 ±5.6^{ab}	140.0 ±49.7^{a}	383.5 ±194.1^{b}
Eny7.11o	4.2±2.1^{a}	68.6±32.3^{a}	87.9±47.1^{b}	1.1±0.5^{a}	69.2±39.1^{a}	130.6±63.8^{b}	2.1 ±1.6^{a}	57.2 ±31.2^{a}	43.8±18.2^{b}	5.5 ±3.0^{ab}	195.0 ±101.8^{a}	262.9 ±127.5^{b}
V5W2	12.5±3.9^{a}	88.8±36.2^{a}	35.5±16.8^{b}	6.3±2.3^{a}	116.6±49.9^{a}	53.5±18.8^{b}	5.5 ±1.9^{a}	114.7±47.6^{a}	19.1±6.7^{b}	24.5 ±7.9^{a}	320.2 ±128.2^{a}	109.0 ±41.9^{b}
P-value	0.0756	0.7151	0.0034	0.0756	0.8610	0.0003	0.0662	0.7920	0.0001	0.0226	0.8561	0.0003
Experiment 2												
Control	19.3±1.6^{a}	228.6±73.4^{a}	443.0±25.6^{a}	33.0±1.9^{a}	453.8±165.6^{a}	381.1±67.4^{a}	13.0±1.1^{a}	159.8±56.4^{a}	255.8±132.2^{a}	65.3±2.9^{a}	842.2±258.4^{a}	1080.0±174.1^{a}
Eny1.31i	9.2±4.6^{a}	84.6±38.3a	536.9±247.3^{a}	12.8±6.5^{a}	97.3±47.1^{a}	408.2±199.1^{a}	7.0±4.0^{a}	44.1±21.4^{a}	319.7±189.3^{a}	29.1±15.2^{a}	226.1±104.3^{a}	1265.0±627.5^{a}
Eny7.11o	18.1±5.1^{a}	195.7±56.7^{a}	887.5±350.2^{a}	26.8±7.6^{a}	343.1±110.4^{a}	509.3±140.2^{a}	14.4±5.5^{a}	135.5±39.6^{a}	344.5±100.6^{a}	59.3±16.9^{a}	674.3±202.5^{a}	1741.4±544.4^{a}
V5W2	17.2±5.4^{a}	222.5±68.6^{a}	436.2±188.9^{a}	28.8±8.8^{a}	256.8±88.3^{a}	278.1±84.8^{a}	13.8±5.1^{a}	102.6±37.4^{a}	221.3±70.6^{a}	59.8±17.3^{a}	582.1±181.8^{a}	935.7±305.3^{a}
P-value	0.7740	0.6775	0.6416	0.7719	0.1001	0.6665	0.0814	0.8551	0.7416	0.8745	0.3213	0.7778

*Total *R. similis* density (females + males + juveniles). In columns within an experiment, means followed by the same letter (superscript) are not different at $P<0.05$ according to Tukey's studentized range test. Values represent the mean ± S.E., n= 15.

Table 6. Percentage root necrosis, reproduction rates (Pf/Pi) of *Radopholus similis* and endophytic colonization in roots of tissue culture banana plants cv. Enyeru (*Musa* spp. AAA-EA) 25, 50 and 75 days after inoculation of individual primary roots with 50 *Radopholus similis* females.

Treatment	Root necrosis (%) Days after nematode inoculation			Reproduction ratio (*Pf/Pi*) Days after nematode inoculation			Root colonization (%) Days after nematode inoculation		
	25	50	75	25	50	75	25	50	75
Experiment 1									
Control	26.4 ±11.5^{a}	8.4 ±2.9^{b}	11.7 ±2.5^{a}	0.1 ±0.0^{b}	6.7 ±2.6^{a}	22.4 ±3.9^{a}	0.0 ±0.0^{a}	26.8 ±12.1^{a}	0.0 ±0.0^{a}
Eny1.31i	22.7 ±6.4^{a}	18.4 ±1.0^{a}	12.9 ±1.7^{a}	0.2 ±0.1^{ab}	2.8 ±0.9^{a}	7.6 ±3.8^{b}	13.8 ±7.3^{a}	39.5 ±6.3^{a}	50.0 ±41.6^{a}
Eny7.11o	32.5 ±9.9^{a}	13.2 ±3.1^{ab}	5.7 ±1.5^{a}	0.1 ±0.1^{ab}	3.9 ±2.0^{a}	5.2 ±2.5^{b}	61.1 ±26.4^{a}	21.6 ±7.7^{a}	0.0 ±0.0^{a}
V5W2	20.5 ±7.1^{a}	19.7 ±0.1^{a}	12.1±2.6^{a}	0.4 ±0.1^{a}	6.4 ±2.5^{a}	2.1 ±0.8^{b}	16.6 ±8.3^{a}	38.8 ±8.0^{a}	33.3 ±14.2^{a}
P-value	0.7525	0.0047	0.0630	0.0218	0.8583	0.0004	0.1257	0.5924	0.1796
Experiment 2									
Control	20.8 ±5.8^{a}	100.0 ±0.0^{a}	97.5 ±1.7^{a}	1.3 ±0.1^{a}	16.8 ±5.1^{a}	21.6 ±3.4^{a}	0.0 ±0.0^{b}	12.5 ±7.2^{a}	0.0 ±0.0^{b}
Eny1.31i	15.8 ±7.6^{a}	81.6 ±9.5^{a}	98.3 ±1.6^{a}	0.5 ±0.3^{a}	4.5 ±2.1^{a}	25.3 ±12.5^{a}	45.2 ±5.7^{a}	48.2 ±12.0^{a}	43.7 ±21.3^{a}
Eny7.11o	29.1 ±7.0^{a}	95.0 ±1.8^{a}	95.0 ±3.1^{a}	1.1 ±0.3^{a}	13.4 ±4.1^{a}	34.8 ±10.8^{a}	52.1 ±10.4^{a}	36.4 ±9.6^{a}	66.6 ±5.4^{a}
V5W2	31.6 ±8.7^{a}	76.1±10.5^{a}	91.1 ±5.4^{a}	1.1 ±0.3^{a}	11.6 ±3.6^{a}	18.7 ±6.1^{a}	53.5 ±9.7^{a}	46.1 ±7.5^{a}	53.3 ±6.2^{a}
P-value	0.4214	0.1392	0.2826	0.0895	0.7271	0.4813	0.0002	0.5616	0.0004

In columns within an experiment, means followed by the same letter are not significantly different at $P<0.05$ according to Tukey's studentized range test. Values represent the mean ± S.E., n= 15.

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Figure 1. Banana tissue culture plants growing in an aquaculture system in 250-ml plastic pots filled with nutrient solution.

Figure 2. Experimental set up used to test for host preference and attraction of *Radopholus similis* to either root segments from endophyte-inoculated or un-inoculated banana plants by means of the detached root bioassay (figure not drawn to scale).

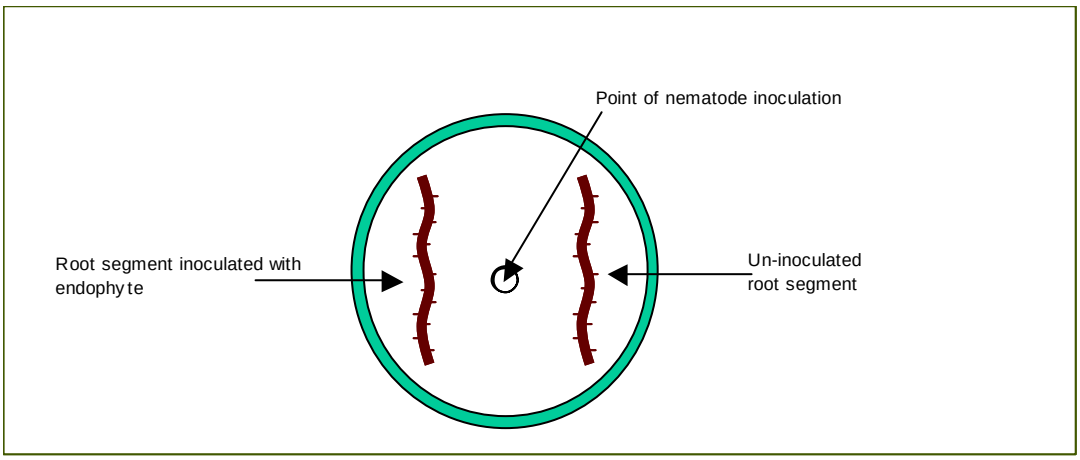


Figure 3. The polyvinyl chloride (PVC) tube apparatus used for *Radopholus similis* host preference and attraction bioassays involving intact endophyte-inoculated and un-inoculated (control) plants (A), and a schematic presentation of the PVC apparatus (B) (figure not drawn to scale).

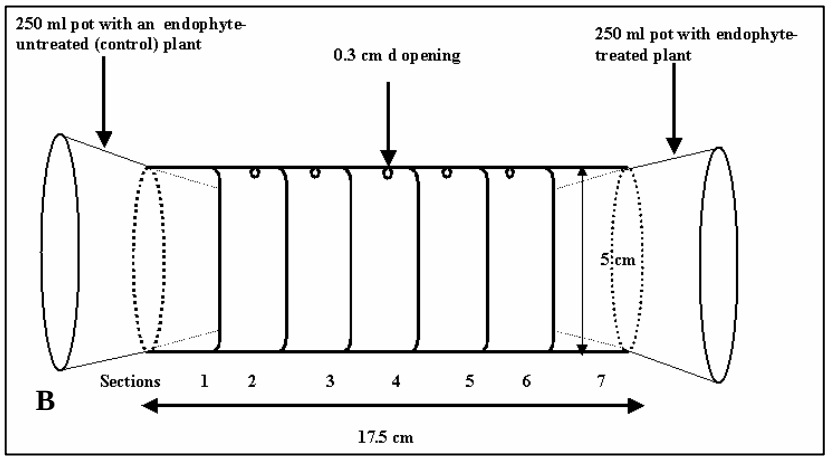


Figure 4. Experimental set up used to assess penetration of endophyte-inoculated and un-inoculated banana roots by *Radopholus similis* in vitro. (A), comprising a rectangular plastic container in which the plants were placed with three roots from each plant placed in Petri dishes. Nematode inoculation was done directly on the root sections inside the Petri dishes and extraction performed 24 hrs later. (B) represents a schematic presentation of the experimental set up (figure not drawn to scale).

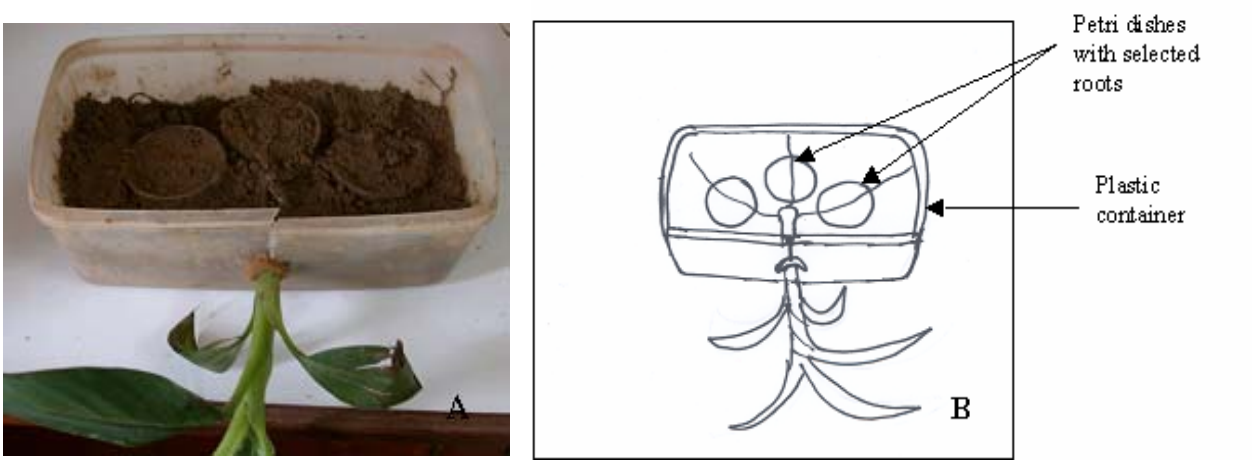


Figure 5. Experimental set up used to assess *Radopholus similis* reproduction in individual roots (A) and an excavated pot with the nematode inoculated root segment inside the plastic pot flanked by two root pieces (B).

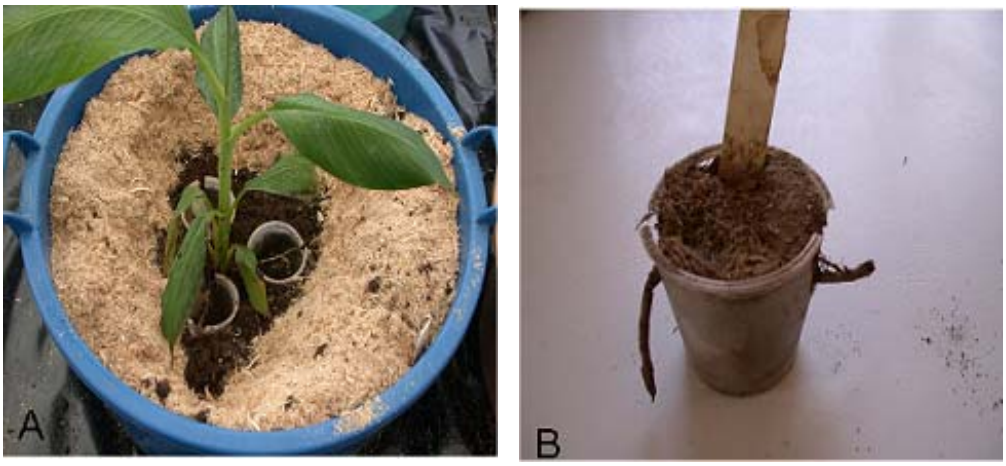
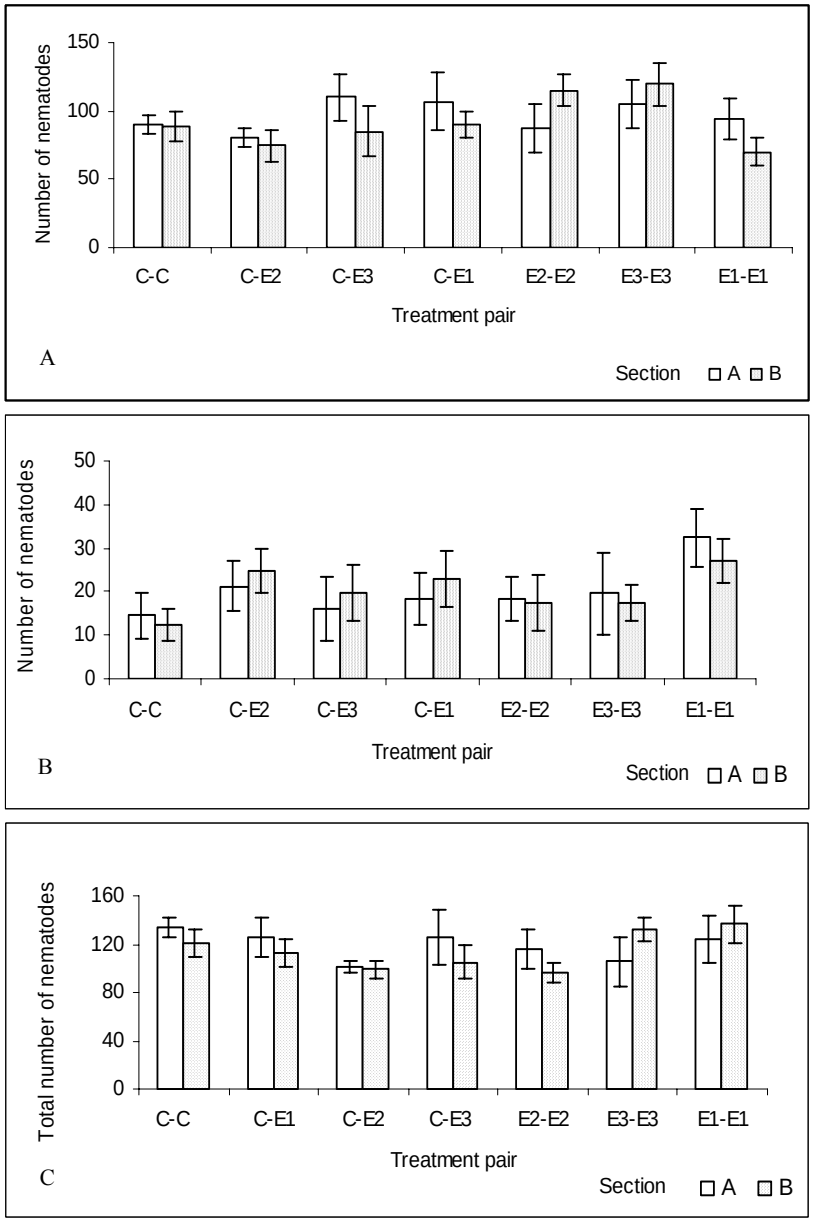
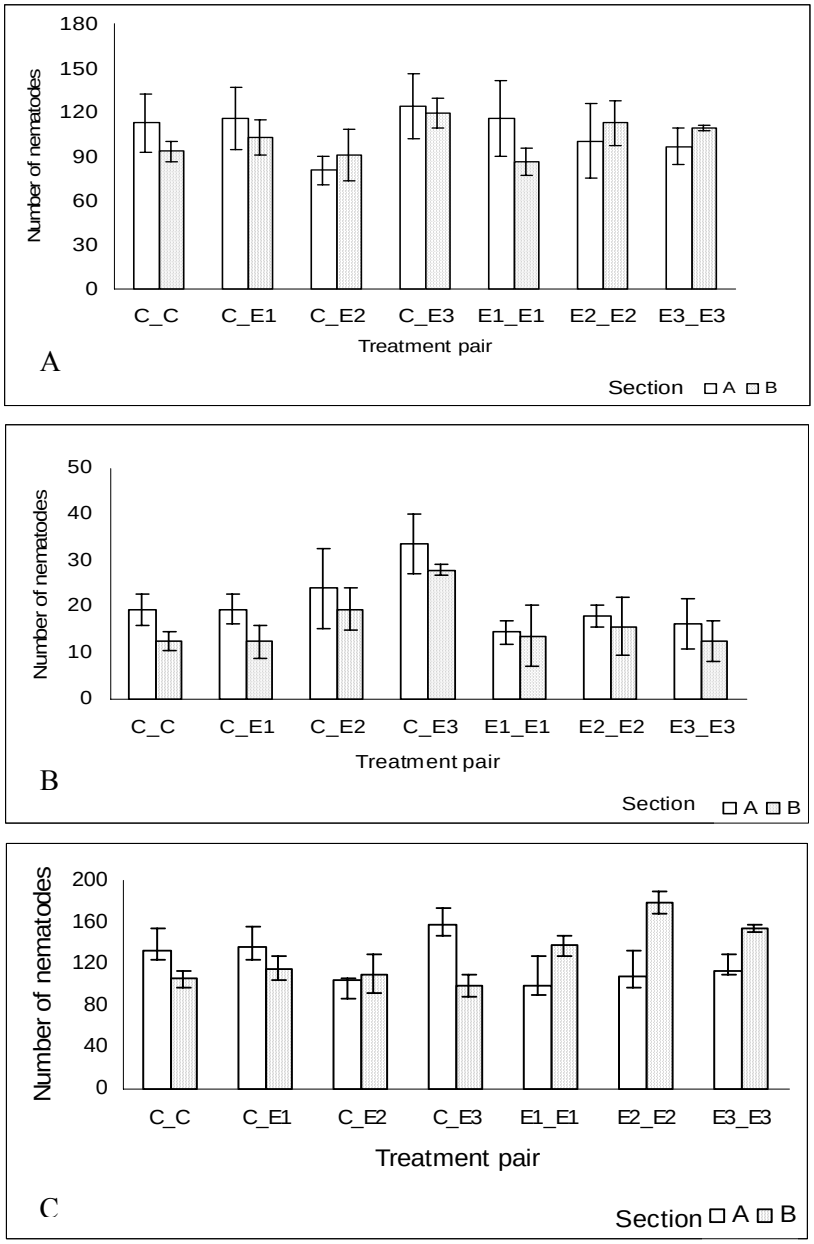


Figure 6. Average number of *Radopholus similis* that migrated towards root segments excised from endophyte-inoculated or un-inoculated banana roots in the detached root bioassay (Bioassay 1). Total nematodes recovered from the sand (A), number from the root segments (B) and the total nematodes from both the sand and root segment (C) in opposite sides of a Petri dish containing each respective treatment pair.



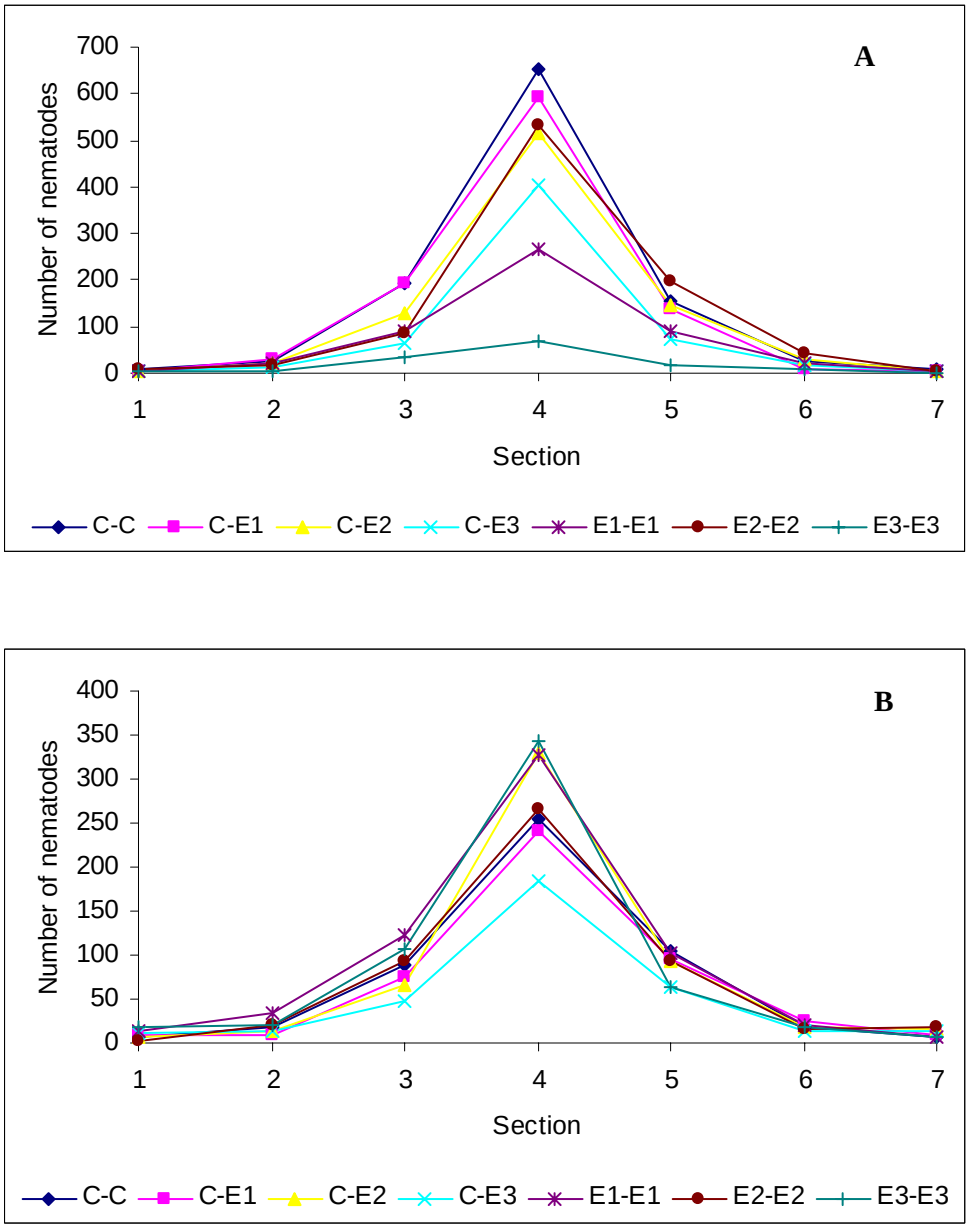
C=control, E1=endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*, E3= endophyte isolate *Eny7.11o*. Root segments from either endophyte-inoculated or un-inoculated plants were paired in 90-mm-diameter Petri dishes. The first treatment in each treatment pair was placed in section A and the second in section B. Nematodes were inoculated at the middle of the Petri dish equidistant from the two root segments. Nematode extraction was carried out 24 hrs later from both sand and the root segment in both sections of the Petri dish. Error bars represent standard errors of the mean, n=4

Figure 7: Average number of *Radopholus similis* that migrated towards root segments excised from endophyte-inoculated or un-inoculated banana roots in the detached root bioassay (Bioassay 2). Total nematodes recovered from the sand (A), number from the root segments (B) and the total nematodes from both the sand and root segment (C) in opposite sides of a Petri dish containing each respective treatment pair.



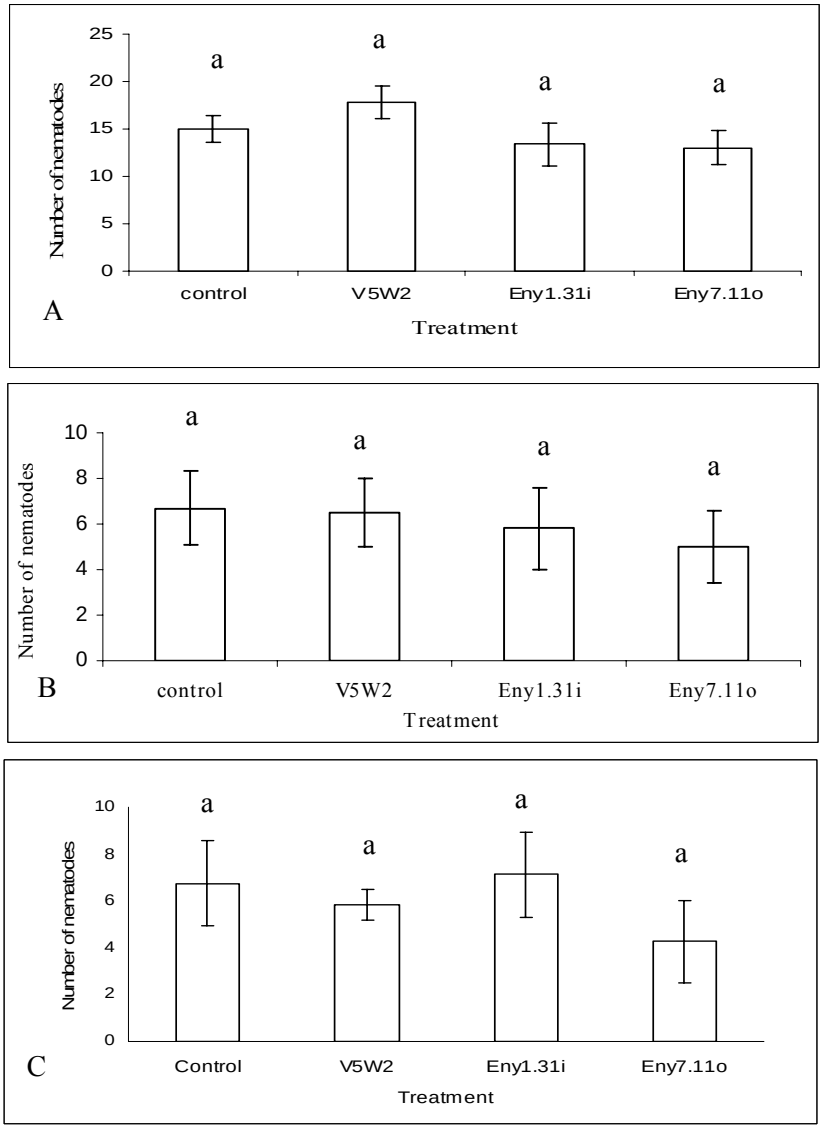
C=control, E1=endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*, E3= endophyte isolate *Eny7.11o*. Root segments from either endophyte-inoculated or un-inoculated plants were paired in 90-mm-diameter Petri dishes. The first treatment in each treatment pair was placed in section A and the second in section B. Nematodes were inoculated at the middle of the Petri dish equidistant from the two root segments. Nematode extraction was carried out 24 hrs later from both sand and the root segment in both sections of the Petri dish. Error bars represent standard errors of the mean, n=4

Figure 8: Distribution of nematodes in the polyvinyl chloride (PVC) sections showing migration of nematodes towards endophyte-inoculated and un-inoculated banana plants paired in a PVC tube apparatus divided into seven sections (A, bioassay 1; B, bioassay 2). *Radopholus similis* (2000 mixed stages) were introduced in the middle section of the PVC apparatus (section 4) equal distances from both plants placed in sections 1 and 7, which contained the either an endophyte-inoculated or un-inoculated plant.



C=control, E1= endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*, E3= endophyte isolte *Eny7.11o*.

Figure 9. Number of nematodes present in 8-cm-long root segments of endophyte-inoculated and un-inoculated banana plants 24 hrs after direct inoculation of root segments with 50 *Radopholus similis* females in the *in vitro* root penetration experiments. A, B and C are repeat experiments.



Control plants were not treated with any fungal isolate; endophyte-inoculated plants were treated with any of the three endophyte isolates viz. *V5W2*, *Eny1.31i* and *Eny7.11o*. Error bars represent standard errors of the mean, n=15. Bars with the same letters are not significantly different at *P*=0.05 according to Tukey's studentized range test.

Chapter 5

Mechanisms of action of endophytic *Fusarium oxysporum* against *Radopholus similis* in banana plants

Abstract

The mechanisms through which endophytic *Fusarium oxysporum* inhibits *Radopholus similis* damage to banana roots were studied by analyzing production of extracellular enzymes by the fungus, and induction of resistance mechanisms in the plant. Nine isolates of *F. oxysporum* produced proteases on gelatine-amended medium but none showed chitinase or lipase activity on chitin agar and medium amended with Tween 20, respectively. In split-root experiments, *R. similis* nematode numbers were reduced when the banana roots were treated with the endophytic *F. oxysporum* isolates *V5W2*, *Eny1.31i* and *Eny7.11o*. Histological analysis of banana roots and rhizomes showed higher levels of phenols in endophyte-treated (isolate *V5W2*) than in untreated plants. Cell wall-bound phenolics were more abundant in rhizomes than in the roots, and in the central cylinder of the rhizome than in the cortex regions. HPLC analysis did not show any significant differences between metabolite profiles of endophyte-treated and untreated plants. However, four unidentified compounds were found in both endophyte-treated and untreated plant extracts. Although not significantly different, the quantities of unknown compound 1 and 3 with retention times of 2.39 and 33.3 min, respectively, were found only in the endophyte-treated compared to the untreated plants. Known phenolic compounds identified in equal quantities in both endophyte-treated and untreated plants were 3, 4-dihydroxybenzoic, hydroxybenzoic, ferulic, syringic and vanillic acids. *p*-Coumaric acid was detected in rhizomes of plants treated with *V5W2* and *R. similis*. The results of the current study indicate that the systemic production of phenolic compounds in the host plant may constitute one of the main mechanims through which endophytic *F. oxysporum* suppresses *R. similis* in banana plants.

Introduction

The banana burrowing nematode, *Radopholus similis* (Thorne) Cobb is the most economically important nematode species-affecting banana in Uganda and the world (Sarah, 1989; Sarah *et al*., 1996; Speijer *et al*., 1999; Gowen *et al*., 2005). In Uganda, nematode-infected banana plants can have yield losses of between 30 and 50% (Speijer *et al*., 1999; Speijer and Kajumba, 2000). The nematode-induced losses are a result of reduction in the number of standing leaves, flower production, bunch weight, and an increase in the number of dead roots, root necrosis, plant toppling, and the time between successive harvests (Sarah *et al*., 1996; Speijer *et al*., 1999; Speijer and Kajumba, 2000; Talwana *et al*., 2003).

Management of *R. similis* has mainly relied on cultural practices such as the use of clean planting material obtained through paring and hot-water treatment of nematode infected plants (Speijer *et al*., 1995; Gold *et al*., 1998), planting of tissue culture plants (Mateille *et al*., 1994; Sarah, 2000), mulching (McIntyre *et al*., 2000; Talwana *et al*., 2003) and use of legume intercrops (McIntyre *et al*., 2001). Nematode resistant banana cultivars have also been identified and may be used in breeding programmes (Fogain and Gowen, 1997; Sarah *et al*., 1997; Collingborn *et al*., 2000). However, none of these methods offer permanent nematode control. An integrated nematode management approach involving a combination of several complementary methods would be best suited for control of *R. similis* in banana.

Biological control of *R. similis* using endophytic *Fusarium oxysporum* Schlecht.: Fries. is a promising management option that can be used to complement other nematode management strategies. Endophytic *F. oxysporum* reduced *R. similis* densities and damage in earlier studies (Chapter 3; Pocasangre, 2000; Niere, 2001). They suppress the nematodes in a number of ways by utilizing mechanisms that may act alone or in combination. One of the main mechanisms for *in vitro* inhibition of nematodes is the production of antagonistic compounds that cause nematode paralysis and mortality (Chapter 2; Hallman and Sikora 1994a,b; Niere, 2001; Dubois *et al*., 2004). Treatment of tissue culture banana plants with endophytes did not influence the nematode host preferences and root penetration by *R. similis*, but had an effect on nematode reproduction (Chapter 4). The reduction in nematode reproduction may be due to induced resistance a mechanism that has previously been reported as responsible for suppression of nematodes in plants (Sikora *et al*., 2003). Non-pathogenic *F. oxysporum* also

reduced the severity of Fusarium wilt diseases through induced resistance in tomato (*Lycopsersicon esculentum* L.) (Fuchs *et al*., 1997), cucumber (*Cucumis sativus* L.) (Mandeel and Baker, 1991), chickpea (*Cicer arietinum* L.) (Hervás *et al*., 1995) and banana (*Musa* spp.) (Nel *et al*., 2006). Another mechanism that may play a role in nematode control by endophytes is the direct parasitism of nematodes by the fungi.

Fungal endophytes may induce plant resistance responses by means of structural/morphological and physiological/biochemical changes in the plant. Biochemical responses include the synthesis of defence-related chemicals, such as phenolic compounds, against pest and pathogens (Ramamoorthy *et al*., 2001). Phenolic compounds may occur as constitutive molecules present in healthy plants or as substances synthesized by plants in response to bacterial or fungal infection (Mansfield, 1983), and are well recognized as plant resistance factors against nematodes (Giebel, 1974; 1982; Bajaj *et al*., 1983; Peng and Moens, 2004; Zinov'eva *et al*., 2004; Pegard *et al*., 2005). Banana cultivars resistant to *R. similis* were reported to contain higher amounts of constitutive phenolics compared to susceptible cultivars (Fogain and Gowen, 1996; Valette *et al*., 1998; Collingborn *et al*., 2000; Dochez, 2004). Schulz *et al*. (1999) also demonstrated that higher amounts of phenolic metabolites were produced in barley (*Hordeum vulgare* L.) inoculated with an endophytic *Fusarium* sp.

Direct parasitism is accomplished through the hydrolytic activity of extracellular enzymes produced by the nematode-antagonistic fungi (Stirling, 1991). For direct parasitism of nematodes to occur, the fungus must penetrate the nematode cuticle, a rigid and flexible exoskeleton composed mainly of proteins (Inglis, 1983). The nematode egg shell consists mainly of a chitinous and lipid layer (Perry and Trett, 1986; Bird and Bird, 1991). Production of extracellular enzymes by nematode–parasitic fungi has been demonstrated for nematophagous fungi like *Arthrobotryis oligospora* Fresenius (Minglian *et al*., 2004) and *Verticillium chlamydosporium* Goddard (Segers *et al*., 1994; Tikhonov *et al*., 2002).

Understanding the mechanism(s) of action involved in the biological control of *R. similis* in banana by endophytic *F. oxysporum* is important for successful application in the field. The objectives of this study were therefore, to (i) determine the production of extracellular enzymes such as chitinase, lipase and protease by nine endophytic *F. oxysporum* isolates on solid medium, (ii) assess induction of systemic resistance in banana plants against *R. similis* in

split root experiments by three endophytic *F. oxysporum* isolates and (iii) assess the accumulation of phenolic compounds in endophyte-treated banana plants as an indicator of induced resistance through histological and histochemical analysis.

Material and methods

Site description

All experiments were carried out at the International Institute of Tropical Agriculture (IITA) Research Station in Namulonge-Uganda, approximately 30 km Northeast of Kampala, Uganda. The site is situated at 1150 m above sea level, 32º 34'N latitude, with a mean annual rainfall of 1255 mm and an average temperature of 22ºC.

Fungal isolates, nematode cultures and banana plants

The endophytic *F. oxysporum* isolates used in this study were previously isolated from roots and rhizomes of apparently healthy banana plants in Uganda (Schuster *et al*., 1995), and are preserved in soil tubes at IITA. Pure *R. similis* inoculum was obtained from carrot disc cultures maintained at IITA (Chapter 1; Speijer and De Waele, 1997). Tissue culture banana plants of the local East African highland banana *cv*. 'Enyeru' (*Musa* spp. AAA-EA) were propagated from sword suckers (Vuylsteke, 1998). The plants were grown in a nutrient solution containing 1 g / L of Poly-Feed (Haifa Chemicals, Haifa Bay, Israel) and acclimatised in a humidity chamber (Chapter 3) for 1 month with weekly renewal of the nutrient solution.

Production of extra cellular enzymes on solid medium

Nine *F. oxysporum* isolates (*V5W2*, *Eny1.31i*, *Eny7.11o*, *V4W5*, *V2W2*, *V1W7*, *Emb2.4o*, *III4W1* and *III3W3*) were assayed for the production of extra cellular enzymes on solid medium amended with enzyme-specific substrates. Fungal isolates were pre-grown on synthetic nutrient agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar/L distilled water) in 65-mm-

diameter Petri dishes under laboratory conditions (room temperature of *ca*. 25°C and a photoperiod of 12 hrs light and 12 hrs darkness) for 1 week. The SNA medium was supplemented with 10 mg chlortetracycline, 100 mg penicillin G and 50 mg streptomycin-sulphate/L to prevent bacterial contamination.

Chitinase activity of the *F. oxysporum* isolates was assessed using 0.4% chitin agar (4 g chitin powder (Sigma-Aldrich St. Louis MO, USA), 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.5H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.001g $ZnSO_4$, 0.001g $MnCl_2$ and 20 g agar / L distilled water) (Hsu & Lockwood, 1975). One-week-old fungal isolates growing on SNA were point inoculated in the middle of Petri dishes containing chitin agar. A 3-mm-diameter cork borer was used to remove a disc of agar from the middle of the chitin agar plates and the hole replaced with a similar sized mycelial agar disc of the fungal cultures. To test for the production of lipases, fungal isolates from 1-week-old cultures on SNA were point inoculated on medium containing sorbitan monolaurate (Tween 20, Sigma, MO, USA) comprising of 10 ml Tween 20 and 20 g agar/L of distilled water. For assessment of protease activity, fungal isolates were point inoculated on gelatine medium comprising of 26.6 g gelatine (Sigma) and 14 g agar / L distilled water.

Ten Petri dishes were used for each of the isolates and enzymes; five with medium amended with the substrate and five without the substrate (controls). The medium in the control plates comprised of each of the above-mentioned ingredients except the enzyme–specific substrate. All petri dishes were incubated for 1 week under laboratory conditions. Cultures were examined on a daily basis for the presence of a clear zone (halo) around the fungal colony. The diameters of the clear zone and of fungal colonies were measured, and the difference between the areas of the clear zone and the fungal colony calculated to provide an estimate of the levels of enzyme production by the different isolates (Alves *et al*., 2002).

Split-root experiments for assessing induced resistance

Split-root experiments were conducted in the screen house to determine whether three *F. oxysporum* isolates (*V5W2*, *Eny1.31i* and *Eny7.11o*) induced systemic resistance in banana roots against *R. similis*. One-month-old tissue culture banana plants cv. Enyeru (*Musa* spp. AAA-EA) growing in nutrient solution in the humidity chamber (Chapter 4) were removed

from their pots and transplanted into 3-L plastic bags containing steam-sterilized loamy forest soil, and grown for another month. The 2-months-old the banana plants were then gently removed from their bags and each root system separated into two equal halves. Each half was planted separately into adjacent 300-ml pots, filled with sterile loamy forest soil (Fig. 1). The paired pots were marked A (inducer half) and B (responder half). The unsplit upper portion of the root system and rhizome were wrapped in moistened cotton wool to prevent dehydration, and then enclosed in an inverted pot from which the bottom part has been removed (Ogallo and McClure, 1996). The cotton wool was kept moist by spraying with tap water using a household sprayer.

Inoculation of plants with the endophytes started 1 week after replanting of the plants into the split-root systems. Fungal spore suspensions were prepared in half strength potato dextrose broth (PDB) (Sigma-Aldrich) obtained by dissolving 12 g of PDB in 1 L of distilled water. One hundred-ml aliquots of PDB were dispensed into 250-ml Erlenmeyer flasks and sterilized. After cooling, flasks were inoculated with 4 to 5 disks of agar of each fungal isolate. Un-inoculated PDB was used as the control treatment. Duplicate flasks were prepared for each fungal isolate and the control. Inoculated flasks were incubated in the laboratory for 7 days to allow for fungal growth and sporulation. Fungal spore suspensions were filtered through a 1-mm-diameter sieve to remove mycelial fragments. The spore suspensions were then adjusted to provide a final spore count of 1.5×10^6 spores/ml.

One week after transplanting, 1 ml of the spore suspensions was applied to the inducer half of the split root system. The soil around the roots was removed and the spore suspensions applied directly to the exposed roots. One week later, a 2-ml nematode inoculum containing 500 mixed stages of *R. similis* was added to the responder half of the split-root system so that both the fungal isolates and nematodes were spatially separated in adjacent pots on the same plant (Fig. 1). To inoculate plants with nematodes, soil was removed from around the roots and the nematode suspension pipetted directly onto the roots. The roots were then covered with soil. The experiment was repeated twice. The number of replications per treatment was 7, 10 and 14 in experiments 1, 2 and 3, respectively. Plants were arranged in the screen house in a completely randomized design (CRD).

One month after nematode inoculation the plants were harvested, and the nematode numbers and the extent of root necrosis determined. Root necrosis was determined from five randomly selected roots of each plant. The roots were split longitudinally and scored for percentage necrosis (Chapter 3; Speijer and De Waele 1997). The roots used for necrosis assessment were subsequently cut into smaller pieces, weighed and then macerated in a Waring blender (Waring, Connecticut, USA) at low speed for 15 s. Nematode extraction was carried out overnight according to a modified Baermann method (Chapter 3; Hooper *et al*., 2005). To check for cross-contamination and confirm spatial separation of the endophytes from the nematodes, nematode extraction and fungal reisolation was conducted on roots from both pots A and B.

To determine colonization of plant roots by the fungal isolates, three healthy primary roots were randomly selected from each pot at harvest and surface sterilized in 75% ethanol for 1 min, followed by sterilization in 2% NaOCl for 30 s. Root pieces were blotted dry on sterile tissue paper and cut into *ca*. 0.25-cm-long segments. Six sterilized segments per root were randomly selected and placed on SNA in 65-mm-diameter Petri dishes. The plates were incubated in the laboratory for 7 days under laboratory conditions. *Fusarium oxysporum* colonies growing from the root pieces were identified as described in Chapter 3 under a light microscope (magnification x 400) (Nelson *et al*., 1983). The number of root pieces with *F. oxysporum* colonies were recorded and the percentage recovery of the fungus calculated.

Analysis of phenolic compounds in endophyte-treated plants

The amount of phenolic compounds in rhizome and root tissues was studied as an indication of induced resistance in endophyte-treated tissue culture banana plants. Fungal inoculum was produced on sterile millet seed (Strauss and Labuschagne, 1995). Two hundred g of millet seed in 500-ml Erlenmeyer flasks were soaked in distilled water overnight and autoclaved twice (121ºC for 15 min) on successive days. The flasks were subsequently inoculated with five mycelial disks of 1-week-old cultures of isolate *V5W2* grown on SNA. The flasks were then incubated at room temperature at *ca*. 25ºC in the laboratory for 3 weeks. Flasks were shaken daily to disperse the inoculum throughout the seeds. Uninoculated millet seed was included as the control treatment.

Plants were removed from the humidity chamber and their roots cut back to *ca*.10 cm long. The plants were then potted in steam-sterilized loamy forest soil in 300-ml plastic pots and placed on a table in the screen house. The experiment included five treatments: a positive control that was sprayed until run-off with 50 mM di-hydrogen potassium phosphate (KH_2PO4), a known chemical inducer of resistance in plants (Manandhar *et al*., 1998); a negative control comprising plants treated with sterile millet seed inoculum (10% w/v); plants treated with isolate *V5W2*-colonized millet seed inoculum only (10% w/v); plants treated with *R. similis* only and plants treated with both *V5W2*-colonized millet seed inoculum (10% w/v) and *R. similis*. Treatments with *R. similis* were inoculated with a 2-ml suspension of 500 mixed stages of *R. similis*. The endophyte was inoculated at the beginning of the experiment and the nematodes at 1 week after endophyte inoculation.

Each treatment consisted of 25 1-month-old plants, which were arranged in a completely randomized design (CRD). In a time course study, five plants from each treatment were harvested at 0, 1, 2, 3 and 4 weeks after endophyte inoculation for analysis of total phenolic compounds. The roots and rhizomes of each plant sampled were washed free of soil under running tap water. From each plant, three primary roots were selected and a 1-cm piece cut from the basal part of the root (part of root proximal to the rhizome). The rhizome was split longitudinally into two equal parts. One half of the rhizome and the three 1-cm root pieces were fixed in a fixative comprising of 70% ethanol, pure acetic acid and 35% formaldehyde in the ratio 18:1:1 in 10-ml vials for histological analysis (Dochez, 2004). The other half of the rhizome and remaining roots were preserved at -20ºC for histochemical analysis of phenolic compounds.

Histological analysis

The fixed root and rhizome samples were trimmed back to ca. 0.25 cm long pieces prior to processing. The samples were dehydrated in an alcohol series of 70%, 80%, 90% and 100% alcohol for 2 hrs at each series and subsequently cleared in two steps of xylene. The dehydrated samples were impregnated in paraffin wax (50ºC melting temp.), embedded in paraffin wax (80ºC melting temp.) and mounted in wooden blocks (50 x 40 mm) for sectioning. Six µm thick ransverse sections of roots and rhizomes were subsequently made using a microtome (Baird & Tatlock London Ltd, Chadwall, UK). Three sections from each

root and rhizome piece were mounted on microscope slides, dewaxed in xylene and rehydrated in four steps of descending alcohol series (100%, 90%, 80% and 70%) (Fogain and Gowen, 1996).

The rehydrated sections were stained for phenolic compounds by flooding the sections with 2% ferric chloride dissolved in 95% ethanol for 5 min, and counterstained with Orange G for 1 min. The sections were rinsed in 95% isopropanol and cleared in xylene. Sections stained for lignin were flooded with 1% safranin dissolved in water for 5 min, rinsed briefly in distilled water and counterstained in 5% light green in water for 3 min (Fogain and Gowen, 1996). After staining for phenolic compounds and lignin, the slides were dehydrated in a series of ascending concentrations of alcohol with four steps (90%, 95% and two stages of 100%). Sections on slides were mounted in a synthetic mounting medium (DPX mountant, BDH, Kampala, Uganda) and covered with a cover slip.

Stained sections were observed under a light microscope at X400 magnification and the number of cells with phenolic compounds recorded separately for central cylinder (vascular bundles) and the cortex region of both the root and rhizome. Preformed phenolic cells were recorded as those with granular precipitates dispersed throughout the cell vacuole (Mace, 1963; Fogain and Gowen, 1996). Fully formed phenolic cells were recorded as the cells appearing as one large amorphous mass of granular bodies (Fogain and Gowen, 1996). From each treatment at each time period, 15 root and 15 rhizome sections were examined. The number of preformed and fully formed phenolic cells were scored on a scale of 0 to 5 where 0 = zero cells, 1 = 1 to 4 cells, 2 = 5 to 10 cells, 3 = 11 to 15 cells, 4 = 16 to 20 cells and 5 = more than 20 stained cells (Dochez, 2004). For sections stained for lignin, the presence of lignified cell walls and the location of the lignified cells (central cylinder, cortex or endodermis) were recorded. Stained root and rhizome sections were photographed using a Zeiss Axioplan 2 light microscope (Carl-Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam HR, Carl-Zeiss).

Histochemical analysis

Extraction of phenolic compounds

Phenolic compounds were extracted from the root and rhizome samples preserved at -20ºC according to the method described by De Ascensão and Dubery (2003). Half a gram of frozen root and rhizome samples were ground in liquid nitrogen and transferred to 1-ml Eppendorf tubes. Nine hundred ml of 80% methanol was added and the mixture vortexed for 30 s. The mixture was homogenized for 1 hr in a rotary shaker and centrifuged at 12000 rpm for 10 min. The supernatant was transferred into new Eppendorf tubes and the extraction procedure repeated overnight. The supernatants from the first and second extraction were pooled and left on the bench to evaporate to ca. 1 ml of crude extracts. The extraction procedure was conducted in the laboratories at IITA-Uganda and the extracts preserved at 4ºC. Histochemical analysis was carried out at the University of Pretoria, South Africa. The extracts were transported from IITA-Uganda to South Africa in cooler boxes at ca. 4ºC and refrigerated immediately upon arrival.

Analysis of total soluble phenolic compounds by means of the Folin method

Total phenolic content was determined using the Folin method, which utilizes the Folin-Ciocaltaeu (FC) reagent (Sigma) (Swain and Hills, 1959). Reaction mixtures were prepared in 96-well Elisa plates (Merck, Darmstadt, Germany). Five µl of each crude extract was mixed with 175 µl distilled water and 25 µl FC reagent and left for 3 min, before adding 50 µl of saturated $NaCO_3$ and incubation at 40ºC for 30 min. After incubation, absorbance was read at 690 nm using an ELISA reader (Multiskan Ascent, Version 1.3.1, Labsystems, Helsinki, Finland). For each root and rhizome sample, four absorbance readings were obtained and the average absorbance calculated. The absorbance of a blank consisting of distilled water was subtracted from all sample readings. Gallic acid was used to prepare a standard curve for estimation of the amount of soluble phenolics in each sample (Sivakumar *et al*., 2005). The concentration of phenolic compounds in the crude root and rhizome extracts was subsequently calculated from the standard curve and expressed as µg gallic acid/g fresh weight.

Identification of phenolic compounds by high performance liquid chromatography

Phenolic compounds were identified and quantified using high performance liquid chromatography (HPLC). Three root and three rhizome samples from different plants sampled 2 weeks after endophyte inoculation were selected from each treatment for HPLC analysis. The crude extracts were first hydrolyzed with hydrochloric acid (De Ascensão and Dubery, 2003). For hydrolysis, 100 µl of the crude extract was mixed with 10 µl pure HCl, incubated at 96ºC for 1 hr and extracted two times with anhydrous diethyl ether. The extract was evaporated to dryness and re-dissolved in 50-µl methanol. The hydrolyzed samples were assayed on a Hewlett- Packard (HP) HPLC system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA) equipped with a 20 µl loop injection valve (Agilent Technologies) and connected with a UV detector at 280, 325 and 340 nm. A Luna 3u C18 (Phenomenex, Palo Alto, CA, USA) reverse phase column (250 x 4.60 mm) was used. Acetronitrile and water (pH 2.6 acidified with phosphoric acid, H_3PO_4) were used as the solvents with a gradient program from 7% acetronitrile/water at 0 min, 20% at 20 min, 23% at 28 min, 27% at 40 min, 29% at 45 min, 33% at 47 min and 80% at 50 min. Twenty µl of the hydrolyzed extracts were injected and chromatogramed with a flow rate of 1 ml/min. Data were analyzed using the HP software provided with the HPLC equipment. The phenolic compounds in the extracts were identified by comparison with the reference compounds: gallic acid, caffeic acid, ferulic acid, syringic acid, quercetin, umbelliferone, naringin, hydroxy benzoic acid, 3,4-dihydroxy benzoic acid, sinapic acid, vanillic acid, *ρ*-coumaric acid, salicylic acid, scopoletin, catechin, kaempferol, chlorogenic acid, luteolin and fisetin obtained from Sigma.

Data analysis

All data were tested for normality and homogeneity of variances using Shapiro-Wilkinson, Levene Welch and Kolmogorov-Smirnov tests. Normal probability plots, box plots and stem leaf plots were additionally used to confirm normality of data and equality of variances. If not normally distributed, various transformations were tested until the most suitable transformation was obtained. For the enzyme production assays, statistical analysis was

performed on the averages of the size of the clear zones (calculated from the difference in size between the fungal colony and the clear zone) using one-way ANOVA.

Nematode counts in the three split-root experiments were calculated per 100 g of roots and square-root (x + 0.5) transformed prior to analysis. Percentage root necrosis and percent endophytic colonization were arcsine-square root (sqrt) x +0.5 transformed prior to analysis. One-way ANOVA was used to determine differences among repeat experiments. When differences were observed between experiments, data from each experiment was analyzed separately. In each experiment, one-way ANOVA was conducted to assess variability among treatments.

For the histological analysis of phenolic cells, the scores for pre-formed and fully formed phenolic cells were averaged for each treatment-week combination. Before statistical analysis, data was sqrt transformed. One-way ANOVA was used to evaluate differences among treatments, time (weeks) and plant part (rhizome or root). Two-way ANOVA was used to evaluate interaction effects between treatments, time and plant part. Interaction effects were subsequently evaluated using least square means. Differences in the number of phenolic cells between the central cylinder and cortex regions were evaluated using paired t-tests.

Data from histochemical analysis of soluble phenolic compounds with the FC reagent was expressed as µg gallic acid/gram of fresh weight and log (x +1) transformed prior to analysis. One-way ANOVA was used to determine main effects of treatment, time and plant part. Two-way ANOVA was used to evaluate interaction effects between treatments, time and plant part using least square means. HPLC data was analyzed qualitatively by comparing the presence and absence of peaks in chromatograms obtained with the different treatments. Quantities of unidentified compounds, estimated from the area under the peaks (in milliabsorption units [mAU/s], were used to evaluate quantitative differences among treatments. For the known phenolic compounds identified by comparison with the reference standards, the amount in µg/ml was used for comparison between endophyte and non-endophyte treatments. Data of the amounts of the unidentified compounds was log (x+1)-transformed prior to analysis. One-way ANOVA was used to assess differences among treatments. For all experiments, differences between means were separated using Tukey's studentized range test (SAS Institute, 1989).

Results

Production of extra cellular enzymes

The nine *F. oxysporum* isolates tested did not show chitinase or lipase activity in solid medium, as no clear zone formed around the fungal colonies 1 week after incubation. All the isolates, however, showed positive protease activity. A clear zone formed around the fungal colonies 2 days after the fungus was placed on gelatine-amended medium (Fig. 2). The measurements of the fungal colony diameter and the diameter of the clear zone were conducted on the third day of incubation. Measurements could not be done on the second day since the distinction between the fungal colony and the clear zone could not be easily discerned. From the fourth day onwards the clear zone was not visible anymore. No clear zones were observed in the control plates in which the fungus was grown without the enzyme substrates. The diameters of the clear zone did not differ between the different fungal isolates (P=0.0889). However, based on the diameters of the halo, isolates *III3W3*, *Emb2.40*, *Eny1.31i* and *V4W5* had produced more protease activity compared to isolates *V5W2*, *Eny7.11o*, *V1W7*, *III4W1* and *V2W2* (Fig. 3).

Split-root experiments for assessment of induced resistance

The three *F. oxysporum* endophytic isolates did not reduce the number of *R. similis* significantly, with the exception in the number of females in Experiment 2 (Table 1). The total number of nematodes was also reduced significantly by isolates *Eny1.3li* and *V5W2* in Experiment 2. Generally, the responder half of endophyte-treated roots supported fewer females, juveniles, males and total nematodes than the control roots. Damage due to nematodes was not significantly different between the fungal isolates and the control treatment (P=0.6934) (Table 1).

The split-root system provided spatial separation of the nematodes from the endophytes on the same plant. No nematodes occurred in the uninoculated halves of the split-root systems. However, endophytic *F. oxysporum* was re-isolated from the untreated roots, indicating possible contamination of untreated halves. Percentage root colonization in the inducer half of

the split root systems differed significantly between the endophyte and control treatments (P=0.0003). In the inducer half, root colonization by isolates *Eny1.31i*, *V5W2* and *Eny7.11o* were 75.0 ± 5.9%, 59.5 ± 6.4% and 52.7 ± 5.4%, respectively. Root colonization in the control treatment ranged from 16.6 ± 7.4% to 37.6 ± 7.2% (data not presented). Root colonization by endophytic *F. oxysporum* was 40.8 ± 6.2, 33.3 ± 6.6, 25.7 ± 5.7 and 36.5 ± 5.2% in the responder roots when the inducer roots were treated with the fungal isolates *Eny1.31i*, *V5W2*, *Eny7.11o* and the uninoculated broth (control), respectively.

Analysis of phenolic compounds

Histological analysis

Phenolic cells were observed in the central cylinder and cortex of roots and rhizomes of all banana plants, whether they were treated or not treated with the endophytic *F. oxysporum* isolate *V5W2* (Fig. 4 and 5). Significantly more phenolic cells, however, were formed in the central cylinder than in the cortex of roots and rhizomes (Fig. 4 and 6) (P=0.0027). The number of phenolic cells that formed in the central cylinder of root and rhizome sections differed significantly between the different treatments applied (P=0.0009) the time since treatment (P<0.0001), and the plant parts investigated (root or rhizome) (P<0.0001) (Fig. 4). Root and rhizome sections from plants treated with the endophytic *F. oxysporum* isolate *V5W2* + *R. similis* had significantly more phenolic cells in their central cylinder compared to endophyte-untreated plants (Fig. 4). In the cortex, however, most phenolic cells were formed in plants treated with 50 mM K_2HPO_4. The number of phenolic cells in the central cylinder of plants treated with *V5W2* only and *V5W2* + *R. similis* increased from week 0 to week 4, but reached its highest levels in the cortex 2 weeks after inoculation. More phenolic cells formed in *V5W2* + *R. similis* than in the negative control plants at each time interval, except in the rhizome cortex. While phenolic cells were present in plants treated with *R. similis* only, the numbers recorded were lower than in plants treated with the endophyte. No lignification of cell walls was demonstrated except in the endodermis of a few roots of endophyte-treated plants (data not shown).

Preformed phenolic cells with granular precipitates dispersed in the cell vacuoles were formed in the central cylinder and cortex of roots and rhizomes of both plants treated and not treated

with the endophytic *F. oxysporum* isolate *V5W2* (Fig. 6F). The endophyte treatment, however, resulted in higher numbers of preformed phenolic cells when compared to non-endophyte treated tissue (P=0.0059). The number of preformed phenolic cells in the roots and rhizomes increased significantly with time after endophyte inoculation (P<0.0001) and differed between plant parts (rhizomes and roots) (P=0.0343). The number of preformed phenolic cells in the central cylinder of both rhizomes and roots occured in descending order in plants treated with isolate *V5W2* only, *V5W2* + *R. similis*, K_2HPO_4, negative control and *R. similis* only (Fig. 5A and B). In plants treated with isolate *V5W2* only or with *V5W2* and *R. similis*, the number of preformed phenolic cells in the rhizomes increased from 0 weeks after inoculation, and reached a maximum after 2 weeks (Fig. 5A). In the roots, the maximum number of phenolic cells was observed mostly 1 or 2 weeks after inoculation (Fig. 5B). Although not significantly different, plants treated with *V5W2* and *V5W2* with *R. similis* had more preformed phenolic cells than the negative control plants and plants treated with *R. similis* only. Rhizomes had significantly more preformed phenolic cells (P<0.0001) than roots (Fig. 5C and D). More preformed phenolic cells were found in the central cylinder compared to the cortex regions (P<0.0001) (Fig. 5A and C, Fig. 5B and D).

Histochemical analysis

Analysis of total soluble phenolics

The amount of total soluble phenolics produced in the banana rhizome (P=0.0019) and roots (P=0.0011) differed significantly between endophyte and non-endophyte treated plants (Fig. 7). No differences in total soluble phenolics, however, were observed in rhizomes of plants treated with *V5W2* only and plants treated with *V5W2* and *R. similis* (P=0.5390). Total soluble phenolics also did not differ significantly in the roots of plants treated with endophytes and 50 mM K_2HPO_4 (P=0.7285). In all rhizome treatments, the amount of total soluble phenolics in rhizomes increased from week 0 to week 2, and then decreased in weeks 3 and 4 (Fig. 7A). The amount of total soluble phenolics in plants treated with *V5W2* only and plants treated with *V5W2* and *R. similis* increased by 16.8% and 41.9% from week 0 to week 2, respectively. In the roots, however, the total soluble phenolics dropped rapidly in both endophyte and non-endophyte treated plants from week 0 to the second week, after which it remained relatively stable until week 4 (Fig. 7 B).

HPLC analysis of phenolic compounds

HPLC separation of phenolic compounds extracted from rhizomes and roots of plants treated with the endophytic *F. oxysporum* isolate *V5W2* and *R. similis* revealed the elution of four major peaks (Fig. 8). These peaks did not represent compounds of any of the known reference standards. Based on spectral analysis, they had spectrums similar to that of ferulic acid with a maximum absorbance at 280 and 325 nm, and thus belong to the hydrocinnamics group of phenolic compounds (Harborne, 1991). Unknown compound 1 with a retention time of 2.39 min was found in extracts from plants treated with 50 mM K_2HPO_4, *V5W2* and *V5W2* and *R. similis* but in undetectable amounts in the negative control and *R. similis*-treated plants (Fig. 8). No significant differences were found in the size of the peaks (unknown compounds 2, 3 and 4) between endophyte and non-endophyte treatments. Extracts from endophyte-treated plants, however, were higher for compounds 3 and 4, although not significantly different from non-endophyte treatments (Fig. 9). The amounts of compounds 2 (retention time 7.83 min), 3 (retention time 17.7 min), and 4 (retention time 33.3 min), were significantly higher in the plants treated with 50 mM K_2PO_4 and plants treated with *R. similis* only compared to endophyte-treated plants. Quantities of unknown compounds 2 (P=0.0484) and 3 (P=0.0401) were significantly more in the rhizome compared to the roots, unlike unknown compound 3 (P=0.1831) (Fig. 9).

The known phenolic compounds in rhizome and root extracts did not differ among the endophyte and non-endophyte treatments (P=0.4525) (Table 2) with the exception of hydroxybenzoic and ρ-coumaric acid, which were detected in the rhizome of plants treated with both *V5W2* and *R. similis*, but not in the negative control. Gallic acid was detected in the rhizome of plants treated with 50mM K_2HPO_4. Other compounds, such as 3, 4 dihydroxybenzoic acid, vanillic acid, ferulic acid and syringic acid were present in both endophyte-treated and untreated plants (Table 2).

Discussion

Endophyte treatment of banana roots with endophytic *F. oxysporum* appeared to trigger defence mechanisms that could reduce reproduction of *R. similis* (Chapter 4). It did not, however, reduce the numbers of nematodes infecting the roots or reduce nematode-inflicted damage to the roots. According to the current investigation, the enhanced production of phenolic compounds in cells, primarily in the central cylinder of the roots and rhizome due to endophyte infection is a major indicator of induced host resistance response. These phenolic depositions appeared to be induced, as the number of phenolic cells increased over time. Induced resistance in plants might not be the only mechanisms of action whereby endophytic *F. oxysporum* result in biological control of nematodes, as the endophytic isolates also showed protease activity *in vitro* and also produced toxic secondary metabolites (Chapter 2).

Extracellular hydrolytic enzymes target the external and internal structures of nematodes and their eggs (Wuyts *et al*., 2004). In this study, all nine endophytic *F. oxysporum* isolates produced proteases, but none showed chitinolytic or lipolytic activity. The production of proteases might be partly responsible for mortality of the motile stages of *R. similis* when they were treated with fungal culture filtrates (Chapter 2), since the nematode cuticle is mainly composed of proteins (Inglis, 1983). Vu *et al*. (2004) previously demonstrated a lack of direct parasitism of *R. similis* by endophytic *Fusarium* isolates, suggesting that other secondary metabolites might be involved in the killing of nematodes. The lack of chitinolytic and lipolytic activity suggests that direct parasitism of nematode eggs, composed mainly of chitin and lipids (Bird and Bird, 1991), by *F. oxysporum* is unlikely. Poor hatching of *R. similis* eggs treated with fungal filtrates (Chapter 2) should, therefore, rather be attributed to other secondary metabolites and toxins. To confirm the role of extracellular enzymes and toxins in biological control, specific secondary metabolites need to be purified from fungal cultures and assayed against nematodes and their eggs, and observations made by means of scanning electron microscopy (Bonants *et al*., 1995).

For direct parasitism of the nematodes by fungal hyphae to occur, contact between the nematode and the fungus for a sufficient duration is required. Paparu (2005) demonstrated that colonization of banana roots by endophytic *F. oxysporum* isolates was extensive in the hypodermal cells and cortex. All developmental stages of *R. similis* also occur in the cortex of

plant roots (Araya and De Waele, 2001; Gowen and Quénéhervé, 2005). Despite occupying the same niche inside roots, direct parasitism of the destructive stages and eggs of *R. similis* by endophytic fungi may not happen due to the migratory nature of the nematode. Though direct parasitism may not necessarily represent a substantial part of nematode control by endophytes, the association with other modes of action, for instance the production of toxic metabolites and induced resistance, may improve their efficacy and levels of plant protection.

Split-root systems have been used to investigate induced systemic resistance by non-pathogenic isolates of *F. oxysporum* in tomato (Ogallo and McClure, 1996; Fuchs *et al*., 1997; Larkin and Fravel, 1999) and cucumber (Mandeel and Baker, 1991). Using split-root experiments, several researchers have reported induction of systemic resistance against root-knot nematodes, *Meloidogyne* spp. (Aalten *et al*., 1998; Siddiqui and Shaukat, 2002; 2003; 2004) and the potato cyst nematode, *Globodera pallida* (Sikora and Reitz, 1998; Reitz *et al*., 2000). In this study, split-root experiments did not convincingly demonstrate the ability of endophytic *F. oxysporum* isolates to induce systemic resistance in banana plants against *R. similis*. In one experiment, *R. similis* numbers were significantly reduced by *isolates V5W2*, *Eny1.31i* and *Eny7.11o* compared to the control treatment, but not in the other two experiments. The number of nematodes in these experiments, however, was still lower than in the control treatments. This indicates that there was some measure of induced systemic resistance in the plants against the nematode. The lack of significance between the two split-root experiments may have been due to the small sample size (low numbers of replications per treatment) and high variation between replicates within a treatment. Contamination by other endophytic *F. oxysporum* may also have buffered the effects of the inoculated isolates resulting in lack of significant results.

This study provides the first indication of ISR by endophytic *F. oxysporum* against *R. similis* in banana. While endophytic *F. oxysporum* isolates have been used to suppress *R. similis* in banana before (Pocasangre 2000; Sikora *et al*., 2000; Niere 2001; Chapter 3 and 4), none of these studies demonstrated endophyte-induced resistance. Endophytic isolates of *F. oxysporum* and *Pseudomonas fluorescens* Migula proved to induce systemically acquired resistance in Cavendish banana against *F. oxysporum* f.sp. *cubense*, the causal agent of Fusarium wilt (Belgrove and Viljoen, personal communication). Similarly, Aalten *et al*. (1998) concluded that rhizosphere strains of fluorescent *Pseudomonas* spp. elicited induced

systemic resistance responses in banana plants that reduced *R. similis* and *Meloidogyne* spp. numbers in the roots.

Contamination of untreated split-roots by *F. oxysporum* may denote that the effects of endophyte treatment on *R. similis* cannot be ascribed to ISR. For ISR to occur, spatial separation of the inducing agents and the nematode must be maintained (Siddiqui and Shaukat, 2002). Despite the presence of endophytic *F. oxysporum* in untreated split-roots, nematode reproduction in plants treated with endophytes was substantially less than in the case of the endophyte-untreated roots in one experiment, suggesting possible ISR. However, the lack of significant reduction in nematode numbers and root damage between endophyte-treated and non-treated plants in the other two experiments can either be ascribed to contamination by *F. oxysporum*, or a lack of ISR. The most effective way to confirm ISR would be to conduct the experiments in a controlled environment that prevents introduction of other fungi to the untreated plants. From the current study it is clear that further investigations are required on the threshold root colonization as well as on persistence of systemic resistance in banana against *R. similis*. Hallman *et al*. (1997) previously demonstrated that, even when colonization rates of roots by endophytes decline over time, the plant may retain the induced protection over time.

No significant difference was observed in the number of fully formed and preformed phenolic cells in the rhizomes and roots of banana plants immediately after endophyte inoculation. The increase in the number of phenolic cells from week 1 to 4, however, may be attributed to increased (induced) synthesis due to endophyte and nematode infection. The number of phenolic cells in *R. similis*-infected plants was lower than in endophyte-treated plants, indicating a positive response to endophyte infection. The higher number of phenolic cells formed when both endophyte and nematode were inoculated on banana roots, compared to when they were inoculated separately, indicates that the joint infection induced greater plant defence responses. This could be explained by the report of Kloepper *et al*. (1992) that induced plants often do not produce defence chemicals until challenged by a pest or pathogen. The presence of constitutive phenols has been associated with resistance in banana cultivars against *R. similis* (Fogain and Gowen, 1996; Collingborn *et al*., 2000; Dochez, 2004). In future experiments, it may be useful to additionally include a resistant cultivar for comparative purposes.

No significant differences were obtained in total soluble phenolics between endophyte-treated and untreated plants, but higher concentrations occurred in the endophyte-treated plants compared to non-treated plants over time. The high concentration of phenolic compounds in the roots immediately after endophyte inoculation was unexpected. This may be due to transplanting shock and some injury of the plants during handling, as the plants were sampled immediately after establishment of the experiment. An increase in the levels of phenolic compounds is known to occur due to both biotic and abiotic stress factors (Beckman, 2000).

Qualitative analysis by HPLC revealed four major unknown compounds in root and rhizome extracts of plants in endophyte and non-endophyte treatments. One compound was only found in plants treated with the chemical inducer and also in the endophyte–treated plants but in very low amounts in the negative controls and *R. similis*-treated plants indicating induced synthesis of this compound. The areas under the peaks of two unknown compounds were slightly larger in the endophyte-treatments compared to the controls, a possible indication of their importance in the defence mechanism of the plants triggered by the fungal endophyte. Further characterization of these compounds is required to elucidate their identity. Nevertheless, quantitative differences observed between endophyte-inoculated and uninoculated plants suggest that the presence of the endophyte triggers the plant to increase synthesis of these compounds. The results obtained in the current study further indicate that the presence of the four unidentified compounds are not entirely due to endophyte infection as they were detected in both endophyte-inoculated and uninoculated plants. There is strong evidence indicating that phenolic compounds are involved in plant resistance against nematodes (Hung and Rohde, 1973; Mahajan *et al*., 1985). The endophyte-induced phenolics in banana plants most likely play a significant role in the suppression of *R. similis*.

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Figure 1. Illustration of the split-root system used for assessment of induced resistance by endophytic *Fusarium oxysporum* isolates against *Radopholus similis*. Two-month-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA) in split-root systems within adjacent pots (A) and the split-root system with the upper undivided portion of roots and rhizome section wrapped in cotton wool (B). The fungal isolates or uninoculated broth and nematodes were applied in the halves designated a (inducer half), and b (responder half), respectively.

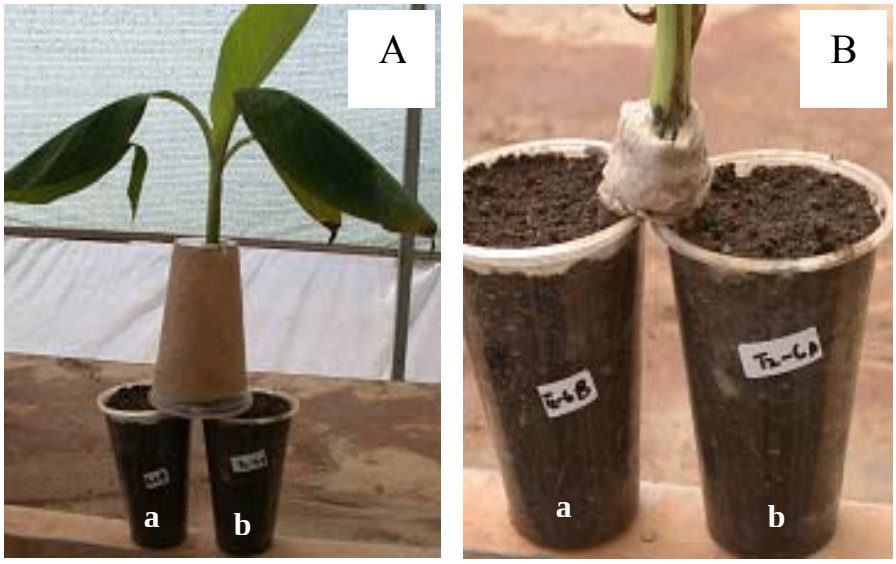


Figure 2. Protease activity of endophytic *Fusarium oxysporum* (isolate Eny1.31i) in a 65-mm-diameter Petri dish 3 days after inoculation on gelatine-amended medium. The clear zone (halo) indicates positive protease activity.

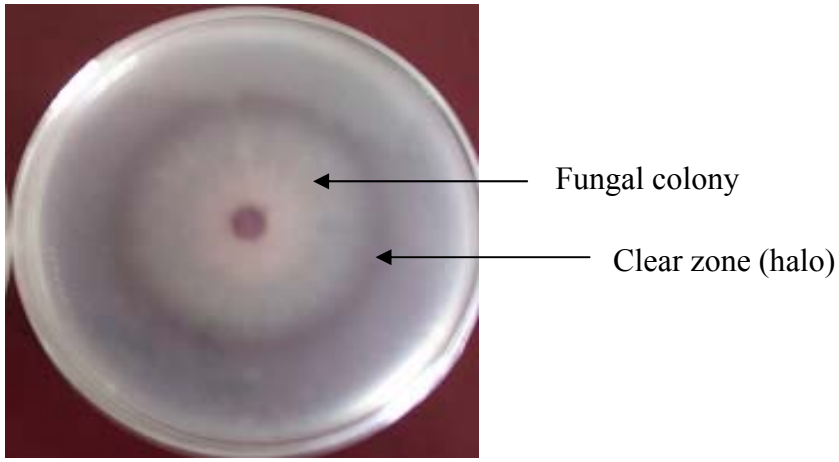
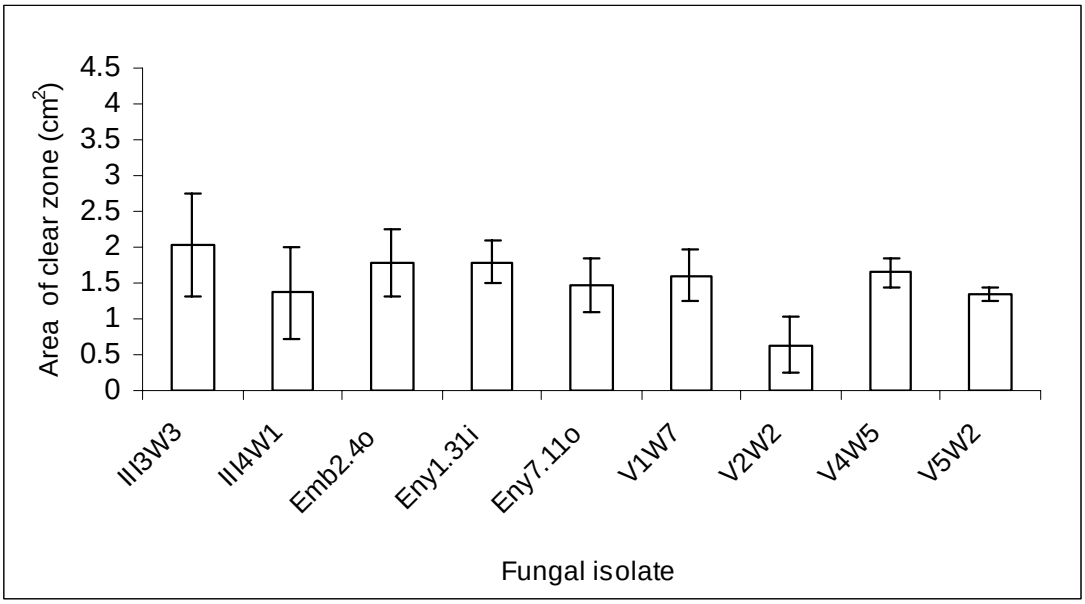
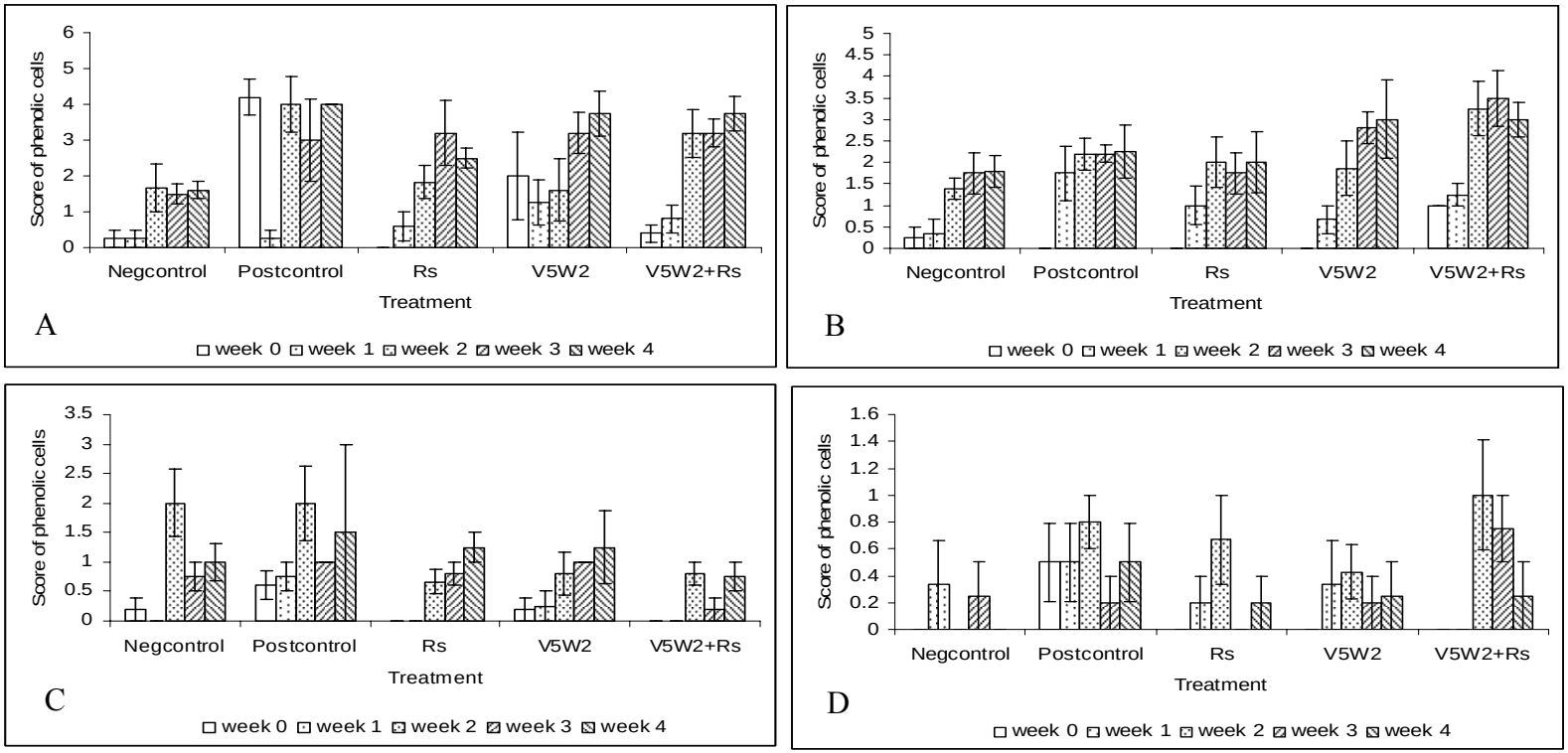


Figure 3. Level of protease activity exhibited by nine endophytic *Fusarium oxysporum* isolates 3 days after inoculation on gelatine-amended medium. The levels of protease activity were estimated using the diameter of the halo zone (cm) compared to the fungal colony diameter.



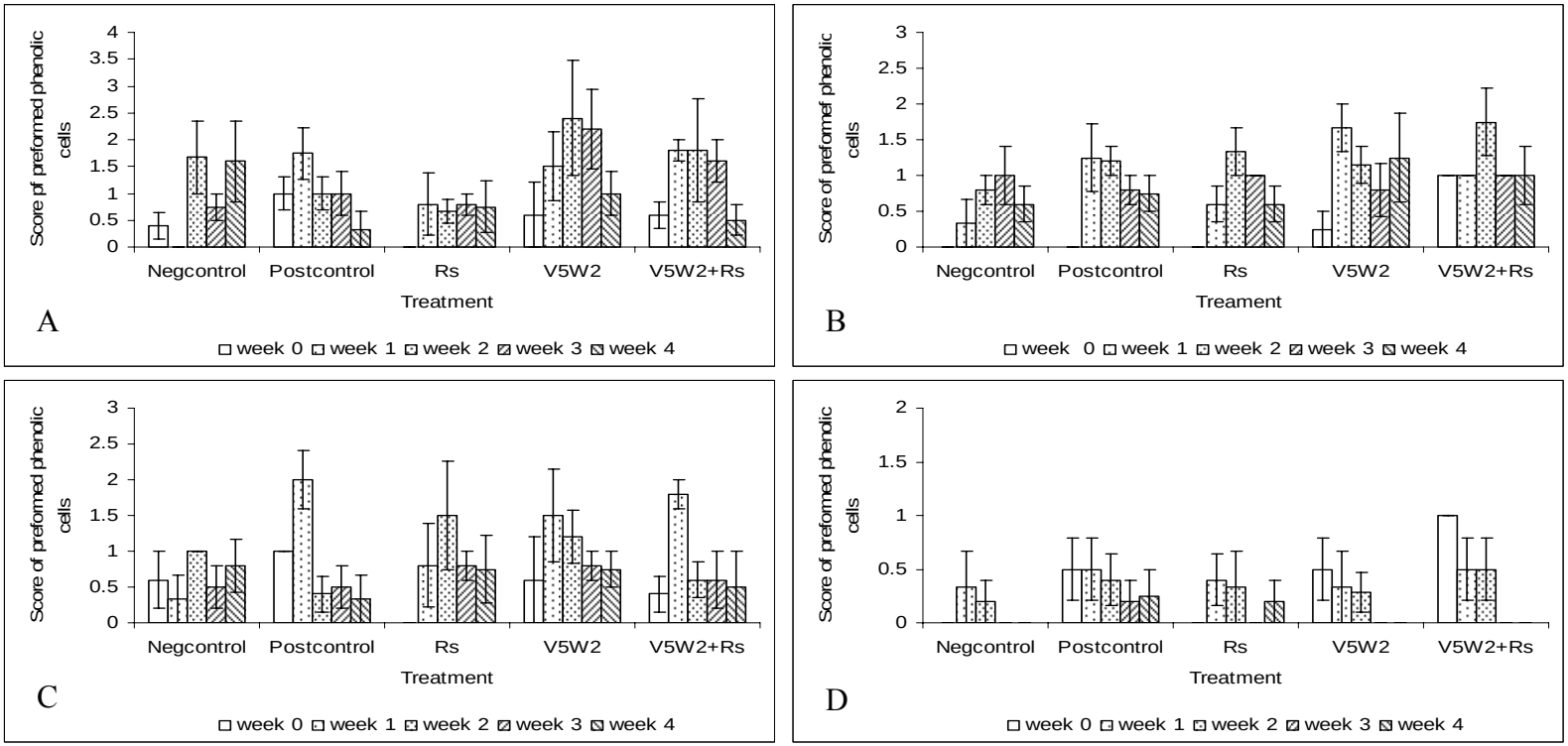
Error bars represent standard errors of the mean (n=5).

Figure 4. Fully formed phenolic cells in the central cylinder of transverse sections of rhizomes (A) and root (B), and in the cortex region of rhizomes (C) and roots (D), 0 to 4 weeks after treatment of plants with or without endophytes and/or nematodes. Formation of phenolic cells were scored on a scale of 0-5, where 0 = zero, 1 = 1 to 4, 2 = 5 to 10, 3 = 11 to 15, 4 = 16 to 20 and 5 = more than 20 phenolic cells.



Negcontrol=negative control (sterile millet seed), Postcontrol=Positive control (50 mM K_2HPO_4); V5W2= endophyte isolate V5W2; Rs= *Radopholus similis*. Error bars represent standard errors of the mean, n=15.

Figure 5. Preformed phenolic cells in the central cylinder of transverse sections of rhizomes (A) and root (B); and the cortex region of rhizomes (C) and roots (D), 0 to 4 weeks after treatment of plants with or without endophytes and/or nematodes. Formation of phenolic cells were scored on a scale of 0-5, where 0 = zero, 1 = 1 to 4, 2 = 5 to 10, 3 = 11 to 15, 4 = 16 to 20 and 5 = more than 20 phenolic cells.



Negcontrol=negative control (sterile millet seed); Postcontrol=Positive control (50 mM K2HPO4); V5W2= endophyte isolate V5W2; Rs= *Radopholus similis*. Error bars represent standard errors of the mean, n=15.

Figure 6. Histological analysis of phenolic cells showing brown stained phenolic cells in transverse sections of rhizomes obtained from tissue culture banana plants treated with (A) sterile millet seed (negative control), (B) 50 mM K_2HPO_4 (positive control), (C) *Radopholus similis*, (D) *V5W2*, and (E) *V5W2* and *Radopholus similis* 2 weeks after inoculation with *V5W2* and 1 week after nematode inoculation. Preformed phenolic cells in the cortex region of rhizome sections treated with both *V5W2* and *Radopholus similis* (F).

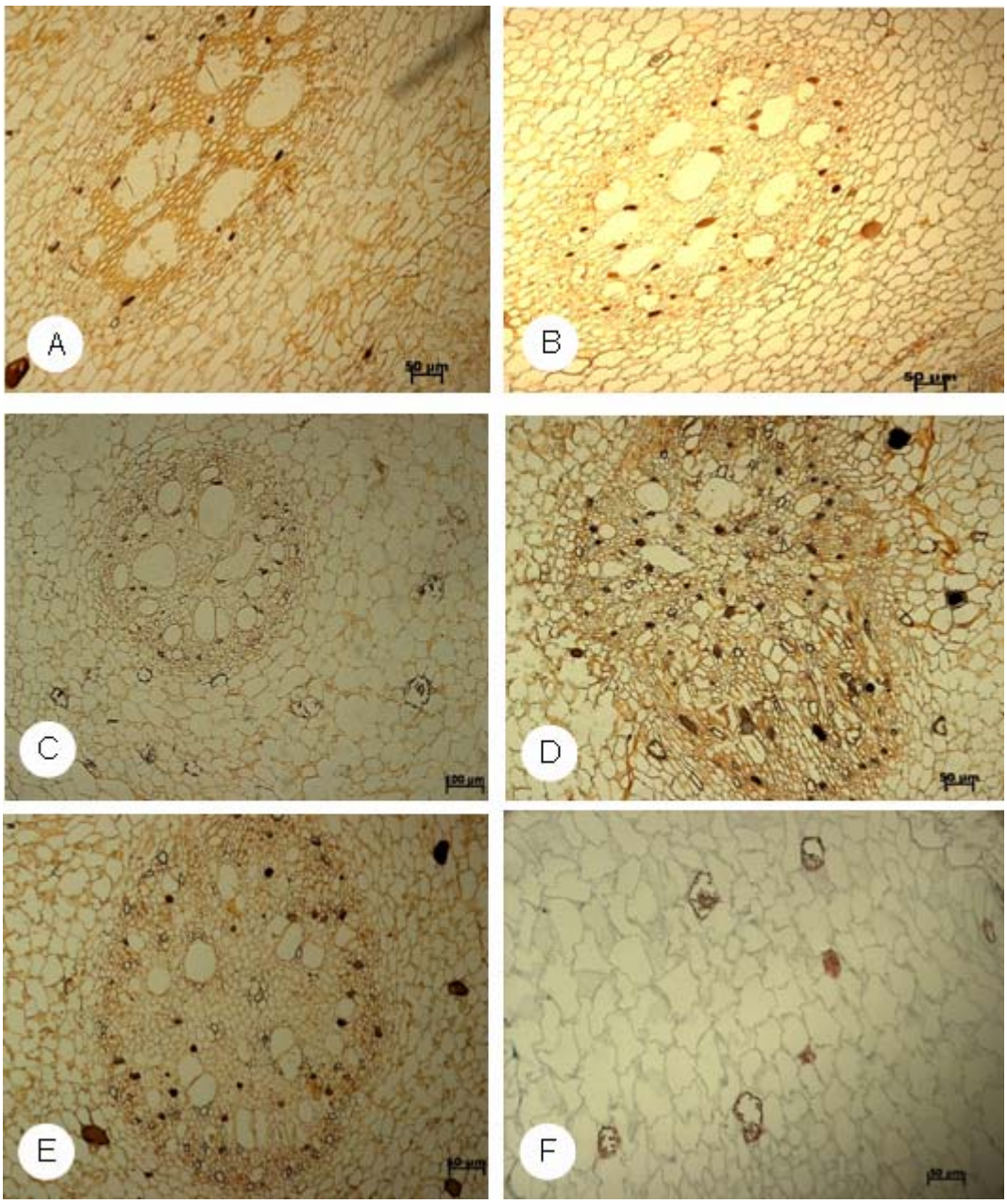
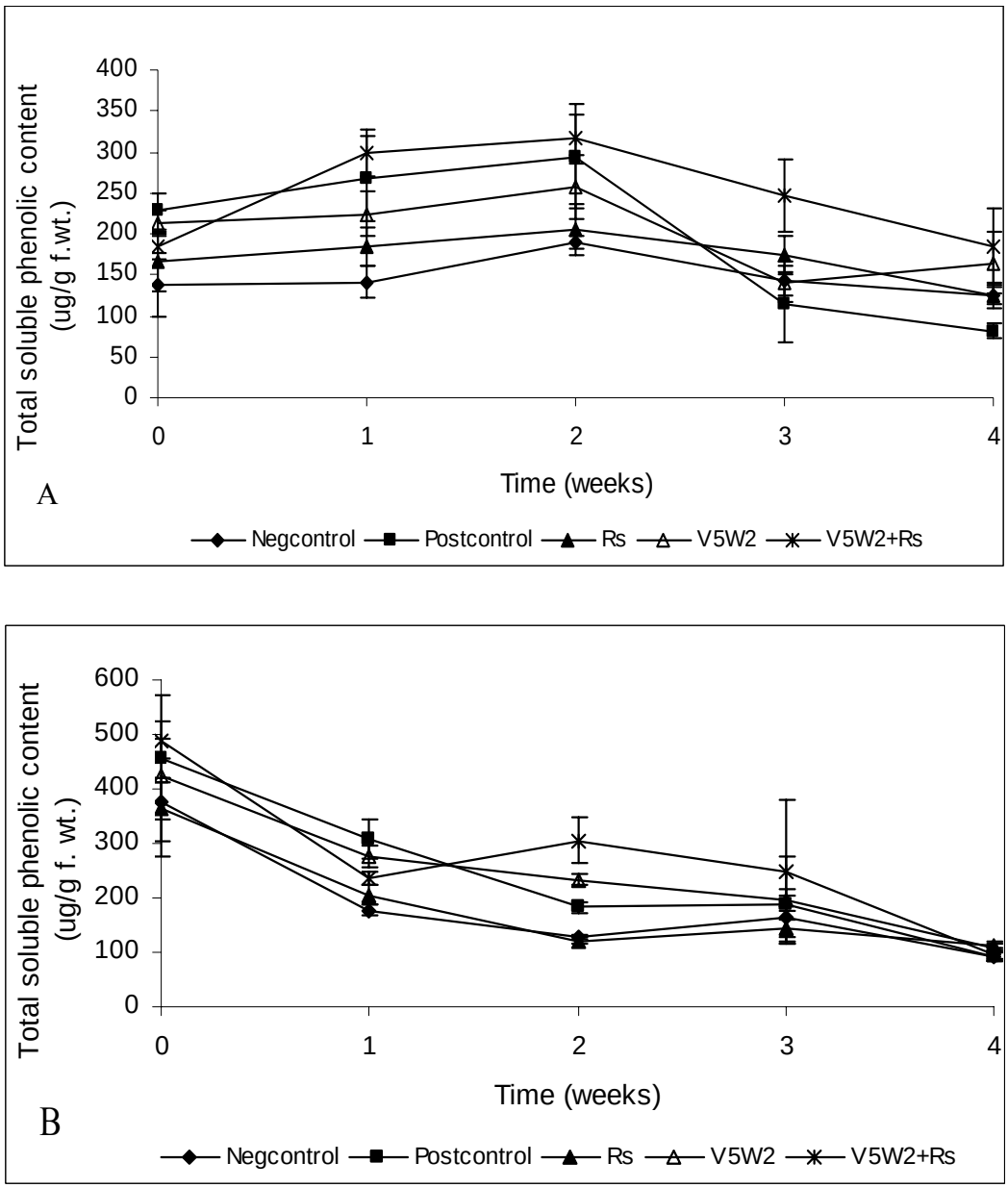


Figure 7. Total soluble phenolics (µg equivalent gallic acid/g fresh weight) in rhizomes (A) and roots (B) of banana plants treated with sterile millet seed (negative control), 50 mM K_2HPO_4 (positive control), *Radopholus similis* only, *Fusarium oxysporum* isolate *V5W2* only, and *V5W2* and *Radopholus similis*.



Negcontrol=negative control (sterile millet seed); Postcontrol=Positive control (50 mM K_2HPO_4); Rs= *Radopholus similis*. Error bars represent standard errors of the mean (n=5).

Figure 8. HPLC chromatograms at 280 nm absorbance of soluble phenolic compounds in rhizome extracts from tissue culture banana plants treated with (A) sterile millet seed, (B) 50 mM K_2HPO_4, (C) *Radopholus similis*, (D) endophytic *Fusarium oxysporum* isolate V5W2 and (E) both *V5W2* and *R. similis*, 2 weeks after endophyte inoculation. Peaks labelled 1-4 are the major compounds that were used for quantitative comparison between treatments.

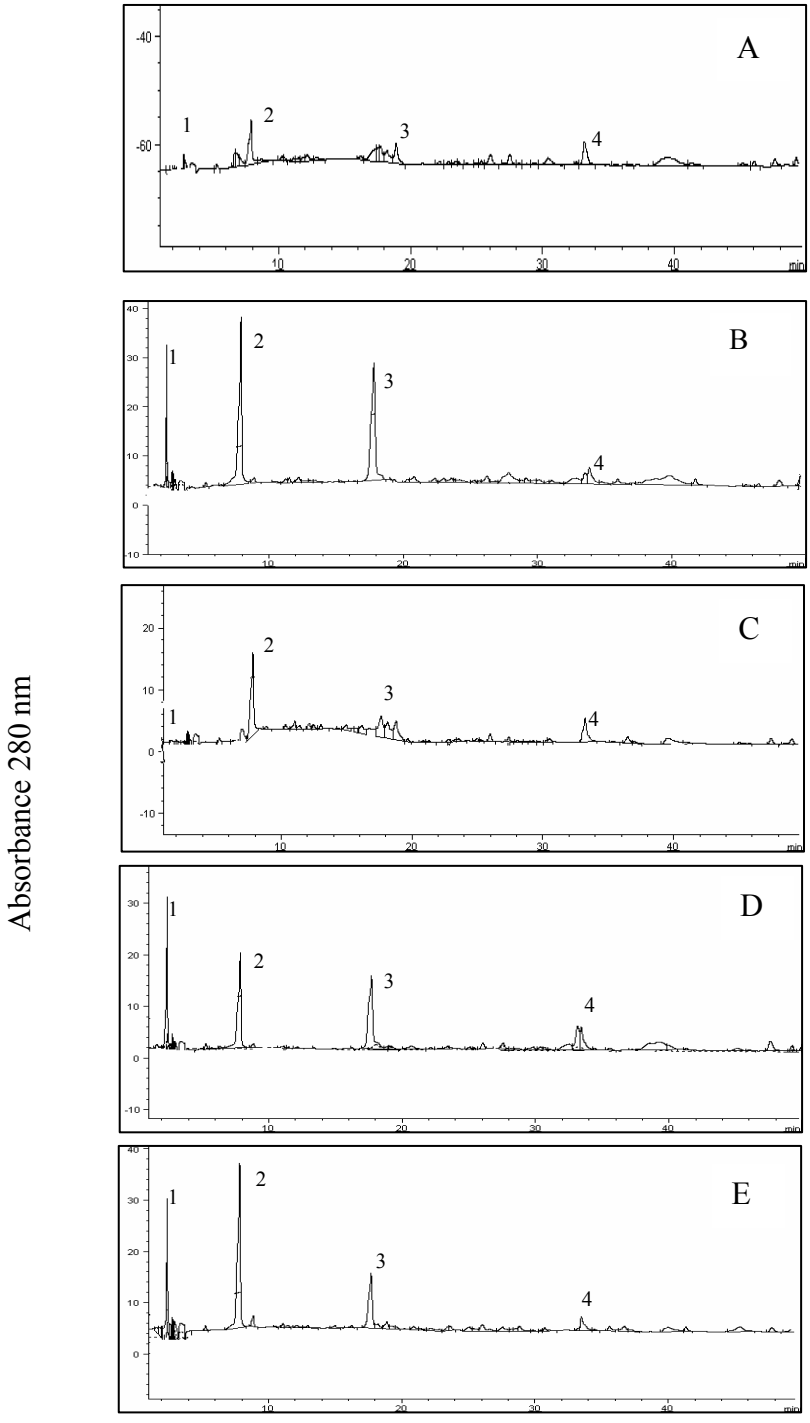
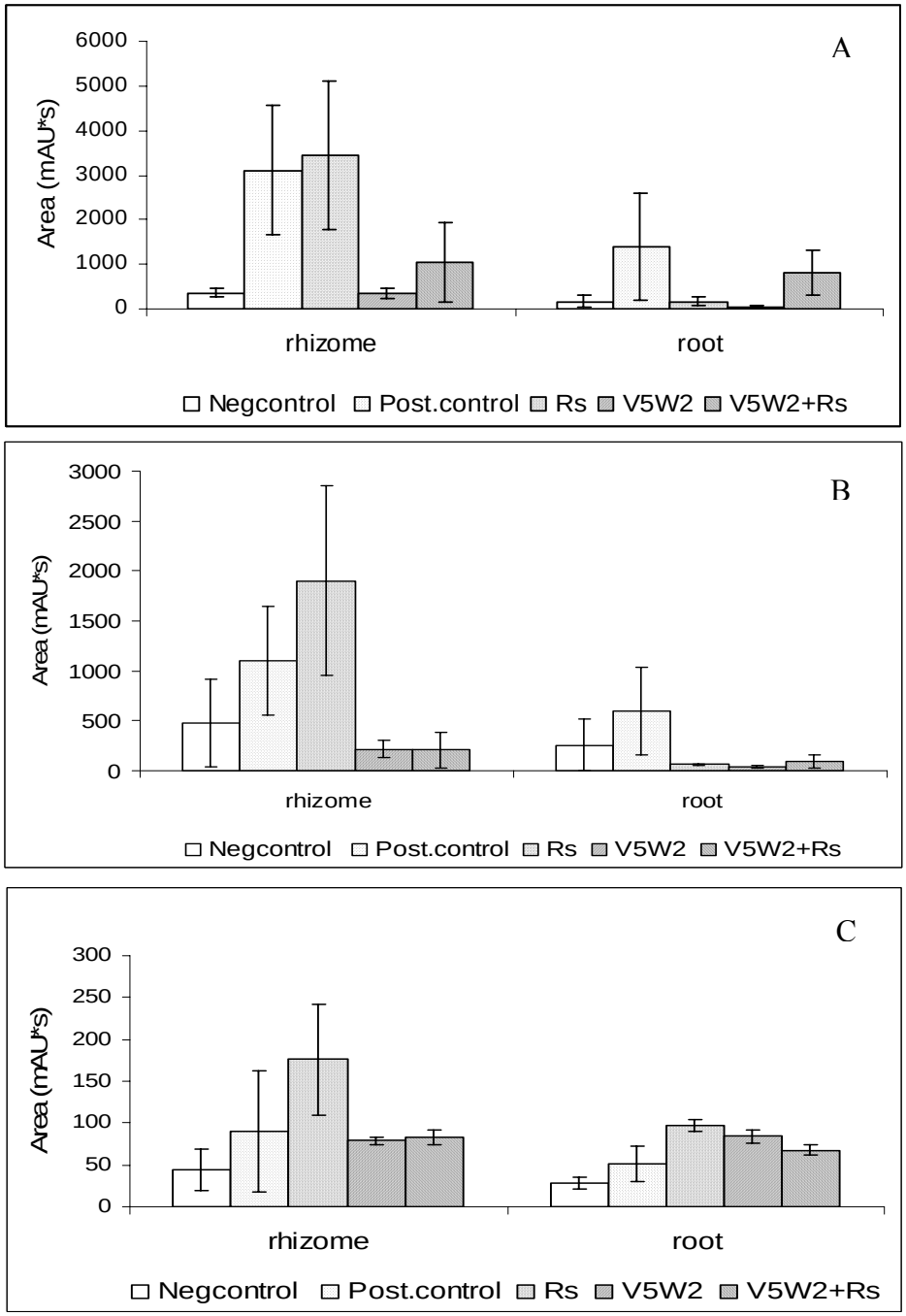


Figure 9. Three unknown compounds in rhizome and root extracts of tissue culture banana plants 2 weeks after inoculation with the endophytic *Fusarium oxysporum* isolate *V5W2*, and 1 week after inoculation with *Radopholus similis*. (A, unknown compound 2; B, unknown compound 3 and C; unknown compound 4)



Negcontrol=negative control (sterile millet seed); Postcontrol=Positive control (50 mM K_2HPO_4); V5W2= endophyte isolate V5W2; Rs= *Radopholus similis*.

Error bars represent standard errors of the mean, n=3.

Table 1. Number of *Radopholus similis* females, males, juveniles and total nematode density in 100 g roots, and percentage root necrosis in the responder roots, 1 month after inoculation of the inducer roots with endophytic *Fusarium oxysporum* isolates.

	Females	Males	Juveniles	Total*	Necrosis (%)
Experiment 1					
Control	582.8 ± 220.8 a	56.9 ± 18.6 a	2322.7 ± 425.9 a	2962.4 ± 534.0 a	26.6 ± 5.4 a
Env1.31i	189.7 ± 66.3 (67.4) a	56.3 ± 24.8 (1.1) a	2276.7 ± 652.0 (1.9) a	2522.7 ± 711.4 (14.8) a	28.7 ± 7.1 a
Env7.11o	364.1 ± 100.3 (35.5) a	76.6 ± 16.9 (34.6) a	1503.1 ± 246.0 (35.2) a	1943.9 ± 297.8 (34.3) a	20.6 ± 3.9 a
V5W2	412.9 ± 168.4 (29.5) a	65.6 ± 29.3 (15.2) a	1613.3 ± 512.0 (30.5) a	2091.8 ± 690.2 (29.4) a	20.7 ± 6.6 a
P Value	0.3475	0.6843	0.6735	0.6693	0.6934
Experiment 2					
Control	693.5 ± 62.7 a	3.5 ± 3.5 a	478.0 ± 48.7 a	1175.1 ± 98.7 a	23.8 ± 3.3 a
Env1.31i	104.5 ± 26.4 (84.9) c	9.1 ± 4.0 (160.5) a	277.3 ± 72.0 (41.9) ab	390.9 ± 88.8 (66.7) c	28.3 ± 2.9 a
Env7.11o	247.1 ± 47.6 (64.3) b	6.0 ± 2.7 (69.6) a	613.2 ± 160.2 (28.2) a	866.3 ± 193.6 (26.2) ab	28.5 ± 3.4 a
V5W2	51.0 ± 16.8 (92.6) c	2.7 ± 1.4 (22.7) a	131.7 ± 36.5 (72.5) b	185.3 ± 47.0 (84.2) c	25.6 ± 2.7 a
P Value	<0.0001	0.6843	0.0041	<0.0001	0.6609
Experiment 3					
Control	212.6 ± 53.9 a	116.2 ± 57.0 a	1743.4 ± 548.8 a	2072.2 ± 691.1 a	12.4 ± 1.9 ab
Env1.31i	137.2 ± 21.5 (35.4) a	72.6 ± 16.7 (37.5) a	746.7 ± 81.8 (57.2) a	956.5 ± 106.0 (53.8) a	12.6 ± 3.2 b
Env7.11o	186.8 ± 40.6 (12.1) a	87.1 ± 9.7 (25.1) a	1639.4 ± 434.1 (5.96) a	1913.3 ± 477.6 (7.7) a	21.8 ± 2.8 a
V5W2	170.7 ± 63.0 (19.6) a	63.0 ± 29.5 (45.7) a	1085.5 ± 248.1 (37.7) a	1319.2 ± 332.0 (36.4) a	10.1 ± 2.1 b
P Value	0.7473	0.4284	0.1620	0.2048	0.0096

*Total nematode density (females+males+juveniles). In each column within an experiment, means followed by the same letter are not different according to Tukey's studentized range test. Figures in parenthesis are percentage reduction of nematode numbers compared to the control treatment.

Table 2. Known phenolic compounds in rhizome and root extracts of tissue culture banana plants identified by HPLC 2 weeks after inoculation with endophytic *Fusarium oxysporum* isolate *V5W2*.

		Amount (µg/ml)	
Compound	Treatment	Corm extracts	Root extracts
3,4-dihydroxybenzoic acid	Negative control	2.1 ± 0.0	49.6 ± 15.5
(11.131)	Positive control	29.4 ± 0.0	19.5 ± 6.5
	R. similis	106.0 ± 86.0	48.7 ± 27.1
	V5W2	16.0 ± 5.9	22.9 ± 2.9
	V5W2+R. similis	25.0 ± 8.9	21.7 ± 14.5
Ferulic acid	Negative control	20.0 ± 0.0	nd
(27.521)	Positive control	nd	nd
	R. similis	nd	nd
	V5W2	20.0 ± 0.0	15.3 ± 0.0
	V5W2+R. similis	4.7 ± 0.0	nd
Hydroxybenzoic acid	Negative control	nd	nd
(16.219)	Positive control	20.0 ± 0.0	nd
	R. similis	nd	20.0 ± 0.0
	V5W2	20.0 ± 0.0	nd
	V5W2+R. similis	nd	nd
Syringic acid	Negative control	nd	127.6 ± 124.0
(18.915)	Positive control	2.2 ± 0.0	67.6 ± 0.0
	R. similis	nd	114.2 ± 60.8
	V5W2	20.0 ± 0.0	nd
	V5W2+R. similis	91.3 ± 41.9	20.0 ± 0.0
Vanillic acid	Negative control	20.0 ± 0.0	23.8 ± 3.8
(18.259)	Positive control	nd	39.8 ± 19.8
	R. similis	20.0 ± 0.0	44.2 ± 16.7
	V5W2	20.0 ± 0.0	22.1 ± 2.0
	V5W2+R. similis	20.0 ± 0.0	12.8 ± 7.1
Gallic acid	Negative control	nd	nd
(6.359)	Positive control	5.86 ± 0.0	nd
	R. similis	nd	nd
	V5W2	nd	nd
	V5W2+R. similis	nd	nd
p-Coumaric acid	Negative control	nd	nd
(25.305)	Positive control	20.0 ± 0.0	20.0 ± 0.0
	R. similis	nd	nd
	V5W2	nd	nd
	V5W2+R. similis	20.0 ± 0.0	nd

Negative control =sterile millet seed; Positive control=(50 mM K_2HPO_4; *V5W2= F. oxysporum* endophyte isolate; Rs= *Radopholus similis*. Values represent the mean and standard error of the mean (n=3). Figures in brackets below each compound represent the retention time. nd= not detected.

Chapter 6

Genetic diversity of endophytic *Fusarium* spp. associated with Cavendish banana in South Africa

Abstract

Endophytic *Fusarium* spp. are commonly associated with banana plants. Nothing is known regarding tissue specificity and the genetic diversity within endophytic *Fusarium* spp. from banana plants. Endophytic *Fusarium* spp. from roots, rhizomes and pseudostem bases of Cavendish banana plants from three sites in South Africa were assessed using morphological and molecular analyses. *Fusarium oxysporum* was the predominant species isolated, followed by *Fusarium solani* and *Fusarium semitectum*. Tissue specificity was observed with *F. oxysporum* and *F. solani* being predominantly isolated from the roots whilst *F. semitectum, F. sacchari* and *Fusarium subglutinans* were predominantly isolated from rhizomes and pseudostem bases. PCR-RFLP analysis of the IGS region of the rDNA divided 46 isolates of *F. oxysporum* obtained from roots into nine different genotype groups. Distance analysis of AFLP data of 57 *Fusarium* isolates resolved the isolates into two major clades: one consisting of the isolated *F. oxysporum* and the other of *F. sacchari*. The *F. solani* isolates formed three clades clearly separated from the other two species. *Fusarium oxysporum* isolates further grouped according to plant part origin while the *F. sacchari* and *F. solani* isolates grouped randomly. The results obtained demonstrate tissue specificity of endophytic *Fusarium* in Cavendish banana and also a wide inter- and intraspecific genetic variation among endophytic *Fusarium* isolates of banana in South Africa.

Introduction

Endophytes are microorganisms that spend at least part of their life cycle inside plant tissues without causing any apparent disease symptoms (Carroll, 1988). The presence of endophytic fungi has been demonstrated in many plants including important agricultural crops like banana (Brown *et al*., 1998; Pereira *et al*., 1999; Pocasangre *et al*., 1999; Dubois *et al*., 2004), maize (Fisher *et al*., 1992), rice (Fisher and Petrini, 1992) and tomato (Hallman and Sikora, 1994). Fungal endophytes are known to produce bioactive products that may play important ecological and biological roles in the host plant (Tan and Zhou, 2001; Strobel, 2003; Schulz and Boyle, 2005). In mutualistic associations, endophyte-infected plants are protected from attack by some insects, nematodes and fungi, while in return the endophyte is provided with shelter and nutrition by the host plant (Latch, 1993; Saikkonen *et al*., 1998; Azevedo *et al*., 2000; Schardl *et al*., 2004).

Fusarium spp. are cosmopolitan fungi that contain many pathogenic forms causing diseases to a wide variety of economically important crops, such as banana (Stover, 1981). The genus also comprises nonpathogenic strains, some of which occur as endophytes colonizing different plant tissues (Niere, 2001; Tan and Zhou, 2001; Sikora *et al*., 2003). Several researchers have reported association between endophytic *Fusarium* and banana plants (Pereira *et al*., 1999; Pocasangre *et al*., 1999; Dubois *et al*., 2004). *Fusarium oxysporum* has been identified as the predominant species establishing endophytic relationships with banana plants. The ability of endophytic *F. oxysporum* isolates to protect banana plants against pests and diseases has been demonstrated in laboratory and screen house experiments (Pocasangre *et al*., 1999; Dubois *et al*., 2004; Gold and Dubois 2005, Nel *et al*., 2006b). Thus, mutualistic associations between endophytic *Fusarium* spp. and banana plants may be viewed as a promising form of biological protection.

The international banana trade is dominated by the Cavendish–type bananas (INIBAP, 2003). Apart from the export trade, Cavendish bananas are grown in many other countries for local consumption only. Opportunities for improving production of Cavendish bananas are often constrained by pests and diseases. The main pests are the banana weevil *Cosmopolites sordidus* and the banana nematode *Radopholus similis* (Daneel *et al*., 2004; de Graaf *et al*., 2004). The main diseases of Cavendish bananas are Fusarium wilt (Panama disease) caused by *Fusarium oxysporum* f. sp. *cubense* (Moore *et al*., 1995) and black

Sigatoka, caused by *Mycosphaerella fijiensis* (Mourichon *et al*., 1997; Surridge *et al*., 2003). These pests and diseases are difficult and costly to control, and can amount to yield losses of up to 100% of severely infested fields (Moore *et al*., 1995; Mourichon *et al*., 1997).

Genetic diversity exists between and within pathogenic and nonpathogenic populations of *F. oxysporum*, and various molecular methods can be used to detect such variation (Gordon and Okamoto 1991; Edel *et al*., 1995). The amplification of variable ribosomal DNA (rDNA) regions allows for discrimination at the genus, species and intraspecific level (Edel *et al*., 1995). Of particular importance is the intergenic spacer (IGS) region that shows considerable divergence within closely related species (Appel and Gordon 1994; 1995). The use of PCR amplified rDNA regions as substrates for restriction fragment length polymorphisms (RFLPs) is one of the widely used methods use to evaluate genetic diversity in *F. oxysporum* populations. By using PCR-RFLP analysis of the IGS region, Woo *et al*. (1996) identified genetic differences between worldwide collections of pathogenic and nonpathogenic isolates of *F. oxysporum* of the bean wilt pathogen *F. oxysporum* f. sp. *phaseoli*. Appel and Gordon (1995) were able to resolve intraspecific variation among 56 soil isolates of *F. oxysporum*, and Nel *et al*. (2006a) reported variation among isolates of *F. oxysporum* from the banana rhizosphere. Several researchers have also reported genetic diversity of endophytic *F. oxysporum* from various plants based on analysis of the IGS region. In addition, diversity among endophytic isolates of *F. oxysporum* from symptomless tomato roots (Elias *et al*., 1991) and from roots and hypocotyls of beans (Alves-Santos *et al*., 1999) was demonstrated.

Amplified fragment length polymorphism (AFLP) is based on the selective amplification of a high number of restriction fragments and is highly reproducible (Vos *et al*., 1995). It is also useful in delineating genetic differences between and within species (Vos *et al*., 1995). For example, AFLPs were shown to relate five distinct clusters with five different *Fusarium* taxa in a phylogenetic study (Abdel-Satar *et al*., 2003). Groenewald *et al*. (2006) further demonstrated that AFLPs could be used to subdivide *F. oxysporum* f.sp. *cubense* in several different clades which correspond to vegetative compatibility. Inter- and intraspecific variation obtained with AFLPs can also be used to construct genetic markers for different microorganisms (Kema *et al*., 2002).

Despite the known association of banana plants with endophytic *Fusarium* spp. genetic diversity of these endophytes, and their relation to different plant parts has been poorly

studied. The objectives of this study were, therefore, to isolate and identify endophytic *Fusarium* spp. from field-grown Cavendish bananas, to examine the tissue specificity of such *Fusarium* spp., and to determine the genetic diversity within these *Fusarium* endophytes of banana.

Materials and methods

Collection of plant material

Fresh root, rhizome and pseudostem base samples were collected from apparently healthy Cavendish banana plants growing in banana weevil- and nematode-infested plantations in Ramsgate, KwaZulu-Natal (KZN) province and Tzaneen, Limpopo province of South Africa in May and June, 2003 respectively. Neither of these plantations were affected by Fusarium wilt of banana. Five and 10 plants were sampled from Ramsgate and Tzaneen, respectively. From each plant, five 10-cm-long primary roots and five 100-cm^3 pieces of the rhizomes and pseudostem bases were randomly taken. Samples were packed in polythene bags, placed in cooler boxes and transported to the Forestry and Agricultural Biotechnology Institute (FABI), located at the University of Pretoria in South Africa, for isolation of endophytic *Fusarium* spp.

Isolation of endophytic Fusarium spp.

Roots were washed under running tap water to remove adhering soil, and surface sterilized in 75 % ethanol for 1 min followed by 2 % sodium hypochlorite solution for 30 s (Petrini, 1986), followed by a double rinse in sterile distilled water (SDW). After rinsing, the samples were dried by blotting them on sterile tissue paper. From each surface sterilized root, five 1-cm long pieces were randomly cut and placed on *Fusarium*-specific rose bengal agar (12 g agar, 10 ml glycerine, 0.5 g L-alanine, 1.0 g pentachloronitrobenzene, 0.5 g Rose Bengal and 1.0 g urea / L SDW) in 65-mm-diameter Petri dishes. The medium was amended with 0.25 g l^{-1} chloramphenicol to prevent bacterial growth. The pseudostem bases, inner and outer rhizome samples from each plant, were surface sterilized by dipping in 95% ethanol, followed by flaming. From each surface sterilized pseudostem base, inner and outer rhizome sample, five 1-cm^3 pieces were randomly removed using a flamed scalpel blade, and also

placed on rose bengal medium amended with antibiotics. All Petri dishes were incubated at 25 °C and checked for fungal growth for 7 days. All colonies of putative *Fusarium* spp. were sub-cultured on half strength potato dextrose agar (19 g PDA, 8 agar / L SDW) (PDA, Difco, Detroit, USA) and incubated for 7 days at 25 °C.

Morphological identification of Fusarium spp.

Single-spore cultures were prepared for all *Fusarium* colonies according to Nelson *et al*. (1983). From the single-spore cultures, mycelial disks were transferred to carnation leaf agar (CLA) and half strength PDA in 65-mm and 90-mm diameter Petri dishes, respectively. CLA was prepared by placing a dry sterile carnation leaf on 2 % water agar (Nelson and Toussoun 1986). The cultures on CLA medium were incubated for 7 to 28 days at 25 °C before being examined for conidium and conidiophore morphology, and chlamydospore production. Cultures on half strength PDA were examined for growth rates and morphological characteristics such as colony appearance (presence or absence of aerial mycelia, surface texture, and pigmentation) after incubation at 25 °C for 10 days. Identification of *Fusarium* isolates to species level was done according to Nelson *et al*. (1983). Only one isolate for each *Fusarium* species recovered per root, rhizomes or pseudostem base tissue per plant was preserved for further analysis to limit duplication of isolates. Isolates were preserved on sterile filter papers and also in 15 % glycerol and stored at 4 °C and –80 °C respectively. All the isolates used in this study are maintained in the FABI culture collection.

Statistical analysis of data

Isolation frequencies of different *Fusarium* spp. among plant parts and between geographic origins were analyzed using categorical logistic regression. Only species for which more than one isolate was obtained were included in the analysis. Likelihood ratio tests were performed to investigate differences within factors (site, *Fusarium* sp. and plant part). If different, means were separated using 95 % confidence intervals and significant α levels of 0.0051 after the Dunn-Sidak correction (Sokal and Rolf 1995; Ury, 1976) using the SAS system (SAS, 1989).

Molecular characterization of Fusarium spp.

DNA extraction from fungal mycelia

To obtain DNA, single spore isolates of *Fusarium* were grown at 25 °C on PDA medium in 65-mm-diameter Petri dishes for 7 days. Fungal mycelium was scrapped from the surface of the medium using a flame-sterilized scapel and transferred into 1.5-ml Eppendorf tubes. DNA was extracted according to Raeder and Broda (1985). To each tube, 300 µl of DNA extraction buffer (200 mM Tris/HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA (pH 8.0), 0.5% SDS in SDW) was added. The mycelium was homogenised in the extraction buffer with a pestle and frozen in liquid nitrogen. The cell lysate was extracted with 700 µl of phenol-chloroform (1:1 v/v) and centrifuged at 14000 rpm for 7 min at 4 °C. The supernatant was then transferred to new tubes and the extraction procedure repeated three more times. To the final supernatant (~250 µl), 25 µl of 3 M sodium acetate (pH 5.5) and 500 µl of ice cold 100 % ethanol were added, and the mixture centrifuged at 14000 rpm for 10 min at 4°C to yield the DNA pellet. The DNA pellet was washed with 70 % ethanol and dried *in vacuo* for 5 min at 45°C, redissolved in 60 µl TE buffer (10 mM Tris/HCl (pH 8.0) and 1 mM EDTA (pH 8.0) in SDW) and treated with RNAse (Sigma-Aldrich, MO, USA) to degrade RNAThe DNA concentrations were determined using a spectrophotometer and diluted to a final working concentration of 20 ng μl^{-1}.

Sequence analysis

Part of the transfer elongation factor 1-α (TEF) region of selected isolates was amplified using the Ef1 and Ef2 primers (O'Donell *et al*., 1998). The PCR reaction conditions were as follows; initial denaturation of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 45 s at 60 ºC, 90 s at 72 ºC and a final extension of 5 min at 72 ºC. PCR reactions were carried out in a mastercycler (Epperndorf, Hamburg, Germany). The resulting amplicons were electrophoresed in 1% TBE agarose gels and visualized using ethidium bromide staining and UV light. The amplified DNA fragments were purified using a High Pure PCR Product Purification kit (Roche Diagnostics, Johannesburg, South Africa) according to the manufacturers instructions. Sequencing was performed in 10 µl reactions each containing 4 µl of purified PCR products, 1 µl of Ef1 or Ef2, 2 µl of Big dye III (Applied Biosystems, Foster City, CA, USA), 1 µl 5x dilution buffer and 2-µl dH_2O. PCR conditions were 25 cycles of 10 s at 96 ºC, 5 s at 50 ºC and 4 min at 60 ºC. Sequencing products were

precipitated and electophoresed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The obtained sequences were edited and aligned using the Sequence Navigator software program (Applied Biosystems). Final alignments were done manually and the sequences compared with GenBank sequence databases using nucleotide Blast on the National Center for Biotechnology Information (NCBI) website.

PCR-RFLP analysis of the IGS region of F. oxysporum *isolates*

All *F. oxysporum* isolates obtained from banana roots were subjected to PCR-RFLP analyses in order to group them into IGS genotypes for subsequent AFLP analyses of a smaller, representative number of isolates. Two oligonucleotide primers, forward primer PNFo (5' CCCGCCTGGCTGCGTCCGACTC 3') and reverse primer PN22 (5' CAAGCATATGACTACTGGC 3') were used to amplify a fragment of the IGS region of the rDNA (Edel *et al*., 1995). The primers were synthesized by Inqaba Biotechnical Industries (Hatfield, Pretoria, South Africa). PCR amplifications were performed in a total volume of 50 µl by mixing 50 ng of template DNA with 0.1 µM of each primer, 2.5 mM of each of dATP, dCTP, dGTP and dTTP, 1 unit *Taq* DNA polymerase and 1x PCR reaction buffer (10 mM Tris/HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml of bovine serum albumin in dH_2O) (Roche Diagnostics). Amplifications were performed in a mastercycler (Epperndorf) with 30 cycles of 90 s at 95 °C, 60 s at 50 °C and 90 s at 72 °C. Five microliters of PCR products was used to verify successful amplification of DNA by electrophoresis in 2% (w/v) agarose gels in 1x TAE buffer for 1 hr at 80 V. Aliquots of 10 µl of PCR products were digested for 4 hrs at 37 °C with 5 units of each of four restriction endonucleases: *Hae*III, *Hinf*I, *Msp*I and *Rsa*I (Roche Diagnostics). Restriction fragments were separated by electrophoresis in 4% agarose gels in 1x TAE buffer for 1 hr at 60 V. Fragments were run against a 100 base pair (bp) molecular marker for size estimation. Each unique banding pattern obtained with the different enzymes was assigned a letter, and each isolate assigned a composite IGS genotype defined by a combination of the patterns (letters) obtained with the four enzymes (Appel and Gordon, 1995).

AFLP analysis of Fusarium *spp.*

A total of 57 *Fusarium* spp. isolates from three banana-growing regions (Ramsgate, Tzaneen and Kiepersol in the Mpumulanga province) in South Africa were studied. Of these, 31 and 16 isolates were obtained from banana plants in Tzaneen and Ramsgate in the current study, respectively. Ten isolates previously collected from symptomless banana plants in Fusarium wilt-suppressive soils in Kiepersol, were obtained from the culture collection at FABI and included in the AFLP analysis. Isolates from Kiepersol had been confirmed as nonpathogenic to banana. Of the 57 isolates, 34 were *F. oxysporum*, 8 were *Fusarium solani*, 14 were *Fusarium sacchari* and 1 *Fusarium. equiseti* (Table 1).

A high-throughput AFLP analysis procedure using infrared dye-labeled primers and an automated DNA analyzer was used (Myburg *et al*., 2001). The restriction, ligation and preamplification reactions were performed as described by Vos *et al*. (1995). Genomic DNA (20 ng) was digested with two units each of *Eco*RI and *Mse*I (Roche Diagnostics) and incubated for 3 hr at 37 °C. For adaptor ligation, 30 µl of digested DNA was added to 10 µl of the restriction-ligation buffer (50 ρmol/µl of *Mse*I adaptor, 5 ρmol/µl of *Eco*RI adaptor and one unit of DNA ligase) and incubated for 3 hr at 37 °C. Restriction-ligation mixtures were diluted 10-fold with low TE buffer (10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA (pH 8.0) in dH2O) to serve as a template for preamplification. Preamplification reactions were performed in a total volume of 30 µl containing: 5 µl of diluted restriction-ligation products, 0.6 units *Taq* polymerase, 3 µl PCR reaction buffer, 0.3 µM of *Eco*RI and *Mse*I primer without any additional selective nucleotides, and 2.5 mM of each of the four dNTPs. The PCR program consisted of 25 cycles of 30 s at 72 °C, 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C, with an additional 1 s per cycle increase in the extension time. Electrophoresis to verify successful preamplification was carried out in 1.2% agarose gels in 0.5x TBE for 1.5 hr at 60 V. Depending on the intensity of the smear, preamplification products were diluted either 10- or 20-fold with low TE buffer and served as the templates for the final amplification step.

Three primer combinations were used for final selective amplification. The *Eco*RI primers were 5′-end labeled with infrared dye IRDye™ 700 or 800 (LI-COR). The primer combinations used were *Eco*RI-AT / *Mse*I-CC, *Eco*RI-CC / *Mse*I-TA and *Eco*RI-TC / *Mse*I-AA. Final amplifications were performed in a total volume of 20 µl containing 5 µl diluted preamplification product, 2 µl PCR reaction buffer, 1.6 µl of 2.5 mM of each of the four

dNTPs, 0.04 µM IRDye 700-labeled *EcoR*1 primer or 0.04 µM IRDye 800-labelled *EcoR*I primer, 0.25 µM *Mse*I primer and 1.2 units *Taq* polymerase. A total of 37 amplification cycles were carried out. The first amplification cycle was carried out for 10 s at 90 °C, 30 s at 65 °C and 1 min at 72 °C. In each of the following 12 cycles, the annealing temperature was reduced by 0.7 °C per cycle. The last 23 cycles were carried out for 10 s at 94 °C, 30 s at 56 °C and 1 min, extended 1 s per cycle, at 72 °C. A final extension cycle was carried out for 1 min at 72 °C. All amplification steps were carried out in a mastercycler (Eppendorf).

Electrophoresis and detection of AFLP fragments

Electrophoresis and detection of AFLP fragments was performed on a two-dye automated DNA sequencer Model 4200s (LI-COR, Lincoln, NE, USA). AFLP fragments were resolved in 25 cm gels containing 8% polyacrylamide gel solution (Long Ranger Gel Solution, Cambrex Bioscience, Rockland, USA), 7.0 M urea and 1x TBE buffer. Ten microliters of formamide loading buffer (95% deionised formamide, 20 mM EDTA, 1 mg/ml Bromophenol blue and 0.5 ml dH_20) was added to 20 µl final AFLP products in 0.2 ml PCR tubes and the tubes wrapped in aluminum foil. All samples were denatured for 3 min at 90 °C and transferred to ice before loading. Disposable 64-well paper combs (KB^{Plus}, LI-COR) (0.25 mm spacer thickness) were used for gel loading. 0.8 µl of each of the diluted preamplification products was loaded into a single lane. The first and last lanes of the gels were loaded with a denatured 50-700 bp molecular weight marker labeled with the corresponding infrared dye (LI-COR). Gel images were transferred automatically from the DNA analyzer to the Saga Application server (LI-COR) where analyses and band scoring were performed.

Scoring of AFLP images

Digital AFLP gel images were scored to obtain binary (band presence or absence) data using $Saga^{MX}$ automated AFLP analysis software (Keygene, Wageningen, the Netherlands). Lane definition and band sizing were performed as described in the $Saga^{MX}$ user manual. Semi-automated scoring was performed by manually clicking on polymorphic fragments present or absent in any of the 60 lanes. After scoring, data were exported directly to a spreadsheet program and reformatted for use in mapping software. Binary data (0 = absence, 1 = presence) obtained from band scores of the gel images obtained with the three different primer pairs was combined. The combined AFLP data was subjected to distance analysis

using the Phylogenetic Analysis Using Parsimony (PAUP) Version 4.0 b10 (Swofford 2002) software (Applied Biosystems, Foster City, CA, USA) which included neighbour joining as a tree building algorithm and a bootstrap of a 1000 replicates (Felsenetein, 1985) for confidence support. The *F. equiseti* isolate was used as the outgroup.

Results

Isolation frequencies of endophytic Fusarium spp. from Cavendish banana plants

The frequency of isolation and diversity of endophytic *Fusarium* spp. was assessed in roots, rhizomes and pseudostem bases of healthy Cavendish banana plants. We isolated a total of 140 isolates belonging to eight *Fusarium* species. Of these 140 isolates, 57.2% were from the roots, 22.7% from the rhizomes, and 19.9% were obtained from the pseudostem bases. *Fusarium oxysporum* was isolated at the highest frequency among the endophytic *Fusarium* isolates. Other *Fusarium* spp. that were isolated included, in decreasing frequencies, *F. solani, F. semitectum, F. sacchari*, *F. subglutinans, F. dimerum*, *F. equiseti* and *F. proliferatum* (Table 2). Mixed infections from an individual plant were common; as many as six different *Fusarium* spp. were found in a single plant. The highest number of *Fusarium* species recovered from a single plant was four and six in Ramsgate and Tzaneen, respectively. From the isolations, an individual *Fusarium* species could be recovered from all plants within one site; *F. oxysporum* and *F. solani* were found in all plants from Ramsgate and Tzaneen, respectively.

The frequency of isolation of different *Fusarium* spp. was influenced by the plant part ($P<0.0001$) and geographic site ($P<0.0001$) from which they were isolated. The frequency of isolation of different endophytic *Fusarium* species also differed within plant parts; roots ($P<0.0001$), rhizomes ($P=0.0115$) and pseudostem bases ($P=0.0301$). A strong interaction between species frequency and plant part ($P<0.0001$) was observed. *Fusarium oxysporum* and *F. solani* were predominantly isolated from the roots, *F. semitectum* and *F. sacchari*, from rhizomes and *F. subglutinans* from pseudostem bases. The composition of different *Fusarium* species differed significantly within geographic sites (Ramsgate, $P<0.0001$ and Tzaneen, $P<0.0001$) with a strong interaction between species composition and geographic site ($P=0.0127$). In Ramsgate, *F. oxysporum* was the most frequently isolated species while in Tzaneen, *F. oxysporum* and *F. solani* were recovered at the highest frequencies. *Fusarium*

sacchari, *F. semitectum* and *F. subglutinans* were also found in higher frequencies in banana plants from Tzaneen than from banana plants from Ramsgate.

Molecular characterization of endophytic Fusarium spp.

Sequence analysis of the TEF 1-α region of selected Fusarium isolates

PCR amplification of a part of the transfer elongation factor 1-α (TEF) of selected isolates using the Ef1 and Ef2 primers yielded a fragment of ~700 bp. The obtained sequences were used to confirm morphological species identification and have been submitted to GenBank, Accession numbers DQ465925 – DQ465954 (Table 1).

PCR-RFLP analysis of the IGS region of Fusarium oxysporum *isolates*

A fragment of approximately 1.7 kbp was amplified from each of the 46 isolates of *F. oxysporum* obtained from banana roots in Ramsgate. Depending on the restriction enzyme used, 1-5 distinct restriction fragment patterns were obtained. Five patterns were realized for *Hae*III, three for *Msp*I, two for *Rsa*I and one for *Hinf*I (Fig. 1). Among the 46 isolates of *F. oxysporum*, a total of 9 IGS genotype groups were identified (Table 3). Isolates from each site generally clustered within the same IGS genotype, although some isolates from the different sites shared the same IGS genotypes. The most common IGS genotype, AAAA, included 10 isolates from Ramsgate and 16 isolates from Tzaneen. Five genotypes out of the nine contained isolates only from Ramsgate. One genotype contained isolates only from Tzaneen and three genotypes contained isolates from both sites (Table 3). Depending on the number of isolates within a particular IGS genotype, a total of 19 representative isolates were selected for further analysis using AFLPs (Table 3). Seven were from genotype 1, three from genotype 5, two each from genotypes 3 and 9, and one each from genotypes 2, 4, 6, 7 and 8.

AFLP analysis of Fusarium spp.

AFLP analysis of *Fusarium* spp. from banana roots, corms and pseudostem bases, using three primer combinations, produced approximately 150 polymorphic bands useful for comparison of genetic diversity. Distance analyses of the AFLP data obtained with the three primer pairs clearly divided the isolates into two distinct clades, one comprising of *F.*

oxysporum isolates and the second comprising of *F. sacchari* isolates. The *F. solani* isolates did not group into one distinct clade, but rather formed two clades, which were clearly separated from the *F. oxysporum* and *F. sacchari* clades (Fig. 2).

The clade comprising of *F. oxysporum* isolates was further divided into 3 distinct sub clades. The clustering of *F. oxysporum* isolates was closely related to the plant part origin than to geographic origin. The first 2 sub-clades comprised of isolates obtained from the roots while the third sub-clade comprised of isolates from the rhizomes and pseudostem bases. Within the root isolates sub-clade, *F. oxysporum* isolates from Kiepersol (sub-clade 2) clearly formed a distinct group while the *F. oxysporum* isolates from Ramsgate and Tzaneen clustered randomly within sub-clades 1 and 3. The *F. oxysporum* isolates obtained from roots were found to be highly diverse as shown in the cladogram where several sub-clades were formed (Fig. 2). The *F. sacchari* clade was further divided into 2 sub-clades, all isolates in the two sub-clades were obtained from the rhizomes and pseudostem bases except for isolate SAT22, which was obtained from roots and which grouped separately from the other isolates. The *F. solani* isolates grouped into two distinct sub-clades with no correlation to plant part or geographic origin. One *F. solani* isolate (SAK39) was separated from the rest of the isolates clades.

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Discussion

Cavendish banana plants in South Africa host a wide array of fungal endophytes. The isolation of a total of 140 isolates belonging to eight different *Fusarium* spp. from roots, rhizomes and pseudostem bases from only 15 Cavendish banana plants in two sites demonstrates the richness of *Fusarium* endophytes and compares favorably to results from earlier investigations involving fungal endophytes of banana. In Thailand, a total of 285 fungal endophytes belonging to 15 different genera were isolated from 49 banana plants of the cv. 'Pisang-Awak' (*Musa* spp. ABB) in nine sites (Niere, 2001). Of these, 79 were isolates of *Fusarium* species. High levels of endophytic fungal diversity from recently harvested plants of East African highland banana, which showed little or no weevil damage in western Uganda, have also been reported (Griesbach, 2000), while a similar study conducted on bananas in central America showed extensive endophytic fungal diversity (Pocasangre *et al*., 1999). Though the current study focused only on endophytic *Fusarium* spp. infecting Cavendish banana plants, the results obtained here and in earlier studies indicate that banana plants are a rich source of fungal endophytes. The role of endophytes in the host plant however remains largely unknown but may have an ecological and biological significance. It has been speculated that since endophytic fungi establish intimate relationships with their host plants, they may therefore, be effective in the management of the major banana pests and pathogens at the site of attack. Introduction of fungal endophytes into tissue culture derived banana plantlets may also be done to enhance the plant's vigor and provide protection against pests and diseases prior to field planting (Pereira *et al*., 1999; Griesbach, 2000; Niere, 2001; Sikora *et al*., 2003).

Fusarium oxysporum, *F. solani*, *F. semitectum* and *F. sacchari* were the most frequently isolated of *Fusarium* spp. from Cavendish bananas in South Africa. All three species can be fungal pathogens of agricultural crops, but only *F. oxysporum* is known to attack bananas (Booth, 1971). Non-pathogenic isolates of *F. oxysporum* have been reported as endophytes of banana before, and have been assessed for their ability to reduce damage caused by pathogenic isolates of *F. oxysporum* and nematodes (Niere, 2001; Pocasangre *et al*., 1999; Nel *et al*., 2006b). *Fusarium solani* and *F. semitectum* are pathogens of crops such as cotton and sorghum (Ciegler *et al*., 1982; Saubois *et al*., 1999), respectively whilst *F. sacchari* is a pathogen of sugarcane (Ganguly, 1964; Rao and Agnihotri, 2000). Because of the frequency of their isolation, and the tissue that they were isolated from, it is possible that these three

species may have important mutualistic relationships with banana. Interestingly, endophytic isolates of *F. sacchari* from sugarcane have been shown to inhibit development of the sugarcane borer moth *in vitro* (Mc Farlane and Rutherford, 2005). Since *F. sacchari* is non-pathogenic to banana, the role of this fungus in the plant remains unknown and requires further investigation.

In the current study, we obtained more isolates of *Fusarium* spp. from banana roots than from rhizomes or pseudostem bases. These results are in agreement with other studies in which roots appear to harbor more endophytic fungi than other plant parts (Niere, 2001), which may indicate that the roots are potentially colonized by fungi from the soil. *Fusarium oxysporum* was the dominant species found in the roots and *F. semitectum* and *F. sacchari* in rhizomes of Cavendish bananas. Similarly, *F. oxysporum* was found to be the most dominant species in roots and *F. semitectum* in rhizomes of the banana cv. 'Pisang-Awak' (Niere, 2001). Photita *et al*. (2001) reported presence of lower numbers of endophytic fungal isolates from pseudostems in comparison to the veins, interveins and midribs of wild banana plants in Thailand. Differences in the isolation frequencies and the interaction observed between *Fusarium* spp. and different plant parts indicate that endophytic *Fusarium* spp. might be adapted to different banana plant parts. Tissue specificity of endophytic fungi has been reported for conifer needles (Carroll and Carroll 1978), and may be due to the adaptation by particular endophytes to the micro-ecological and physiological conditions present in the different plant organs (Petrini, 1996). Tissue specificity may also be a reflection of tissue preferences of individual dominating taxa (Rodrigues and Samuels 1990). The observed tissue specificity of various *Fusarium* endophytes may be used in biological control programs to target establishment of the endophytes most suited to the plants parts that require protection.

PCR-RFLP analyses grouped endophytic *F. oxysporum* isolates obtained from banana roots from Ramsgate and Tzaneen into 9 IGS genotypes. Although PCR-RFLP may not be the most suitable method to study genetic diversity, it has been used in several studies to resolve genetic differences among *F. oxysporum* strains (Appel and Gordon 1995; Nel *et al*., 2006a). In the current investigation, the PCR-RFLP technique was useful in identifying genetic differences among *F. oxysporum* isolates obtained from the roots and aided in selecting representative isolates for further AFLP analyses. In previous investigations using IGS-RFLP, 120 isolates of *F. oxysporum* from roots and hypocotyls of beans were grouped into

four haplotypes (Alves-Santos *et al*., 1999). Similarly, Appel and Gordon (1995) grouped 56 isolates of *F. oxysporum* into 13 IGS haplotypes.

In the current study, IGS genotypes that occurred at highest frequencies contained isolates from both collection sites, while groups that consisted solely of isolates from either of the two sites occurred at low frequencies. The greater number of IGS genotypes found in Ramsgate, compared to those in Tzaneen, despite being isolated from fewer plants, indicates a greater genetic diversity within isolates from Ramsgate. One possible explanation for this finding is that, because bananas have been cultivated in southern KZN for longer than in Tzaneen, more non-pathogens might have developed a mutualistic relationship with bananas in KZN.

IGS genotype grouping as opposed to AFLP's did not reveal a clear-cut correlation between *F. oxysporum* isolates, and their geographic origin or plant part origin. Some isolates from one site were restricted to a single IGS genotype, and others from both sites (Ramsgate and Tzaneen) shared the same IGS groups. Isolates from a particular site that occurred in the same IGS groups were not necessarily from the same plant or plant part. In fact, our results showed that the influence of plant part origin on IGS genotype grouping was very minimal. Several researchers have reported conflicting results in this regard. For example, genetic variation between isolates of *F. oxysporum* f. sp. *elaidis* have been linked to geographic origins with isolates originating from a specific site grouping together (Flood *et al*., 1992), while there was no correlation between grouping and the geographic origin of 49 strains of *F. oxysporum* isolated from pea plants (Skovgaard *et al*., 2002). Sharing of IGS genotypes by isolates obtained from different sites has previously been reported before (Appel and Gordon, 1995). Since this study was on endophytic fungi in two separated regions and from plantations that were initiated from tissue culture banana plants, we can rule out the possibility of introduction of the endophytes with planting material or long distance dispersal of soil particles and fungal spores. Absence or limited long distance dispersal of conidia may restrict gene flow, allowing for populations to evolve independently, and thus contributing to development of discrete fungal populations (Appel and Gordon, 1994). Probably, isolates from the two sites, which shared IGS genotypes, represent clonal lineages that occur irrespective of the geographic distances separating them (Appel and Gordon, 1995).

AFLP analysis proved useful in separating 57 isolates of *Fusarium* into two distinct clades according to the species, with *F. oxysporum* and *F. sacchari* clearly separated from the *F. solani* clades. Using AFLP, Abdel-Satar *et al*. (2003) were able to resolve five different *Fusarium* spp. into five distinct clusters according to each species. AFLP analysis successfully grouped the *F. oxysporum* isolates according to the plant part origin. The correlation between *F. oxysporum* isolates and the plant part origin further indicates species tissue specificity. In the current study however, AFLP analysis was not efficient in correlating the isolates of *F. oxysporum* with their geographic origin .The only exception was observed with isolates of *F. oxysporum* from Kiepersol, which formed a distinct sub-clade within the *F. oxysporum* main clade as would be expected of endophytes that co-evolve with plants over time, and that are selected to persist and form compatible long-lasting associations with bananas. In a previous investigation involving *F. oxysporum* f. sp. *lentis* no correlation was observed between 32 isolates and their geographic origin (Belabid *et al*., 2004). The findings obtained in this study are difficult to explain, but may be due to differences in plantation age in the different sites. Banana cultivation in Kiepersol has been going on for longer periods of time than in Ramsgate and Tzaneen (Altus Viljoen pers. comm.) and this may explain why isolates from this site grouped together. In well-established agronomic systems, limited genetic diversity has been observed between plants and their endophytic counterparts possibly due to a constant environment, which ensures continual persistence of the association. However, short-term establishments for annual crops, as opposed to perennial crops such as banana, may prevent the formation of stable compatible plant-endophyte combinations (Saikkonen *et al*., 2004). The *F. solani* isolates, which grouped into two distinct clades probably represent phylogenetic species within the *F. solani* species complex (O'Donnell, 2000; Geiser *et al*., 2004).

Endophytic fungi have recently gained popularity as possible biological control agents of crop pests and diseases and may act in several ways; through metabolite production, competition for nutrients and ecological niches, and/or induced resistance (Sikora *et al*., 2003; Schulz and Boyle 2005). Whether variation in endophyte genotypes can be linked to biologically important traits, such as pathogenicity or aggressiveness against pests and diseases and variations in metabolite production remains to be determined (Woo *et al*., 1998). AFLP analysis has also been useful in generating molecular markers that are linked to genes of biological interest in plants such as barley (Karakousis *et al*., 2003) and the wheat fungus, *Mycosphaerella graminicola* (Kema *et al*., 2002) might therefore be useful in

developing molecular markers for studying the dynamics and persistence of introduced agents and in tracking of the isolates in plants.

The current study has demonstrated the diversity in *Fusarium* endophytes. We have shown that different *Fusarium* spp. may occur as endophytes in crops other than those that they are known to be pathogenic to, possibly establishing mutualistic associations with the host plants. Different endophytic *Fusarium* species appear to have preferences for certain plant parts. The potential for using endophytic *Fusarium* spp. as an alternative or complementary pest and disease control option in banana is promising and requires further investigation.

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Figure 1. Schematic representation of PCR-restriction fragment length polymorphism patterns after digestion of the intergenic spacer region of the ribosomal DNA of 46 isolates of endophytic *Fusarium oxysporum* with the restriction enzymes *Hae*III, *Msp*I, *Rsa*I and *Hinf*I.

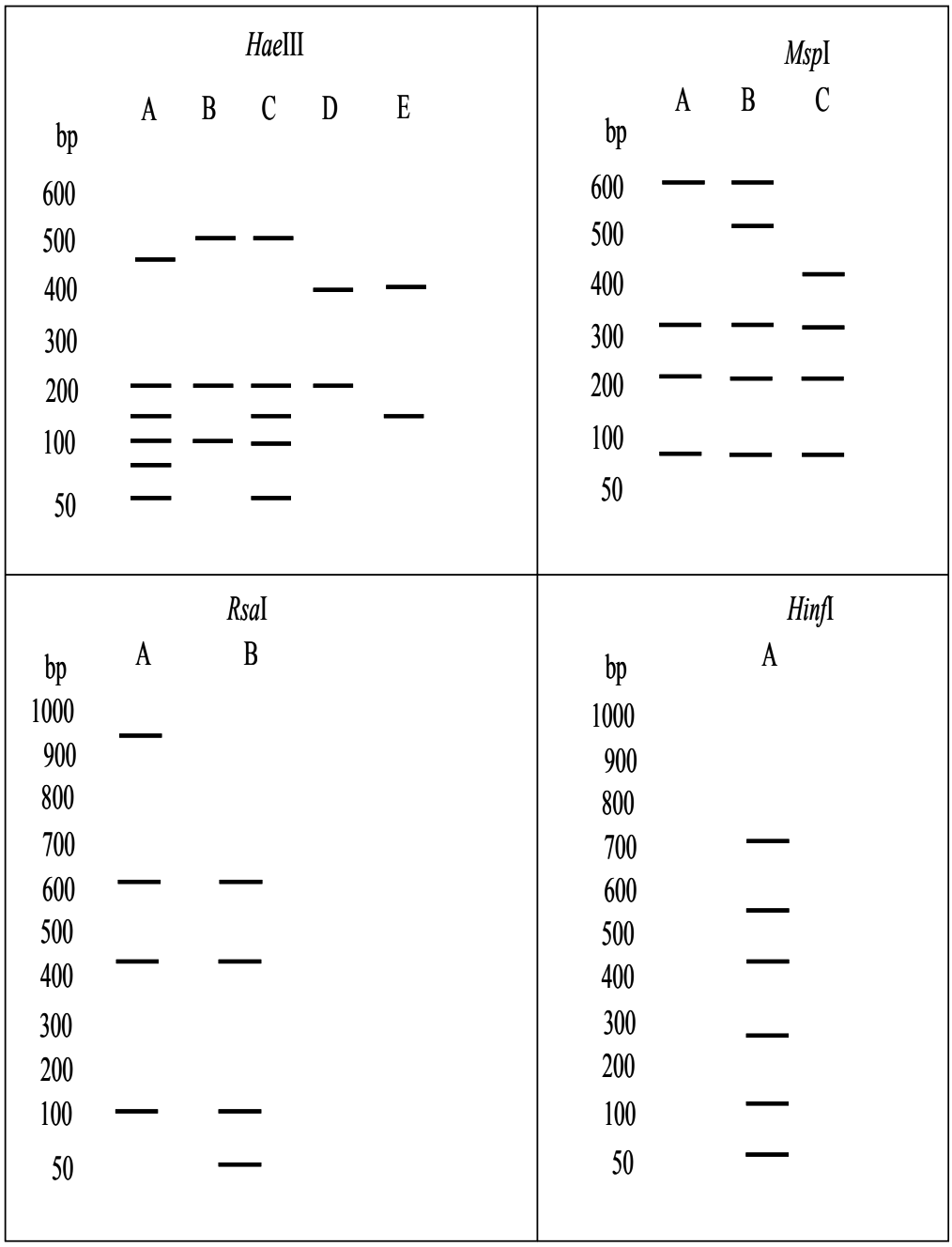

Figure 2. Phylogram inferred from amplified fragment length polymorphism analysis showing genetic relationship of 57 endophytic Fusarium isolates from banana root, rhizome and pseudostem base tissues. Distance analysis included neighbor joining as a tree building algorithm and a bootstrap of 1000 replicates. Bootstrap values are indicated above nodes, values less than 50% are not shown. The Fusarium isolates were obtained from Tzaneen, Ramsgate, and Kiepersol in South Africa and are designated with the prefix SAK, SAT, and KIP respectively

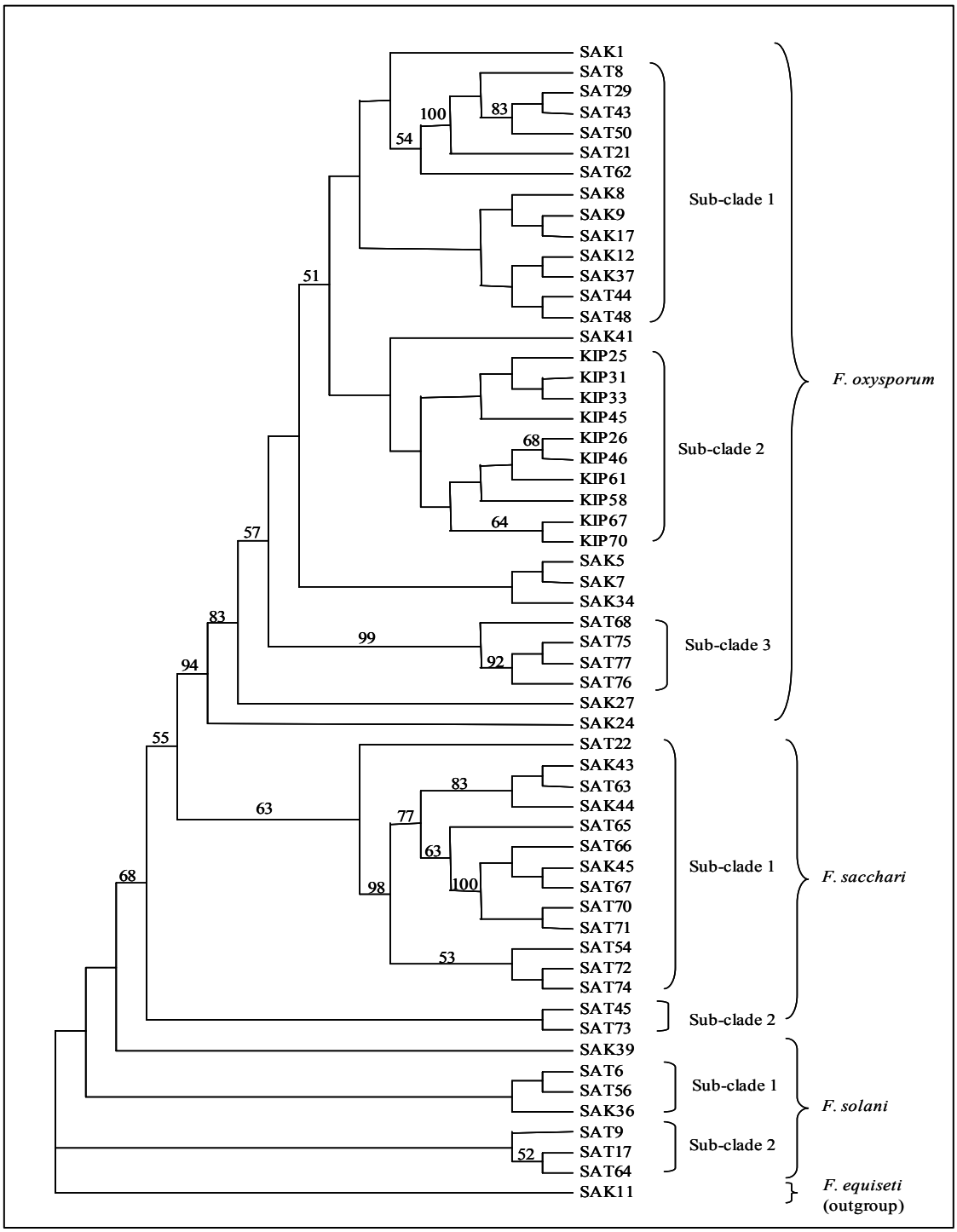

Table 1. Geographic origin of 57 endophytic isolates of *Fusarium* spp. from roots, rhizomes and pseudostem base tissues of Cavendish banana plants (*Musa* spp. AAA) in South Africa used for amplified fragment length polymorphism analysis.

Collectors no.[a]	CAV no.[b]	Species	GenBank accession number	Geographic origin[c]	Plant tissue
KIP25	CAV 542	*F. oxysporum*	DQ465926	Kiepersol	Root
KIP26	CAV 543	*F. oxysporum*		Kiepersol	Root
KIP31	CAV 546	*F. oxysporum*		Kiepersol	Root
KIP33	CAV 548	*F. oxysporum*		Kiepersol	Root
KIP45	CAV 552	*F. oxysporum*	DQ465927	Kiepersol	Root
KIP46	CAV 553	*F. oxysporum*		Kiepersol	Root
KIP58	CAV 559	*F. oxysporum*		Kiepersol	Root
KIP61	CAV 561	*F. oxysporum*		Kiepersol	Root
KIP67	CAV 563	*F. oxysporum*		Kiepersol	Root
KIP70	CAV 566	*F. oxysporum*		Kiepersol	Root
SAK1	CAV 722	*F. oxysporum*		Ramsgate	Root
SAK12		*F. oxysporum*		Ramsgate	Root
SAK17		*F. oxysporum*		Ramsgate	Root
SAK24	CAV 735	*F. oxysporum*	DQ465929	Tzaneen	Outer rhizome
SAK27		*F. oxysporum*		Ramsgate	Root
SAK34		*F. oxysporum*		Ramsgate	Root
SAK37		*F. oxysporum*		Ramsgate	Root
SAK41	CAV 754	*F. oxysporum*		Ramsgate	Root
SAK5	CAV 725	*F. oxysporum*	DQ465928	Ramsgate	Root
SAK7	CAV 727	*F. oxysporum*		Ramsgate	Root
SAK8	CAV 728	*F. oxysporum*		Ramsgate	Root
SAK9		*F. oxysporum*		Ramsgate	Root
SAT21	CAV 761	*F. oxysporum*		Tzaneen	Root
SAT29	CAV 766	*F. oxysporum*		Tzaneen	Root
SAT43	CAV 771	*F. oxysporum*		Tzaneen	Root
SAT44	CAV 772	*F. oxysporum*		Tzaneen	Root
SAT48	CAV 774	*F. oxysporum*	DQ465930	Tzaneen	Root
SAT50	CAV 776	*F. oxysporum*		Tzaneen	Root
SAT62	CAV 784	*F. oxysporum*		Tzaneen	Root
SAT68	CAV 189	*F. oxysporum*		Tzaneen	Inner rhizome
SAT75		*F. oxysporum*	DQ465931	Tzaneen	Pseudostem base
SAT76		*F. oxysporum*	DQ465932	Tzaneen	Pseudostem base
SAT77		*F. oxysporum*	DQ465933	Tzaneen	Pseudostem base
SAT8	CAV 750	*F. oxysporum*		Tzaneen	Root
SAK43		*F. sacchari*	DQ465944	Ramsgate	Inner rhizome
SAK44	CAV 1673	*F. sacchari*	DQ465943	Ramsgate	Pseudostem base
SAK45		*F. sacchari*	DQ465945	Tzaneen	Pseudostem base
SAT22	CAV 762	*F. sacchari*	DQ465934	Tzaneen	Root
SAT54	CAV 778	*F. sacchari*	DQ465935	Tzaneen	Outer rhizome
SAT63	CAV 1665	*F. sacchari*	DQ465936	Tzaneen	Inner rhizome
SAT65	CAV 1666	*F. sacchari*	DQ465937	Tzaneen	Pseudostem base
SAT66	CAV 1667	*F. sacchari*	DQ465938	Tzaneen	Pseudostem base

SAT67	CAV 1668	*F. sacchari*	DQ465939	Tzaneen	Inner rhizome
SAT71	CAV 1671	*F. sacchari*	DQ465941	Tzaneen	Inner rhizome
SAT72	CAV 1672	*F. sacchari*	DQ465942	Tzaneen	Inner rhizome
SAT73		*F. sacchari*	DQ465946	Tzaneen	Inner rhizome
SAT74		*F. sacchari*	DQ465947	Tzaneen	Pseudostem base
SAT70	CAV 1670	*F. sacchari*	DQ465940	Tzaneen	Inner rhizome
SAK36	CAV 740	*F. solani*	DQ465948	Ramsgate	Root
SAK39	CAV 742	*F. solani*	DQ465949	Ramsgate	Root
SAT17	CAV 759	*F. solani*	DQ465952	Tzaneen	Root
SAT45	CAV 773	*F. solani*		Tzaneen	Root
SAT56	CAV 780	*F. solani*	DQ465953	Tzaneen	Root
SAT6	CAV 748	*F. solani*	DQ465950	Tzaneen	Root
SAT64		*F. solani*	DQ465954	Tzaneen	Pseudostem base
SAT9	CAV 751	*F. solani*	DQ465951	Tzaneen	Root
SAK11	CAV 729	*F. equiseti*	DQ465925	Ramsgate	Outer rhizome

[a] Collectors numbers designates the isolate code given after primary isolation.

[b] CAV numbers designate codes for the fungal cultures deposited in the FABI culture collection, University of Pretoria, Pretoria, South Africa.

[c]Geographic site from which banana plants were sampled; endophytic *F. oxysporum* isolates from Kiepersol were not isolated in the current study but were obtained from the FABI culture collection.

Table 2. Incidence (%) of endophytic *Fusarium* spp. isolated from different plant parts of Cavendish bananas (*Musa* spp. AAA) in Ramsgate and Tzaneen, South Africa.

Species	Plant part									Geographic origin						Total[b]	
	Root			Rhizomes[a]			Pseudostem bases			Ramsgate			Tzaneen				
	n	%		n	%		n	%		n	%		n	%		n	%
F. oxysporum	45	32.8	a	3	2.1	a	3	2.1	a	24	17.1	a	28	20.0	c	52	37.4
F. solani	23	16.4	b	8	5.7	a	3	2.1	a	7	5.0	b	27	19.2	c	34	24.2
F. sacchari	1	0.7	c	5	3.6	a	8	5.7	a	2	1.4	b	12	8.5	ab	14	10.0
F. semitectum	0	0.0	c	10	7.1	a	5	3.6	a	5	3.5	b	10	7.1	ab	15	10.7
F. subglutinans	1	0.7	c	2	1.4	a	8	5.7	a	1	0.7	b	9	6.4	ab	10	7.1
F. dimerum	10	7.1	b	1	0.7	a	0	0.0	b	1	0.7	b	10	7.1	a	11	7.8
F. equiseti	0	0.0		3	2.1	a	0	0.0	b	2	1.4	b	1	0.7		3	2.1
F. proliferatum	0	0.0		0	0.0		1	0.7		0	0.0		1	0.7		1	0.7
Total	80	57.7		32	22.7		28	19.9		42	29.8		98	69.7		140	100

Within each plant part or site, incidence (isolation frequencies) of the different *Fusarium* spp. followed by the same letter are not statistically different at P<0.0051 after the Dunn Sidak correction.

Table 3. Intergenic spacer (IGS) region groups of 46 *Fusarium oxysporum* isolates from Cavendish banana (*Musa* spp. AAA) roots from two banana growing regions in South Africa that were subjected to PCR-restriction fragment length polymorphism analysis with four restriction enzymes *Hae*III, *Msp*I, *Rsa*I and *Hinf*I.

		Percentage[b]	Restriction enzyme			
IGS group	Isolates[a]		*Hae*III	*Msp*I	*Rsa*I	*Hinf*I
1	SAK1*, 3, 4, 16, 20, 22, 27*, 30, 35 & 37 SAT 2, 10, 16, 19, 21*, 28, 29*, 31, 34, 41, 43*, 49, 50*, 52, 61 & 62*	56.5	A	A	A	A
2	SAK 12* & 14	4.3	A	A	B	A
3	SAK 8* & SAT 8*	4.3	A	B	A	A
4	SAK 2, 5 & 15*	6.5	B	A	A	A
5	SAK 7*, 17*, 23 & 24* SAT 3, 14 & 48*	15.2	B	A	B	A
6	SAK 6 & 9*	4.3	C	B	A	A
7	SAK 41*	2.1	D	A	A	A
8	SAK 34*	2.1	D	A	B	A
9	SAT 12 & 44*	4.3	E	C	A	A

[a]Designation of isolates used in the PCR-RFLP analysis. Isolates with the prefix SAK were isolated from Ramsgate, SATfrom Tzaneen. *Fusarium oxysporum* isolates with an asterisk were selected from each IGS genotype for AFLP analysis.

[b] Percentage of total isolates in that particular IGS genotype

Summary

Radopholus similis is one of the key pests of banana in the East African highlands. Nematode damage results in large crop and yield losses. Although control of this pest had mainly relied on the use of clean planting material, re-infestation of plants in the field remains a critical concern. Alternative management options such as the use of fungal endophytes may be used to provide protection and extend plant life. In this thesis, the objective was to identify endophytic isolates of *Fusarium* spp. and especially *F. oxysporum* with potential for *R. similis* control in tissue culture banana plantlets and to study the interactions between the host plant, nematode and the endophyte. Isolates were screened both *in vitro* and *in vivo* and the most promising isolates were studied further to elucidate when, where and how the endophytes suppressed *R. similis*.

In the first phase of this research, a total of 35 endophytic *Fusarium* spp. isolates were screened for the production of secondary metabolites antagonistic to *R. similis in vitro*. All isolates tested demonstrated some level of *in vitro* antagonistic activity on the mobility of *R. similis* males, females and juveniles. The percentage of immobilized nematodes increased with increase in the length of exposure time and the concentration of the culture filtrates. An interesting finding of this research was that male *R. similis* were more sensitive to culture filtrate treatment than females. The method of using culture filtrates to identify isolates with antagonistic effects against the nematode has the advantage of being rapid, thus saving time and allows for selection of the most promising isolates for *in planta* evaluation. The actual metabolites produced by the isolates in culture were not determined in this study and warrant further investigations.

In the second part of this research, nine *F. oxysporum* isolates that showed good *in vitro* antagonistic activity against *R. similis* were screened for nematode suppression in tissue culture banana plants. A local banana cultivar susceptible to *R. similis* was used in nine screen house experiments. Endophyte treatment did not affect nematode population densities significantly although although there was a tendency for nematode populations in endophyte-treated plants to be lower than in the untreated control plants. Banana plant growth and nematode damage were also not influenced by endophyte treatment except in a few

experiments. The results obtained showed the effect of individual isolates on plant growth, nematode damage and density varied widely across repeat experiments. Despite the inconsistencies, three isolates, *V5W2*, *Eny1.13i* and *Eny7.11o* were identified as the most effective isolates. The reproducibility of the results obtained is questionable as control plants were also found to be infected by endophytic *F. oxysporum*. This research also raises a big question pertaining to the ability to monitor and track re-isolated endophytes. Thus proper endophyte marking techniques need to be developed for future experiments to compare the re-isolated endophytes with the original ones. Although the levels of nematode control afforded by endophytes may not be very high, the use of endophytes in tissue culture plants may complement the clean nature of the plants thereby boosting their ability to resist or tolerate nematode attack in the field.

The endophytes tested during the current study have shown ability to suppress *R. similis* in tissue culture banana plants. However, it is not known how, where and when the endophytes are most effective against the nematodes. In the third part of this research, the effects of three isolates, *V5W2*, *Eny1.31i* and *Eny7.11o* on the host preferences, root penetration and reproduction of *R. similis* in tissue culture banana plants were evaluated under laboratory and screen house conditions. Host preference tests provided evidence that nematodes were equally attracted to both endophyte-treated and untreated plants. Similarly, root penetration of banana plant roots by *R. similis* did not differ between endophyte-treated and untreated plants. Nematode reproduction which was assessed over a period coinciding with three nematode generations was however, influenced by endophyte treatment. Differences were observed during the second and third nematode generations with nematode reproduction rates being higher in the untreated compared to endophyte-treated plants. The results obtained demonstrate that the mechanism by which the fungal isolates control *R. similis* in the plant is mainly post-infectional, acting only after the plants have been infected and may possibly be through induced resistance in the plant against the nematode.

In the fourth part of this research, potential mechanism(s) through which endophytic isolates of *F. oxysporum* result in nematode suppression in tissue culture banana plants were studied. Nine isolates of *F. oxysporum* showed positive results for protease enzyme production but none showed chitinase and lipase activity. In split-root experiments, the densities of *R. similis* females, males, juveniles and the total nematode density were substantially lower in one half

of the split-root systems when the corresponding half was treated with one of the endophytic *F. oxysporum* isolates *V5W2*, *Eny1.131i* or *Eny7.11o*. To further investigate the role of induced resistance in nematode management by endophytes, the amounts and types of phenolic compounds were assessed in plants inoculated with isolate *V5W2*. Histological analysis showed the presence of phenolic cells in the cortex and central cylinder regions of roots and rhizomes. Although constitutive phenols occurred both in roots and corms of plants treated with isolate *V5W2* as well as untreated plants, the quantities increased over time in the endophyte-inoculated plants compared to the uninoculated plants. Higher amounts of total soluble phenolics were found in endophyte-treated than in untreated plants. Corm and root metabolite profiles of endophyte-treated and untreated plants did not differ. HPLC analysis revealed four major unidentified compounds, which were present in endophyte-treated as well as untreated plants. These results demonstrate that induction of systemic resistance by endophytic isolates of *F. oxysporum* may play a role in *R. similis* management and that treatment of plants with endophytes triggers the plant to increase synthesis of phenolic compounds. Further research needs to be conducted to identify the unknown compounds.

In the last part of this research, endophytic *Fusarium* spp. from roots, rhizomes and pseudostem bases of apparently healthy Cavendish banana plants from three sites in South Africa were assessed using morphological and PCR-RFLP and AFLP analyses. The objectives were to identify *Fusarium* endophytes in banana plants, study their relation to specific plant parts and determine the genetic diversity within and between the endophytes. From the isolations, *Fusarium oxysporum* was the predominant species isolated, followed by *Fusarium solani* and *Fusarium semitectum*. Tissue specificity was observed with *F. oxysporum* and *F. solani* being predominantly isolated from the roots whilst *F. semitectum, F. sacchari* and *F. subglutinans* were predominantly isolated from rhizomes and pseudostem bases. The *F. oxysporum* isolates from the roots were grouped into nine genotypes using PCR-RFLP analysis of the IGS region of the rDNA. Distance analysis of AFLP data of 57 *Fusarium* isolates resolved the isolates into two major clades: one consisting of the isolated *F. oxysporum* and the other of *F. sacchari*. *Fusarium oxysporum* isolates further grouped according to plant part origin while the *F. sacchari* and *F. solani* isolates grouped randomly. The results obtained demonstrated tissue specificity of endophytic *Fusarium in* Cavendish banana and also a wide inter- and intraspecific genetic variation among endophytic *Fusarium* isolates of banana in South Africa. The observed tissue specificity of various *Fusarium*

endophytes may be used in future biological control programs to target establishment of the endophytes most suited to the plants parts that require protection.

Chapter 4

Effect of endophytic *Fusarium oxysporum* isolates on host preference, attraction, root penetration and reproduction of *Radopholus similis* in tissue culture banana plants

Abstract

The effects of three endophytic *Fusarium oxysporum* isolates, *V5W2*, *Eny1.31i* and *Eny7.11o*, on host preference and attraction, root penetration and reproduction of *Radopholus similis* in tissue culture banana plants *cv*. Enyeru (*Musa* spp., AAA-EA) were evaluated under laboratory and screen house conditions. Treatment of tissue culture banana plants with the three isolates did not alter host preferences and attraction of *R. similis*, as similar numbers of nematodes migrated towards plants and root segments from both endophyte-inoculated and uninoculated plants. The number of *R. similis* that penetrated the roots of endophyte-inoculated and un-inoculated plants was not influenced by the three endophytic fungal isolates, either in the laboratory or in the screen house. *Radopholus similis* reproduction was, however, highly influenced by endophyte infection. Differences in *R. similis* reproduction rates between endophyte-inoculated and un-inoculated plants were observed at 50 and 75 days after nematode inoculation. No differences were observed 25 days after inoculation. Endophyte-un-inoculated plants supported higher nematode densities than endophyte-inoculated plants, and the nematode reproduction rates in control plants were 10, 4 and 3 times higher than in plants treated with isolates *V5W2*, *Eny7.11o* and *Eny1.31i* at 75 days after inoculation, respectively. Isolate *V5W2* resulted in the highest reduction in *R. similis* reproduction for all nematode stages (females, males and juveniles). The results of this study imply that the early processes of banana root infection by *R. similis* are not affected by endophytes and that the main endophyte effects are post-infectional and occur only during the nematode reproduction phase.

Introduction

Radopholus similis (Cobb) Thorne is a migratory endoparasite that completes its life cycle in the roots and rhizomes of banana plants (*Musa* spp.) in 20 to 25 days (Gowen and Quénéhervé, 2005). Only female and juvenile stages are infective, as males have degenerate stylets. *Radopholus similis* penetrates banana roots at the root apex mainly, but penetration can also occur at any position along the root (Sarah *et al*., 1996). After root penetration, the nematodes occupy the intercellular spaces of the cortical parenchyma. Nematodes may migrate both within and between cells in the root cortex to feed on cell cytoplasm. This results in collapsed cell walls, cavities and tunnels in the root (Sarah *et al*., 1996). Symptoms of nematode damage become visible as reddish brown necrotic patches that are confined to the cortex region. In the rhizome, necrosis appears as a reddish-brown discoloration that begins where roots attach to the rhizome (Speijer and De Waele, 1997). Nematode feeding destroys root and rhizome tissue, reduces water and mineral uptake, and results in a reduction of plant growth and development. This leads to a severe reduction of bunch weight and a significant increase in time between successive harvests (Sarah *et al.,* 1996; Gowen *et al*., 2005).

During their initial life stages nematodes migrate through the rhizosphere to the plant roots (Kaplan and Keen, 1980). Host recognition involves signals from plant roots that influence egg hatch, attraction towards roots and root penetration (Zhao *et al*., 2000). Plant-parasitic nematodes are attracted to plant roots by a variety of factors, which may operate over considerable distances (Prot, 1980; Prot and Van Gundy, 1980; Zuckerman and Jansson, 1984; Spiegel *et al*., 2001; Luc *et al*., 2005). The major factors of nematode attraction to plant roots are chemostatic factors and secretions emanating from the host plant, while minor factors include thermal, vibratory and tactile stimuli (Prot, 1980; Zuckerman and Jansson, 1984; Perry, 1996). Following attraction, nematodes penetrate the host plant cells by using their stylets where they feed and reproduce.

Differences in the response of the host plant to nematode infection have been used to classify plants as resistant, tolerant, intolerant, host or non-host. Resistant plants allow for nematode penetration but not reproduction. Tolerant plants suffer little damage even when heavily infested, while susceptible plants suffer heavy damage under light nematode infestations. Plants are further classified as hosts or non-hosts depending on whether nematode

reproduction occurs or not. Plants that allow high nematode reproduction are classified as good hosts, while those that allow for low nematode reproduction are classified as non-hosts (Luc *et al*., 2005).

Antagonistic microorganisms can reduce early root infection by nematodes. Oostendorp and Sikora (1989; 1990) reported a reduction in egg hatch and early root infection of sugar beet (*Beta Vulgaris* L.) by the sugar beet nematode *Heterodera schachtii* Schmidt after seed treatment with antagonistic rhizobacteria. However, the application of rhizobacteria to the root surface of sugar beet seedlings did not alter migration of *H. schachtii* second stage juveniles. The authors concluded that bacterial alteration of root exudates might have influenced nematode hatch, attraction and root penetration behavior.

Endophytic *Fusarium oxysporum* Schlecht.: Fries reduced *R. similis* populations in tissue culture banana plants (Chapter 3; Pocasangre, 2000; Niere, 2001; Gold and Dubois, 2005). The mechanism through which nematode populations were reduced, however, is unknown. The objectives of this study, therefore, were to determine the effect of endophytic *F. oxysporum* isolates on (i) host preferences and relative attractiveness of endophyte-inoculated plants to *R. similis* (ii) root penetration by *R. similis* and (iii) reproduction of *R. similis* in tissue culture banana plants.

Materials and methods

Site description

Experiments were carried out in the laboratory or screen house at the International Institute of Tropical Agriculture (IITA) Research Station in Namulonge-Uganda, approximately 30 km Northeast of Kampala, Uganda. The screen house air temperatures ranged from 19.1 to 32.8ºC, while the soil temperature in the polythene bags ranged from 20.4 to 26.3ºC. The site is situated at 1150 m above sea level, 32º 34'N latitude, with a mean annual rainfall of 1255 mm and an average temperature of 22ºC.

Fungal isolates and nematode cultures

Three *F. oxysporum* isolates (*V5W2*, *Eny1.31i* and *Eny7.11o*) were included in all experiments. These isolates were obtained from healthy East African highland cooking banana plants roots and rhizomes in Uganda (Schuster *et al*., 1995) and are preserved in soil tubes (Niere, 2001). The isolates were selected on their ability to consistently reduce nematode population build-up in banana plants in *in vivo* screening experiments (Chapter 3). Pure *R. similis* cultures maintained on carrot disks were used as the source of nematode inoculum (Chapter 1; Speijer and De Waele, 1997).

Tissue culture plants

Tissue culture banana plants of the cultivar Enyeru (*Musa* spp. AAA-EA) were used in this study. The plants were micropropagated from sword suckers using standard shoot-tip culture protocols for banana (Vuylsteke, 1998). When ready for weaning, plants were transferred to an aquaculture system comprised of 250-ml lidded plastic pots filled with 200 ml of nutrient solution to allow for root development prior to inoculation with the endophytes (Fig. 1). The nutrient solution was prepared by dissolving a commercial fertilizer in sterilized (autoclaved at 121ºC for 15 min) tap water. In the root penetration experiments, plants were fertilized with Multifeed Classic (Gouws and Scheepers Ltd., Witfield, South Africa) at a rate of 2.5 g/L (Table 1). For the *R. similis* host preference, attraction and reproduction experiments, plants were grown in 1 g/L of Poly-Feed (Haifa Chemicals, Haifa Bay, Israel) (Table 1). In each pot, a plant was supported by the lid with the roots submerged in the nutrient solution. Strips of sterile sponge were used to stabilize the plants through a hole made in the middle of the lids. To simulate dark soil conditions and to discourage growth of algae on the roots, the pots were wrapped with a brown paper bag. The plastic pots then were transferred to a humidity chamber (Chapter 3) for a 1-month acclimatization period, during which the nutrient solution was renewed weekly.

Inoculation of plants with fungal isolates

The fungal isolates were pre-grown on synthetic nutrient agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar/L distilled water) in 65-mm-diameter Petri dishes at ± 25°C and 12 hrs light, 12 hrs darkness photoperiod (laboratory conditions) for 1 week. The SNA medium was supplemented with 10 mg chlortetracycline, 100 mg penicillin G and 50 mg streptomycin-sulphate/L to prevent bacterial contamination. Half strength potato dextrose broth (PDB) (Sigma-Aldrich, MO, USA) was prepared by dissolving 12 g of PDB in 1 L of distilled water. One hundred-ml aliquots of PDB were dispensed into 250-ml Erlenmeyer flasks and sterilized. After cooling, flasks were inoculated with 4 to 5 disks of agar of each fungal isolate. Uninoculated PDB was used as the control treatment. Duplicate flasks were prepared for each fungal isolate and the control. Inoculated flasks were incubated in the laboratory for 7 days to allow for fungal growth and sporulation. Fungal spore suspensions were filtered through a 1-mm-diameter sieve to remove mycelial fragments. The spore suspensions were then adjusted to provide a final spore count of 1.5×10^6 spores/ml.

For the inoculation of banana tissue culture plants with endophytic *F. oxysporum* isolates, 1-month-old plants were removed from the nutrient solution and the roots cut back to 10 cm in length. Plants were selected for uniformity according to size and subdivided among the different treatments. To inoculate the plants with the different fungal isolates, the root systems were dipped in the different fungal spore suspensions for 2 hrs. Control plants were dipped in sterile un-inoculated PDB for the same duration of time. After fungal inoculation, plants were transplanted in steam-pasteurized heavy loamy soil either into 3-L polythene bags for the root penetration and reproduction experiments, or into 250-ml plastic pots for the host preferences and attraction experiments.

To determine colonization of plant roots by the fungal isolates, three healthy primary roots were randomly selected from each plant at harvest and surface sterilized in 75% ethanol for 1 min, followed by sterilization in 2% NaOCl for 30 s. Root pieces were blotted dry on sterile tissue paper and cut into ca. 0.25 cm-long segments. Six segments per root were randomly selected and placed on SNA in 65-mm-diameter Petri dishes. The plates were incubated in the laboratory for 7 days under laboratory conditions. Fungal colonies growing from the root

pieces were identified under a light microscope (magnification x 400) as *F. oxysporum* based on the presence of microconidia carried in false heads on short phialides, the shape of macroconidia and the presence of chlamydospores (Nelson *et al*., 1983). The number of root pieces with *F. oxysporum* colonies were recorded and the percentage recovery of the fungus calculated.

Host preference and attraction experiments

Two experiments were conducted to investigate host preference and relative attractiveness of endophyte-inoculated and un-inoculated (control) banana plants to *R. similis*. Each experiment was repeated once. These included a detached root bioassay where root segments from endophyte-inoculated and uninoculated banana plants were paired in a Petri dish, and an intact plant bioassay using endophyte-inoculated and un-inoculated banana plants paired in a polyvinyl chloride (PVC) tube apparatus. Both experiments had seven treatments that involved combinations of the three endophyte-inoculated *V5W2* (E1), *Eny1.31i* (E2) and *Eny7.11o* (E3) and uninoculated (C) banana plants in the following pairs: C-C, C-E1, C-E2, C-E3, E1-E1, E2-E2 and E3-E3. The detached root bioassay and the intact plant bioassay were replicated four and five times, respectively.

Detached root bioassay for host preference and attraction

Endophyte-inoculated and uninoculated plants were uprooted 1 month after being transplanted into the 250-ml pots and washed free of soil. In the laboratory, healthy roots of the same age and size were selected from the plants and cut into 1-cm-long segments. For each replication, root segments were obtained from the same plant. Root segments from endophyte-inoculated and uninoculated plants were paired and placed on opposite sides in 90-mm-diameter Petri dishes filled with a 50-mm thick layer of moistened sterile sand (Fig. 2). The bottom of the Petri dish was divided into two equal sections, A and B, and two root segments from either an endophyte-inoculated or uninoculated plant placed in each of the sections. The distance between the root segments was 70 mm, and each root segment was placed 10 mm away from the wall of the Petri dish. Petri dishes were covered and left to stand for 12 hrs. Approximately 500 *R. similis* mixed stages (females, males and juveniles) in 0.3 ml water were inoculated in the middle of the Petri dish equal distances from the root

segments using a micropipette. After 24 hrs, nematodes were extracted from the sand and from root segments on either side of the inoculation point by means of the modified Baermann technique (Hooper *et al*., 2005). Extraction from the sand was carried out for 48 hrs by spreading the sand on the tissue paper in the modified Baermann funnel apparatus. Nematodes were extracted for 24 hrs from the root segments following maceration in a Waring blender (Waring, Connecticut, USA) at low speed for 15 s.

Intact plant bioassay for host preference and attraction

A PVC tube apparatus was designed to determine whether *R. similis* is differentially attracted to endophyte-inoculated or uninoculated (control) banana plant roots (Prot, 1979). The PVC apparatus consisted of a 22.5–cm-long and 5-cm-diameter PVC tube divided into seven sections, each 2.5 cm long (Fig. 3). The sections were filled with moistened sterile heavy loam soil and joined together with adhesive tape. The use of PVC pipes not only reduced the amount of soil from which nematodes had to be extracted, but also restricted nematode movement from the point of inoculation to either of the two direction.

Endophyte-inoculated and uninoculated plants transplanted into 250-ml plastic pots were grown until they were 8 weeks old. An endophyte-inoculated plant was then paired with an uninoculated plant on opposite sides of the PVC apparatus. The bottom parts of the pots were first removed to expose the roots, and the pots were then fixed to the ends of the PVC tubes in section number 1 and number 7 (Fig 3). Treatments were paired in such a way that the first plant which is either endophyte-inoculated (E1, E2 or E3) or uninoculated (C) in each treatment pair was placed in PVC section number 1 and the last in section number 7. A 3-mm-diameter opening was drilled at the top of the middle of each PVC section to facilitate watering and nematode inoculation.

One week after transplanting the banana plants into the PVC apparatus, 2000 mixed stages of *R. similis* were pipetted into the soil in the middle section (section 4) that was an equal distance from both plants. The tubes were maintained horizontally at laboratory conditions. The PVC sections were separated 5 days after introduction of the nematodes by cutting through the adhesive tape using a knife. The number of nematodes in each section was determined by extracting the nematodes from the soil over 48 hrs using the modified

Baermann funnel method (Hooper *et al*., 2005). Nematode extraction from the plant's roots in sections number 1 and 7 was conducted for 24 hrs after maceration in a Waring blender for 15 s. Nematode suspensions were concentrated on a 28-µm sieve prior to counting.

Root penetration experiments

To study the effect of endophytes on root penetration by *R. similis*, two experiments were conducted on 8-week-old banana plants inoculated with three different fungal endophytes (*V5W2*, *Eny1.31i* and *Eny7.11o*). The first experiment involved the *in vitro* root penetration of endophyte-inoculated and un-inoculated plants by nematodes in the laboratory. The second experiment was an *in vivo* root penetration experiment in the screen house, conducted as a time course study. Nematode inoculation was performed on the whole root system and plants were sampled to determine the number of nematodes that had penetrated into the roots 3, 6 10 and 14 days after inoculation. Six plants were randomly harvested after each time interval. Each experiment was repeated twice.

In vitro *root penetration experiments*

Endophyte-inoculated and un-inoculated banana plants were uprooted from their polythene bags and their roots washed free of soil. These plants were then transplanted to rectangular plastic trays (90 x 143 x 230 mm) that were filled with *ca*. 500 cm^3 moistened sterile sand (Fig 4). A hole was cut on the side of the trays, and a slit made from the rim to the hole to facilitate placement of the plant. A strip of sponge was used to stabilise the plants and avoid damage to their pseudostems. Three 65-mm-diameter Petri dishes in which two V-shaped cuts were made on the opposite sides were placed inside the trays and filled with moistened sterile sand. Three healthy primary roots of the same size and age were randomly selected from the transplanted plants, and each one of roots placed inside one of the three Petri dishes.

A nematode suspension containing a total of 50 *R. similis* mixed stages in *ca*. 0.1 ml of water was pipetted directly on the root segments in each Petri dishes 24 hrs after transfer of the plants. The roots were then covered with sterile sand. The plastic trays were covered with a lid to prevent dehydration of the plants and root segments. Inoculated root segments were excised after 24 hrs and stained with acid fuschin to observe nematodes that had penetrated

the roots (Byrd *et al*., 1983). After staining, root segments were flattened between two microscope slides and the number of nematodes visible in the root tissue was recorded. There were five plants for each treatment, with three roots selected per plant, each representing a single replication (i.e. a total of 15 replications per treatment).

In vivo root penetration experiments

Eight-week-old endophyte-inoculated and un-inoculated banana plants grown in 3-L polythene bags were inoculated with a nematode inoculum containing 500 mixed stages of *R. similis* per 2 ml water as follows. Three holes were made at the base of the plants around the roots. To each hole, *ca*. 0.6 ml of the nematode suspension was pipetted, and the hole covered with soil. Six plants from each treatment were then uprooted 3, 6, 10 and 14 days after nematode inoculation. Nematodes were extracted from 5 g of each root samples by first macerating them in a Waring blender at low speed for 15 s, and subsequently collecting the nematodes for 24 hrs using a modified Baermann technique (Hooper *et al*., 2005). Nematode suspensions were transferred into sample bottles and kept in the fridge at 4ºC until counted. Prior to counting, nematode suspensions were concentrated on a 28-µm sieve.

Radopholus similis reproduction experiments

A single-root inoculation technique developed for screening banana plants for resistance to *R. similis* (De Schutter *et al*., 2001) was adopted to study the effects of endophytes on *R. similis* reproduction. One-month-old endophyte-inoculated and un-inoculated plants were grown for a further 2 months in 3-L plastic polythene bags after acclimatization in the humidity chamber. The 3-month-old plants were then uprooted and their roots separated. The plants were placed into 24 x 54 cm plastic buckets filled three quarters with sterile sawdust (Fig. 5). Three equally developed primary roots were selected from each plant and planted in 150-ml plastic pots filled with moistened sterile sand. After transplanting, the roots not planted in the pots were covered with sterile soil. A nematode suspension containing 50 *R. similis* in approximately 0.3 ml of sterile water was pipetted directly onto each of the root segments planted inside the plastic pots. The inoculated roots were then also covered with moistened sterile sand. Following inoculation, the banana plants were maintained in the screen house (20.4 to 26.3ºC) and watered two times per week.

Nematodes were extracted from inoculated banana roots 25, 50 and 75 days after nematode inoculation. At each harvest date, five plants were randomly selected and the plastic pots excavated from the sawdust. The root segments inside the pots were removed and washed with tap water. The percentage root necrosis was determined by slicing each root segment lengthwise and estimating the percentage necrotic area (Speijer and De Waele, 1997). The sliced root segment was then cut into 0.5-cm pieces and macerated in a Waring blender at low speed for 15 s. Nematodes were extracted from the macerated root tissue over 24 hrs using the modified Baermann funnel method (Hooper *et al*., 2005). All *R. similis* stages (females, males, juveniles) were counted. Nematode reproduction rates (RR) were calculated by dividing the final population density (*Pf*) with the initial population density (*Pi*). Reisolation of fungal endophytes was conducted on the root segments adjacent to nematode inoculated root segment as described above.

Data analysis

Nematode data in each experiment were tested for normality and homogeneity of variances using the Shapiro-Wilkinson, Levene-Welch and Kolmogorov tests. Normal probability plots, box plots and stem leaf plots were additionally used to confirm normality of data. If not normally distributed with equal variances across treatments, various transformations were tested until the most suitable transformation was obtained.

For the host preference and nematode attraction bioassays using detached roots in Petri dishes, statistical analysis was performed on untransformed data. One-way ANOVA was used to demonstrate differences in the total number of nematodes in each treatment pair. If different, treatment means were separated using Tukey's Studentized range test. Within each treatment pair, differences between treatments were evaluated using a paired t-test.

For the bioassay using intact plants in the PVC tube apparatus and for the *R. similis* reproduction experiments, nematode counts were square root (sqrt) (x+0.5)-transformed prior to analysis. Within each treatment, differences between the proportion of nematodes attracted to either endophyte-inoculated or uninoculated plants were evaluated using a paired t-test. For the reproduction experiments, a two-way ANOVA was conducted to demonstrate variability among treatments (control and fungal isolates) and days after nematode inoculation. Prior to

analysis, the nematode RR was sqrt-transformed x+0.5 to normalise data. Percentage root necrosis and colonization were arcsine sqrt-transformed x+0.5 prior to analysis. If different, means were separated using Tukey's studentized range test (SAS Institute, 1989).

Results

Host preference and attraction experiments

Detached root bioassay for host preference and attraction

Similar numbers of nematodes were attracted to banana roots, whether they were inoculated or uninoculated with *F. oxysporum* endophytes (Fig. 6 and 7) Comparison between the total numbers of *R. similis* that migrated towards opposite sections of the Petri dish containing either an endophyte-inoculated or un-inoculated root segment revealed no significant differences between any of the treatments pair (Table 2). When two endophyte-uninoculated root segments (treatment pair C-C) were paired in opposite sides of a Petri dish, the number of nematodes attracted to either root segment did not differ significantly (Table 2). Likewise, when an endophyte-uninoculated root segment was paired with an endophyte-inoculated root segment (treatment pairs C-E1, C-E2 and C-E3), the number of nematodes that migrated towards either root segment did not differ significantly. When two endophyte-inoculated root segments were paired (treatment combinations E1-E1, E2-E2 and E3-E3), no differences were observed in the number of *R. similis* that migrated towards either root segment (Table 2). The only exception was observed in experiment 2, when two root segments infected with isolate *Eny1.31i* (E2-E2) and isolate *Eny7.11o* (E3-E3) were paired, where significant differences in the number of *R. similis* that migrated to either root segment were observed (Table 2).

Intact plant bioassay for host preference and attraction

THE EXPERIMENTS USED TO DETERMINE NEMATODE ATTRACTION SHOWED THAT THE NUMBER OF NEMATODES (EXPRESSED AS A PERCENTAGE OF THE TOTAL NEMATODES RECOVERED PER TREATMENT) THAT MIGRATED FROM THE POINT OF INOCULATION IN PVC TUBES DID NOT DIFFER BETWEEN THE TREATMENT PAIRS. IN EXPERIMENT 1 (*P*=0.1544), THE PROPORTION OF NEMATODES THAT MIGRATED RANGED FROM 10.2 ± 3.5% IN TREATMENT PAIR E3-E3 (TWO *ENY7.11O*-INFECTED PLANTS PAIRED) TO 35.9 ± 3.5% IN C-C (WHEN TWO CONTROL PLANTS WERE PAIRED). IN BIOASSAY 2 (*P*=0.3776) THE PROPORTION OF NEMATODES THAT MIGRATED FROM THE POINT OF INOCULATION RANGED FROM 40.4 ± 1.6 WHEN TWO *ENY7.11O*-TREATED PLANTS WERE PAIRED TO 57.7 ± 10.1% WHEN A CONTROL PLANT WAS PAIRED WITH A PLANT TREATED WITH *ENY.11O*. PAIRED T-TESTS DEMONSTRATED THAT THE PROPORTION OF

NEMATODES THAT MIGRATED TO OPPOSITE SIDES OF THE INOCULATION POINT TOWARDS EITHER AN ENDOPHYTE-INOCULATED PLANT OR UNINOCULATED PLANT DID NOT DIFFER AMONG THE TREATMENT PAIRS IN BOTH BIOASSAYS EXCEPT IN THE TREATMENT WERE TWO *ENY7.11O*-INFECTED PLANTS WERE PAIRED (TABLE 3).

In both bioassays, the total number of nematodes present in each section of the PVC tube differed significantly between the sections ($P<0.0001$) and was influenced by the distance from the nematode inoculation point. In all treatment pairs, most nematodes were found in the middle section (section 4), the point of nematode inoculation (Fig. 8 A and B), with less nematodes in sections further from the point of nematode inoculation. The number of nematodes recorded in sections at the same distance from the point of nematode inoculation was not different within a given treatment ($P<0.05$).

Root penetration experiments

In vitro experiments

No significant difference was found in the number of nematodes that penetrated root segments of endophyte-inoculated and uninoculated plants 24 hrs after inoculation with *R. similis* ($P=0.6064$, 0.2528 and 0.8752 for experiment 1, 2 and 3 respectively) (Fig. 9). Nematodes were observed in the cortex region of the root segments only and not the stele.

In vivo experiments

No significant differences in the penetration of banana roots by *R. similis* were observed between the endophyte-inoculated and uninoculated banana plants planted in polythene bags (Table 4). There were also no significant differences in the number of nematodes that penetrated the roots 3, 6, 10 and 14 days after nematode inoculation for any of the treatments.

Radopholus similis reproduction

Endophyte treatment significantly reduced the number of *R. similis* females, males, juveniles and the total nematode density at 75 days after nematode inoculation (DAI) in the first

experiment, but not the second one (Table 5). In the former, the percentage reduction in *R. similis* females was 65.0%, 67.4% and 35.5% after 75 days, whilst juvenile numbers compared to the control were lower by 55.2%, 77.4% and 90.8% in plants infected with *Eny1.31i*, *Eny7.11o* and *V5W2*, respectively. The reduction in the number of *R. similis* males was 63.4%, 83.6% and 92.8% and the reduction in the total nematode density was 65.7%, 76.5% and 90.2% in plants treated with the fungal isolates *Eny1.31i*, *Eny7.11o* and *V5W2* respectively compared to control plants (Table 5). In the second experiment, only isolate *V5W2* resulted in less females, males, juveniles and the total nematode density although this did not differ significantly from the control. Isolates *Eny1.31i* and *Eny7.11o*-treated plants had more nematode than the controls 75 DAI (Table 5).

No differences were observed in the number of females, juveniles, males and the total nematode density between endophyte-inoculated and un-inoculated plants at 25 and 50 DAI, (Table 5). Although not significantly different, *R. similis* female numbers were lower by 71.1, 40.5 and 23.0% in plants infected with the fungal isolates *Eny1.31i*, *Eny7.11o* and *V5W2* 50 DAI, respectively, compared to the un-inoculated plants. Similarly, the number of *R. similis* juveniles was lower by 50.7%, 51.7% and 18.6% in plants treated with the fungal isolates *Eny1.31i*, *Eny7.11o* and *V5W2*, respectively (Table 5). The most important reduction in the second experiment, although not significant, was achieved with *Eny1.31i* that reduced nematode numbers by 62.9% after 25 DAI, and after 50 days, juvenile numbers by 78.5%, 24.3% and 43.4%, and males were reduced by 74.2, 15.2 and 37.7% in plants treated with isolates *Eny1.31i*, *Eny7.11o* and *V5W2* respectively.

The DAI affected the female, male, juvenile and total *R. similis* density significantly influenced ($P<0.0001$) in both endophyte-inoculated and uninoculated plants. No differences were observed in the nematode numbers at 50 and 75 DAI in both endophyte-treated plants while the number of nematodes in both endophyte-inoculated and un-inoculated plants at 25 DAI were significantly lower than those at 50 and 75 DAI ($P<0.0001$).

Treatment with endophytes did not significantly reduce the damage caused by nematodes to banana roots (percentage root necrosis) ($P=0.5593$), but the damage was reduced significant over time ($P=0.0239$) (Table 6). At 25 and 75 DAI, plants had similar levels of damage, whether they were inoculated with endophytes or not. The only significant differences in

percentage root necrosis were observed in experiment 1 50 DAI where plants treated with *V5W2* and *Eny1.31i* had significantly more necrosis than control plants, but not in experiment 2 (Table 6).

Nematode reproduction rate (RR) was significantly reduced by endophyte treatment after 75 days in experiment 1, but not experiment 2 (Table 6). At 25 and 50 DAI, no significant differences were observed in the RR between endophyte-inoculated and un-inoculated plants. For both endophyte-inoculated and un-inoculated plants, the nematode RR increased with an increase in DAI, except for *V5W2*-treated plants where the RR was higher at 50 DAI than at 75 DAI (Table 7).

Endophytic colonization of banana roots

In the *in vivo* root penetration experiments, the percentage colonization of plants treated with isolates *Eny1.31i* (16.9 ± 6.8%), *Eny7.11o* (15.5 ± 5.9%) and *V5W2* (20.5 ± 5.5%) was higher than in the control plants (3.6 ± 2.9%). Root colonization increased with increase in the days after nematode inoculation (DAI). In experiments 1 and 3, colonization was significantly influenced by treatment (P=0.0003) but not by DAI (P=0.6210). Lowest root colonization was recorded in endophyte-un-inoculated plants compared to colonization rates in roots inoculated with the various fungal isolates.

At all harvest dates in the first *R. similis* reproduction experiment, percentage colonization of roots by endophytic *F. oxysporum* did not differ between endophyte-inoculated and un-inoculated plants (Table 6). Significant differences, however, were found between un-inoculated and endophyte-inoculated plants 25 (P=0.0002) and 75 (P=0.0004) DAI. Although *F. oxysporum* was re-isolated from control plants 50 DAI, no isolations were made 25 and 50 DAI in either experiment. Still, the difference between the control and endophyte treatments was not significant, even where control yielded 0% re-isolation, and the treated plants 61% re-isolation.

Discussion

This study has demonstrated that endophyte-inoculated roots of tissue culture banana plants did not influence host preferences, attraction and penetration by *R. similis*. This is evident from the lack of repulsion or attraction of *R. similis* in the host preference and attraction experiments. Similar penetration rates into plant roots indicate that the initial processes of host recognition and root penetration, and invasion are not affected by the endophytes tested. The results suggest that, once in the field, roots of plants infected with the endophytic *F. oxysporum* isolates used in this study will still be infected by *R. similis*. However, endophytes inhibit reproduction of the nematode over several generations.

The results obtained in the current study are in contrast to those obtained with other fungal endophytes. Bernard and Gwinn (1991) reported that more nematodes (*Pratylenchus scribneri* Steiner) migrated towards endophyte-free tall fescue (*Festuca arundinacea* Schreb) root segments when both endophyte-infected and endophyte-free root segments were paired in a petri dish. Evidence that biological control agents other than endophytes affect early root infection by nematodes was demonstrated by Oostendorp and Sikora (1989; 1990). These authors reported a reduction in egg hatch and early root infection of sugar beet by the sugar beet nematode, *H. schachtii* after seed treatment with antagonistic rhizobacteria. Treatment of sugar beet seedlings with the rhizobacteria did not alter migration of *H. schachtii* second stage juveniles. These examples, however, involve different crops and nematode species and thus the response in banana may be different.

The results obtained in this study are in contrast to results obtained by Pocasangre (2000) who reported reduction in root penetration by *R. similis* in Cavendish banana plants treated with endophytic isolates of *Fusarium* spp. The contradiction in results may be due to the fact that the banana cultivars, *R. similis* populations and endophytic isolates in the two studies were different and experiments were conducted under different environmental conditions. In grasses, the presence of morphological barriers such as thickening of the endodermis in tall fescue (*Festuca arundinacea* Shreb.) infected by the endophyte *Neotyphodium coenophialium* ([Morgan-Jones and Gams] Glenn, Bacon, and Hanlin) was associated with reduced root penetration of root-knot nematodes *Meloidogyne marylandi* Jepson and Golden (Gwinn and Bernard, 1993) but not of the migratory nematode *Pratylenchus scribneri* Steiner. Reduced

root penetration subsequently led to reduced growth and reproduction of *M. marylandi* in the endophyte-infected grasses. In the current study, penetration into roots of endophyte-treated as well as untreated plants may be an indication that the endophytes did not induce morphological and structural changes that limit nematode penetration.

The complete life cycle of *R. similis* takes 20-25 days a t a temperature range of 24-32ºC (Loos, 1962). In the current study, nematodes reproduced both in endophyte-inoculated and un-inoculated plants. The nematode reproduction rates were, however, lower in endophyte-inoculated plants compared to uninoculated plants. Endophyte-inoculation did not have an effect on the first *R. similis* generation; reduction in nematode populations was observed at the second and third generation only. It is probable that, at 25 DAI, the nematodes that were found in the roots were those that survived after initial inoculation. The lower reproduction rates of *R. similis* in endophyte-treated plants during the second and third generations may probably be due to inhibition of nematode migration inside the plant roots, interference with the feeding processes or reduction in nematode reproduction capacity (Oostendorp and Sikora, 1989; 1990; Jones and Bernard, 1997). These conclusions, however, require further investigation. Another implication of these results is that endophytes may protect banana plants against *R. similis* for multiple nematode generations. Banana is a perennial crop and protection for several seasons by endophytes would greatly benefit the plant (Sikora *et al*., 2003). Hunt *et al*. (2005) defined resistant plants as those that allow for nematode penetration but restrict nematode reproduction. Based on this definition, endophyte infection of tissue culture banana plants restricted nematode reproduction and thus conferred some degree of resistance to the host plant. The lack of differences in *R. similis* reproduction between endophyte-inoculated and uninoculated plants in one of the experiments may have been due to differences in the vigour of both the plants and nematode inoculum used. Large variation in the data may also be responsible for the observed lack of differences. Nevertheless, endophyte-inoculated plants supported less nematodes than uninoculated plants.

Endophytic colonization was higher in the endophyte-inoculated plants than in the non-inoculated plants, but not significantly different. The true identity of the re-isolated endophytic *F. oxysporum* strains, however, is unknown, since endophytic *F. oxysporum* strains have also been re-isolated from the control treatment. The endophytic *F. oxysporum* from non-inoculated plants could have come from the soil, water or screen house

environment. Before it is possible to deduce whether the inoculated *F. oxysporum* indeed contributed to the reduction in reproduction rates, and also to the non-significance of endophyte treatment in host preference, attraction and penetration, it would be necessary to conduct these experiments under greenhouse conditions that prevent the re-introduction of endophytes into control plants. Colonization of control plants by *F. oxysporum*, however, is a common occurrence in screen house studies, and has been reported before by Niere (2001) and Paparu (2005). Niere (2001) reported colonization of uninoculated plants of up to 40%, while Paparu (2005) reported up to 15% colonization of uninoculated plants.

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Table 1. Nutrient composition of Multifeed Classic (Gouws and Scheepers Ltd., Witfield, South Africa) and Polyfeed fertilizers (Haifa Chemicals, Haifa Bay, Israel) used to enhance root development of tissue culture plants prior to fungal inoculation.

Ingredient	Multifeed Classic (g/kg)	Polyfeed (g/kg)
Nitrogen	190	0.19
Phosphorus	82	0.19
Potassium	158	0.19
Iron	0.75	1
Manganese	0.3	0.5
Boron	1	0.2
Zinc	0.35	0.15
Copper	0.075	0.11
Molybdenum	0.07	0.07
Magnesium	0.9	0

Table 2. Paired *t*-tests for the differences in the total number of *Radopholus similis* present in the sand, roots and in both sand and roots, which migrated towards root segments obtained from endophyte-inoculated or un-inoculated plants paired in a Petri dish.

		Bioassay 1						Bioassay 2					
		Sand		Root		Sand + Root		Sand		Root		Sand + Root	
Treatment pair *	Difference**	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|	T value	Pr>|t|
C-C	A-B	2.37	0.0986	0.28	0.7999	2.23	0.1119	1.29	0.2861	-0.34	0.7587	1.09	0.3538
C-E1	A-B	0.64	0.5684	-2.71	0.0729	0.45	0.6860	0.41	0.7116	1.36	0.2671	0.65	0.5641
C-E2	A-B	0.63	0.5723	-0.90	0.4339	0.35	0.7492	-0.49	0.6749	-0.8	0.4798	-1.31	0.2809
C-E3	A-B	1.28	0.2918	-0.42	0.7016	0.92	0.4236	1.43	0.2474	-0.72	0.5239	1.26	0.2982
E1-E1	A-B	1.62	0.2033	1.68	0.1920	2.11	0.1253	1.36	0.2666	-0.61	0.5848	1.16	0.3315
E2-E2	A-B	-1.77	0.1749	0.11	0.9173	-2.06	0.1312	-4.01	0.0279	-0.78	0.4917	-3.46	0.0407
E3-E3	A-B	-0.50	0.6512	0.28	0.7987	-0.42	0.7017	-3.35	0.044	0.73	0.5158	-2.97	0.0589

*C= control, E1=endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*. E3=endophyte isolate *Eny7.11o*, root segments from either control or endophyte-infected plants were paired in a Petri dish as indicated above.

**The Petri dishes were divided into two sections, A and B, with the first and second treatment in each treatment pair placed in section A and B respectively (Fig. 2).

Table 3: Paired *t*-tests for the differences between the proportion of nematodes that migrated towards an endophyte-inoculated or un-inoculated plant in the intact plant host preference and attraction bioassays conducted using PVC tube apparatus.

		Bioassay 1		Bioassay 2	
Treatment pair*	Difference**	T Value	P>|t|	T Value	P>|t|
C-C	A-B	2.17	0.0958	-0.59	0.5877
C-E1	A-B	0.76	0.4879	-1.40	0.2344
C-E2	A-B	-1.05	0.3517	-1.70	0.1644
C-E3	A-B	0.26	0.8042	0.34	0.7507
E1-E1	A-B	-0.09	0.9305	0.91	0.4321
E2-E2	A-B	-2.49	0.0673	-0.37	0.7234
E3-E3	A-B	4.54	0.0105	2.79	0.0493

*C= control, E1= endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*. E3= endophyte isolate *Eny7.11o*.

**Control and endophyte-infected plants were paired in a polyvinyl chloride (PVC) tube divided into 7 sections (Fig. 3), with the first and second treatment in each treatment pair placed on section 1 and 7, respectively. Nematodes were inoculated at the middle section (4) of the tube equidistant from the two plants. A and B represents sections on either side of the nematode inoculation point and correspond to the plant (treatment) on that side of the inoculation point.

Table 4. Average number of *Radopholus similis* in 5 g banana root samples of endophyte-inoculated and un-inoculated plants 3, 6, 10 and 14 days after nematode inoculation in in vivo root penetration experiments in the screen house.

Treatment	Days after nematode inoculation				*P*-Value[2]
	3	6	10	14	
Experiment 1					
Control	13.5 ± 0.5	19.2 ± 0.6	15.6 ± 1.9	14.8 ± 5.7	0.1028
V5W2	9.2 ± 4.6	6.6 ± 2.1	13.6 ± 0.6	8.3 ± 5.9	0.7772
Eny1.31i	5.8 ± 1.1	8.8 ± 2.8	7.6 ± 3.4	13.1 ± 3.7	0.8447
Eny7.11o	6.1 ± 2.4	4.8 ± 1.4	7.6 ± 3.4	13.1 ± 3.7	0.3514
P-value[1]	0.6771	0.6340	0.1301	0.7300	
Experiment 2					
Control	4.1 ± 1.1	5.2 ± 2.2	3.4 ± 1.3	1.8 ± 0.3	0.5584
V5W2	1.7 ± 0.4	4.2 ± 1.1	6.0 ± 1.6	3.9 ± 0.8	0.2099
Eny1.31i	1.0 ± 0.5	10.9 ± 9.5	4.0 ± 0.6	3.8 ± 1.1	0.5859
Eny7.11o	2.8 ± 0.9	1.5 ± 0.4	5.9 ± 3.3	3.3 ± 0.8	0.3147
P-value[1]	0.1179	0.7025	0.6780	0.1909	
Experiment 3					
Control	6.0 ± 1.4	4.2 ± 1.3	5.5 ± 1.4	13.7 ± 5.4	0.1531
V5W2	2.8 ± 0.6	5.5 ± 1.4	6.2 ± 2.9	26.5 ± 11.9	0.0693
Eny1.31i	5.3 ± 3.1	3.3 ± 0.5	5.5 ± 1.7	4.6 ± 1.8	0.6586
Eny7.11o	12.1 ± 6.7	2.2 ± 0.4	4.5 ± 2.4	4.8 ± 0.6	0.4473
P-value[1]	0.5981	0.2858	0.7552	0.0643	

P-Value[1] is the *P*-value for treatments along columns in each experiment. *P*-Value[2] is the *P*-value for days after inoculation along rows within each treatment.

Table 5. Effect of endophyte treatment of tissue culture banana plants cv. Enyeru (*Musa* spp. AAA-EA) with fungal isolates *V5W2*, *Eny1.31i* and *Eny7.11o* on the reproduction of *Radopholus similis* 25, 50 and 75 days after inoculation of individual primary roots with 50 *Radopholus similis* females.

Treatment	Females			Juveniles			Males			Total*		
	Days after nematode inoculation			Days after nematode inoculation			Days after nematode inoculation			Days after nematode inoculation		
	25	50	75	25	50	75	25	50	75	25	50	75
Experiment 1												
Control	1.1 ±0.3^{a}	115.4±44.7^{a}	270.0±55.1^{a}	1.0±0.3^{a}	143.3±53.7^{a}	581.8±110.9^{a}	0.1 ±0.1^{a}	80.4±33.7^{a}	268.5±47.8^{a}	2.0 ±0.6^{b}	339.2 ±130.6^{a}	1120.6 ±196.7^{a}
Eny1.31i	7.5±3.3^{a}	33.4 ±11.3^{a}	94.5±47.1^{b}	4.3±1.9^{a}	70.6±28.7^{a}	190.5±98.7^{b}	2.1 ±0.8^{a}	36.0±13.2^{a}	98.5±56.5^{b}	12.8 ±5.6^{ab}	140.0 ±49.7^{a}	383.5 ±194.1^{b}
Eny7.11o	4.2±2.1^{a}	68.6±32.3^{a}	87.9±47.1^{b}	1.1±0.5^{a}	69.2±39.1^{a}	130.6±63.8^{b}	2.1 ±1.6^{a}	57.2 ±31.2^{a}	43.8±18.2^{b}	5.5 ±3.0^{ab}	195.0 ±101.8^{a}	262.9 ±127.5^{b}
V5W2	12.5±3.9^{a}	88.8±36.2^{a}	35.5±16.8^{b}	6.3±2.3^{a}	116.6±49.9^{a}	53.5±18.8^{b}	5.5 ±1.9^{a}	114.7±47.6^{a}	19.1±6.7^{b}	24.5 ±7.9^{a}	320.2 ±128.2^{a}	109.0 ±41.9^{b}
P-value	0.0756	0.7151	0.0034	0.0756	0.8610	0.0003	0.0662	0.7920	0.0001	0.0226	0.8561	0.0003
Experiment 2												
Control	19.3±1.6^{a}	228.6±73.4^{a}	443.0±25.6^{a}	33.0±1.9^{a}	453.8±165.6^{a}	381.1±67.4^{a}	13.0±1.1^{a}	159.8±56.4^{a}	255.8±132.2^{a}	65.3±2.9^{a}	842.2±258.4^{a}	1080.0±174.1^{a}
Eny1.31i	9.2±4.6^{a}	84.6±38.3a	536.9±247.3^{a}	12.8±6.5^{a}	97.3±47.1^{a}	408.2±199.1^{a}	7.0±4.0^{a}	44.1±21.4^{a}	319.7±189.3^{a}	29.1±15.2^{a}	226.1±104.3^{a}	1265.0±627.5^{a}
Eny7.11o	18.1±5.1^{a}	195.7±56.7^{a}	887.5±350.2^{a}	26.8±7.6^{a}	343.1±110.4^{a}	509.3±140.2^{a}	14.4±5.5^{a}	135.5±39.6^{a}	344.5±100.6^{a}	59.3±16.9^{a}	674.3±202.5^{a}	1741.4±544.4^{a}
V5W2	17.2±5.4^{a}	222.5±68.6^{a}	436.2±188.9^{a}	28.8±8.8^{a}	256.8±88.3^{a}	278.1±84.8^{a}	13.8±5.1^{a}	102.6±37.4^{a}	221.3±70.6^{a}	59.8±17.3^{a}	582.1±181.8^{a}	935.7±305.3^{a}
P-value	0.7740	0.6775	0.6416	0.7719	0.1001	0.6665	0.0814	0.8551	0.7416	0.8745	0.3213	0.7778

*Total *R. similis* density (females + males + juveniles). In columns within an experiment, means followed by the same letter (superscript) are not different at $P<0.05$ according to Tukey's studentized range test. Values represent the mean ± S.E., n= 15.

Table 6. Percentage root necrosis, reproduction rates (Pf/Pi) of *Radopholus similis* and endophytic colonization in roots of tissue culture banana plants cv. Enyeru (*Musa* spp. AAA-EA) 25, 50 and 75 days after inoculation of individual primary roots with 50 *Radopholus similis* females.

Treatment	Root necrosis (%) Days after nematode inoculation			Reproduction ratio (*Pf/Pi*) Days after nematode inoculation			Root colonization (%) Days after nematode inoculation		
	25	50	75	25	50	75	25	50	75
Experiment 1									
Control	26.4 ±11.5^{a}	8.4 ±2.9^{b}	11.7 ±2.5^{a}	0.1 ±0.0^{b}	6.7 ±2.6^{a}	22.4 ±3.9^{a}	0.0 ±0.0^{a}	26.8 ±12.1^{a}	0.0 ±0.0^{a}
Eny1.31i	22.7 ±6.4^{a}	18.4 ±1.0^{a}	12.9 ±1.7^{a}	0.2 ±0.1^{ab}	2.8 ±0.9^{a}	7.6 ±3.8^{b}	13.8 ±7.3^{a}	39.5 ±6.3^{a}	50.0 ±41.6^{a}
Eny7.11o	32.5 ±9.9^{a}	13.2 ±3.1^{ab}	5.7 ±1.5^{a}	0.1 ±0.1^{ab}	3.9 ±2.0^{a}	5.2 ±2.5^{b}	61.1 ±26.4^{a}	21.6 ±7.7^{a}	0.0 ±0.0^{a}
V5W2	20.5 ±7.1^{a}	19.7 ±0.1^{a}	12.1±2.6^{a}	0.4 ±0.1^{a}	6.4 ±2.5^{a}	2.1 ±0.8^{b}	16.6 ±8.3^{a}	38.8 ±8.0^{a}	33.3 ±14.2^{a}
P-value	0.7525	0.0047	0.0630	0.0218	0.8583	0.0004	0.1257	0.5924	0.1796
Experiment 2									
Control	20.8 ±5.8^{a}	100.0 ±0.0^{a}	97.5 ±1.7^{a}	1.3 ±0.1^{a}	16.8 ±5.1^{a}	21.6 ±3.4^{a}	0.0 ±0.0^{b}	12.5 ±7.2^{a}	0.0 ±0.0^{b}
Eny1.31i	15.8 ±7.6^{a}	81.6 ±9.5^{a}	98.3 ±1.6^{a}	0.5 ±0.3^{a}	4.5 ±2.1^{a}	25.3 ±12.5^{a}	45.2 ±5.7^{a}	48.2 ±12.0^{a}	43.7 ±21.3^{a}
Eny7.11o	29.1 ±7.0^{a}	95.0 ±1.8^{a}	95.0 ±3.1^{a}	1.1 ±0.3^{a}	13.4 ±4.1^{a}	34.8 ±10.8^{a}	52.1 ±10.4^{a}	36.4 ±9.6^{a}	66.6 ±5.4^{a}
V5W2	31.6 ±8.7^{a}	76.1±10.5^{a}	91.1 ±5.4^{a}	1.1 ±0.3^{a}	11.6 ±3.6^{a}	18.7 ±6.1^{a}	53.5 ±9.7^{a}	46.1 ±7.5^{a}	53.3 ±6.2^{a}
P-value	0.4214	0.1392	0.2826	0.0895	0.7271	0.4813	0.0002	0.5616	0.0004

In columns within an experiment, means followed by the same letter are not significantly different at $P<0.05$ according to Tukey's studentized range test. Values represent the mean ± S.E., n= 15.

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Figure 1. Banana tissue culture plants growing in an aquaculture system in 250-ml plastic pots filled with nutrient solution.

Figure 2. Experimental set up used to test for host preference and attraction of *Radopholus similis* to either root segments from endophyte-inoculated or un-inoculated banana plants by means of the detached root bioassay (figure not drawn to scale).

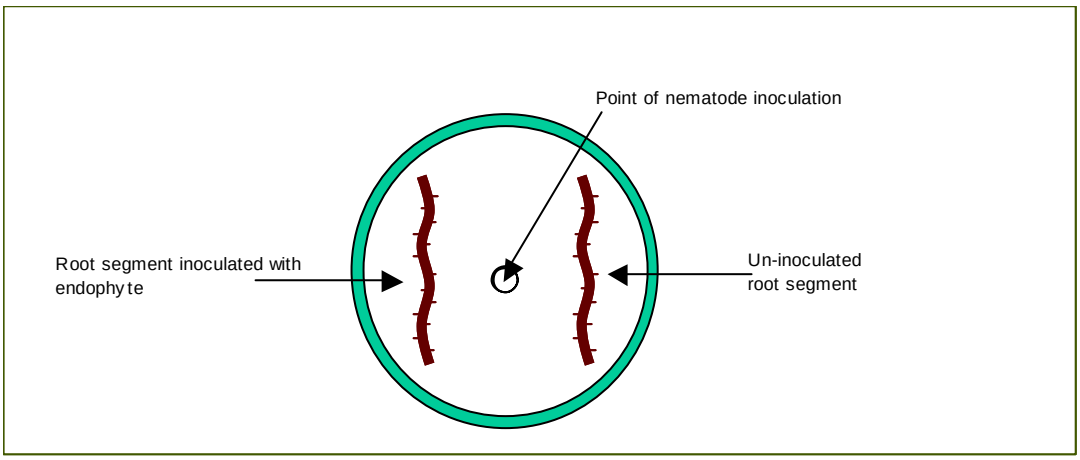


Figure 3. The polyvinyl chloride (PVC) tube apparatus used for *Radopholus similis* host preference and attraction bioassays involving intact endophyte-inoculated and un-inoculated (control) plants (A), and a schematic presentation of the PVC apparatus (B) (figure not drawn to scale).

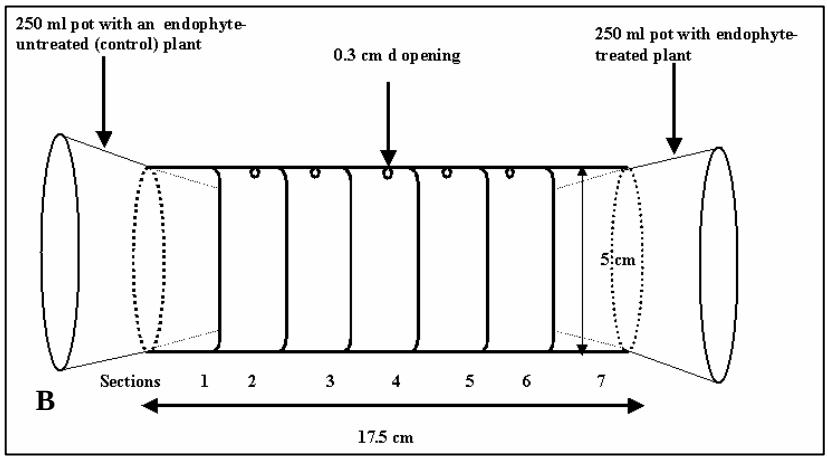


Figure 4. Experimental set up used to assess penetration of endophyte-inoculated and un-inoculated banana roots by *Radopholus similis* in vitro. (A), comprising a rectangular plastic container in which the plants were placed with three roots from each plant placed in Petri dishes. Nematode inoculation was done directly on the root sections inside the Petri dishes and extraction performed 24 hrs later. (B) represents a schematic presentation of the experimental set up (figure not drawn to scale).

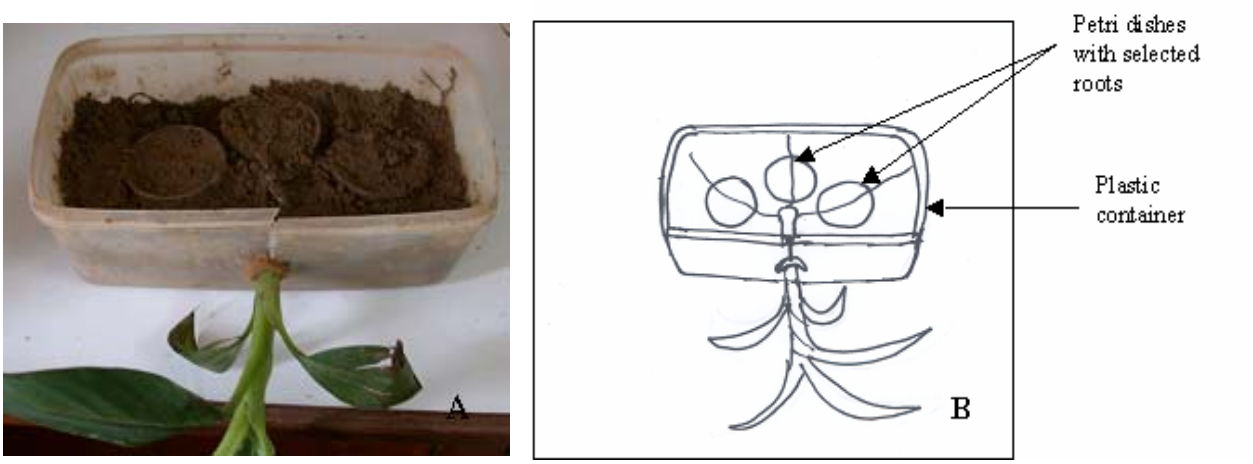


Figure 5. Experimental set up used to assess *Radopholus similis* reproduction in individual roots (A) and an excavated pot with the nematode inoculated root segment inside the plastic pot flanked by two root pieces (B).

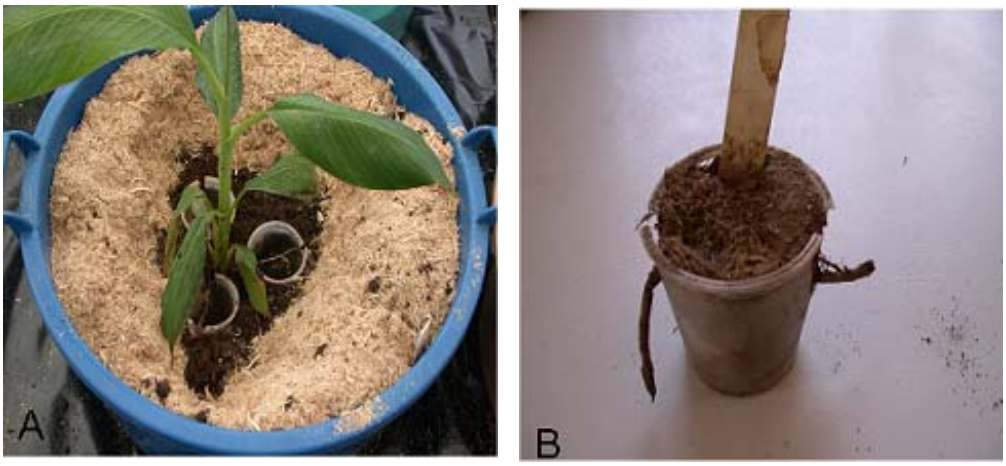
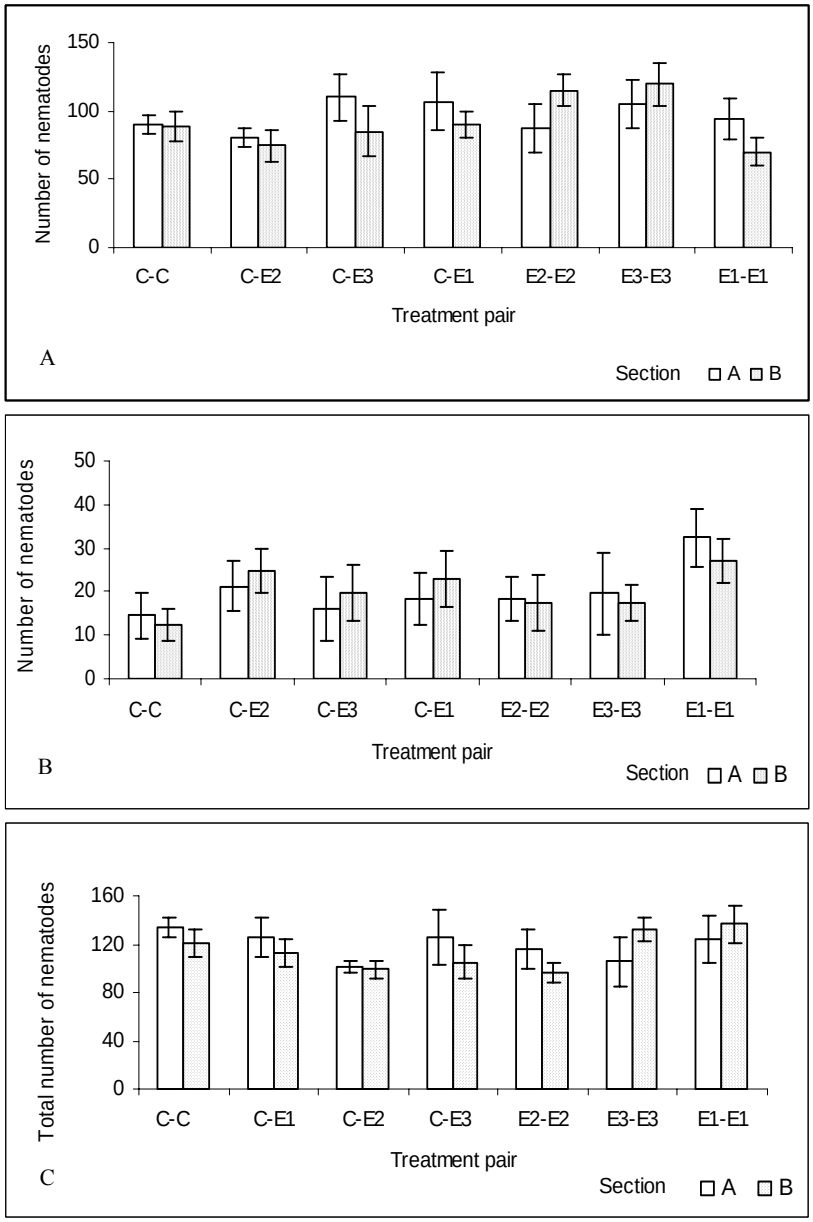
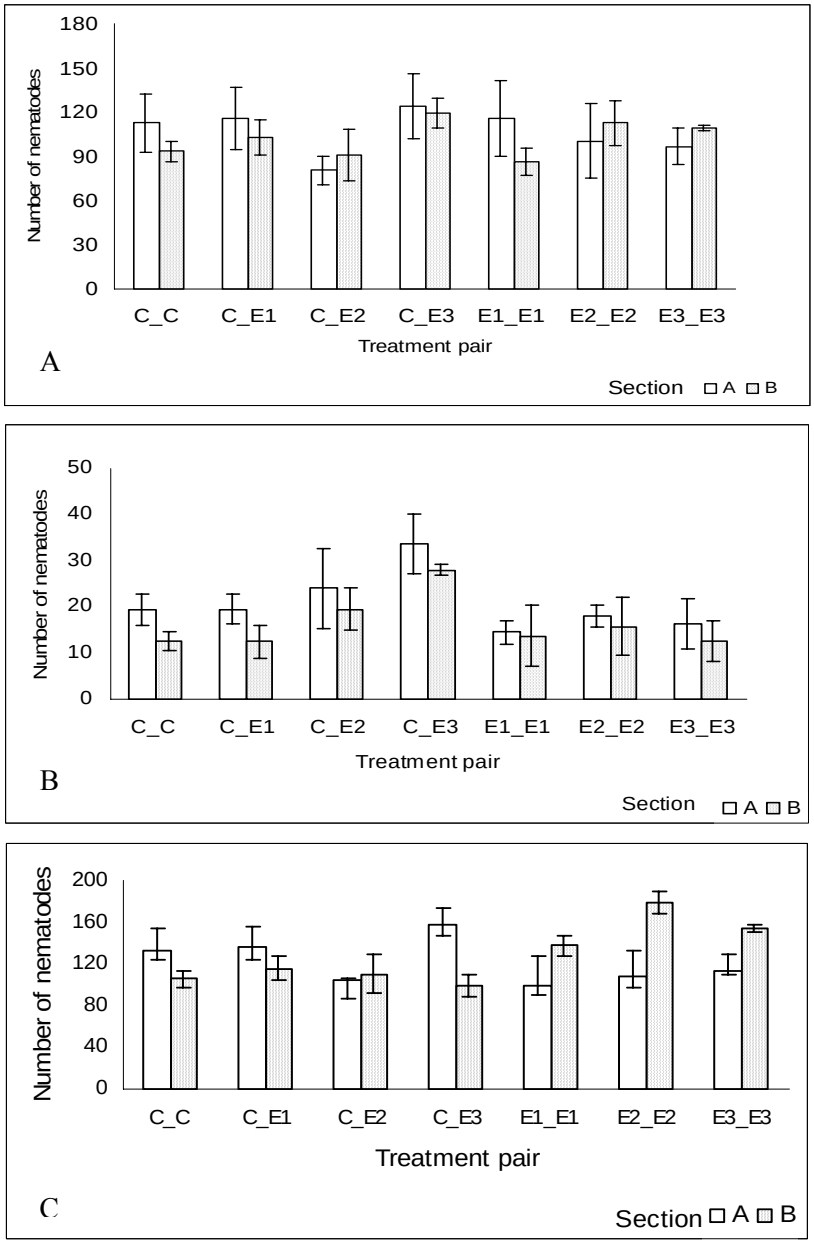


Figure 6. Average number of *Radopholus similis* that migrated towards root segments excised from endophyte-inoculated or un-inoculated banana roots in the detached root bioassay (Bioassay 1). Total nematodes recovered from the sand (A), number from the root segments (B) and the total nematodes from both the sand and root segment (C) in opposite sides of a Petri dish containing each respective treatment pair.



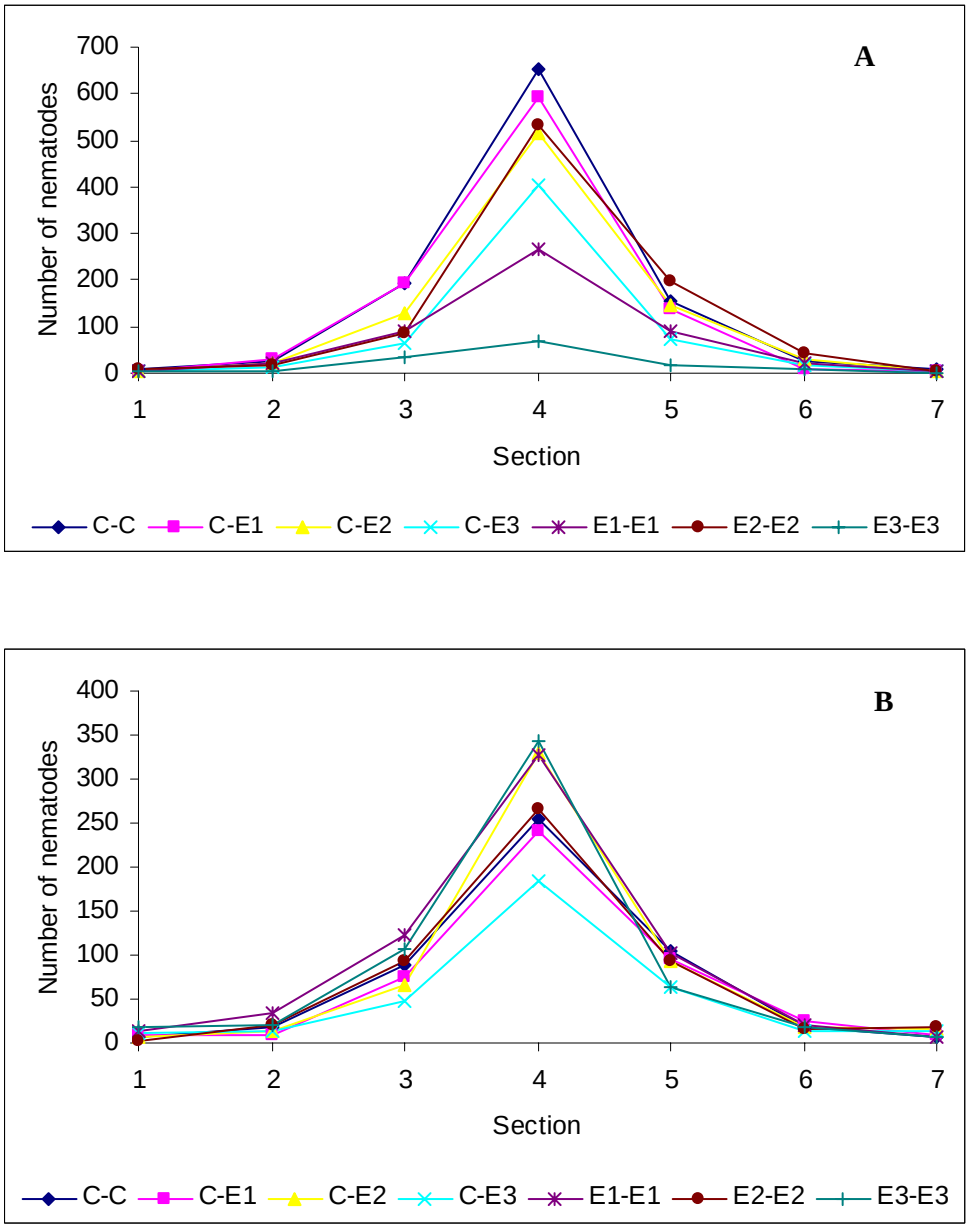
C=control, E1=endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*, E3= endophyte isolate *Eny7.11o*. Root segments from either endophyte-inoculated or un-inoculated plants were paired in 90-mm-diameter Petri dishes. The first treatment in each treatment pair was placed in section A and the second in section B. Nematodes were inoculated at the middle of the Petri dish equidistant from the two root segments. Nematode extraction was carried out 24 hrs later from both sand and the root segment in both sections of the Petri dish. Error bars represent standard errors of the mean, n=4

Figure 7: Average number of *Radopholus similis* that migrated towards root segments excised from endophyte-inoculated or un-inoculated banana roots in the detached root bioassay (Bioassay 2). Total nematodes recovered from the sand (A), number from the root segments (B) and the total nematodes from both the sand and root segment (C) in opposite sides of a Petri dish containing each respective treatment pair.



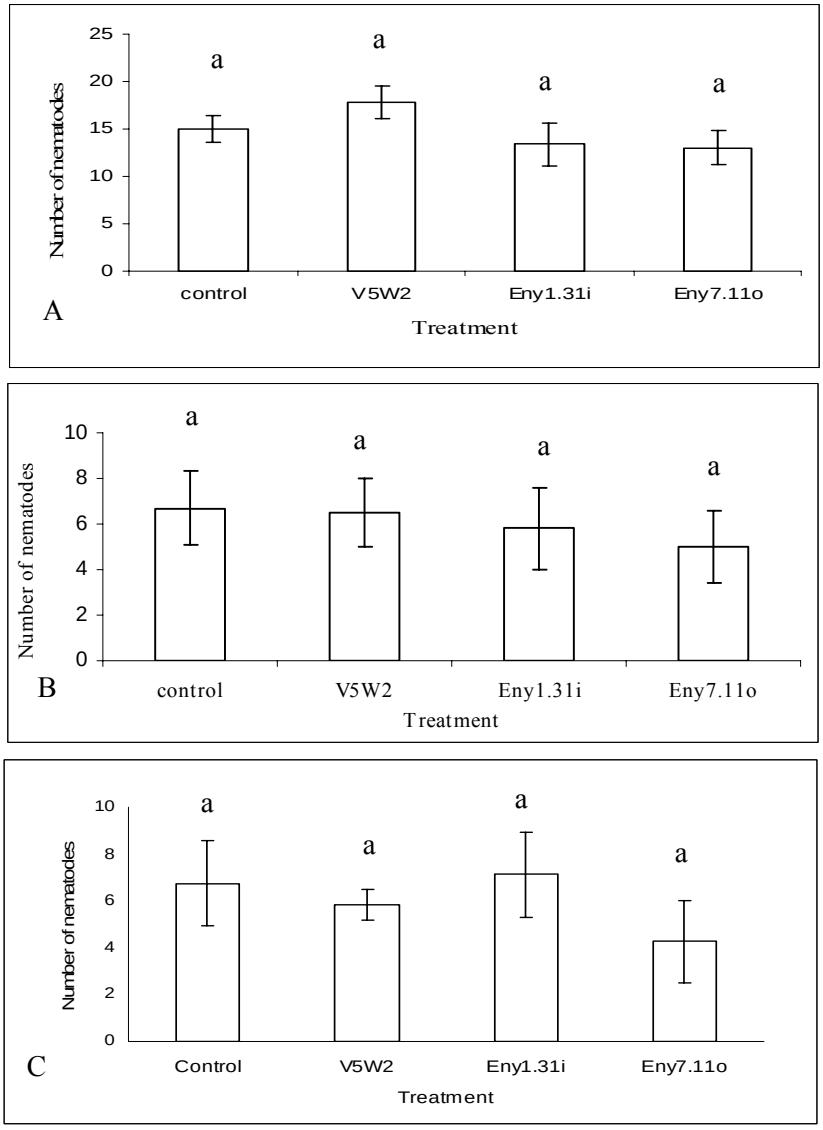
C=control, E1=endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*, E3= endophyte isolate *Eny7.11o*. Root segments from either endophyte-inoculated or un-inoculated plants were paired in 90-mm-diameter Petri dishes. The first treatment in each treatment pair was placed in section A and the second in section B. Nematodes were inoculated at the middle of the Petri dish equidistant from the two root segments. Nematode extraction was carried out 24 hrs later from both sand and the root segment in both sections of the Petri dish. Error bars represent standard errors of the mean, n=4

Figure 8: Distribution of nematodes in the polyvinyl chloride (PVC) sections showing migration of nematodes towards endophyte-inoculated and un-inoculated banana plants paired in a PVC tube apparatus divided into seven sections (A, bioassay 1; B, bioassay 2). *Radopholus similis* (2000 mixed stages) were introduced in the middle section of the PVC apparatus (section 4) equal distances from both plants placed in sections 1 and 7, which contained the either an endophyte-inoculated or un-inoculated plant.



C=control, E1= endophyte isolate *V5W2*, E2= endophyte isolate *Eny1.31i*, E3= endophyte isolte *Eny7.11o*.

Figure 9. Number of nematodes present in 8-cm-long root segments of endophyte-inoculated and un-inoculated banana plants 24 hrs after direct inoculation of root segments with 50 *Radopholus similis* females in the *in vitro* root penetration experiments. A, B and C are repeat experiments.



Control plants were not treated with any fungal isolate; endophyte-inoculated plants were treated with any of the three endophyte isolates viz. *V5W2*, *Eny1.31i* and *Eny7.11o*. Error bars represent standard errors of the mean, n=15. Bars with the same letters are not significantly different at *P*=0.05 according to Tukey's studentized range test.

Chapter 5

Mechanisms of action of endophytic *Fusarium oxysporum* against *Radopholus similis* in banana plants

Abstract

The mechanisms through which endophytic *Fusarium oxysporum* inhibits *Radopholus similis* damage to banana roots were studied by analyzing production of extracellular enzymes by the fungus, and induction of resistance mechanisms in the plant. Nine isolates of *F. oxysporum* produced proteases on gelatine-amended medium but none showed chitinase or lipase activity on chitin agar and medium amended with Tween 20, respectively. In split-root experiments, *R. similis* nematode numbers were reduced when the banana roots were treated with the endophytic *F. oxysporum* isolates *V5W2*, *Eny1.31i* and *Eny7.11o*. Histological analysis of banana roots and rhizomes showed higher levels of phenols in endophyte-treated (isolate *V5W2*) than in untreated plants. Cell wall-bound phenolics were more abundant in rhizomes than in the roots, and in the central cylinder of the rhizome than in the cortex regions. HPLC analysis did not show any significant differences between metabolite profiles of endophyte-treated and untreated plants. However, four unidentified compounds were found in both endophyte-treated and untreated plant extracts. Although not significantly different, the quantities of unknown compound 1 and 3 with retention times of 2.39 and 33.3 min, respectively, were found only in the endophyte-treated compared to the untreated plants. Known phenolic compounds identified in equal quantities in both endophyte-treated and untreated plants were 3, 4-dihydroxybenzoic, hydroxybenzoic, ferulic, syringic and vanillic acids. *p*-Coumaric acid was detected in rhizomes of plants treated with *V5W2* and *R. similis*. The results of the current study indicate that the systemic production of phenolic compounds in the host plant may constitute one of the main mechanims through which endophytic *F. oxysporum* suppresses *R. similis* in banana plants.

Introduction

The banana burrowing nematode, *Radopholus similis* (Thorne) Cobb is the most economically important nematode species-affecting banana in Uganda and the world (Sarah, 1989; Sarah *et al*., 1996; Speijer *et al*., 1999; Gowen *et al*., 2005). In Uganda, nematode-infected banana plants can have yield losses of between 30 and 50% (Speijer *et al*., 1999; Speijer and Kajumba, 2000). The nematode-induced losses are a result of reduction in the number of standing leaves, flower production, bunch weight, and an increase in the number of dead roots, root necrosis, plant toppling, and the time between successive harvests (Sarah *et al*., 1996; Speijer *et al*., 1999; Speijer and Kajumba, 2000; Talwana *et al*., 2003).

Management of *R. similis* has mainly relied on cultural practices such as the use of clean planting material obtained through paring and hot-water treatment of nematode infected plants (Speijer *et al*., 1995; Gold *et al*., 1998), planting of tissue culture plants (Mateille *et al*., 1994; Sarah, 2000), mulching (McIntyre *et al*., 2000; Talwana *et al*., 2003) and use of legume intercrops (McIntyre *et al*., 2001). Nematode resistant banana cultivars have also been identified and may be used in breeding programmes (Fogain and Gowen, 1997; Sarah *et al*., 1997; Collingborn *et al*., 2000). However, none of these methods offer permanent nematode control. An integrated nematode management approach involving a combination of several complementary methods would be best suited for control of *R. similis* in banana.

Biological control of *R. similis* using endophytic *Fusarium oxysporum* Schlecht.: Fries. is a promising management option that can be used to complement other nematode management strategies. Endophytic *F. oxysporum* reduced *R. similis* densities and damage in earlier studies (Chapter 3; Pocasangre, 2000; Niere, 2001). They suppress the nematodes in a number of ways by utilizing mechanisms that may act alone or in combination. One of the main mechanisms for *in vitro* inhibition of nematodes is the production of antagonistic compounds that cause nematode paralysis and mortality (Chapter 2; Hallman and Sikora 1994a,b; Niere, 2001; Dubois *et al*., 2004). Treatment of tissue culture banana plants with endophytes did not influence the nematode host preferences and root penetration by *R. similis*, but had an effect on nematode reproduction (Chapter 4). The reduction in nematode reproduction may be due to induced resistance a mechanism that has previously been reported as responsible for suppression of nematodes in plants (Sikora *et al*., 2003). Non-pathogenic *F. oxysporum* also

reduced the severity of Fusarium wilt diseases through induced resistance in tomato (*Lycopsersicon esculentum* L.) (Fuchs *et al*., 1997), cucumber (*Cucumis sativus* L.) (Mandeel and Baker, 1991), chickpea (*Cicer arietinum* L.) (Hervás *et al*., 1995) and banana (*Musa* spp.) (Nel *et al*., 2006). Another mechanism that may play a role in nematode control by endophytes is the direct parasitism of nematodes by the fungi.

Fungal endophytes may induce plant resistance responses by means of structural/morphological and physiological/biochemical changes in the plant. Biochemical responses include the synthesis of defence-related chemicals, such as phenolic compounds, against pest and pathogens (Ramamoorthy *et al*., 2001). Phenolic compounds may occur as constitutive molecules present in healthy plants or as substances synthesized by plants in response to bacterial or fungal infection (Mansfield, 1983), and are well recognized as plant resistance factors against nematodes (Giebel, 1974; 1982; Bajaj *et al*., 1983; Peng and Moens, 2004; Zinov'eva *et al*., 2004; Pegard *et al*., 2005). Banana cultivars resistant to *R. similis* were reported to contain higher amounts of constitutive phenolics compared to susceptible cultivars (Fogain and Gowen, 1996; Valette *et al*., 1998; Collingborn *et al*., 2000; Dochez, 2004). Schulz *et al*. (1999) also demonstrated that higher amounts of phenolic metabolites were produced in barley (*Hordeum vulgare* L.) inoculated with an endophytic *Fusarium* sp.

Direct parasitism is accomplished through the hydrolytic activity of extracellular enzymes produced by the nematode-antagonistic fungi (Stirling, 1991). For direct parasitism of nematodes to occur, the fungus must penetrate the nematode cuticle, a rigid and flexible exoskeleton composed mainly of proteins (Inglis, 1983). The nematode egg shell consists mainly of a chitinous and lipid layer (Perry and Trett, 1986; Bird and Bird, 1991). Production of extracellular enzymes by nematode–parasitic fungi has been demonstrated for nematophagous fungi like *Arthrobotryis oligospora* Fresenius (Minglian *et al*., 2004) and *Verticillium chlamydosporium* Goddard (Segers *et al*., 1994; Tikhonov *et al*., 2002).

Understanding the mechanism(s) of action involved in the biological control of *R. similis* in banana by endophytic *F. oxysporum* is important for successful application in the field. The objectives of this study were therefore, to (i) determine the production of extracellular enzymes such as chitinase, lipase and protease by nine endophytic *F. oxysporum* isolates on solid medium, (ii) assess induction of systemic resistance in banana plants against *R. similis* in

split root experiments by three endophytic *F. oxysporum* isolates and (iii) assess the accumulation of phenolic compounds in endophyte-treated banana plants as an indicator of induced resistance through histological and histochemical analysis.

Material and methods

Site description

All experiments were carried out at the International Institute of Tropical Agriculture (IITA) Research Station in Namulonge-Uganda, approximately 30 km Northeast of Kampala, Uganda. The site is situated at 1150 m above sea level, 32º 34'N latitude, with a mean annual rainfall of 1255 mm and an average temperature of 22ºC.

Fungal isolates, nematode cultures and banana plants

The endophytic *F. oxysporum* isolates used in this study were previously isolated from roots and rhizomes of apparently healthy banana plants in Uganda (Schuster *et al*., 1995), and are preserved in soil tubes at IITA. Pure *R. similis* inoculum was obtained from carrot disc cultures maintained at IITA (Chapter 1; Speijer and De Waele, 1997). Tissue culture banana plants of the local East African highland banana *cv*. 'Enyeru' (*Musa* spp. AAA-EA) were propagated from sword suckers (Vuylsteke, 1998). The plants were grown in a nutrient solution containing 1 g / L of Poly-Feed (Haifa Chemicals, Haifa Bay, Israel) and acclimatised in a humidity chamber (Chapter 3) for 1 month with weekly renewal of the nutrient solution.

Production of extra cellular enzymes on solid medium

Nine *F. oxysporum* isolates (*V5W2*, *Eny1.31i*, *Eny7.11o*, *V4W5*, *V2W2*, *V1W7*, *Emb2.4o*, *III4W1* and *III3W3*) were assayed for the production of extra cellular enzymes on solid medium amended with enzyme-specific substrates. Fungal isolates were pre-grown on synthetic nutrient agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar/L distilled water) in 65-mm-

diameter Petri dishes under laboratory conditions (room temperature of *ca*. 25°C and a photoperiod of 12 hrs light and 12 hrs darkness) for 1 week. The SNA medium was supplemented with 10 mg chlortetracycline, 100 mg penicillin G and 50 mg streptomycin-sulphate/L to prevent bacterial contamination.

Chitinase activity of the *F. oxysporum* isolates was assessed using 0.4% chitin agar (4 g chitin powder (Sigma-Aldrich St. Louis MO, USA), 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.5H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.001g $ZnSO_4$, 0.001g $MnCl_2$ and 20 g agar / L distilled water) (Hsu & Lockwood, 1975). One-week-old fungal isolates growing on SNA were point inoculated in the middle of Petri dishes containing chitin agar. A 3-mm-diameter cork borer was used to remove a disc of agar from the middle of the chitin agar plates and the hole replaced with a similar sized mycelial agar disc of the fungal cultures. To test for the production of lipases, fungal isolates from 1-week-old cultures on SNA were point inoculated on medium containing sorbitan monolaurate (Tween 20, Sigma, MO, USA) comprising of 10 ml Tween 20 and 20 g agar/L of distilled water. For assessment of protease activity, fungal isolates were point inoculated on gelatine medium comprising of 26.6 g gelatine (Sigma) and 14 g agar / L distilled water.

Ten Petri dishes were used for each of the isolates and enzymes; five with medium amended with the substrate and five without the substrate (controls). The medium in the control plates comprised of each of the above-mentioned ingredients except the enzyme–specific substrate. All petri dishes were incubated for 1 week under laboratory conditions. Cultures were examined on a daily basis for the presence of a clear zone (halo) around the fungal colony. The diameters of the clear zone and of fungal colonies were measured, and the difference between the areas of the clear zone and the fungal colony calculated to provide an estimate of the levels of enzyme production by the different isolates (Alves *et al*., 2002).

Split-root experiments for assessing induced resistance

Split-root experiments were conducted in the screen house to determine whether three *F. oxysporum* isolates (*V5W2*, *Eny1.31i* and *Eny7.11o*) induced systemic resistance in banana roots against *R. similis*. One-month-old tissue culture banana plants cv. Enyeru (*Musa* spp. AAA-EA) growing in nutrient solution in the humidity chamber (Chapter 4) were removed

from their pots and transplanted into 3-L plastic bags containing steam-sterilized loamy forest soil, and grown for another month. The 2-months-old the banana plants were then gently removed from their bags and each root system separated into two equal halves. Each half was planted separately into adjacent 300-ml pots, filled with sterile loamy forest soil (Fig. 1). The paired pots were marked A (inducer half) and B (responder half). The unsplit upper portion of the root system and rhizome were wrapped in moistened cotton wool to prevent dehydration, and then enclosed in an inverted pot from which the bottom part has been removed (Ogallo and McClure, 1996). The cotton wool was kept moist by spraying with tap water using a household sprayer.

Inoculation of plants with the endophytes started 1 week after replanting of the plants into the split-root systems. Fungal spore suspensions were prepared in half strength potato dextrose broth (PDB) (Sigma-Aldrich) obtained by dissolving 12 g of PDB in 1 L of distilled water. One hundred-ml aliquots of PDB were dispensed into 250-ml Erlenmeyer flasks and sterilized. After cooling, flasks were inoculated with 4 to 5 disks of agar of each fungal isolate. Un-inoculated PDB was used as the control treatment. Duplicate flasks were prepared for each fungal isolate and the control. Inoculated flasks were incubated in the laboratory for 7 days to allow for fungal growth and sporulation. Fungal spore suspensions were filtered through a 1-mm-diameter sieve to remove mycelial fragments. The spore suspensions were then adjusted to provide a final spore count of 1.5×10^6 spores/ml.

One week after transplanting, 1 ml of the spore suspensions was applied to the inducer half of the split root system. The soil around the roots was removed and the spore suspensions applied directly to the exposed roots. One week later, a 2-ml nematode inoculum containing 500 mixed stages of *R. similis* was added to the responder half of the split-root system so that both the fungal isolates and nematodes were spatially separated in adjacent pots on the same plant (Fig. 1). To inoculate plants with nematodes, soil was removed from around the roots and the nematode suspension pipetted directly onto the roots. The roots were then covered with soil. The experiment was repeated twice. The number of replications per treatment was 7, 10 and 14 in experiments 1, 2 and 3, respectively. Plants were arranged in the screen house in a completely randomized design (CRD).

One month after nematode inoculation the plants were harvested, and the nematode numbers and the extent of root necrosis determined. Root necrosis was determined from five randomly selected roots of each plant. The roots were split longitudinally and scored for percentage necrosis (Chapter 3; Speijer and De Waele 1997). The roots used for necrosis assessment were subsequently cut into smaller pieces, weighed and then macerated in a Waring blender (Waring, Connecticut, USA) at low speed for 15 s. Nematode extraction was carried out overnight according to a modified Baermann method (Chapter 3; Hooper *et al*., 2005). To check for cross-contamination and confirm spatial separation of the endophytes from the nematodes, nematode extraction and fungal reisolation was conducted on roots from both pots A and B.

To determine colonization of plant roots by the fungal isolates, three healthy primary roots were randomly selected from each pot at harvest and surface sterilized in 75% ethanol for 1 min, followed by sterilization in 2% NaOCl for 30 s. Root pieces were blotted dry on sterile tissue paper and cut into *ca*. 0.25-cm-long segments. Six sterilized segments per root were randomly selected and placed on SNA in 65-mm-diameter Petri dishes. The plates were incubated in the laboratory for 7 days under laboratory conditions. *Fusarium oxysporum* colonies growing from the root pieces were identified as described in Chapter 3 under a light microscope (magnification x 400) (Nelson *et al*., 1983). The number of root pieces with *F. oxysporum* colonies were recorded and the percentage recovery of the fungus calculated.

Analysis of phenolic compounds in endophyte-treated plants

The amount of phenolic compounds in rhizome and root tissues was studied as an indication of induced resistance in endophyte-treated tissue culture banana plants. Fungal inoculum was produced on sterile millet seed (Strauss and Labuschagne, 1995). Two hundred g of millet seed in 500-ml Erlenmeyer flasks were soaked in distilled water overnight and autoclaved twice (121ºC for 15 min) on successive days. The flasks were subsequently inoculated with five mycelial disks of 1-week-old cultures of isolate *V5W2* grown on SNA. The flasks were then incubated at room temperature at *ca*. 25ºC in the laboratory for 3 weeks. Flasks were shaken daily to disperse the inoculum throughout the seeds. Uninoculated millet seed was included as the control treatment.

Plants were removed from the humidity chamber and their roots cut back to *ca*.10 cm long. The plants were then potted in steam-sterilized loamy forest soil in 300-ml plastic pots and placed on a table in the screen house. The experiment included five treatments: a positive control that was sprayed until run-off with 50 mM di-hydrogen potassium phosphate (KH_2PO4), a known chemical inducer of resistance in plants (Manandhar *et al*., 1998); a negative control comprising plants treated with sterile millet seed inoculum (10% w/v); plants treated with isolate *V5W2*-colonized millet seed inoculum only (10% w/v); plants treated with *R. similis* only and plants treated with both *V5W2*-colonized millet seed inoculum (10% w/v) and *R. similis*. Treatments with *R. similis* were inoculated with a 2-ml suspension of 500 mixed stages of *R. similis*. The endophyte was inoculated at the beginning of the experiment and the nematodes at 1 week after endophyte inoculation.

Each treatment consisted of 25 1-month-old plants, which were arranged in a completely randomized design (CRD). In a time course study, five plants from each treatment were harvested at 0, 1, 2, 3 and 4 weeks after endophyte inoculation for analysis of total phenolic compounds. The roots and rhizomes of each plant sampled were washed free of soil under running tap water. From each plant, three primary roots were selected and a 1-cm piece cut from the basal part of the root (part of root proximal to the rhizome). The rhizome was split longitudinally into two equal parts. One half of the rhizome and the three 1-cm root pieces were fixed in a fixative comprising of 70% ethanol, pure acetic acid and 35% formaldehyde in the ratio 18:1:1 in 10-ml vials for histological analysis (Dochez, 2004). The other half of the rhizome and remaining roots were preserved at -20ºC for histochemical analysis of phenolic compounds.

Histological analysis

The fixed root and rhizome samples were trimmed back to ca. 0.25 cm long pieces prior to processing. The samples were dehydrated in an alcohol series of 70%, 80%, 90% and 100% alcohol for 2 hrs at each series and subsequently cleared in two steps of xylene. The dehydrated samples were impregnated in paraffin wax (50ºC melting temp.), embedded in paraffin wax (80ºC melting temp.) and mounted in wooden blocks (50 x 40 mm) for sectioning. Six µm thick ransverse sections of roots and rhizomes were subsequently made using a microtome (Baird & Tatlock London Ltd, Chadwall, UK). Three sections from each

root and rhizome piece were mounted on microscope slides, dewaxed in xylene and rehydrated in four steps of descending alcohol series (100%, 90%, 80% and 70%) (Fogain and Gowen, 1996).

The rehydrated sections were stained for phenolic compounds by flooding the sections with 2% ferric chloride dissolved in 95% ethanol for 5 min, and counterstained with Orange G for 1 min. The sections were rinsed in 95% isopropanol and cleared in xylene. Sections stained for lignin were flooded with 1% safranin dissolved in water for 5 min, rinsed briefly in distilled water and counterstained in 5% light green in water for 3 min (Fogain and Gowen, 1996). After staining for phenolic compounds and lignin, the slides were dehydrated in a series of ascending concentrations of alcohol with four steps (90%, 95% and two stages of 100%). Sections on slides were mounted in a synthetic mounting medium (DPX mountant, BDH, Kampala, Uganda) and covered with a cover slip.

Stained sections were observed under a light microscope at X400 magnification and the number of cells with phenolic compounds recorded separately for central cylinder (vascular bundles) and the cortex region of both the root and rhizome. Preformed phenolic cells were recorded as those with granular precipitates dispersed throughout the cell vacuole (Mace, 1963; Fogain and Gowen, 1996). Fully formed phenolic cells were recorded as the cells appearing as one large amorphous mass of granular bodies (Fogain and Gowen, 1996). From each treatment at each time period, 15 root and 15 rhizome sections were examined. The number of preformed and fully formed phenolic cells were scored on a scale of 0 to 5 where 0 = zero cells, 1 = 1 to 4 cells, 2 = 5 to 10 cells, 3 = 11 to 15 cells, 4 = 16 to 20 cells and 5 = more than 20 stained cells (Dochez, 2004). For sections stained for lignin, the presence of lignified cell walls and the location of the lignified cells (central cylinder, cortex or endodermis) were recorded. Stained root and rhizome sections were photographed using a Zeiss Axioplan 2 light microscope (Carl-Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam HR, Carl-Zeiss).

Histochemical analysis

Extraction of phenolic compounds

Phenolic compounds were extracted from the root and rhizome samples preserved at -20ºC according to the method described by De Ascensão and Dubery (2003). Half a gram of frozen root and rhizome samples were ground in liquid nitrogen and transferred to 1-ml Eppendorf tubes. Nine hundred ml of 80% methanol was added and the mixture vortexed for 30 s. The mixture was homogenized for 1 hr in a rotary shaker and centrifuged at 12000 rpm for 10 min. The supernatant was transferred into new Eppendorf tubes and the extraction procedure repeated overnight. The supernatants from the first and second extraction were pooled and left on the bench to evaporate to ca. 1 ml of crude extracts. The extraction procedure was conducted in the laboratories at IITA-Uganda and the extracts preserved at 4ºC. Histochemical analysis was carried out at the University of Pretoria, South Africa. The extracts were transported from IITA-Uganda to South Africa in cooler boxes at ca. 4ºC and refrigerated immediately upon arrival.

Analysis of total soluble phenolic compounds by means of the Folin method

Total phenolic content was determined using the Folin method, which utilizes the Folin-Ciocaltaeu (FC) reagent (Sigma) (Swain and Hills, 1959). Reaction mixtures were prepared in 96-well Elisa plates (Merck, Darmstadt, Germany). Five µl of each crude extract was mixed with 175 µl distilled water and 25 µl FC reagent and left for 3 min, before adding 50 µl of saturated $NaCO_3$ and incubation at 40ºC for 30 min. After incubation, absorbance was read at 690 nm using an ELISA reader (Multiskan Ascent, Version 1.3.1, Labsystems, Helsinki, Finland). For each root and rhizome sample, four absorbance readings were obtained and the average absorbance calculated. The absorbance of a blank consisting of distilled water was subtracted from all sample readings. Gallic acid was used to prepare a standard curve for estimation of the amount of soluble phenolics in each sample (Sivakumar *et al*., 2005). The concentration of phenolic compounds in the crude root and rhizome extracts was subsequently calculated from the standard curve and expressed as µg gallic acid/g fresh weight.

Identification of phenolic compounds by high performance liquid chromatography

Phenolic compounds were identified and quantified using high performance liquid chromatography (HPLC). Three root and three rhizome samples from different plants sampled 2 weeks after endophyte inoculation were selected from each treatment for HPLC analysis. The crude extracts were first hydrolyzed with hydrochloric acid (De Ascensão and Dubery, 2003). For hydrolysis, 100 µl of the crude extract was mixed with 10 µl pure HCl, incubated at 96ºC for 1 hr and extracted two times with anhydrous diethyl ether. The extract was evaporated to dryness and re-dissolved in 50-µl methanol. The hydrolyzed samples were assayed on a Hewlett- Packard (HP) HPLC system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA) equipped with a 20 µl loop injection valve (Agilent Technologies) and connected with a UV detector at 280, 325 and 340 nm. A Luna 3u C18 (Phenomenex, Palo Alto, CA, USA) reverse phase column (250 x 4.60 mm) was used. Acetronitrile and water (pH 2.6 acidified with phosphoric acid, H_3PO_4) were used as the solvents with a gradient program from 7% acetronitrile/water at 0 min, 20% at 20 min, 23% at 28 min, 27% at 40 min, 29% at 45 min, 33% at 47 min and 80% at 50 min. Twenty µl of the hydrolyzed extracts were injected and chromatogramed with a flow rate of 1 ml/min. Data were analyzed using the HP software provided with the HPLC equipment. The phenolic compounds in the extracts were identified by comparison with the reference compounds: gallic acid, caffeic acid, ferulic acid, syringic acid, quercetin, umbelliferone, naringin, hydroxy benzoic acid, 3,4-dihydroxy benzoic acid, sinapic acid, vanillic acid, *ρ*-coumaric acid, salicylic acid, scopoletin, catechin, kaempferol, chlorogenic acid, luteolin and fisetin obtained from Sigma.

Data analysis

All data were tested for normality and homogeneity of variances using Shapiro-Wilkinson, Levene Welch and Kolmogorov-Smirnov tests. Normal probability plots, box plots and stem leaf plots were additionally used to confirm normality of data and equality of variances. If not normally distributed, various transformations were tested until the most suitable transformation was obtained. For the enzyme production assays, statistical analysis was

performed on the averages of the size of the clear zones (calculated from the difference in size between the fungal colony and the clear zone) using one-way ANOVA.

Nematode counts in the three split-root experiments were calculated per 100 g of roots and square-root (x + 0.5) transformed prior to analysis. Percentage root necrosis and percent endophytic colonization were arcsine-square root (sqrt) x +0.5 transformed prior to analysis. One-way ANOVA was used to determine differences among repeat experiments. When differences were observed between experiments, data from each experiment was analyzed separately. In each experiment, one-way ANOVA was conducted to assess variability among treatments.

For the histological analysis of phenolic cells, the scores for pre-formed and fully formed phenolic cells were averaged for each treatment-week combination. Before statistical analysis, data was sqrt transformed. One-way ANOVA was used to evaluate differences among treatments, time (weeks) and plant part (rhizome or root). Two-way ANOVA was used to evaluate interaction effects between treatments, time and plant part. Interaction effects were subsequently evaluated using least square means. Differences in the number of phenolic cells between the central cylinder and cortex regions were evaluated using paired t-tests.

Data from histochemical analysis of soluble phenolic compounds with the FC reagent was expressed as µg gallic acid/gram of fresh weight and log (x +1) transformed prior to analysis. One-way ANOVA was used to determine main effects of treatment, time and plant part. Two-way ANOVA was used to evaluate interaction effects between treatments, time and plant part using least square means. HPLC data was analyzed qualitatively by comparing the presence and absence of peaks in chromatograms obtained with the different treatments. Quantities of unidentified compounds, estimated from the area under the peaks (in milliabsorption units [mAU/s], were used to evaluate quantitative differences among treatments. For the known phenolic compounds identified by comparison with the reference standards, the amount in µg/ml was used for comparison between endophyte and non-endophyte treatments. Data of the amounts of the unidentified compounds was log (x+1)-transformed prior to analysis. One-way ANOVA was used to assess differences among treatments. For all experiments, differences between means were separated using Tukey's studentized range test (SAS Institute, 1989).

Results

Production of extra cellular enzymes

The nine *F. oxysporum* isolates tested did not show chitinase or lipase activity in solid medium, as no clear zone formed around the fungal colonies 1 week after incubation. All the isolates, however, showed positive protease activity. A clear zone formed around the fungal colonies 2 days after the fungus was placed on gelatine-amended medium (Fig. 2). The measurements of the fungal colony diameter and the diameter of the clear zone were conducted on the third day of incubation. Measurements could not be done on the second day since the distinction between the fungal colony and the clear zone could not be easily discerned. From the fourth day onwards the clear zone was not visible anymore. No clear zones were observed in the control plates in which the fungus was grown without the enzyme substrates. The diameters of the clear zone did not differ between the different fungal isolates (P=0.0889). However, based on the diameters of the halo, isolates *III3W3*, *Emb2.40*, *Eny1.31i* and *V4W5* had produced more protease activity compared to isolates *V5W2*, *Eny7.11o*, *V1W7*, *III4W1* and *V2W2* (Fig. 3).

Split-root experiments for assessment of induced resistance

The three *F. oxysporum* endophytic isolates did not reduce the number of *R. similis* significantly, with the exception in the number of females in Experiment 2 (Table 1). The total number of nematodes was also reduced significantly by isolates *Eny1.3li* and *V5W2* in Experiment 2. Generally, the responder half of endophyte-treated roots supported fewer females, juveniles, males and total nematodes than the control roots. Damage due to nematodes was not significantly different between the fungal isolates and the control treatment (P=0.6934) (Table 1).

The split-root system provided spatial separation of the nematodes from the endophytes on the same plant. No nematodes occurred in the uninoculated halves of the split-root systems. However, endophytic *F. oxysporum* was re-isolated from the untreated roots, indicating possible contamination of untreated halves. Percentage root colonization in the inducer half of

the split root systems differed significantly between the endophyte and control treatments (P=0.0003). In the inducer half, root colonization by isolates *Eny1.31i*, *V5W2* and *Eny7.11o* were 75.0 ± 5.9%, 59.5 ± 6.4% and 52.7 ± 5.4%, respectively. Root colonization in the control treatment ranged from 16.6 ± 7.4% to 37.6 ± 7.2% (data not presented). Root colonization by endophytic *F. oxysporum* was 40.8 ± 6.2, 33.3 ± 6.6, 25.7 ± 5.7 and 36.5 ± 5.2% in the responder roots when the inducer roots were treated with the fungal isolates *Eny1.31i*, *V5W2*, *Eny7.11o* and the uninoculated broth (control), respectively.

Analysis of phenolic compounds

Histological analysis

Phenolic cells were observed in the central cylinder and cortex of roots and rhizomes of all banana plants, whether they were treated or not treated with the endophytic *F. oxysporum* isolate *V5W2* (Fig. 4 and 5). Significantly more phenolic cells, however, were formed in the central cylinder than in the cortex of roots and rhizomes (Fig. 4 and 6) (P=0.0027). The number of phenolic cells that formed in the central cylinder of root and rhizome sections differed significantly between the different treatments applied (P=0.0009) the time since treatment (P<0.0001), and the plant parts investigated (root or rhizome) (P<0.0001) (Fig. 4). Root and rhizome sections from plants treated with the endophytic *F. oxysporum* isolate *V5W2* + *R. similis* had significantly more phenolic cells in their central cylinder compared to endophyte-untreated plants (Fig. 4). In the cortex, however, most phenolic cells were formed in plants treated with 50 mM K_2HPO_4. The number of phenolic cells in the central cylinder of plants treated with *V5W2* only and *V5W2* + *R. similis* increased from week 0 to week 4, but reached its highest levels in the cortex 2 weeks after inoculation. More phenolic cells formed in *V5W2* + *R. similis* than in the negative control plants at each time interval, except in the rhizome cortex. While phenolic cells were present in plants treated with *R. similis* only, the numbers recorded were lower than in plants treated with the endophyte. No lignification of cell walls was demonstrated except in the endodermis of a few roots of endophyte-treated plants (data not shown).

Preformed phenolic cells with granular precipitates dispersed in the cell vacuoles were formed in the central cylinder and cortex of roots and rhizomes of both plants treated and not treated

with the endophytic *F. oxysporum* isolate *V5W2* (Fig. 6F). The endophyte treatment, however, resulted in higher numbers of preformed phenolic cells when compared to non-endophyte treated tissue (P=0.0059). The number of preformed phenolic cells in the roots and rhizomes increased significantly with time after endophyte inoculation (P<0.0001) and differed between plant parts (rhizomes and roots) (P=0.0343). The number of preformed phenolic cells in the central cylinder of both rhizomes and roots occured in descending order in plants treated with isolate *V5W2* only, *V5W2* + *R. similis*, K_2HPO_4, negative control and *R. similis* only (Fig. 5A and B). In plants treated with isolate *V5W2* only or with *V5W2* and *R. similis*, the number of preformed phenolic cells in the rhizomes increased from 0 weeks after inoculation, and reached a maximum after 2 weeks (Fig. 5A). In the roots, the maximum number of phenolic cells was observed mostly 1 or 2 weeks after inoculation (Fig. 5B). Although not significantly different, plants treated with *V5W2* and *V5W2* with *R. similis* had more preformed phenolic cells than the negative control plants and plants treated with *R. similis* only. Rhizomes had significantly more preformed phenolic cells (P<0.0001) than roots (Fig. 5C and D). More preformed phenolic cells were found in the central cylinder compared to the cortex regions (P<0.0001) (Fig. 5A and C, Fig. 5B and D).

Histochemical analysis

Analysis of total soluble phenolics

The amount of total soluble phenolics produced in the banana rhizome (P=0.0019) and roots (P=0.0011) differed significantly between endophyte and non-endophyte treated plants (Fig. 7). No differences in total soluble phenolics, however, were observed in rhizomes of plants treated with *V5W2* only and plants treated with *V5W2* and *R. similis* (P=0.5390). Total soluble phenolics also did not differ significantly in the roots of plants treated with endophytes and 50 mM K_2HPO_4 (P=0.7285). In all rhizome treatments, the amount of total soluble phenolics in rhizomes increased from week 0 to week 2, and then decreased in weeks 3 and 4 (Fig. 7A). The amount of total soluble phenolics in plants treated with *V5W2* only and plants treated with *V5W2* and *R. similis* increased by 16.8% and 41.9% from week 0 to week 2, respectively. In the roots, however, the total soluble phenolics dropped rapidly in both endophyte and non-endophyte treated plants from week 0 to the second week, after which it remained relatively stable until week 4 (Fig. 7 B).

HPLC analysis of phenolic compounds

HPLC separation of phenolic compounds extracted from rhizomes and roots of plants treated with the endophytic *F. oxysporum* isolate *V5W2* and *R. similis* revealed the elution of four major peaks (Fig. 8). These peaks did not represent compounds of any of the known reference standards. Based on spectral analysis, they had spectrums similar to that of ferulic acid with a maximum absorbance at 280 and 325 nm, and thus belong to the hydrocinnamics group of phenolic compounds (Harborne, 1991). Unknown compound 1 with a retention time of 2.39 min was found in extracts from plants treated with 50 mM K_2HPO_4, *V5W2* and *V5W2* and *R. similis* but in undetectable amounts in the negative control and *R. similis*-treated plants (Fig. 8). No significant differences were found in the size of the peaks (unknown compounds 2, 3 and 4) between endophyte and non-endophyte treatments. Extracts from endophyte-treated plants, however, were higher for compounds 3 and 4, although not significantly different from non-endophyte treatments (Fig. 9). The amounts of compounds 2 (retention time 7.83 min), 3 (retention time 17.7 min), and 4 (retention time 33.3 min), were significantly higher in the plants treated with 50 mM K_2PO_4 and plants treated with *R. similis* only compared to endophyte-treated plants. Quantities of unknown compounds 2 (P=0.0484) and 3 (P=0.0401) were significantly more in the rhizome compared to the roots, unlike unknown compound 3 (P=0.1831) (Fig. 9).

The known phenolic compounds in rhizome and root extracts did not differ among the endophyte and non-endophyte treatments (P=0.4525) (Table 2) with the exception of hydroxybenzoic and ρ-coumaric acid, which were detected in the rhizome of plants treated with both *V5W2* and *R. similis*, but not in the negative control. Gallic acid was detected in the rhizome of plants treated with 50mM K_2HPO_4. Other compounds, such as 3, 4 dihydroxybenzoic acid, vanillic acid, ferulic acid and syringic acid were present in both endophyte-treated and untreated plants (Table 2).

Discussion

Endophyte treatment of banana roots with endophytic *F. oxysporum* appeared to trigger defence mechanisms that could reduce reproduction of *R. similis* (Chapter 4). It did not, however, reduce the numbers of nematodes infecting the roots or reduce nematode-inflicted damage to the roots. According to the current investigation, the enhanced production of phenolic compounds in cells, primarily in the central cylinder of the roots and rhizome due to endophyte infection is a major indicator of induced host resistance response. These phenolic depositions appeared to be induced, as the number of phenolic cells increased over time. Induced resistance in plants might not be the only mechanisms of action whereby endophytic *F. oxysporum* result in biological control of nematodes, as the endophytic isolates also showed protease activity *in vitro* and also produced toxic secondary metabolites (Chapter 2).

Extracellular hydrolytic enzymes target the external and internal structures of nematodes and their eggs (Wuyts *et al*., 2004). In this study, all nine endophytic *F. oxysporum* isolates produced proteases, but none showed chitinolytic or lipolytic activity. The production of proteases might be partly responsible for mortality of the motile stages of *R. similis* when they were treated with fungal culture filtrates (Chapter 2), since the nematode cuticle is mainly composed of proteins (Inglis, 1983). Vu *et al*. (2004) previously demonstrated a lack of direct parasitism of *R. similis* by endophytic *Fusarium* isolates, suggesting that other secondary metabolites might be involved in the killing of nematodes. The lack of chitinolytic and lipolytic activity suggests that direct parasitism of nematode eggs, composed mainly of chitin and lipids (Bird and Bird, 1991), by *F. oxysporum* is unlikely. Poor hatching of *R. similis* eggs treated with fungal filtrates (Chapter 2) should, therefore, rather be attributed to other secondary metabolites and toxins. To confirm the role of extracellular enzymes and toxins in biological control, specific secondary metabolites need to be purified from fungal cultures and assayed against nematodes and their eggs, and observations made by means of scanning electron microscopy (Bonants *et al*., 1995).

For direct parasitism of the nematodes by fungal hyphae to occur, contact between the nematode and the fungus for a sufficient duration is required. Paparu (2005) demonstrated that colonization of banana roots by endophytic *F. oxysporum* isolates was extensive in the hypodermal cells and cortex. All developmental stages of *R. similis* also occur in the cortex of

plant roots (Araya and De Waele, 2001; Gowen and Quénéhervé, 2005). Despite occupying the same niche inside roots, direct parasitism of the destructive stages and eggs of *R. similis* by endophytic fungi may not happen due to the migratory nature of the nematode. Though direct parasitism may not necessarily represent a substantial part of nematode control by endophytes, the association with other modes of action, for instance the production of toxic metabolites and induced resistance, may improve their efficacy and levels of plant protection.

Split-root systems have been used to investigate induced systemic resistance by non-pathogenic isolates of *F. oxysporum* in tomato (Ogallo and McClure, 1996; Fuchs *et al*., 1997; Larkin and Fravel, 1999) and cucumber (Mandeel and Baker, 1991). Using split-root experiments, several researchers have reported induction of systemic resistance against root-knot nematodes, *Meloidogyne* spp. (Aalten *et al*., 1998; Siddiqui and Shaukat, 2002; 2003; 2004) and the potato cyst nematode, *Globodera pallida* (Sikora and Reitz, 1998; Reitz *et al*., 2000). In this study, split-root experiments did not convincingly demonstrate the ability of endophytic *F. oxysporum* isolates to induce systemic resistance in banana plants against *R. similis*. In one experiment, *R. similis* numbers were significantly reduced by *isolates V5W2*, *Eny1.31i* and *Eny7.11o* compared to the control treatment, but not in the other two experiments. The number of nematodes in these experiments, however, was still lower than in the control treatments. This indicates that there was some measure of induced systemic resistance in the plants against the nematode. The lack of significance between the two split-root experiments may have been due to the small sample size (low numbers of replications per treatment) and high variation between replicates within a treatment. Contamination by other endophytic *F. oxysporum* may also have buffered the effects of the inoculated isolates resulting in lack of significant results.

This study provides the first indication of ISR by endophytic *F. oxysporum* against *R. similis* in banana. While endophytic *F. oxysporum* isolates have been used to suppress *R. similis* in banana before (Pocasangre 2000; Sikora *et al*., 2000; Niere 2001; Chapter 3 and 4), none of these studies demonstrated endophyte-induced resistance. Endophytic isolates of *F. oxysporum* and *Pseudomonas fluorescens* Migula proved to induce systemically acquired resistance in Cavendish banana against *F. oxysporum* f.sp. *cubense*, the causal agent of Fusarium wilt (Belgrove and Viljoen, personal communication). Similarly, Aalten *et al*. (1998) concluded that rhizosphere strains of fluorescent *Pseudomonas* spp. elicited induced

systemic resistance responses in banana plants that reduced *R. similis* and *Meloidogyne* spp. numbers in the roots.

Contamination of untreated split-roots by *F. oxysporum* may denote that the effects of endophyte treatment on *R. similis* cannot be ascribed to ISR. For ISR to occur, spatial separation of the inducing agents and the nematode must be maintained (Siddiqui and Shaukat, 2002). Despite the presence of endophytic *F. oxysporum* in untreated split-roots, nematode reproduction in plants treated with endophytes was substantially less than in the case of the endophyte-untreated roots in one experiment, suggesting possible ISR. However, the lack of significant reduction in nematode numbers and root damage between endophyte-treated and non-treated plants in the other two experiments can either be ascribed to contamination by *F. oxysporum*, or a lack of ISR. The most effective way to confirm ISR would be to conduct the experiments in a controlled environment that prevents introduction of other fungi to the untreated plants. From the current study it is clear that further investigations are required on the threshold root colonization as well as on persistence of systemic resistance in banana against *R. similis*. Hallman *et al*. (1997) previously demonstrated that, even when colonization rates of roots by endophytes decline over time, the plant may retain the induced protection over time.

No significant difference was observed in the number of fully formed and preformed phenolic cells in the rhizomes and roots of banana plants immediately after endophyte inoculation. The increase in the number of phenolic cells from week 1 to 4, however, may be attributed to increased (induced) synthesis due to endophyte and nematode infection. The number of phenolic cells in *R. similis*-infected plants was lower than in endophyte-treated plants, indicating a positive response to endophyte infection. The higher number of phenolic cells formed when both endophyte and nematode were inoculated on banana roots, compared to when they were inoculated separately, indicates that the joint infection induced greater plant defence responses. This could be explained by the report of Kloepper *et al*. (1992) that induced plants often do not produce defence chemicals until challenged by a pest or pathogen. The presence of constitutive phenols has been associated with resistance in banana cultivars against *R. similis* (Fogain and Gowen, 1996; Collingborn *et al*., 2000; Dochez, 2004). In future experiments, it may be useful to additionally include a resistant cultivar for comparative purposes.

No significant differences were obtained in total soluble phenolics between endophyte-treated and untreated plants, but higher concentrations occurred in the endophyte-treated plants compared to non-treated plants over time. The high concentration of phenolic compounds in the roots immediately after endophyte inoculation was unexpected. This may be due to transplanting shock and some injury of the plants during handling, as the plants were sampled immediately after establishment of the experiment. An increase in the levels of phenolic compounds is known to occur due to both biotic and abiotic stress factors (Beckman, 2000).

Qualitative analysis by HPLC revealed four major unknown compounds in root and rhizome extracts of plants in endophyte and non-endophyte treatments. One compound was only found in plants treated with the chemical inducer and also in the endophyte–treated plants but in very low amounts in the negative controls and *R. similis*-treated plants indicating induced synthesis of this compound. The areas under the peaks of two unknown compounds were slightly larger in the endophyte-treatments compared to the controls, a possible indication of their importance in the defence mechanism of the plants triggered by the fungal endophyte. Further characterization of these compounds is required to elucidate their identity. Nevertheless, quantitative differences observed between endophyte-inoculated and uninoculated plants suggest that the presence of the endophyte triggers the plant to increase synthesis of these compounds. The results obtained in the current study further indicate that the presence of the four unidentified compounds are not entirely due to endophyte infection as they were detected in both endophyte-inoculated and uninoculated plants. There is strong evidence indicating that phenolic compounds are involved in plant resistance against nematodes (Hung and Rohde, 1973; Mahajan *et al*., 1985). The endophyte-induced phenolics in banana plants most likely play a significant role in the suppression of *R. similis*.

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Figure 1. Illustration of the split-root system used for assessment of induced resistance by endophytic *Fusarium oxysporum* isolates against *Radopholus similis*. Two-month-old tissue culture banana plants of the cv. Enyeru (*Musa* spp. AAA-EA) in split-root systems within adjacent pots (A) and the split-root system with the upper undivided portion of roots and rhizome section wrapped in cotton wool (B). The fungal isolates or uninoculated broth and nematodes were applied in the halves designated a (inducer half), and b (responder half), respectively.

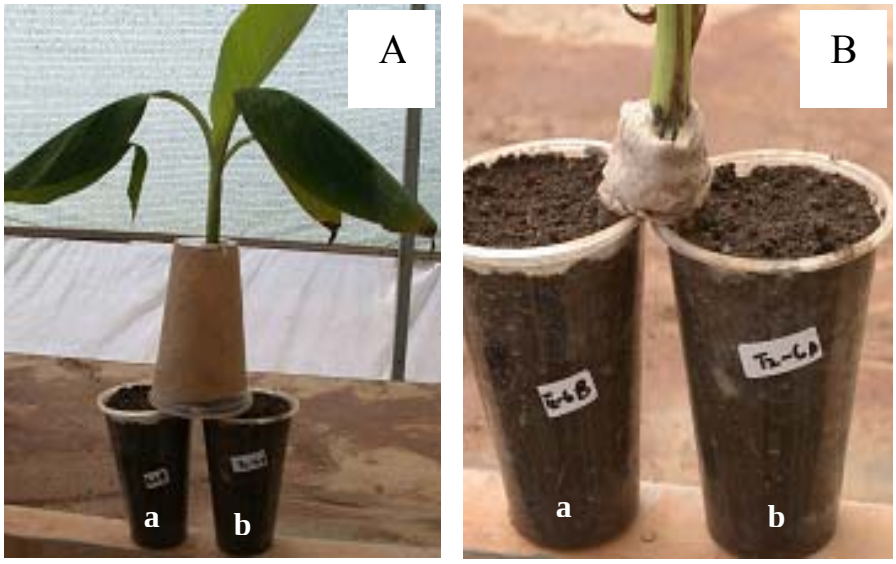


Figure 2. Protease activity of endophytic *Fusarium oxysporum* (isolate Eny1.31i) in a 65-mm-diameter Petri dish 3 days after inoculation on gelatine-amended medium. The clear zone (halo) indicates positive protease activity.

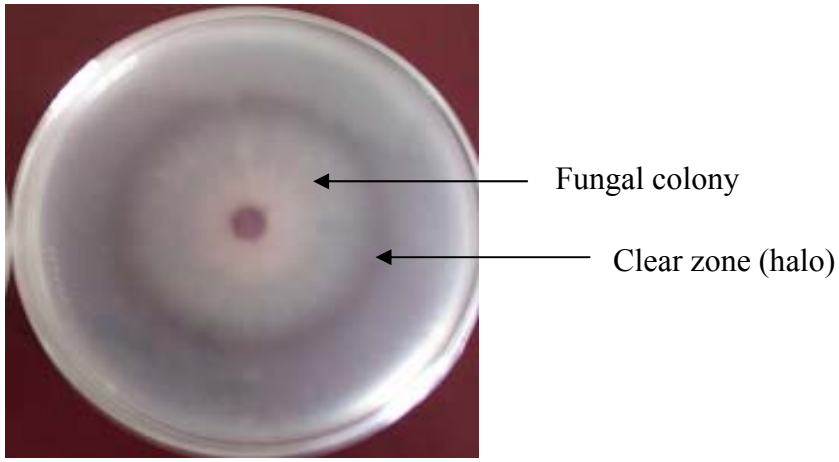
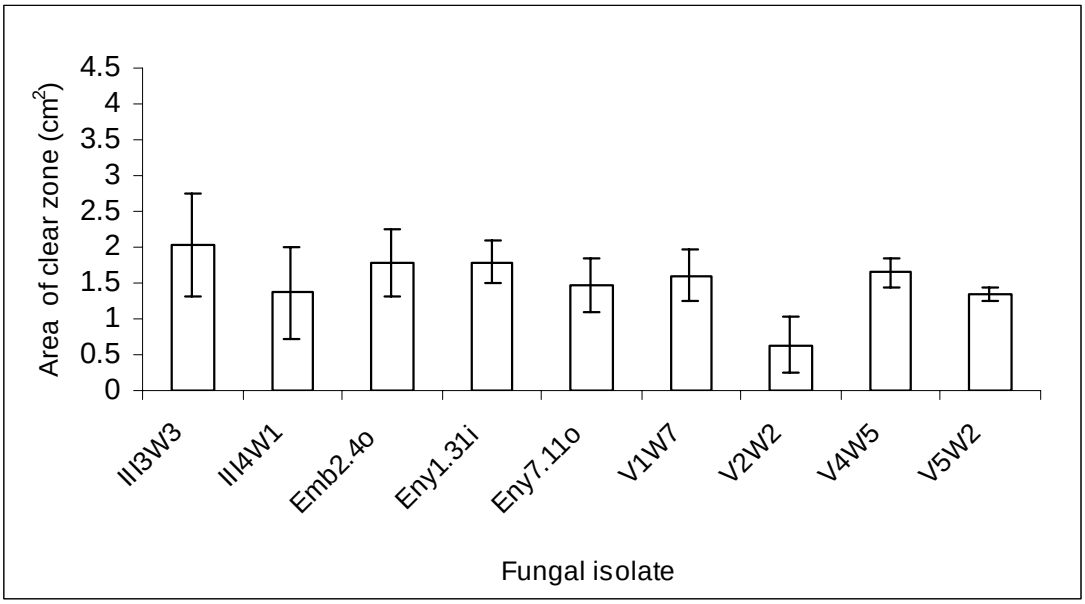
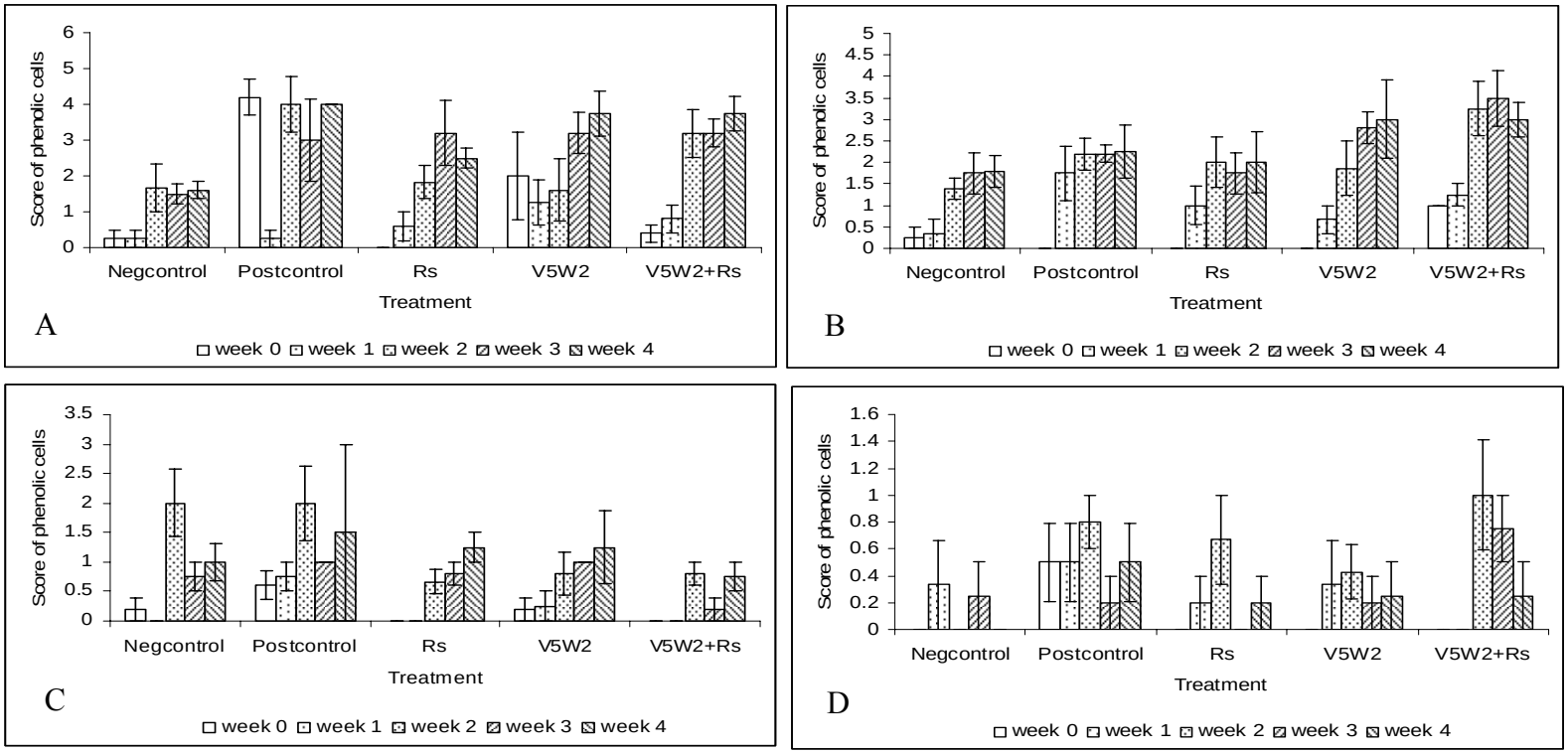


Figure 3. Level of protease activity exhibited by nine endophytic *Fusarium oxysporum* isolates 3 days after inoculation on gelatine-amended medium. The levels of protease activity were estimated using the diameter of the halo zone (cm) compared to the fungal colony diameter.



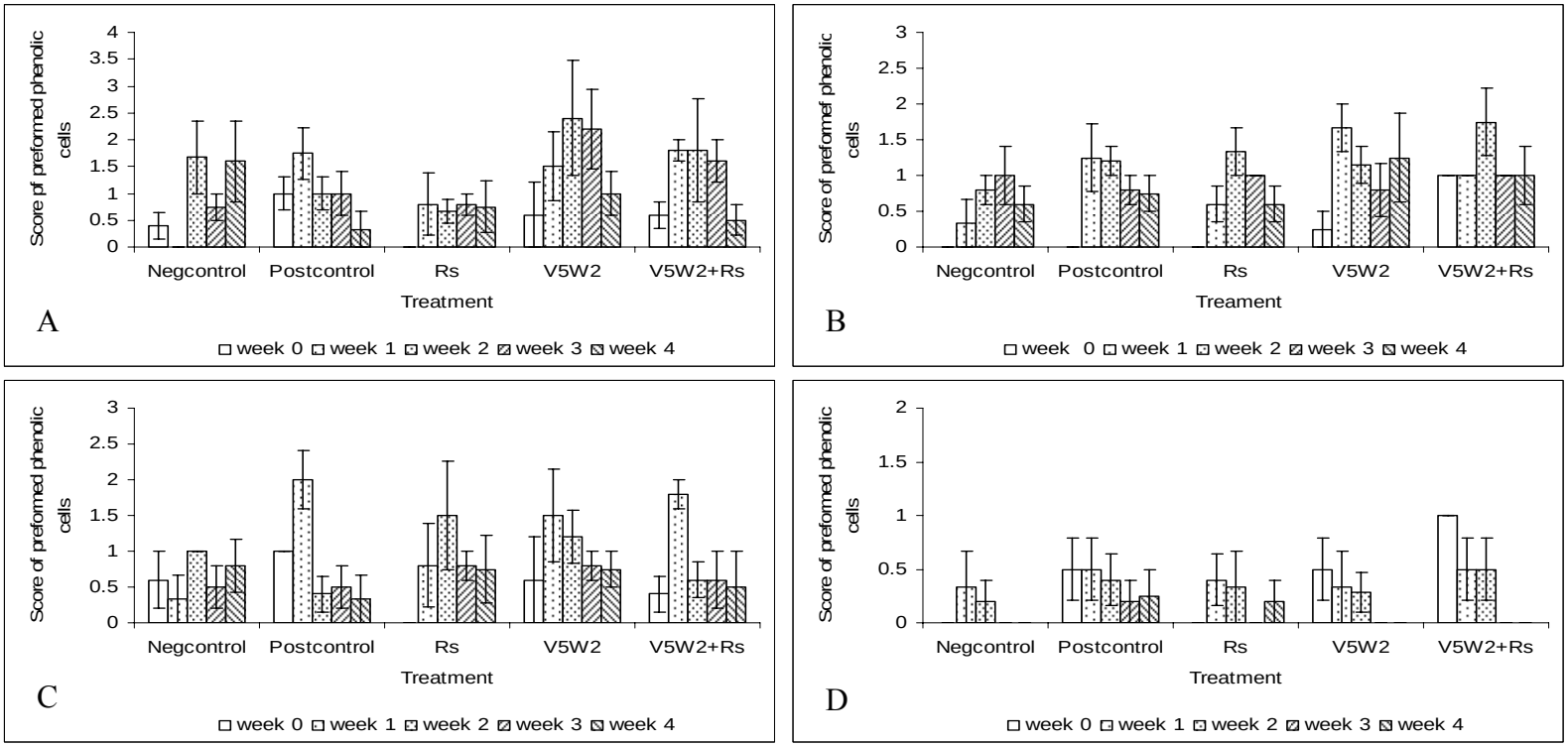
Error bars represent standard errors of the mean (n=5).

Figure 4. Fully formed phenolic cells in the central cylinder of transverse sections of rhizomes (A) and root (B), and in the cortex region of rhizomes (C) and roots (D), 0 to 4 weeks after treatment of plants with or without endophytes and/or nematodes. Formation of phenolic cells were scored on a scale of 0-5, where 0 = zero, 1 = 1 to 4, 2 = 5 to 10, 3 = 11 to 15, 4 = 16 to 20 and 5 = more than 20 phenolic cells.



Negcontrol=negative control (sterile millet seed), Postcontrol=Positive control (50 mM K_2HPO_4); V5W2= endophyte isolate V5W2; Rs= *Radopholus similis*. Error bars represent standard errors of the mean, n=15.

Figure 5. Preformed phenolic cells in the central cylinder of transverse sections of rhizomes (A) and root (B); and the cortex region of rhizomes (C) and roots (D), 0 to 4 weeks after treatment of plants with or without endophytes and/or nematodes. Formation of phenolic cells were scored on a scale of 0-5, where 0 = zero, 1 = 1 to 4, 2 = 5 to 10, 3 = 11 to 15, 4 = 16 to 20 and 5 = more than 20 phenolic cells.



Negcontrol=negative control (sterile millet seed); Postcontrol=Positive control (50 mM K2HPO4); V5W2= endophyte isolate V5W2; Rs= *Radopholus similis*. Error bars represent standard errors of the mean, n=15.

Figure 6. Histological analysis of phenolic cells showing brown stained phenolic cells in transverse sections of rhizomes obtained from tissue culture banana plants treated with (A) sterile millet seed (negative control), (B) 50 mM K_2HPO_4 (positive control), (C) *Radopholus similis*, (D) *V5W2*, and (E) *V5W2* and *Radopholus similis* 2 weeks after inoculation with *V5W2* and 1 week after nematode inoculation. Preformed phenolic cells in the cortex region of rhizome sections treated with both *V5W2* and *Radopholus similis* (F).

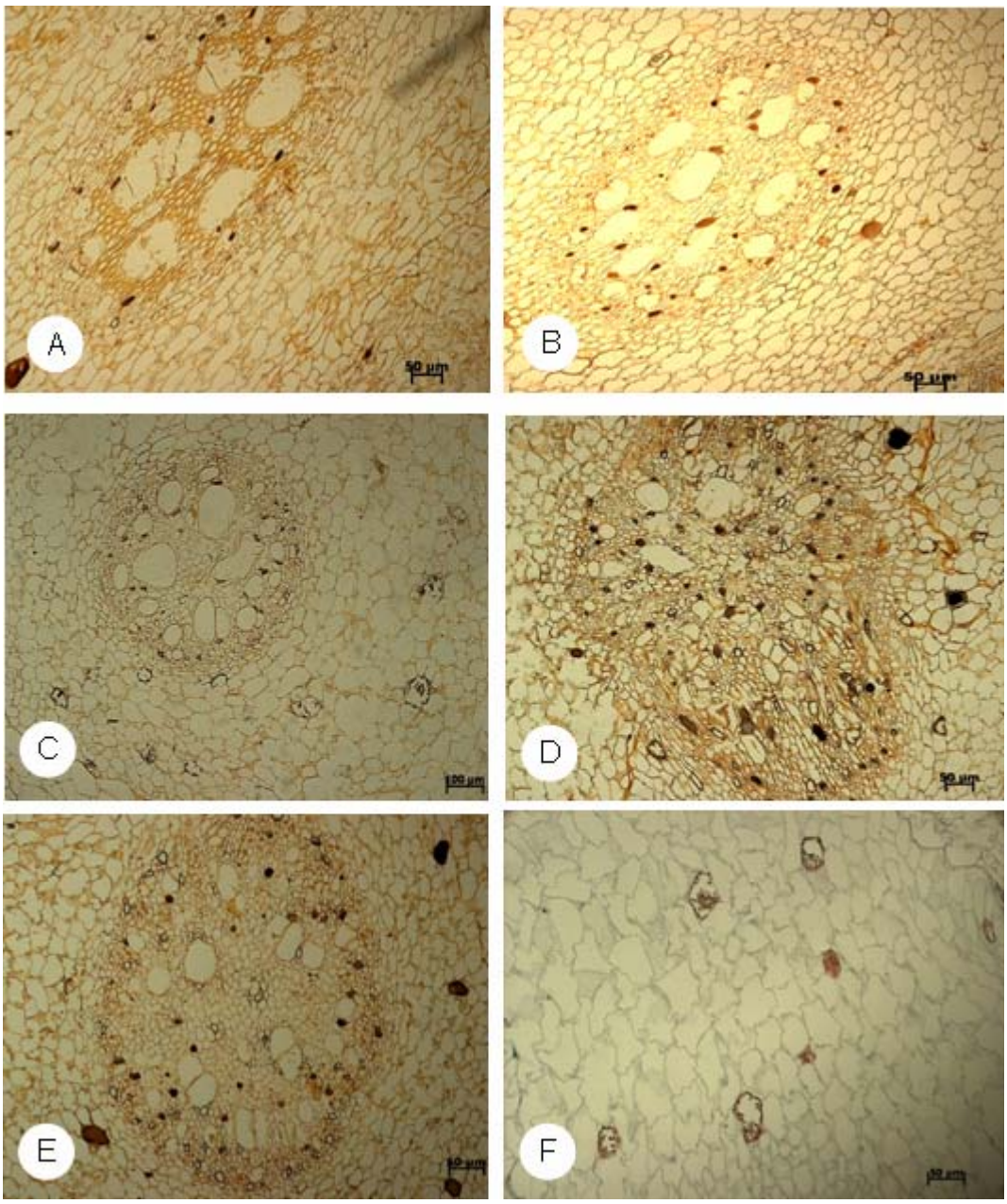
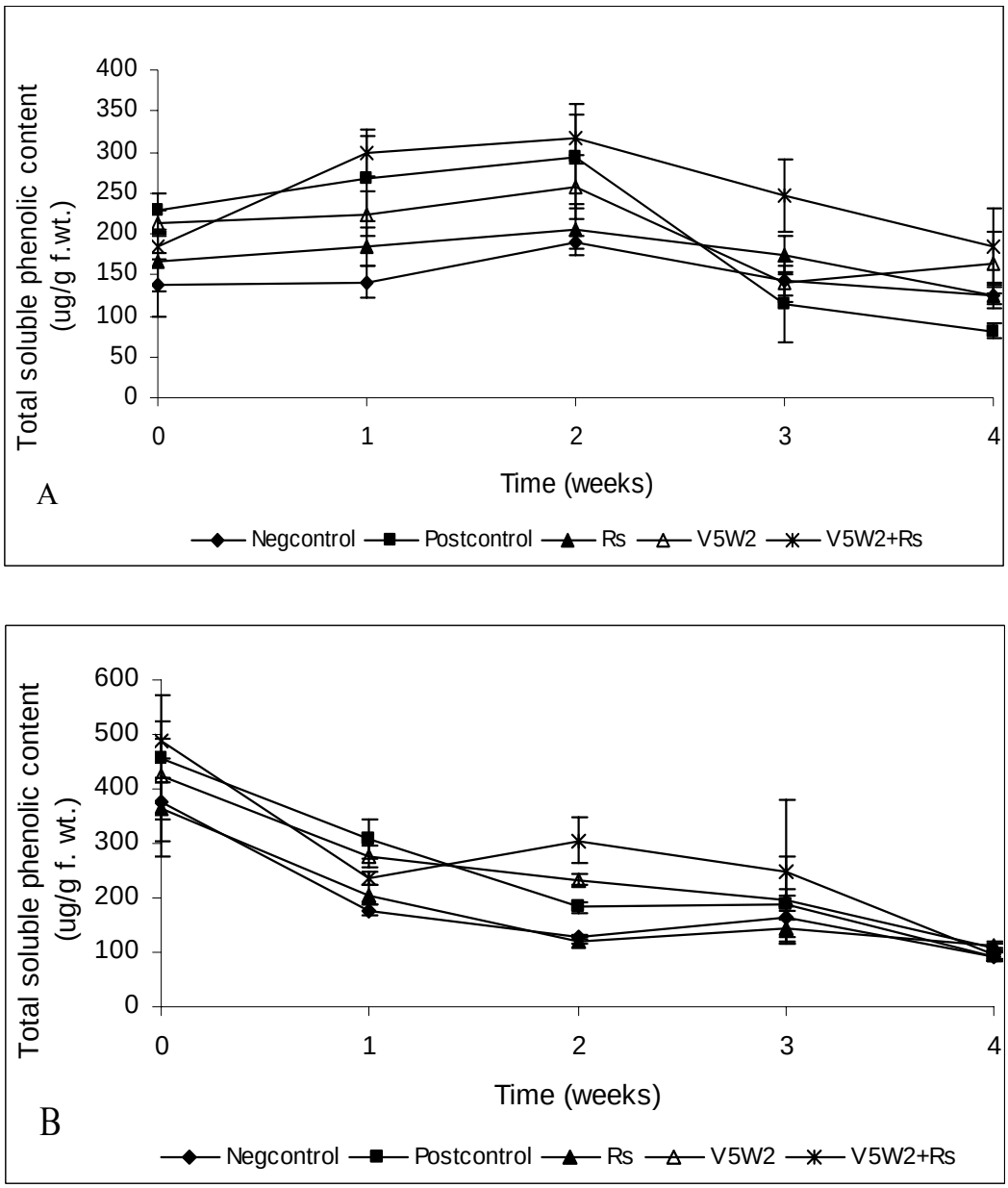


Figure 7. Total soluble phenolics (µg equivalent gallic acid/g fresh weight) in rhizomes (A) and roots (B) of banana plants treated with sterile millet seed (negative control), 50 mM K_2HPO_4 (positive control), *Radopholus similis* only, *Fusarium oxysporum* isolate *V5W2* only, and *V5W2* and *Radopholus similis*.



Negcontrol=negative control (sterile millet seed); Postcontrol=Positive control (50 mM K_2HPO_4); Rs= *Radopholus similis*. Error bars represent standard errors of the mean (n=5).

Figure 8. HPLC chromatograms at 280 nm absorbance of soluble phenolic compounds in rhizome extracts from tissue culture banana plants treated with (A) sterile millet seed, (B) 50 mM K_2HPO_4, (C) *Radopholus similis*, (D) endophytic *Fusarium oxysporum* isolate V5W2 and (E) both *V5W2* and *R. similis*, 2 weeks after endophyte inoculation. Peaks labelled 1-4 are the major compounds that were used for quantitative comparison between treatments.

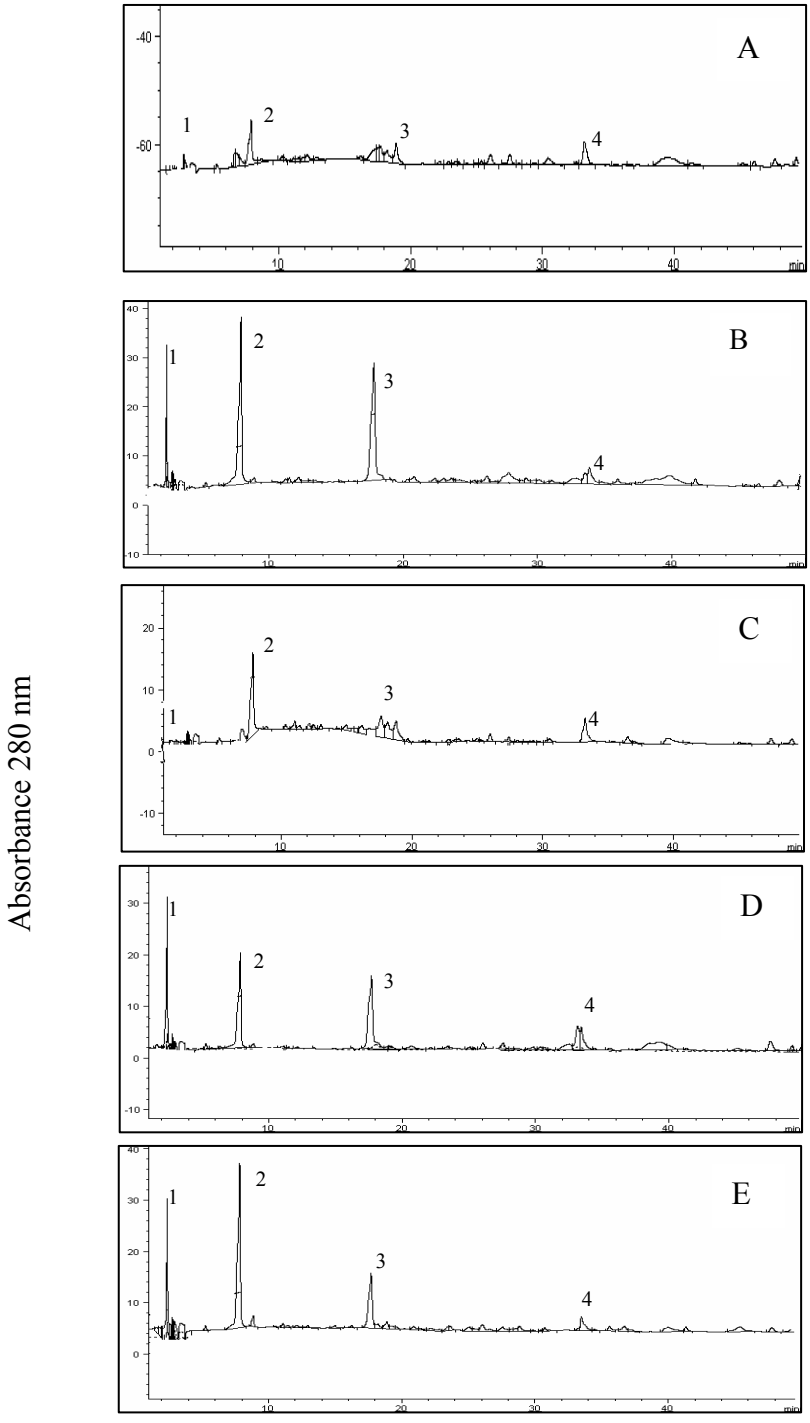
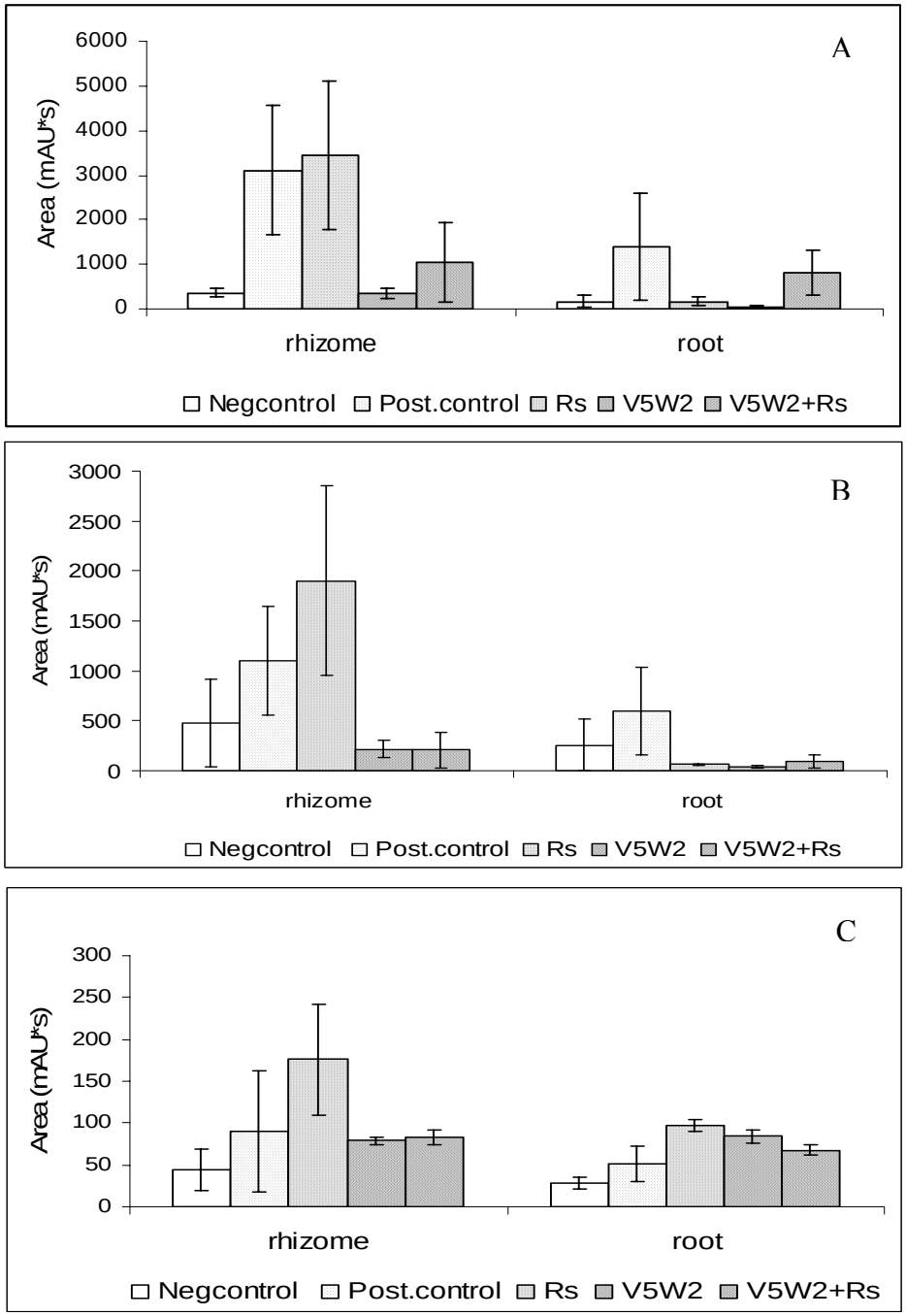


Figure 9. Three unknown compounds in rhizome and root extracts of tissue culture banana plants 2 weeks after inoculation with the endophytic *Fusarium oxysporum* isolate *V5W2*, and 1 week after inoculation with *Radopholus similis*. (A, unknown compound 2; B, unknown compound 3 and C; unknown compound 4)



Negcontrol=negative control (sterile millet seed); Postcontrol=Positive control (50 mM K_2HPO_4); V5W2= endophyte isolate V5W2; Rs= *Radopholus similis*.

Error bars represent standard errors of the mean, n=3.

Table 1. Number of *Radopholus similis* females, males, juveniles and total nematode density in 100 g roots, and percentage root necrosis in the responder roots, 1 month after inoculation of the inducer roots with endophytic *Fusarium oxysporum* isolates.

	Females	Males	Juveniles	Total*	Necrosis (%)
Experiment 1					
Control	582.8 ± 220.8 a	56.9 ± 18.6 a	2322.7 ± 425.9 a	2962.4 ± 534.0 a	26.6 ± 5.4 a
Env1.31i	189.7 ± 66.3 (67.4) a	56.3 ± 24.8 (1.1) a	2276.7 ± 652.0 (1.9) a	2522.7 ± 711.4 (14.8) a	28.7 ± 7.1 a
Env7.11o	364.1 ± 100.3 (35.5) a	76.6 ± 16.9 (34.6) a	1503.1 ± 246.0 (35.2) a	1943.9 ± 297.8 (34.3) a	20.6 ± 3.9 a
V5W2	412.9 ± 168.4 (29.5) a	65.6 ± 29.3 (15.2) a	1613.3 ± 512.0 (30.5) a	2091.8 ± 690.2 (29.4) a	20.7 ± 6.6 a
P Value	0.3475	0.6843	0.6735	0.6693	0.6934
Experiment 2					
Control	693.5 ± 62.7 a	3.5 ± 3.5 a	478.0 ± 48.7 a	1175.1 ± 98.7 a	23.8 ± 3.3 a
Env1.31i	104.5 ± 26.4 (84.9) c	9.1 ± 4.0 (160.5) a	277.3 ± 72.0 (41.9) ab	390.9 ± 88.8 (66.7) c	28.3 ± 2.9 a
Env7.11o	247.1 ± 47.6 (64.3) b	6.0 ± 2.7 (69.6) a	613.2 ± 160.2 (28.2) a	866.3 ± 193.6 (26.2) ab	28.5 ± 3.4 a
V5W2	51.0 ± 16.8 (92.6) c	2.7 ± 1.4 (22.7) a	131.7 ± 36.5 (72.5) b	185.3 ± 47.0 (84.2) c	25.6 ± 2.7 a
P Value	<0.0001	0.6843	0.0041	<0.0001	0.6609
Experiment 3					
Control	212.6 ± 53.9 a	116.2 ± 57.0 a	1743.4 ± 548.8 a	2072.2 ± 691.1 a	12.4 ± 1.9 ab
Env1.31i	137.2 ± 21.5 (35.4) a	72.6 ± 16.7 (37.5) a	746.7 ± 81.8 (57.2) a	956.5 ± 106.0 (53.8) a	12.6 ± 3.2 b
Env7.11o	186.8 ± 40.6 (12.1) a	87.1 ± 9.7 (25.1) a	1639.4 ± 434.1 (5.96) a	1913.3 ± 477.6 (7.7) a	21.8 ± 2.8 a
V5W2	170.7 ± 63.0 (19.6) a	63.0 ± 29.5 (45.7) a	1085.5 ± 248.1 (37.7) a	1319.2 ± 332.0 (36.4) a	10.1 ± 2.1 b
P Value	0.7473	0.4284	0.1620	0.2048	0.0096

*Total nematode density (females+males+juveniles). In each column within an experiment, means followed by the same letter are not different according to Tukey's studentized range test. Figures in parenthesis are percentage reduction of nematode numbers compared to the control treatment.

Table 2. Known phenolic compounds in rhizome and root extracts of tissue culture banana plants identified by HPLC 2 weeks after inoculation with endophytic *Fusarium oxysporum* isolate *V5W2*.

		Amount (µg/ml)	
Compound	Treatment	Corm extracts	Root extracts
3,4-dihydroxybenzoic acid	Negative control	2.1 ± 0.0	49.6 ± 15.5
(11.131)	Positive control	29.4 ± 0.0	19.5 ± 6.5
	R. similis	106.0 ± 86.0	48.7 ± 27.1
	V5W2	16.0 ± 5.9	22.9 ± 2.9
	V5W2+R. similis	25.0 ± 8.9	21.7 ± 14.5
Ferulic acid	Negative control	20.0 ± 0.0	nd
(27.521)	Positive control	nd	nd
	R. similis	nd	nd
	V5W2	20.0 ± 0.0	15.3 ± 0.0
	V5W2+R. similis	4.7 ± 0.0	nd
Hydroxybenzoic acid	Negative control	nd	nd
(16.219)	Positive control	20.0 ± 0.0	nd
	R. similis	nd	20.0 ± 0.0
	V5W2	20.0 ± 0.0	nd
	V5W2+R. similis	nd	nd
Syringic acid	Negative control	nd	127.6 ± 124.0
(18.915)	Positive control	2.2 ± 0.0	67.6 ± 0.0
	R. similis	nd	114.2 ± 60.8
	V5W2	20.0 ± 0.0	nd
	V5W2+R. similis	91.3 ± 41.9	20.0 ± 0.0
Vanillic acid	Negative control	20.0 ± 0.0	23.8 ± 3.8
(18.259)	Positive control	nd	39.8 ± 19.8
	R. similis	20.0 ± 0.0	44.2 ± 16.7
	V5W2	20.0 ± 0.0	22.1 ± 2.0
	V5W2+R. similis	20.0 ± 0.0	12.8 ± 7.1
Gallic acid	Negative control	nd	nd
(6.359)	Positive control	5.86 ± 0.0	nd
	R. similis	nd	nd
	V5W2	nd	nd
	V5W2+R. similis	nd	nd
p-Coumaric acid	Negative control	nd	nd
(25.305)	Positive control	20.0 ± 0.0	20.0 ± 0.0
	R. similis	nd	nd
	V5W2	nd	nd
	V5W2+R. similis	20.0 ± 0.0	nd

Negative control =sterile millet seed; Positive control=(50 mM K_2HPO_4; *V5W2= F. oxysporum* endophyte isolate; Rs= *Radopholus similis*. Values represent the mean and standard error of the mean (n=3). Figures in brackets below each compound represent the retention time. nd= not detected.

Chapter 6

Genetic diversity of endophytic *Fusarium* spp. associated with Cavendish banana in South Africa

Abstract

Endophytic *Fusarium* spp. are commonly associated with banana plants. Nothing is known regarding tissue specificity and the genetic diversity within endophytic *Fusarium* spp. from banana plants. Endophytic *Fusarium* spp. from roots, rhizomes and pseudostem bases of Cavendish banana plants from three sites in South Africa were assessed using morphological and molecular analyses. *Fusarium oxysporum* was the predominant species isolated, followed by *Fusarium solani* and *Fusarium semitectum*. Tissue specificity was observed with *F. oxysporum* and *F. solani* being predominantly isolated from the roots whilst *F. semitectum, F. sacchari* and *Fusarium subglutinans* were predominantly isolated from rhizomes and pseudostem bases. PCR-RFLP analysis of the IGS region of the rDNA divided 46 isolates of *F. oxysporum* obtained from roots into nine different genotype groups. Distance analysis of AFLP data of 57 *Fusarium* isolates resolved the isolates into two major clades: one consisting of the isolated *F. oxysporum* and the other of *F. sacchari*. The *F. solani* isolates formed three clades clearly separated from the other two species. *Fusarium oxysporum* isolates further grouped according to plant part origin while the *F. sacchari* and *F. solani* isolates grouped randomly. The results obtained demonstrate tissue specificity of endophytic *Fusarium* in Cavendish banana and also a wide inter- and intraspecific genetic variation among endophytic *Fusarium* isolates of banana in South Africa.

Introduction

Endophytes are microorganisms that spend at least part of their life cycle inside plant tissues without causing any apparent disease symptoms (Carroll, 1988). The presence of endophytic fungi has been demonstrated in many plants including important agricultural crops like banana (Brown *et al*., 1998; Pereira *et al*., 1999; Pocasangre *et al*., 1999; Dubois *et al*., 2004), maize (Fisher *et al*., 1992), rice (Fisher and Petrini, 1992) and tomato (Hallman and Sikora, 1994). Fungal endophytes are known to produce bioactive products that may play important ecological and biological roles in the host plant (Tan and Zhou, 2001; Strobel, 2003; Schulz and Boyle, 2005). In mutualistic associations, endophyte-infected plants are protected from attack by some insects, nematodes and fungi, while in return the endophyte is provided with shelter and nutrition by the host plant (Latch, 1993; Saikkonen *et al*., 1998; Azevedo *et al*., 2000; Schardl *et al*., 2004).

Fusarium spp. are cosmopolitan fungi that contain many pathogenic forms causing diseases to a wide variety of economically important crops, such as banana (Stover, 1981). The genus also comprises nonpathogenic strains, some of which occur as endophytes colonizing different plant tissues (Niere, 2001; Tan and Zhou, 2001; Sikora *et al*., 2003). Several researchers have reported association between endophytic *Fusarium* and banana plants (Pereira *et al*., 1999; Pocasangre *et al*., 1999; Dubois *et al*., 2004). *Fusarium oxysporum* has been identified as the predominant species establishing endophytic relationships with banana plants. The ability of endophytic *F. oxysporum* isolates to protect banana plants against pests and diseases has been demonstrated in laboratory and screen house experiments (Pocasangre *et al*., 1999; Dubois *et al*., 2004; Gold and Dubois 2005, Nel *et al*., 2006b). Thus, mutualistic associations between endophytic *Fusarium* spp. and banana plants may be viewed as a promising form of biological protection.

The international banana trade is dominated by the Cavendish–type bananas (INIBAP, 2003). Apart from the export trade, Cavendish bananas are grown in many other countries for local consumption only. Opportunities for improving production of Cavendish bananas are often constrained by pests and diseases. The main pests are the banana weevil *Cosmopolites sordidus* and the banana nematode *Radopholus similis* (Daneel *et al*., 2004; de Graaf *et al*., 2004). The main diseases of Cavendish bananas are Fusarium wilt (Panama disease) caused by *Fusarium oxysporum* f. sp. *cubense* (Moore *et al*., 1995) and black

Sigatoka, caused by *Mycosphaerella fijiensis* (Mourichon *et al*., 1997; Surridge *et al*., 2003). These pests and diseases are difficult and costly to control, and can amount to yield losses of up to 100% of severely infested fields (Moore *et al*., 1995; Mourichon *et al*., 1997).

Genetic diversity exists between and within pathogenic and nonpathogenic populations of *F. oxysporum*, and various molecular methods can be used to detect such variation (Gordon and Okamoto 1991; Edel *et al*., 1995). The amplification of variable ribosomal DNA (rDNA) regions allows for discrimination at the genus, species and intraspecific level (Edel *et al*., 1995). Of particular importance is the intergenic spacer (IGS) region that shows considerable divergence within closely related species (Appel and Gordon 1994; 1995). The use of PCR amplified rDNA regions as substrates for restriction fragment length polymorphisms (RFLPs) is one of the widely used methods use to evaluate genetic diversity in *F. oxysporum* populations. By using PCR-RFLP analysis of the IGS region, Woo *et al*. (1996) identified genetic differences between worldwide collections of pathogenic and nonpathogenic isolates of *F. oxysporum* of the bean wilt pathogen *F. oxysporum* f. sp. *phaseoli*. Appel and Gordon (1995) were able to resolve intraspecific variation among 56 soil isolates of *F. oxysporum*, and Nel *et al*. (2006a) reported variation among isolates of *F. oxysporum* from the banana rhizosphere. Several researchers have also reported genetic diversity of endophytic *F. oxysporum* from various plants based on analysis of the IGS region. In addition, diversity among endophytic isolates of *F. oxysporum* from symptomless tomato roots (Elias *et al*., 1991) and from roots and hypocotyls of beans (Alves-Santos *et al*., 1999) was demonstrated.

Amplified fragment length polymorphism (AFLP) is based on the selective amplification of a high number of restriction fragments and is highly reproducible (Vos *et al*., 1995). It is also useful in delineating genetic differences between and within species (Vos *et al*., 1995). For example, AFLPs were shown to relate five distinct clusters with five different *Fusarium* taxa in a phylogenetic study (Abdel-Satar *et al*., 2003). Groenewald *et al*. (2006) further demonstrated that AFLPs could be used to subdivide *F. oxysporum* f.sp. *cubense* in several different clades which correspond to vegetative compatibility. Inter- and intraspecific variation obtained with AFLPs can also be used to construct genetic markers for different microorganisms (Kema *et al*., 2002).

Despite the known association of banana plants with endophytic *Fusarium* spp. genetic diversity of these endophytes, and their relation to different plant parts has been poorly

studied. The objectives of this study were, therefore, to isolate and identify endophytic *Fusarium* spp. from field-grown Cavendish bananas, to examine the tissue specificity of such *Fusarium* spp., and to determine the genetic diversity within these *Fusarium* endophytes of banana.

Materials and methods

Collection of plant material

Fresh root, rhizome and pseudostem base samples were collected from apparently healthy Cavendish banana plants growing in banana weevil- and nematode-infested plantations in Ramsgate, KwaZulu-Natal (KZN) province and Tzaneen, Limpopo province of South Africa in May and June, 2003 respectively. Neither of these plantations were affected by Fusarium wilt of banana. Five and 10 plants were sampled from Ramsgate and Tzaneen, respectively. From each plant, five 10-cm-long primary roots and five 100-cm^3 pieces of the rhizomes and pseudostem bases were randomly taken. Samples were packed in polythene bags, placed in cooler boxes and transported to the Forestry and Agricultural Biotechnology Institute (FABI), located at the University of Pretoria in South Africa, for isolation of endophytic *Fusarium* spp.

Isolation of endophytic Fusarium spp.

Roots were washed under running tap water to remove adhering soil, and surface sterilized in 75 % ethanol for 1 min followed by 2 % sodium hypochlorite solution for 30 s (Petrini, 1986), followed by a double rinse in sterile distilled water (SDW). After rinsing, the samples were dried by blotting them on sterile tissue paper. From each surface sterilized root, five 1-cm long pieces were randomly cut and placed on *Fusarium*-specific rose bengal agar (12 g agar, 10 ml glycerine, 0.5 g L-alanine, 1.0 g pentachloronitrobenzene, 0.5 g Rose Bengal and 1.0 g urea / L SDW) in 65-mm-diameter Petri dishes. The medium was amended with 0.25 g l^{-1} chloramphenicol to prevent bacterial growth. The pseudostem bases, inner and outer rhizome samples from each plant, were surface sterilized by dipping in 95% ethanol, followed by flaming. From each surface sterilized pseudostem base, inner and outer rhizome sample, five 1-cm^3 pieces were randomly removed using a flamed scalpel blade, and also

placed on rose bengal medium amended with antibiotics. All Petri dishes were incubated at 25 °C and checked for fungal growth for 7 days. All colonies of putative *Fusarium* spp. were sub-cultured on half strength potato dextrose agar (19 g PDA, 8 agar / L SDW) (PDA, Difco, Detroit, USA) and incubated for 7 days at 25 °C.

Morphological identification of Fusarium spp.

Single-spore cultures were prepared for all *Fusarium* colonies according to Nelson *et al*. (1983). From the single-spore cultures, mycelial disks were transferred to carnation leaf agar (CLA) and half strength PDA in 65-mm and 90-mm diameter Petri dishes, respectively. CLA was prepared by placing a dry sterile carnation leaf on 2 % water agar (Nelson and Toussoun 1986). The cultures on CLA medium were incubated for 7 to 28 days at 25 °C before being examined for conidium and conidiophore morphology, and chlamydospore production. Cultures on half strength PDA were examined for growth rates and morphological characteristics such as colony appearance (presence or absence of aerial mycelia, surface texture, and pigmentation) after incubation at 25 °C for 10 days. Identification of *Fusarium* isolates to species level was done according to Nelson *et al*. (1983). Only one isolate for each *Fusarium* species recovered per root, rhizomes or pseudostem base tissue per plant was preserved for further analysis to limit duplication of isolates. Isolates were preserved on sterile filter papers and also in 15 % glycerol and stored at 4 °C and –80 °C respectively. All the isolates used in this study are maintained in the FABI culture collection.

Statistical analysis of data

Isolation frequencies of different *Fusarium* spp. among plant parts and between geographic origins were analyzed using categorical logistic regression. Only species for which more than one isolate was obtained were included in the analysis. Likelihood ratio tests were performed to investigate differences within factors (site, *Fusarium* sp. and plant part). If different, means were separated using 95 % confidence intervals and significant α levels of 0.0051 after the Dunn-Sidak correction (Sokal and Rolf 1995; Ury, 1976) using the SAS system (SAS, 1989).

Molecular characterization of Fusarium spp.

DNA extraction from fungal mycelia

To obtain DNA, single spore isolates of *Fusarium* were grown at 25 °C on PDA medium in 65-mm-diameter Petri dishes for 7 days. Fungal mycelium was scrapped from the surface of the medium using a flame-sterilized scapel and transferred into 1.5-ml Eppendorf tubes. DNA was extracted according to Raeder and Broda (1985). To each tube, 300 µl of DNA extraction buffer (200 mM Tris/HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA (pH 8.0), 0.5% SDS in SDW) was added. The mycelium was homogenised in the extraction buffer with a pestle and frozen in liquid nitrogen. The cell lysate was extracted with 700 µl of phenol-chloroform (1:1 v/v) and centrifuged at 14000 rpm for 7 min at 4 °C. The supernatant was then transferred to new tubes and the extraction procedure repeated three more times. To the final supernatant (~250 µl), 25 µl of 3 M sodium acetate (pH 5.5) and 500 µl of ice cold 100 % ethanol were added, and the mixture centrifuged at 14000 rpm for 10 min at 4°C to yield the DNA pellet. The DNA pellet was washed with 70 % ethanol and dried *in vacuo* for 5 min at 45°C, redissolved in 60 µl TE buffer (10 mM Tris/HCl (pH 8.0) and 1 mM EDTA (pH 8.0) in SDW) and treated with RNAse (Sigma-Aldrich, MO, USA) to degrade RNAThe DNA concentrations were determined using a spectrophotometer and diluted to a final working concentration of 20 ng μl^{-1}.

Sequence analysis

Part of the transfer elongation factor 1-α (TEF) region of selected isolates was amplified using the Ef1 and Ef2 primers (O'Donell *et al*., 1998). The PCR reaction conditions were as follows; initial denaturation of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 45 s at 60 ºC, 90 s at 72 ºC and a final extension of 5 min at 72 ºC. PCR reactions were carried out in a mastercycler (Epperndorf, Hamburg, Germany). The resulting amplicons were electrophoresed in 1% TBE agarose gels and visualized using ethidium bromide staining and UV light. The amplified DNA fragments were purified using a High Pure PCR Product Purification kit (Roche Diagnostics, Johannesburg, South Africa) according to the manufacturers instructions. Sequencing was performed in 10 µl reactions each containing 4 µl of purified PCR products, 1 µl of Ef1 or Ef2, 2 µl of Big dye III (Applied Biosystems, Foster City, CA, USA), 1 µl 5x dilution buffer and 2-µl dH_2O. PCR conditions were 25 cycles of 10 s at 96 ºC, 5 s at 50 ºC and 4 min at 60 ºC. Sequencing products were

precipitated and electophoresed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The obtained sequences were edited and aligned using the Sequence Navigator software program (Applied Biosystems). Final alignments were done manually and the sequences compared with GenBank sequence databases using nucleotide Blast on the National Center for Biotechnology Information (NCBI) website.

PCR-RFLP analysis of the IGS region of F. oxysporum *isolates*

All *F. oxysporum* isolates obtained from banana roots were subjected to PCR-RFLP analyses in order to group them into IGS genotypes for subsequent AFLP analyses of a smaller, representative number of isolates. Two oligonucleotide primers, forward primer PNFo (5' CCCGCCTGGCTGCGTCCGACTC 3') and reverse primer PN22 (5' CAAGCATATGACTACTGGC 3') were used to amplify a fragment of the IGS region of the rDNA (Edel *et al*., 1995). The primers were synthesized by Inqaba Biotechnical Industries (Hatfield, Pretoria, South Africa). PCR amplifications were performed in a total volume of 50 µl by mixing 50 ng of template DNA with 0.1 µM of each primer, 2.5 mM of each of dATP, dCTP, dGTP and dTTP, 1 unit *Taq* DNA polymerase and 1x PCR reaction buffer (10 mM Tris/HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml of bovine serum albumin in dH_2O) (Roche Diagnostics). Amplifications were performed in a mastercycler (Epperndorf) with 30 cycles of 90 s at 95 °C, 60 s at 50 °C and 90 s at 72 °C. Five microliters of PCR products was used to verify successful amplification of DNA by electrophoresis in 2% (w/v) agarose gels in 1x TAE buffer for 1 hr at 80 V. Aliquots of 10 µl of PCR products were digested for 4 hrs at 37 °C with 5 units of each of four restriction endonucleases: *Hae*III, *Hinf*I, *Msp*I and *Rsa*I (Roche Diagnostics). Restriction fragments were separated by electrophoresis in 4% agarose gels in 1x TAE buffer for 1 hr at 60 V. Fragments were run against a 100 base pair (bp) molecular marker for size estimation. Each unique banding pattern obtained with the different enzymes was assigned a letter, and each isolate assigned a composite IGS genotype defined by a combination of the patterns (letters) obtained with the four enzymes (Appel and Gordon, 1995).

AFLP analysis of Fusarium *spp.*

A total of 57 *Fusarium* spp. isolates from three banana-growing regions (Ramsgate, Tzaneen and Kiepersol in the Mpumulanga province) in South Africa were studied. Of these, 31 and 16 isolates were obtained from banana plants in Tzaneen and Ramsgate in the current study, respectively. Ten isolates previously collected from symptomless banana plants in Fusarium wilt-suppressive soils in Kiepersol, were obtained from the culture collection at FABI and included in the AFLP analysis. Isolates from Kiepersol had been confirmed as nonpathogenic to banana. Of the 57 isolates, 34 were *F. oxysporum*, 8 were *Fusarium solani*, 14 were *Fusarium sacchari* and 1 *Fusarium. equiseti* (Table 1).

A high-throughput AFLP analysis procedure using infrared dye-labeled primers and an automated DNA analyzer was used (Myburg *et al*., 2001). The restriction, ligation and preamplification reactions were performed as described by Vos *et al*. (1995). Genomic DNA (20 ng) was digested with two units each of *Eco*RI and *Mse*I (Roche Diagnostics) and incubated for 3 hr at 37 °C. For adaptor ligation, 30 µl of digested DNA was added to 10 µl of the restriction-ligation buffer (50 ρmol/µl of *Mse*I adaptor, 5 ρmol/µl of *Eco*RI adaptor and one unit of DNA ligase) and incubated for 3 hr at 37 °C. Restriction-ligation mixtures were diluted 10-fold with low TE buffer (10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA (pH 8.0) in dH2O) to serve as a template for preamplification. Preamplification reactions were performed in a total volume of 30 µl containing: 5 µl of diluted restriction-ligation products, 0.6 units *Taq* polymerase, 3 µl PCR reaction buffer, 0.3 µM of *Eco*RI and *Mse*I primer without any additional selective nucleotides, and 2.5 mM of each of the four dNTPs. The PCR program consisted of 25 cycles of 30 s at 72 °C, 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C, with an additional 1 s per cycle increase in the extension time. Electrophoresis to verify successful preamplification was carried out in 1.2% agarose gels in 0.5x TBE for 1.5 hr at 60 V. Depending on the intensity of the smear, preamplification products were diluted either 10- or 20-fold with low TE buffer and served as the templates for the final amplification step.

Three primer combinations were used for final selective amplification. The *Eco*RI primers were 5′-end labeled with infrared dye IRDye™ 700 or 800 (LI-COR). The primer combinations used were *Eco*RI-AT / *Mse*I-CC, *Eco*RI-CC / *Mse*I-TA and *Eco*RI-TC / *Mse*I-AA. Final amplifications were performed in a total volume of 20 µl containing 5 µl diluted preamplification product, 2 µl PCR reaction buffer, 1.6 µl of 2.5 mM of each of the four

dNTPs, 0.04 µM IRDye 700-labeled *EcoR*1 primer or 0.04 µM IRDye 800-labelled *EcoR*I primer, 0.25 µM *Mse*I primer and 1.2 units *Taq* polymerase. A total of 37 amplification cycles were carried out. The first amplification cycle was carried out for 10 s at 90 °C, 30 s at 65 °C and 1 min at 72 °C. In each of the following 12 cycles, the annealing temperature was reduced by 0.7 °C per cycle. The last 23 cycles were carried out for 10 s at 94 °C, 30 s at 56 °C and 1 min, extended 1 s per cycle, at 72 °C. A final extension cycle was carried out for 1 min at 72 °C. All amplification steps were carried out in a mastercycler (Eppendorf).

Electrophoresis and detection of AFLP fragments

Electrophoresis and detection of AFLP fragments was performed on a two-dye automated DNA sequencer Model 4200s (LI-COR, Lincoln, NE, USA). AFLP fragments were resolved in 25 cm gels containing 8% polyacrylamide gel solution (Long Ranger Gel Solution, Cambrex Bioscience, Rockland, USA), 7.0 M urea and 1x TBE buffer. Ten microliters of formamide loading buffer (95% deionised formamide, 20 mM EDTA, 1 mg/ml Bromophenol blue and 0.5 ml dH_20) was added to 20 µl final AFLP products in 0.2 ml PCR tubes and the tubes wrapped in aluminum foil. All samples were denatured for 3 min at 90 °C and transferred to ice before loading. Disposable 64-well paper combs (KB^{Plus}, LI-COR) (0.25 mm spacer thickness) were used for gel loading. 0.8 µl of each of the diluted preamplification products was loaded into a single lane. The first and last lanes of the gels were loaded with a denatured 50-700 bp molecular weight marker labeled with the corresponding infrared dye (LI-COR). Gel images were transferred automatically from the DNA analyzer to the Saga Application server (LI-COR) where analyses and band scoring were performed.

Scoring of AFLP images

Digital AFLP gel images were scored to obtain binary (band presence or absence) data using $Saga^{MX}$ automated AFLP analysis software (Keygene, Wageningen, the Netherlands). Lane definition and band sizing were performed as described in the $Saga^{MX}$ user manual. Semi-automated scoring was performed by manually clicking on polymorphic fragments present or absent in any of the 60 lanes. After scoring, data were exported directly to a spreadsheet program and reformatted for use in mapping software. Binary data (0 = absence, 1 = presence) obtained from band scores of the gel images obtained with the three different primer pairs was combined. The combined AFLP data was subjected to distance analysis

using the Phylogenetic Analysis Using Parsimony (PAUP) Version 4.0 b10 (Swofford 2002) software (Applied Biosystems, Foster City, CA, USA) which included neighbour joining as a tree building algorithm and a bootstrap of a 1000 replicates (Felsenetein, 1985) for confidence support. The *F. equiseti* isolate was used as the outgroup.

Results

Isolation frequencies of endophytic Fusarium spp. from Cavendish banana plants

The frequency of isolation and diversity of endophytic *Fusarium* spp. was assessed in roots, rhizomes and pseudostem bases of healthy Cavendish banana plants. We isolated a total of 140 isolates belonging to eight *Fusarium* species. Of these 140 isolates, 57.2% were from the roots, 22.7% from the rhizomes, and 19.9% were obtained from the pseudostem bases. *Fusarium oxysporum* was isolated at the highest frequency among the endophytic *Fusarium* isolates. Other *Fusarium* spp. that were isolated included, in decreasing frequencies, *F. solani, F. semitectum, F. sacchari*, *F. subglutinans, F. dimerum*, *F. equiseti* and *F. proliferatum* (Table 2). Mixed infections from an individual plant were common; as many as six different *Fusarium* spp. were found in a single plant. The highest number of *Fusarium* species recovered from a single plant was four and six in Ramsgate and Tzaneen, respectively. From the isolations, an individual *Fusarium* species could be recovered from all plants within one site; *F. oxysporum* and *F. solani* were found in all plants from Ramsgate and Tzaneen, respectively.

The frequency of isolation of different *Fusarium* spp. was influenced by the plant part ($P<0.0001$) and geographic site ($P<0.0001$) from which they were isolated. The frequency of isolation of different endophytic *Fusarium* species also differed within plant parts; roots ($P<0.0001$), rhizomes ($P=0.0115$) and pseudostem bases ($P=0.0301$). A strong interaction between species frequency and plant part ($P<0.0001$) was observed. *Fusarium oxysporum* and *F. solani* were predominantly isolated from the roots, *F. semitectum* and *F. sacchari*, from rhizomes and *F. subglutinans* from pseudostem bases. The composition of different *Fusarium* species differed significantly within geographic sites (Ramsgate, $P<0.0001$ and Tzaneen, $P<0.0001$) with a strong interaction between species composition and geographic site ($P=0.0127$). In Ramsgate, *F. oxysporum* was the most frequently isolated species while in Tzaneen, *F. oxysporum* and *F. solani* were recovered at the highest frequencies. *Fusarium*

sacchari, *F. semitectum* and *F. subglutinans* were also found in higher frequencies in banana plants from Tzaneen than from banana plants from Ramsgate.

Molecular characterization of endophytic Fusarium spp.

Sequence analysis of the TEF 1-α region of selected Fusarium isolates

PCR amplification of a part of the transfer elongation factor 1-α (TEF) of selected isolates using the Ef1 and Ef2 primers yielded a fragment of ~700 bp. The obtained sequences were used to confirm morphological species identification and have been submitted to GenBank, Accession numbers DQ465925 – DQ465954 (Table 1).

PCR-RFLP analysis of the IGS region of Fusarium oxysporum *isolates*

A fragment of approximately 1.7 kbp was amplified from each of the 46 isolates of *F. oxysporum* obtained from banana roots in Ramsgate. Depending on the restriction enzyme used, 1-5 distinct restriction fragment patterns were obtained. Five patterns were realized for *Hae*III, three for *Msp*I, two for *Rsa*I and one for *Hinf*I (Fig. 1). Among the 46 isolates of *F. oxysporum*, a total of 9 IGS genotype groups were identified (Table 3). Isolates from each site generally clustered within the same IGS genotype, although some isolates from the different sites shared the same IGS genotypes. The most common IGS genotype, AAAA, included 10 isolates from Ramsgate and 16 isolates from Tzaneen. Five genotypes out of the nine contained isolates only from Ramsgate. One genotype contained isolates only from Tzaneen and three genotypes contained isolates from both sites (Table 3). Depending on the number of isolates within a particular IGS genotype, a total of 19 representative isolates were selected for further analysis using AFLPs (Table 3). Seven were from genotype 1, three from genotype 5, two each from genotypes 3 and 9, and one each from genotypes 2, 4, 6, 7 and 8.

AFLP analysis of Fusarium spp.

AFLP analysis of *Fusarium* spp. from banana roots, corms and pseudostem bases, using three primer combinations, produced approximately 150 polymorphic bands useful for comparison of genetic diversity. Distance analyses of the AFLP data obtained with the three primer pairs clearly divided the isolates into two distinct clades, one comprising of *F.*

oxysporum isolates and the second comprising of *F. sacchari* isolates. The *F. solani* isolates did not group into one distinct clade, but rather formed two clades, which were clearly separated from the *F. oxysporum* and *F. sacchari* clades (Fig. 2).

The clade comprising of *F. oxysporum* isolates was further divided into 3 distinct sub clades. The clustering of *F. oxysporum* isolates was closely related to the plant part origin than to geographic origin. The first 2 sub-clades comprised of isolates obtained from the roots while the third sub-clade comprised of isolates from the rhizomes and pseudostem bases. Within the root isolates sub-clade, *F. oxysporum* isolates from Kiepersol (sub-clade 2) clearly formed a distinct group while the *F. oxysporum* isolates from Ramsgate and Tzaneen clustered randomly within sub-clades 1 and 3. The *F. oxysporum* isolates obtained from roots were found to be highly diverse as shown in the cladogram where several sub-clades were formed (Fig. 2). The *F. sacchari* clade was further divided into 2 sub-clades, all isolates in the two sub-clades were obtained from the rhizomes and pseudostem bases except for isolate SAT22, which was obtained from roots and which grouped separately from the other isolates. The *F. solani* isolates grouped into two distinct sub-clades with no correlation to plant part or geographic origin. One *F. solani* isolate (SAK39) was separated from the rest of the isolates clades.

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Discussion

Cavendish banana plants in South Africa host a wide array of fungal endophytes. The isolation of a total of 140 isolates belonging to eight different *Fusarium* spp. from roots, rhizomes and pseudostem bases from only 15 Cavendish banana plants in two sites demonstrates the richness of *Fusarium* endophytes and compares favorably to results from earlier investigations involving fungal endophytes of banana. In Thailand, a total of 285 fungal endophytes belonging to 15 different genera were isolated from 49 banana plants of the cv. 'Pisang-Awak' (*Musa* spp. ABB) in nine sites (Niere, 2001). Of these, 79 were isolates of *Fusarium* species. High levels of endophytic fungal diversity from recently harvested plants of East African highland banana, which showed little or no weevil damage in western Uganda, have also been reported (Griesbach, 2000), while a similar study conducted on bananas in central America showed extensive endophytic fungal diversity (Pocasangre *et al*., 1999). Though the current study focused only on endophytic *Fusarium* spp. infecting Cavendish banana plants, the results obtained here and in earlier studies indicate that banana plants are a rich source of fungal endophytes. The role of endophytes in the host plant however remains largely unknown but may have an ecological and biological significance. It has been speculated that since endophytic fungi establish intimate relationships with their host plants, they may therefore, be effective in the management of the major banana pests and pathogens at the site of attack. Introduction of fungal endophytes into tissue culture derived banana plantlets may also be done to enhance the plant's vigor and provide protection against pests and diseases prior to field planting (Pereira *et al*., 1999; Griesbach, 2000; Niere, 2001; Sikora *et al*., 2003).

Fusarium oxysporum, *F. solani*, *F. semitectum* and *F. sacchari* were the most frequently isolated of *Fusarium* spp. from Cavendish bananas in South Africa. All three species can be fungal pathogens of agricultural crops, but only *F. oxysporum* is known to attack bananas (Booth, 1971). Non-pathogenic isolates of *F. oxysporum* have been reported as endophytes of banana before, and have been assessed for their ability to reduce damage caused by pathogenic isolates of *F. oxysporum* and nematodes (Niere, 2001; Pocasangre *et al*., 1999; Nel *et al*., 2006b). *Fusarium solani* and *F. semitectum* are pathogens of crops such as cotton and sorghum (Ciegler *et al*., 1982; Saubois *et al*., 1999), respectively whilst *F. sacchari* is a pathogen of sugarcane (Ganguly, 1964; Rao and Agnihotri, 2000). Because of the frequency of their isolation, and the tissue that they were isolated from, it is possible that these three

species may have important mutualistic relationships with banana. Interestingly, endophytic isolates of *F. sacchari* from sugarcane have been shown to inhibit development of the sugarcane borer moth *in vitro* (Mc Farlane and Rutherford, 2005). Since *F. sacchari* is non-pathogenic to banana, the role of this fungus in the plant remains unknown and requires further investigation.

In the current study, we obtained more isolates of *Fusarium* spp. from banana roots than from rhizomes or pseudostem bases. These results are in agreement with other studies in which roots appear to harbor more endophytic fungi than other plant parts (Niere, 2001), which may indicate that the roots are potentially colonized by fungi from the soil. *Fusarium oxysporum* was the dominant species found in the roots and *F. semitectum* and *F. sacchari* in rhizomes of Cavendish bananas. Similarly, *F. oxysporum* was found to be the most dominant species in roots and *F. semitectum* in rhizomes of the banana cv. 'Pisang-Awak' (Niere, 2001). Photita *et al*. (2001) reported presence of lower numbers of endophytic fungal isolates from pseudostems in comparison to the veins, interveins and midribs of wild banana plants in Thailand. Differences in the isolation frequencies and the interaction observed between *Fusarium* spp. and different plant parts indicate that endophytic *Fusarium* spp. might be adapted to different banana plant parts. Tissue specificity of endophytic fungi has been reported for conifer needles (Carroll and Carroll 1978), and may be due to the adaptation by particular endophytes to the micro-ecological and physiological conditions present in the different plant organs (Petrini, 1996). Tissue specificity may also be a reflection of tissue preferences of individual dominating taxa (Rodrigues and Samuels 1990). The observed tissue specificity of various *Fusarium* endophytes may be used in biological control programs to target establishment of the endophytes most suited to the plants parts that require protection.

PCR-RFLP analyses grouped endophytic *F. oxysporum* isolates obtained from banana roots from Ramsgate and Tzaneen into 9 IGS genotypes. Although PCR-RFLP may not be the most suitable method to study genetic diversity, it has been used in several studies to resolve genetic differences among *F. oxysporum* strains (Appel and Gordon 1995; Nel *et al*., 2006a). In the current investigation, the PCR-RFLP technique was useful in identifying genetic differences among *F. oxysporum* isolates obtained from the roots and aided in selecting representative isolates for further AFLP analyses. In previous investigations using IGS-RFLP, 120 isolates of *F. oxysporum* from roots and hypocotyls of beans were grouped into

four haplotypes (Alves-Santos *et al*., 1999). Similarly, Appel and Gordon (1995) grouped 56 isolates of *F. oxysporum* into 13 IGS haplotypes.

In the current study, IGS genotypes that occurred at highest frequencies contained isolates from both collection sites, while groups that consisted solely of isolates from either of the two sites occurred at low frequencies. The greater number of IGS genotypes found in Ramsgate, compared to those in Tzaneen, despite being isolated from fewer plants, indicates a greater genetic diversity within isolates from Ramsgate. One possible explanation for this finding is that, because bananas have been cultivated in southern KZN for longer than in Tzaneen, more non-pathogens might have developed a mutualistic relationship with bananas in KZN.

IGS genotype grouping as opposed to AFLP's did not reveal a clear-cut correlation between *F. oxysporum* isolates, and their geographic origin or plant part origin. Some isolates from one site were restricted to a single IGS genotype, and others from both sites (Ramsgate and Tzaneen) shared the same IGS groups. Isolates from a particular site that occurred in the same IGS groups were not necessarily from the same plant or plant part. In fact, our results showed that the influence of plant part origin on IGS genotype grouping was very minimal. Several researchers have reported conflicting results in this regard. For example, genetic variation between isolates of *F. oxysporum* f. sp. *elaidis* have been linked to geographic origins with isolates originating from a specific site grouping together (Flood *et al*., 1992), while there was no correlation between grouping and the geographic origin of 49 strains of *F. oxysporum* isolated from pea plants (Skovgaard *et al*., 2002). Sharing of IGS genotypes by isolates obtained from different sites has previously been reported before (Appel and Gordon, 1995). Since this study was on endophytic fungi in two separated regions and from plantations that were initiated from tissue culture banana plants, we can rule out the possibility of introduction of the endophytes with planting material or long distance dispersal of soil particles and fungal spores. Absence or limited long distance dispersal of conidia may restrict gene flow, allowing for populations to evolve independently, and thus contributing to development of discrete fungal populations (Appel and Gordon, 1994). Probably, isolates from the two sites, which shared IGS genotypes, represent clonal lineages that occur irrespective of the geographic distances separating them (Appel and Gordon, 1995).

AFLP analysis proved useful in separating 57 isolates of *Fusarium* into two distinct clades according to the species, with *F. oxysporum* and *F. sacchari* clearly separated from the *F. solani* clades. Using AFLP, Abdel-Satar *et al*. (2003) were able to resolve five different *Fusarium* spp. into five distinct clusters according to each species. AFLP analysis successfully grouped the *F. oxysporum* isolates according to the plant part origin. The correlation between *F. oxysporum* isolates and the plant part origin further indicates species tissue specificity. In the current study however, AFLP analysis was not efficient in correlating the isolates of *F. oxysporum* with their geographic origin .The only exception was observed with isolates of *F. oxysporum* from Kiepersol, which formed a distinct sub-clade within the *F. oxysporum* main clade as would be expected of endophytes that co-evolve with plants over time, and that are selected to persist and form compatible long-lasting associations with bananas. In a previous investigation involving *F. oxysporum* f. sp. *lentis* no correlation was observed between 32 isolates and their geographic origin (Belabid *et al*., 2004). The findings obtained in this study are difficult to explain, but may be due to differences in plantation age in the different sites. Banana cultivation in Kiepersol has been going on for longer periods of time than in Ramsgate and Tzaneen (Altus Viljoen pers. comm.) and this may explain why isolates from this site grouped together. In well-established agronomic systems, limited genetic diversity has been observed between plants and their endophytic counterparts possibly due to a constant environment, which ensures continual persistence of the association. However, short-term establishments for annual crops, as opposed to perennial crops such as banana, may prevent the formation of stable compatible plant-endophyte combinations (Saikkonen *et al*., 2004). The *F. solani* isolates, which grouped into two distinct clades probably represent phylogenetic species within the *F. solani* species complex (O'Donnell, 2000; Geiser *et al*., 2004).

Endophytic fungi have recently gained popularity as possible biological control agents of crop pests and diseases and may act in several ways; through metabolite production, competition for nutrients and ecological niches, and/or induced resistance (Sikora *et al*., 2003; Schulz and Boyle 2005). Whether variation in endophyte genotypes can be linked to biologically important traits, such as pathogenicity or aggressiveness against pests and diseases and variations in metabolite production remains to be determined (Woo *et al*., 1998). AFLP analysis has also been useful in generating molecular markers that are linked to genes of biological interest in plants such as barley (Karakousis *et al*., 2003) and the wheat fungus, *Mycosphaerella graminicola* (Kema *et al*., 2002) might therefore be useful in

developing molecular markers for studying the dynamics and persistence of introduced agents and in tracking of the isolates in plants.

The current study has demonstrated the diversity in *Fusarium* endophytes. We have shown that different *Fusarium* spp. may occur as endophytes in crops other than those that they are known to be pathogenic to, possibly establishing mutualistic associations with the host plants. Different endophytic *Fusarium* species appear to have preferences for certain plant parts. The potential for using endophytic *Fusarium* spp. as an alternative or complementary pest and disease control option in banana is promising and requires further investigation.

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Figure 1. Schematic representation of PCR-restriction fragment length polymorphism patterns after digestion of the intergenic spacer region of the ribosomal DNA of 46 isolates of endophytic *Fusarium oxysporum* with the restriction enzymes *Hae*III, *Msp*I, *Rsa*I and *Hinf*I.

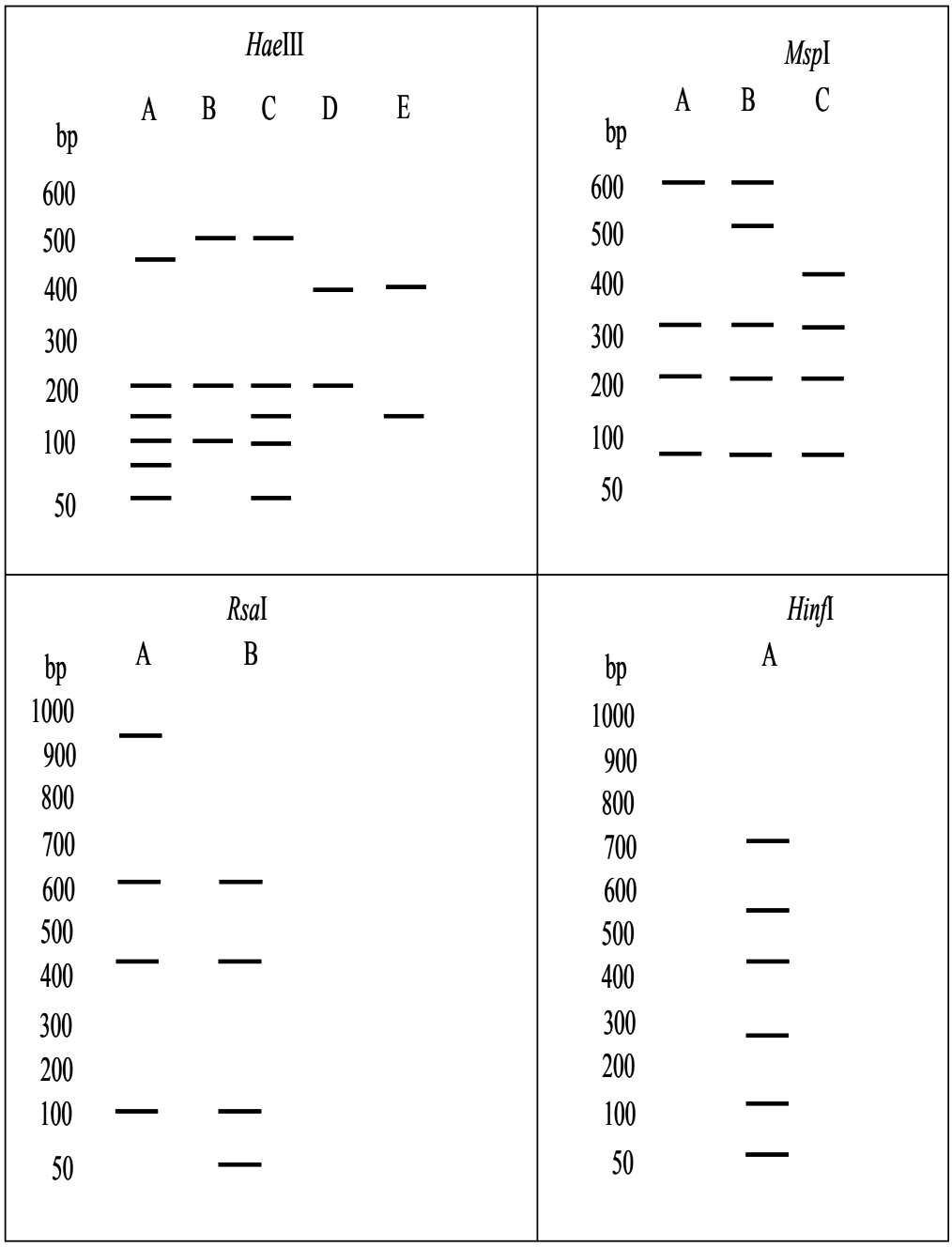

Figure 2. Phylogram inferred from amplified fragment length polymorphism analysis showing genetic relationship of 57 endophytic Fusarium isolates from banana root, rhizome and pseudostem base tissues. Distance analysis included neighbor joining as a tree building algorithm and a bootstrap of 1000 replicates. Bootstrap values are indicated above nodes, values less than 50% are not shown. The Fusarium isolates were obtained from Tzaneen, Ramsgate, and Kiepersol in South Africa and are designated with the prefix SAK, SAT, and KIP respectively

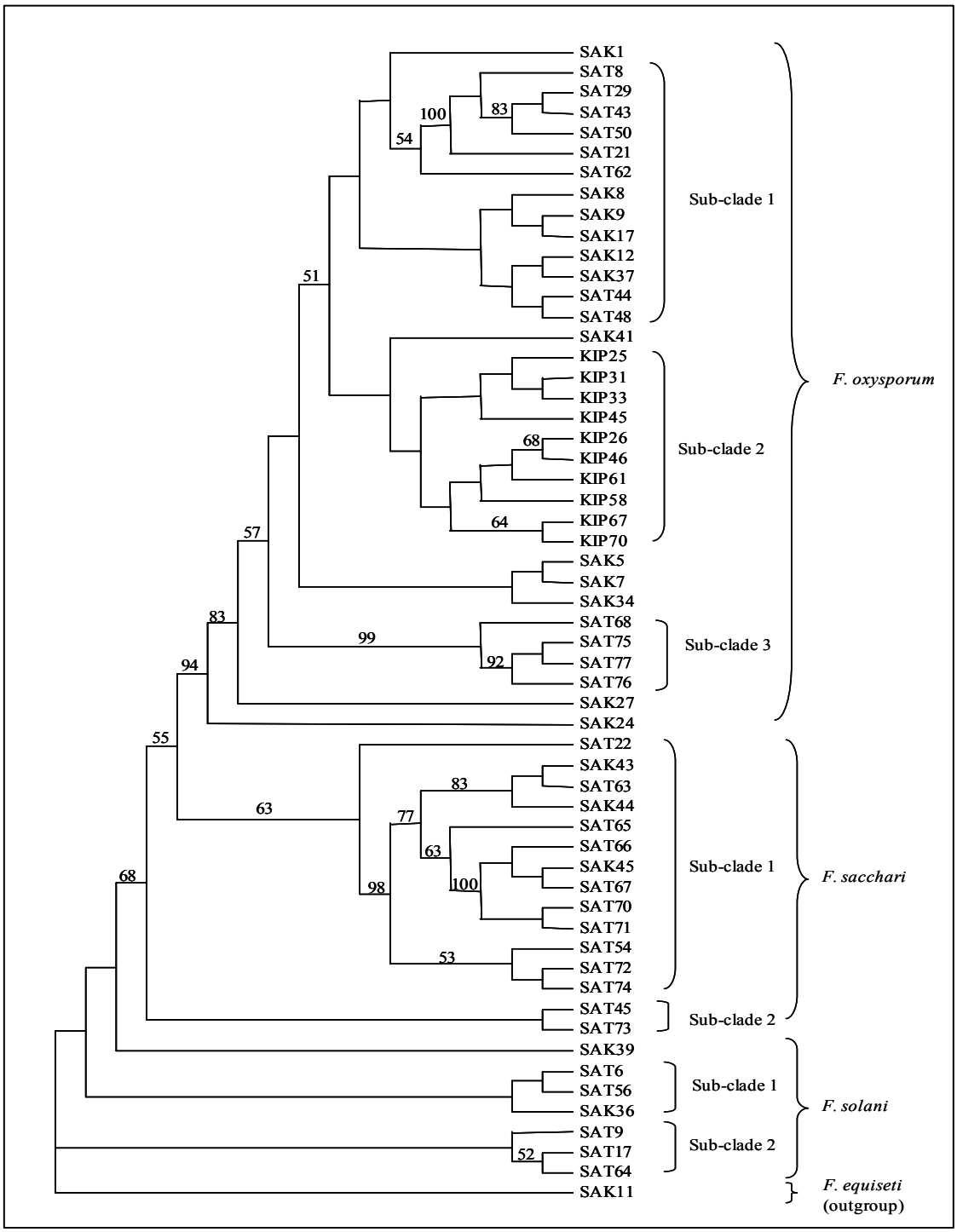

Table 1. Geographic origin of 57 endophytic isolates of *Fusarium* spp. from roots, rhizomes and pseudostem base tissues of Cavendish banana plants (*Musa* spp. AAA) in South Africa used for amplified fragment length polymorphism analysis.

Collectors no.[a]	CAV no.[b]	Species	GenBank accession number	Geographic origin[c]	Plant tissue
KIP25	CAV 542	*F. oxysporum*	DQ465926	Kiepersol	Root
KIP26	CAV 543	*F. oxysporum*		Kiepersol	Root
KIP31	CAV 546	*F. oxysporum*		Kiepersol	Root
KIP33	CAV 548	*F. oxysporum*		Kiepersol	Root
KIP45	CAV 552	*F. oxysporum*	DQ465927	Kiepersol	Root
KIP46	CAV 553	*F. oxysporum*		Kiepersol	Root
KIP58	CAV 559	*F. oxysporum*		Kiepersol	Root
KIP61	CAV 561	*F. oxysporum*		Kiepersol	Root
KIP67	CAV 563	*F. oxysporum*		Kiepersol	Root
KIP70	CAV 566	*F. oxysporum*		Kiepersol	Root
SAK1	CAV 722	*F. oxysporum*		Ramsgate	Root
SAK12		*F. oxysporum*		Ramsgate	Root
SAK17		*F. oxysporum*		Ramsgate	Root
SAK24	CAV 735	*F. oxysporum*	DQ465929	Tzaneen	Outer rhizome
SAK27		*F. oxysporum*		Ramsgate	Root
SAK34		*F. oxysporum*		Ramsgate	Root
SAK37		*F. oxysporum*		Ramsgate	Root
SAK41	CAV 754	*F. oxysporum*		Ramsgate	Root
SAK5	CAV 725	*F. oxysporum*	DQ465928	Ramsgate	Root
SAK7	CAV 727	*F. oxysporum*		Ramsgate	Root
SAK8	CAV 728	*F. oxysporum*		Ramsgate	Root
SAK9		*F. oxysporum*		Ramsgate	Root
SAT21	CAV 761	*F. oxysporum*		Tzaneen	Root
SAT29	CAV 766	*F. oxysporum*		Tzaneen	Root
SAT43	CAV 771	*F. oxysporum*		Tzaneen	Root
SAT44	CAV 772	*F. oxysporum*		Tzaneen	Root
SAT48	CAV 774	*F. oxysporum*	DQ465930	Tzaneen	Root
SAT50	CAV 776	*F. oxysporum*		Tzaneen	Root
SAT62	CAV 784	*F. oxysporum*		Tzaneen	Root
SAT68	CAV 189	*F. oxysporum*		Tzaneen	Inner rhizome
SAT75		*F. oxysporum*	DQ465931	Tzaneen	Pseudostem base
SAT76		*F. oxysporum*	DQ465932	Tzaneen	Pseudostem base
SAT77		*F. oxysporum*	DQ465933	Tzaneen	Pseudostem base
SAT8	CAV 750	*F. oxysporum*		Tzaneen	Root
SAK43		*F. sacchari*	DQ465944	Ramsgate	Inner rhizome
SAK44	CAV 1673	*F. sacchari*	DQ465943	Ramsgate	Pseudostem base
SAK45		*F. sacchari*	DQ465945	Tzaneen	Pseudostem base
SAT22	CAV 762	*F. sacchari*	DQ465934	Tzaneen	Root
SAT54	CAV 778	*F. sacchari*	DQ465935	Tzaneen	Outer rhizome
SAT63	CAV 1665	*F. sacchari*	DQ465936	Tzaneen	Inner rhizome
SAT65	CAV 1666	*F. sacchari*	DQ465937	Tzaneen	Pseudostem base
SAT66	CAV 1667	*F. sacchari*	DQ465938	Tzaneen	Pseudostem base

SAT67	CAV 1668	*F. sacchari*	DQ465939	Tzaneen	Inner rhizome
SAT71	CAV 1671	*F. sacchari*	DQ465941	Tzaneen	Inner rhizome
SAT72	CAV 1672	*F. sacchari*	DQ465942	Tzaneen	Inner rhizome
SAT73		*F. sacchari*	DQ465946	Tzaneen	Inner rhizome
SAT74		*F. sacchari*	DQ465947	Tzaneen	Pseudostem base
SAT70	CAV 1670	*F. sacchari*	DQ465940	Tzaneen	Inner rhizome
SAK36	CAV 740	*F. solani*	DQ465948	Ramsgate	Root
SAK39	CAV 742	*F. solani*	DQ465949	Ramsgate	Root
SAT17	CAV 759	*F. solani*	DQ465952	Tzaneen	Root
SAT45	CAV 773	*F. solani*		Tzaneen	Root
SAT56	CAV 780	*F. solani*	DQ465953	Tzaneen	Root
SAT6	CAV 748	*F. solani*	DQ465950	Tzaneen	Root
SAT64		*F. solani*	DQ465954	Tzaneen	Pseudostem base
SAT9	CAV 751	*F. solani*	DQ465951	Tzaneen	Root
SAK11	CAV 729	*F. equiseti*	DQ465925	Ramsgate	Outer rhizome

[a] Collectors numbers designates the isolate code given after primary isolation.

[b] CAV numbers designate codes for the fungal cultures deposited in the FABI culture collection, University of Pretoria, Pretoria, South Africa.

[c]Geographic site from which banana plants were sampled; endophytic *F. oxysporum* isolates from Kiepersol were not isolated in the current study but were obtained from the FABI culture collection.

Table 2. Incidence (%) of endophytic *Fusarium* spp. isolated from different plant parts of Cavendish bananas (*Musa* spp. AAA) in Ramsgate and Tzaneen, South Africa.

Species	Plant part									Geographic origin						Total[b]	
	Root			Rhizomes[a]			Pseudostem bases			Ramsgate			Tzaneen				
	n	%		n	%		n	%		n	%		n	%		n	%
F. oxysporum	45	32.8	a	3	2.1	a	3	2.1	a	24	17.1	a	28	20.0	c	52	37.4
F. solani	23	16.4	b	8	5.7	a	3	2.1	a	7	5.0	b	27	19.2	c	34	24.2
F. sacchari	1	0.7	c	5	3.6	a	8	5.7	a	2	1.4	b	12	8.5	ab	14	10.0
F. semitectum	0	0.0	c	10	7.1	a	5	3.6	a	5	3.5	b	10	7.1	ab	15	10.7
F. subglutinans	1	0.7	c	2	1.4	a	8	5.7	a	1	0.7	b	9	6.4	ab	10	7.1
F. dimerum	10	7.1	b	1	0.7	a	0	0.0	b	1	0.7	b	10	7.1	a	11	7.8
F. equiseti	0	0.0		3	2.1	a	0	0.0	b	2	1.4	b	1	0.7		3	2.1
F. proliferatum	0	0.0		0	0.0		1	0.7		0	0.0		1	0.7		1	0.7
Total	80	57.7		32	22.7		28	19.9		42	29.8		98	69.7		140	100

Within each plant part or site, incidence (isolation frequencies) of the different *Fusarium* spp. followed by the same letter are not statistically different at P<0.0051 after the Dunn Sidak correction.

Table 3. Intergenic spacer (IGS) region groups of 46 *Fusarium oxysporum* isolates from Cavendish banana (*Musa* spp. AAA) roots from two banana growing regions in South Africa that were subjected to PCR-restriction fragment length polymorphism analysis with four restriction enzymes *Hae*III, *Msp*I, *Rsa*I and *Hinf*I.

		Percentage[b]	Restriction enzyme			
IGS group	Isolates[a]		*Hae*III	*Msp*I	*Rsa*I	*Hinf*I
1	SAK1*, 3, 4, 16, 20, 22, 27*, 30, 35 & 37 SAT 2, 10, 16, 19, 21*, 28, 29*, 31, 34, 41, 43*, 49, 50*, 52, 61 & 62*	56.5	A	A	A	A
2	SAK 12* & 14	4.3	A	A	B	A
3	SAK 8* & SAT 8*	4.3	A	B	A	A
4	SAK 2, 5 & 15*	6.5	B	A	A	A
5	SAK 7*, 17*, 23 & 24* SAT 3, 14 & 48*	15.2	B	A	B	A
6	SAK 6 & 9*	4.3	C	B	A	A
7	SAK 41*	2.1	D	A	A	A
8	SAK 34*	2.1	D	A	B	A
9	SAT 12 & 44*	4.3	E	C	A	A

[a]Designation of isolates used in the PCR-RFLP analysis. Isolates with the prefix SAK were isolated from Ramsgate, SATfrom Tzaneen. *Fusarium oxysporum* isolates with an asterisk were selected from each IGS genotype for AFLP analysis.

[b] Percentage of total isolates in that particular IGS genotype

Summary

Radopholus similis is one of the key pests of banana in the East African highlands. Nematode damage results in large crop and yield losses. Although control of this pest had mainly relied on the use of clean planting material, re-infestation of plants in the field remains a critical concern. Alternative management options such as the use of fungal endophytes may be used to provide protection and extend plant life. In this thesis, the objective was to identify endophytic isolates of *Fusarium* spp. and especially *F. oxysporum* with potential for *R. similis* control in tissue culture banana plantlets and to study the interactions between the host plant, nematode and the endophyte. Isolates were screened both *in vitro* and *in vivo* and the most promising isolates were studied further to elucidate when, where and how the endophytes suppressed *R. similis*.

In the first phase of this research, a total of 35 endophytic *Fusarium* spp. isolates were screened for the production of secondary metabolites antagonistic to *R. similis in vitro*. All isolates tested demonstrated some level of *in vitro* antagonistic activity on the mobility of *R. similis* males, females and juveniles. The percentage of immobilized nematodes increased with increase in the length of exposure time and the concentration of the culture filtrates. An interesting finding of this research was that male *R. similis* were more sensitive to culture filtrate treatment than females. The method of using culture filtrates to identify isolates with antagonistic effects against the nematode has the advantage of being rapid, thus saving time and allows for selection of the most promising isolates for *in planta* evaluation. The actual metabolites produced by the isolates in culture were not determined in this study and warrant further investigations.

In the second part of this research, nine *F. oxysporum* isolates that showed good *in vitro* antagonistic activity against *R. similis* were screened for nematode suppression in tissue culture banana plants. A local banana cultivar susceptible to *R. similis* was used in nine screen house experiments. Endophyte treatment did not affect nematode population densities significantly although although there was a tendency for nematode populations in endophyte-treated plants to be lower than in the untreated control plants. Banana plant growth and nematode damage were also not influenced by endophyte treatment except in a few

experiments. The results obtained showed the effect of individual isolates on plant growth, nematode damage and density varied widely across repeat experiments. Despite the inconsistencies, three isolates, *V5W2*, *Eny1.13i* and *Eny7.11o* were identified as the most effective isolates. The reproducibility of the results obtained is questionable as control plants were also found to be infected by endophytic *F. oxysporum*. This research also raises a big question pertaining to the ability to monitor and track re-isolated endophytes. Thus proper endophyte marking techniques need to be developed for future experiments to compare the re-isolated endophytes with the original ones. Although the levels of nematode control afforded by endophytes may not be very high, the use of endophytes in tissue culture plants may complement the clean nature of the plants thereby boosting their ability to resist or tolerate nematode attack in the field.

The endophytes tested during the current study have shown ability to suppress *R. similis* in tissue culture banana plants. However, it is not known how, where and when the endophytes are most effective against the nematodes. In the third part of this research, the effects of three isolates, *V5W2*, *Eny1.31i* and *Eny7.11o* on the host preferences, root penetration and reproduction of *R. similis* in tissue culture banana plants were evaluated under laboratory and screen house conditions. Host preference tests provided evidence that nematodes were equally attracted to both endophyte-treated and untreated plants. Similarly, root penetration of banana plant roots by *R. similis* did not differ between endophyte-treated and untreated plants. Nematode reproduction which was assessed over a period coinciding with three nematode generations was however, influenced by endophyte treatment. Differences were observed during the second and third nematode generations with nematode reproduction rates being higher in the untreated compared to endophyte-treated plants. The results obtained demonstrate that the mechanism by which the fungal isolates control *R. similis* in the plant is mainly post-infectional, acting only after the plants have been infected and may possibly be through induced resistance in the plant against the nematode.

In the fourth part of this research, potential mechanism(s) through which endophytic isolates of *F. oxysporum* result in nematode suppression in tissue culture banana plants were studied. Nine isolates of *F. oxysporum* showed positive results for protease enzyme production but none showed chitinase and lipase activity. In split-root experiments, the densities of *R. similis* females, males, juveniles and the total nematode density were substantially lower in one half

of the split-root systems when the corresponding half was treated with one of the endophytic *F. oxysporum* isolates *V5W2*, *Eny1.131i* or *Eny7.11o*. To further investigate the role of induced resistance in nematode management by endophytes, the amounts and types of phenolic compounds were assessed in plants inoculated with isolate *V5W2*. Histological analysis showed the presence of phenolic cells in the cortex and central cylinder regions of roots and rhizomes. Although constitutive phenols occurred both in roots and corms of plants treated with isolate *V5W2* as well as untreated plants, the quantities increased over time in the endophyte-inoculated plants compared to the uninoculated plants. Higher amounts of total soluble phenolics were found in endophyte-treated than in untreated plants. Corm and root metabolite profiles of endophyte-treated and untreated plants did not differ. HPLC analysis revealed four major unidentified compounds, which were present in endophyte-treated as well as untreated plants. These results demonstrate that induction of systemic resistance by endophytic isolates of *F. oxysporum* may play a role in *R. similis* management and that treatment of plants with endophytes triggers the plant to increase synthesis of phenolic compounds. Further research needs to be conducted to identify the unknown compounds.

In the last part of this research, endophytic *Fusarium* spp. from roots, rhizomes and pseudostem bases of apparently healthy Cavendish banana plants from three sites in South Africa were assessed using morphological and PCR-RFLP and AFLP analyses. The objectives were to identify *Fusarium* endophytes in banana plants, study their relation to specific plant parts and determine the genetic diversity within and between the endophytes. From the isolations, *Fusarium oxysporum* was the predominant species isolated, followed by *Fusarium solani* and *Fusarium semitectum*. Tissue specificity was observed with *F. oxysporum* and *F. solani* being predominantly isolated from the roots whilst *F. semitectum, F. sacchari* and *F. subglutinans* were predominantly isolated from rhizomes and pseudostem bases. The *F. oxysporum* isolates from the roots were grouped into nine genotypes using PCR-RFLP analysis of the IGS region of the rDNA. Distance analysis of AFLP data of 57 *Fusarium* isolates resolved the isolates into two major clades: one consisting of the isolated *F. oxysporum* and the other of *F. sacchari*. *Fusarium oxysporum* isolates further grouped according to plant part origin while the *F. sacchari* and *F. solani* isolates grouped randomly. The results obtained demonstrated tissue specificity of endophytic *Fusarium in* Cavendish banana and also a wide inter- and intraspecific genetic variation among endophytic *Fusarium* isolates of banana in South Africa. The observed tissue specificity of various *Fusarium*

endophytes may be used in future biological control programs to target establishment of the endophytes most suited to the plants parts that require protection.

Chapter 6

Genetic diversity of endophytic *Fusarium* spp. associated with Cavendish banana in South Africa

Abstract

Endophytic *Fusarium* spp. are commonly associated with banana plants. Nothing is known regarding tissue specificity and the genetic diversity within endophytic *Fusarium* spp. from banana plants. Endophytic *Fusarium* spp. from roots, rhizomes and pseudostem bases of Cavendish banana plants from three sites in South Africa were assessed using morphological and molecular analyses. *Fusarium oxysporum* was the predominant species isolated, followed by *Fusarium solani* and *Fusarium semitectum*. Tissue specificity was observed with *F. oxysporum* and *F. solani* being predominantly isolated from the roots whilst *F. semitectum, F. sacchari* and *Fusarium subglutinans* were predominantly isolated from rhizomes and pseudostem bases. PCR-RFLP analysis of the IGS region of the rDNA divided 46 isolates of *F. oxysporum* obtained from roots into nine different genotype groups. Distance analysis of AFLP data of 57 *Fusarium* isolates resolved the isolates into two major clades: one consisting of the isolated *F. oxysporum* and the other of *F. sacchari*. The *F. solani* isolates formed three clades clearly separated from the other two species. *Fusarium oxysporum* isolates further grouped according to plant part origin while the *F. sacchari* and *F. solani* isolates grouped randomly. The results obtained demonstrate tissue specificity of endophytic *Fusarium* in Cavendish banana and also a wide inter- and intraspecific genetic variation among endophytic *Fusarium* isolates of banana in South Africa.

Introduction

Endophytes are microorganisms that spend at least part of their life cycle inside plant tissues without causing any apparent disease symptoms (Carroll, 1988). The presence of endophytic fungi has been demonstrated in many plants including important agricultural crops like banana (Brown *et al*., 1998; Pereira *et al*., 1999; Pocasangre *et al*., 1999; Dubois *et al*., 2004), maize (Fisher *et al*., 1992), rice (Fisher and Petrini, 1992) and tomato (Hallman and Sikora, 1994). Fungal endophytes are known to produce bioactive products that may play important ecological and biological roles in the host plant (Tan and Zhou, 2001; Strobel, 2003; Schulz and Boyle, 2005). In mutualistic associations, endophyte-infected plants are protected from attack by some insects, nematodes and fungi, while in return the endophyte is provided with shelter and nutrition by the host plant (Latch, 1993; Saikkonen *et al*., 1998; Azevedo *et al*., 2000; Schardl *et al*., 2004).

Fusarium spp. are cosmopolitan fungi that contain many pathogenic forms causing diseases to a wide variety of economically important crops, such as banana (Stover, 1981). The genus also comprises nonpathogenic strains, some of which occur as endophytes colonizing different plant tissues (Niere, 2001; Tan and Zhou, 2001; Sikora *et al*., 2003). Several researchers have reported association between endophytic *Fusarium* and banana plants (Pereira *et al*., 1999; Pocasangre *et al*., 1999; Dubois *et al*., 2004). *Fusarium oxysporum* has been identified as the predominant species establishing endophytic relationships with banana plants. The ability of endophytic *F. oxysporum* isolates to protect banana plants against pests and diseases has been demonstrated in laboratory and screen house experiments (Pocasangre *et al*., 1999; Dubois *et al*., 2004; Gold and Dubois 2005, Nel *et al*., 2006b). Thus, mutualistic associations between endophytic *Fusarium* spp. and banana plants may be viewed as a promising form of biological protection.

The international banana trade is dominated by the Cavendish–type bananas (INIBAP, 2003). Apart from the export trade, Cavendish bananas are grown in many other countries for local consumption only. Opportunities for improving production of Cavendish bananas are often constrained by pests and diseases. The main pests are the banana weevil *Cosmopolites sordidus* and the banana nematode *Radopholus similis* (Daneel *et al*., 2004; de Graaf *et al*., 2004). The main diseases of Cavendish bananas are Fusarium wilt (Panama disease) caused by *Fusarium oxysporum* f. sp. *cubense* (Moore *et al*., 1995) and black

Sigatoka, caused by *Mycosphaerella fijiensis* (Mourichon *et al*., 1997; Surridge *et al*., 2003). These pests and diseases are difficult and costly to control, and can amount to yield losses of up to 100% of severely infested fields (Moore *et al*., 1995; Mourichon *et al*., 1997).

Genetic diversity exists between and within pathogenic and nonpathogenic populations of *F. oxysporum*, and various molecular methods can be used to detect such variation (Gordon and Okamoto 1991; Edel *et al*., 1995). The amplification of variable ribosomal DNA (rDNA) regions allows for discrimination at the genus, species and intraspecific level (Edel *et al*., 1995). Of particular importance is the intergenic spacer (IGS) region that shows considerable divergence within closely related species (Appel and Gordon 1994; 1995). The use of PCR amplified rDNA regions as substrates for restriction fragment length polymorphisms (RFLPs) is one of the widely used methods use to evaluate genetic diversity in *F. oxysporum* populations. By using PCR-RFLP analysis of the IGS region, Woo *et al*. (1996) identified genetic differences between worldwide collections of pathogenic and nonpathogenic isolates of *F. oxysporum* of the bean wilt pathogen *F. oxysporum* f. sp. *phaseoli*. Appel and Gordon (1995) were able to resolve intraspecific variation among 56 soil isolates of *F. oxysporum*, and Nel *et al*. (2006a) reported variation among isolates of *F. oxysporum* from the banana rhizosphere. Several researchers have also reported genetic diversity of endophytic *F. oxysporum* from various plants based on analysis of the IGS region. In addition, diversity among endophytic isolates of *F. oxysporum* from symptomless tomato roots (Elias *et al*., 1991) and from roots and hypocotyls of beans (Alves-Santos *et al*., 1999) was demonstrated.

Amplified fragment length polymorphism (AFLP) is based on the selective amplification of a high number of restriction fragments and is highly reproducible (Vos *et al*., 1995). It is also useful in delineating genetic differences between and within species (Vos *et al*., 1995). For example, AFLPs were shown to relate five distinct clusters with five different *Fusarium* taxa in a phylogenetic study (Abdel-Satar *et al*., 2003). Groenewald *et al*. (2006) further demonstrated that AFLPs could be used to subdivide *F. oxysporum* f.sp. *cubense* in several different clades which correspond to vegetative compatibility. Inter- and intraspecific variation obtained with AFLPs can also be used to construct genetic markers for different microorganisms (Kema *et al*., 2002).

Despite the known association of banana plants with endophytic *Fusarium* spp. genetic diversity of these endophytes, and their relation to different plant parts has been poorly

studied. The objectives of this study were, therefore, to isolate and identify endophytic *Fusarium* spp. from field-grown Cavendish bananas, to examine the tissue specificity of such *Fusarium* spp., and to determine the genetic diversity within these *Fusarium* endophytes of banana.

Materials and methods

Collection of plant material

Fresh root, rhizome and pseudostem base samples were collected from apparently healthy Cavendish banana plants growing in banana weevil- and nematode-infested plantations in Ramsgate, KwaZulu-Natal (KZN) province and Tzaneen, Limpopo province of South Africa in May and June, 2003 respectively. Neither of these plantations were affected by Fusarium wilt of banana. Five and 10 plants were sampled from Ramsgate and Tzaneen, respectively. From each plant, five 10-cm-long primary roots and five 100-cm^3 pieces of the rhizomes and pseudostem bases were randomly taken. Samples were packed in polythene bags, placed in cooler boxes and transported to the Forestry and Agricultural Biotechnology Institute (FABI), located at the University of Pretoria in South Africa, for isolation of endophytic *Fusarium* spp.

Isolation of endophytic Fusarium spp.

Roots were washed under running tap water to remove adhering soil, and surface sterilized in 75 % ethanol for 1 min followed by 2 % sodium hypochlorite solution for 30 s (Petrini, 1986), followed by a double rinse in sterile distilled water (SDW). After rinsing, the samples were dried by blotting them on sterile tissue paper. From each surface sterilized root, five 1-cm long pieces were randomly cut and placed on *Fusarium*-specific rose bengal agar (12 g agar, 10 ml glycerine, 0.5 g L-alanine, 1.0 g pentachloronitrobenzene, 0.5 g Rose Bengal and 1.0 g urea / L SDW) in 65-mm-diameter Petri dishes. The medium was amended with 0.25 g l^{-1} chloramphenicol to prevent bacterial growth. The pseudostem bases, inner and outer rhizome samples from each plant, were surface sterilized by dipping in 95% ethanol, followed by flaming. From each surface sterilized pseudostem base, inner and outer rhizome sample, five 1-cm^3 pieces were randomly removed using a flamed scalpel blade, and also

placed on rose bengal medium amended with antibiotics. All Petri dishes were incubated at 25 °C and checked for fungal growth for 7 days. All colonies of putative *Fusarium* spp. were sub-cultured on half strength potato dextrose agar (19 g PDA, 8 agar / L SDW) (PDA, Difco, Detroit, USA) and incubated for 7 days at 25 °C.

Morphological identification of Fusarium spp.

Single-spore cultures were prepared for all *Fusarium* colonies according to Nelson *et al*. (1983). From the single-spore cultures, mycelial disks were transferred to carnation leaf agar (CLA) and half strength PDA in 65-mm and 90-mm diameter Petri dishes, respectively. CLA was prepared by placing a dry sterile carnation leaf on 2 % water agar (Nelson and Toussoun 1986). The cultures on CLA medium were incubated for 7 to 28 days at 25 °C before being examined for conidium and conidiophore morphology, and chlamydospore production. Cultures on half strength PDA were examined for growth rates and morphological characteristics such as colony appearance (presence or absence of aerial mycelia, surface texture, and pigmentation) after incubation at 25 °C for 10 days. Identification of *Fusarium* isolates to species level was done according to Nelson *et al*. (1983). Only one isolate for each *Fusarium* species recovered per root, rhizomes or pseudostem base tissue per plant was preserved for further analysis to limit duplication of isolates. Isolates were preserved on sterile filter papers and also in 15 % glycerol and stored at 4 °C and –80 °C respectively. All the isolates used in this study are maintained in the FABI culture collection.

Statistical analysis of data

Isolation frequencies of different *Fusarium* spp. among plant parts and between geographic origins were analyzed using categorical logistic regression. Only species for which more than one isolate was obtained were included in the analysis. Likelihood ratio tests were performed to investigate differences within factors (site, *Fusarium* sp. and plant part). If different, means were separated using 95 % confidence intervals and significant α levels of 0.0051 after the Dunn-Sidak correction (Sokal and Rolf 1995; Ury, 1976) using the SAS system (SAS, 1989).

Molecular characterization of Fusarium spp.

DNA extraction from fungal mycelia

To obtain DNA, single spore isolates of *Fusarium* were grown at 25 °C on PDA medium in 65-mm-diameter Petri dishes for 7 days. Fungal mycelium was scrapped from the surface of the medium using a flame-sterilized scapel and transferred into 1.5-ml Eppendorf tubes. DNA was extracted according to Raeder and Broda (1985). To each tube, 300 µl of DNA extraction buffer (200 mM Tris/HCl (pH 8.0), 150 mM NaCl, 25 mM EDTA (pH 8.0), 0.5% SDS in SDW) was added. The mycelium was homogenised in the extraction buffer with a pestle and frozen in liquid nitrogen. The cell lysate was extracted with 700 µl of phenol-chloroform (1:1 v/v) and centrifuged at 14000 rpm for 7 min at 4 °C. The supernatant was then transferred to new tubes and the extraction procedure repeated three more times. To the final supernatant (~250 µl), 25 µl of 3 M sodium acetate (pH 5.5) and 500 µl of ice cold 100 % ethanol were added, and the mixture centrifuged at 14000 rpm for 10 min at 4°C to yield the DNA pellet. The DNA pellet was washed with 70 % ethanol and dried *in vacuo* for 5 min at 45°C, redissolved in 60 µl TE buffer (10 mM Tris/HCl (pH 8.0) and 1 mM EDTA (pH 8.0) in SDW) and treated with RNAse (Sigma-Aldrich, MO, USA) to degrade RNAThe DNA concentrations were determined using a spectrophotometer and diluted to a final working concentration of 20 ng μl^{-1}.

Sequence analysis

Part of the transfer elongation factor 1-α (TEF) region of selected isolates was amplified using the Ef1 and Ef2 primers (O'Donell *et al*., 1998). The PCR reaction conditions were as follows; initial denaturation of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 45 s at 60 ºC, 90 s at 72 ºC and a final extension of 5 min at 72 ºC. PCR reactions were carried out in a mastercycler (Epperndorf, Hamburg, Germany). The resulting amplicons were electrophoresed in 1% TBE agarose gels and visualized using ethidium bromide staining and UV light. The amplified DNA fragments were purified using a High Pure PCR Product Purification kit (Roche Diagnostics, Johannesburg, South Africa) according to the manufacturers instructions. Sequencing was performed in 10 µl reactions each containing 4 µl of purified PCR products, 1 µl of Ef1 or Ef2, 2 µl of Big dye III (Applied Biosystems, Foster City, CA, USA), 1 µl 5x dilution buffer and 2-µl dH_2O. PCR conditions were 25 cycles of 10 s at 96 ºC, 5 s at 50 ºC and 4 min at 60 ºC. Sequencing products were

precipitated and electophoresed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The obtained sequences were edited and aligned using the Sequence Navigator software program (Applied Biosystems). Final alignments were done manually and the sequences compared with GenBank sequence databases using nucleotide Blast on the National Center for Biotechnology Information (NCBI) website.

PCR-RFLP analysis of the IGS region of F. oxysporum *isolates*

All *F. oxysporum* isolates obtained from banana roots were subjected to PCR-RFLP analyses in order to group them into IGS genotypes for subsequent AFLP analyses of a smaller, representative number of isolates. Two oligonucleotide primers, forward primer PNFo (5' CCCGCCTGGCTGCGTCCGACTC 3') and reverse primer PN22 (5' CAAGCATATGACTACTGGC 3') were used to amplify a fragment of the IGS region of the rDNA (Edel *et al*., 1995). The primers were synthesized by Inqaba Biotechnical Industries (Hatfield, Pretoria, South Africa). PCR amplifications were performed in a total volume of 50 µl by mixing 50 ng of template DNA with 0.1 µM of each primer, 2.5 mM of each of dATP, dCTP, dGTP and dTTP, 1 unit *Taq* DNA polymerase and 1x PCR reaction buffer (10 mM Tris/HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml of bovine serum albumin in dH_2O) (Roche Diagnostics). Amplifications were performed in a mastercycler (Epperndorf) with 30 cycles of 90 s at 95 °C, 60 s at 50 °C and 90 s at 72 °C. Five microliters of PCR products was used to verify successful amplification of DNA by electrophoresis in 2% (w/v) agarose gels in 1x TAE buffer for 1 hr at 80 V. Aliquots of 10 µl of PCR products were digested for 4 hrs at 37 °C with 5 units of each of four restriction endonucleases: *Hae*III, *Hinf*I, *Msp*I and *Rsa*I (Roche Diagnostics). Restriction fragments were separated by electrophoresis in 4% agarose gels in 1x TAE buffer for 1 hr at 60 V. Fragments were run against a 100 base pair (bp) molecular marker for size estimation. Each unique banding pattern obtained with the different enzymes was assigned a letter, and each isolate assigned a composite IGS genotype defined by a combination of the patterns (letters) obtained with the four enzymes (Appel and Gordon, 1995).

AFLP analysis of Fusarium *spp.*

A total of 57 *Fusarium* spp. isolates from three banana-growing regions (Ramsgate, Tzaneen and Kiepersol in the Mpumulanga province) in South Africa were studied. Of these, 31 and 16 isolates were obtained from banana plants in Tzaneen and Ramsgate in the current study, respectively. Ten isolates previously collected from symptomless banana plants in Fusarium wilt-suppressive soils in Kiepersol, were obtained from the culture collection at FABI and included in the AFLP analysis. Isolates from Kiepersol had been confirmed as nonpathogenic to banana. Of the 57 isolates, 34 were *F. oxysporum*, 8 were *Fusarium solani*, 14 were *Fusarium sacchari* and 1 *Fusarium. equiseti* (Table 1).

A high-throughput AFLP analysis procedure using infrared dye-labeled primers and an automated DNA analyzer was used (Myburg *et al*., 2001). The restriction, ligation and preamplification reactions were performed as described by Vos *et al*. (1995). Genomic DNA (20 ng) was digested with two units each of *Eco*RI and *Mse*I (Roche Diagnostics) and incubated for 3 hr at 37 °C. For adaptor ligation, 30 µl of digested DNA was added to 10 µl of the restriction-ligation buffer (50 ρmol/µl of *Mse*I adaptor, 5 ρmol/µl of *Eco*RI adaptor and one unit of DNA ligase) and incubated for 3 hr at 37 °C. Restriction-ligation mixtures were diluted 10-fold with low TE buffer (10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA (pH 8.0) in dH2O) to serve as a template for preamplification. Preamplification reactions were performed in a total volume of 30 µl containing: 5 µl of diluted restriction-ligation products, 0.6 units *Taq* polymerase, 3 µl PCR reaction buffer, 0.3 µM of *Eco*RI and *Mse*I primer without any additional selective nucleotides, and 2.5 mM of each of the four dNTPs. The PCR program consisted of 25 cycles of 30 s at 72 °C, 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C, with an additional 1 s per cycle increase in the extension time. Electrophoresis to verify successful preamplification was carried out in 1.2% agarose gels in 0.5x TBE for 1.5 hr at 60 V. Depending on the intensity of the smear, preamplification products were diluted either 10- or 20-fold with low TE buffer and served as the templates for the final amplification step.

Three primer combinations were used for final selective amplification. The *Eco*RI primers were 5′-end labeled with infrared dye IRDye™ 700 or 800 (LI-COR). The primer combinations used were *Eco*RI-AT / *Mse*I-CC, *Eco*RI-CC / *Mse*I-TA and *Eco*RI-TC / *Mse*I-AA. Final amplifications were performed in a total volume of 20 µl containing 5 µl diluted preamplification product, 2 µl PCR reaction buffer, 1.6 µl of 2.5 mM of each of the four

dNTPs, 0.04 µM IRDye 700-labeled *EcoR*1 primer or 0.04 µM IRDye 800-labelled *EcoR*I primer, 0.25 µM *Mse*I primer and 1.2 units *Taq* polymerase. A total of 37 amplification cycles were carried out. The first amplification cycle was carried out for 10 s at 90 °C, 30 s at 65 °C and 1 min at 72 °C. In each of the following 12 cycles, the annealing temperature was reduced by 0.7 °C per cycle. The last 23 cycles were carried out for 10 s at 94 °C, 30 s at 56 °C and 1 min, extended 1 s per cycle, at 72 °C. A final extension cycle was carried out for 1 min at 72 °C. All amplification steps were carried out in a mastercycler (Eppendorf).

Electrophoresis and detection of AFLP fragments

Electrophoresis and detection of AFLP fragments was performed on a two-dye automated DNA sequencer Model 4200s (LI-COR, Lincoln, NE, USA). AFLP fragments were resolved in 25 cm gels containing 8% polyacrylamide gel solution (Long Ranger Gel Solution, Cambrex Bioscience, Rockland, USA), 7.0 M urea and 1x TBE buffer. Ten microliters of formamide loading buffer (95% deionised formamide, 20 mM EDTA, 1 mg/ml Bromophenol blue and 0.5 ml dH_20) was added to 20 µl final AFLP products in 0.2 ml PCR tubes and the tubes wrapped in aluminum foil. All samples were denatured for 3 min at 90 °C and transferred to ice before loading. Disposable 64-well paper combs (KB^{Plus}, LI-COR) (0.25 mm spacer thickness) were used for gel loading. 0.8 µl of each of the diluted preamplification products was loaded into a single lane. The first and last lanes of the gels were loaded with a denatured 50-700 bp molecular weight marker labeled with the corresponding infrared dye (LI-COR). Gel images were transferred automatically from the DNA analyzer to the Saga Application server (LI-COR) where analyses and band scoring were performed.

Scoring of AFLP images

Digital AFLP gel images were scored to obtain binary (band presence or absence) data using $Saga^{MX}$ automated AFLP analysis software (Keygene, Wageningen, the Netherlands). Lane definition and band sizing were performed as described in the $Saga^{MX}$ user manual. Semi-automated scoring was performed by manually clicking on polymorphic fragments present or absent in any of the 60 lanes. After scoring, data were exported directly to a spreadsheet program and reformatted for use in mapping software. Binary data (0 = absence, 1 = presence) obtained from band scores of the gel images obtained with the three different primer pairs was combined. The combined AFLP data was subjected to distance analysis

using the Phylogenetic Analysis Using Parsimony (PAUP) Version 4.0 b10 (Swofford 2002) software (Applied Biosystems, Foster City, CA, USA) which included neighbour joining as a tree building algorithm and a bootstrap of a 1000 replicates (Felsenetein, 1985) for confidence support. The *F. equiseti* isolate was used as the outgroup.

Results

Isolation frequencies of endophytic Fusarium spp. from Cavendish banana plants

The frequency of isolation and diversity of endophytic *Fusarium* spp. was assessed in roots, rhizomes and pseudostem bases of healthy Cavendish banana plants. We isolated a total of 140 isolates belonging to eight *Fusarium* species. Of these 140 isolates, 57.2% were from the roots, 22.7% from the rhizomes, and 19.9% were obtained from the pseudostem bases. *Fusarium oxysporum* was isolated at the highest frequency among the endophytic *Fusarium* isolates. Other *Fusarium* spp. that were isolated included, in decreasing frequencies, *F. solani, F. semitectum, F. sacchari*, *F. subglutinans, F. dimerum*, *F. equiseti* and *F. proliferatum* (Table 2). Mixed infections from an individual plant were common; as many as six different *Fusarium* spp. were found in a single plant. The highest number of *Fusarium* species recovered from a single plant was four and six in Ramsgate and Tzaneen, respectively. From the isolations, an individual *Fusarium* species could be recovered from all plants within one site; *F. oxysporum* and *F. solani* were found in all plants from Ramsgate and Tzaneen, respectively.

The frequency of isolation of different *Fusarium* spp. was influenced by the plant part ($P<0.0001$) and geographic site ($P<0.0001$) from which they were isolated. The frequency of isolation of different endophytic *Fusarium* species also differed within plant parts; roots ($P<0.0001$), rhizomes ($P=0.0115$) and pseudostem bases ($P=0.0301$). A strong interaction between species frequency and plant part ($P<0.0001$) was observed. *Fusarium oxysporum* and *F. solani* were predominantly isolated from the roots, *F. semitectum* and *F. sacchari*, from rhizomes and *F. subglutinans* from pseudostem bases. The composition of different *Fusarium* species differed significantly within geographic sites (Ramsgate, $P<0.0001$ and Tzaneen, $P<0.0001$) with a strong interaction between species composition and geographic site ($P=0.0127$). In Ramsgate, *F. oxysporum* was the most frequently isolated species while in Tzaneen, *F. oxysporum* and *F. solani* were recovered at the highest frequencies. *Fusarium*

sacchari, *F. semitectum* and *F. subglutinans* were also found in higher frequencies in banana plants from Tzaneen than from banana plants from Ramsgate.

Molecular characterization of endophytic Fusarium spp.

Sequence analysis of the TEF 1-α region of selected Fusarium isolates

PCR amplification of a part of the transfer elongation factor 1-α (TEF) of selected isolates using the Ef1 and Ef2 primers yielded a fragment of ~700 bp. The obtained sequences were used to confirm morphological species identification and have been submitted to GenBank, Accession numbers DQ465925 – DQ465954 (Table 1).

PCR-RFLP analysis of the IGS region of Fusarium oxysporum *isolates*

A fragment of approximately 1.7 kbp was amplified from each of the 46 isolates of *F. oxysporum* obtained from banana roots in Ramsgate. Depending on the restriction enzyme used, 1-5 distinct restriction fragment patterns were obtained. Five patterns were realized for *Hae*III, three for *Msp*I, two for *Rsa*I and one for *Hinf*I (Fig. 1). Among the 46 isolates of *F. oxysporum*, a total of 9 IGS genotype groups were identified (Table 3). Isolates from each site generally clustered within the same IGS genotype, although some isolates from the different sites shared the same IGS genotypes. The most common IGS genotype, AAAA, included 10 isolates from Ramsgate and 16 isolates from Tzaneen. Five genotypes out of the nine contained isolates only from Ramsgate. One genotype contained isolates only from Tzaneen and three genotypes contained isolates from both sites (Table 3). Depending on the number of isolates within a particular IGS genotype, a total of 19 representative isolates were selected for further analysis using AFLPs (Table 3). Seven were from genotype 1, three from genotype 5, two each from genotypes 3 and 9, and one each from genotypes 2, 4, 6, 7 and 8.

AFLP analysis of Fusarium spp.

AFLP analysis of *Fusarium* spp. from banana roots, corms and pseudostem bases, using three primer combinations, produced approximately 150 polymorphic bands useful for comparison of genetic diversity. Distance analyses of the AFLP data obtained with the three primer pairs clearly divided the isolates into two distinct clades, one comprising of *F.*

oxysporum isolates and the second comprising of *F. sacchari* isolates. The *F. solani* isolates did not group into one distinct clade, but rather formed two clades, which were clearly separated from the *F. oxysporum* and *F. sacchari* clades (Fig. 2).

The clade comprising of *F. oxysporum* isolates was further divided into 3 distinct sub clades. The clustering of *F. oxysporum* isolates was closely related to the plant part origin than to geographic origin. The first 2 sub-clades comprised of isolates obtained from the roots while the third sub-clade comprised of isolates from the rhizomes and pseudostem bases. Within the root isolates sub-clade, *F. oxysporum* isolates from Kiepersol (sub-clade 2) clearly formed a distinct group while the *F. oxysporum* isolates from Ramsgate and Tzaneen clustered randomly within sub-clades 1 and 3. The *F. oxysporum* isolates obtained from roots were found to be highly diverse as shown in the cladogram where several sub-clades were formed (Fig. 2). The *F. sacchari* clade was further divided into 2 sub-clades, all isolates in the two sub-clades were obtained from the rhizomes and pseudostem bases except for isolate SAT22, which was obtained from roots and which grouped separately from the other isolates. The *F. solani* isolates grouped into two distinct sub-clades with no correlation to plant part or geographic origin. One *F. solani* isolate (SAK39) was separated from the rest of the isolates clades.

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Discussion

Cavendish banana plants in South Africa host a wide array of fungal endophytes. The isolation of a total of 140 isolates belonging to eight different *Fusarium* spp. from roots, rhizomes and pseudostem bases from only 15 Cavendish banana plants in two sites demonstrates the richness of *Fusarium* endophytes and compares favorably to results from earlier investigations involving fungal endophytes of banana. In Thailand, a total of 285 fungal endophytes belonging to 15 different genera were isolated from 49 banana plants of the cv. 'Pisang-Awak' (*Musa* spp. ABB) in nine sites (Niere, 2001). Of these, 79 were isolates of *Fusarium* species. High levels of endophytic fungal diversity from recently harvested plants of East African highland banana, which showed little or no weevil damage in western Uganda, have also been reported (Griesbach, 2000), while a similar study conducted on bananas in central America showed extensive endophytic fungal diversity (Pocasangre *et al*., 1999). Though the current study focused only on endophytic *Fusarium* spp. infecting Cavendish banana plants, the results obtained here and in earlier studies indicate that banana plants are a rich source of fungal endophytes. The role of endophytes in the host plant however remains largely unknown but may have an ecological and biological significance. It has been speculated that since endophytic fungi establish intimate relationships with their host plants, they may therefore, be effective in the management of the major banana pests and pathogens at the site of attack. Introduction of fungal endophytes into tissue culture derived banana plantlets may also be done to enhance the plant's vigor and provide protection against pests and diseases prior to field planting (Pereira *et al*., 1999; Griesbach, 2000; Niere, 2001; Sikora *et al*., 2003).

Fusarium oxysporum, *F. solani*, *F. semitectum* and *F. sacchari* were the most frequently isolated of *Fusarium* spp. from Cavendish bananas in South Africa. All three species can be fungal pathogens of agricultural crops, but only *F. oxysporum* is known to attack bananas (Booth, 1971). Non-pathogenic isolates of *F. oxysporum* have been reported as endophytes of banana before, and have been assessed for their ability to reduce damage caused by pathogenic isolates of *F. oxysporum* and nematodes (Niere, 2001; Pocasangre *et al*., 1999; Nel *et al*., 2006b). *Fusarium solani* and *F. semitectum* are pathogens of crops such as cotton and sorghum (Ciegler *et al*., 1982; Saubois *et al*., 1999), respectively whilst *F. sacchari* is a pathogen of sugarcane (Ganguly, 1964; Rao and Agnihotri, 2000). Because of the frequency of their isolation, and the tissue that they were isolated from, it is possible that these three

species may have important mutualistic relationships with banana. Interestingly, endophytic isolates of *F. sacchari* from sugarcane have been shown to inhibit development of the sugarcane borer moth *in vitro* (Mc Farlane and Rutherford, 2005). Since *F. sacchari* is non-pathogenic to banana, the role of this fungus in the plant remains unknown and requires further investigation.

In the current study, we obtained more isolates of *Fusarium* spp. from banana roots than from rhizomes or pseudostem bases. These results are in agreement with other studies in which roots appear to harbor more endophytic fungi than other plant parts (Niere, 2001), which may indicate that the roots are potentially colonized by fungi from the soil. *Fusarium oxysporum* was the dominant species found in the roots and *F. semitectum* and *F. sacchari* in rhizomes of Cavendish bananas. Similarly, *F. oxysporum* was found to be the most dominant species in roots and *F. semitectum* in rhizomes of the banana cv. 'Pisang-Awak' (Niere, 2001). Photita *et al*. (2001) reported presence of lower numbers of endophytic fungal isolates from pseudostems in comparison to the veins, interveins and midribs of wild banana plants in Thailand. Differences in the isolation frequencies and the interaction observed between *Fusarium* spp. and different plant parts indicate that endophytic *Fusarium* spp. might be adapted to different banana plant parts. Tissue specificity of endophytic fungi has been reported for conifer needles (Carroll and Carroll 1978), and may be due to the adaptation by particular endophytes to the micro-ecological and physiological conditions present in the different plant organs (Petrini, 1996). Tissue specificity may also be a reflection of tissue preferences of individual dominating taxa (Rodrigues and Samuels 1990). The observed tissue specificity of various *Fusarium* endophytes may be used in biological control programs to target establishment of the endophytes most suited to the plants parts that require protection.

PCR-RFLP analyses grouped endophytic *F. oxysporum* isolates obtained from banana roots from Ramsgate and Tzaneen into 9 IGS genotypes. Although PCR-RFLP may not be the most suitable method to study genetic diversity, it has been used in several studies to resolve genetic differences among *F. oxysporum* strains (Appel and Gordon 1995; Nel *et al*., 2006a). In the current investigation, the PCR-RFLP technique was useful in identifying genetic differences among *F. oxysporum* isolates obtained from the roots and aided in selecting representative isolates for further AFLP analyses. In previous investigations using IGS-RFLP, 120 isolates of *F. oxysporum* from roots and hypocotyls of beans were grouped into

four haplotypes (Alves-Santos *et al*., 1999). Similarly, Appel and Gordon (1995) grouped 56 isolates of *F. oxysporum* into 13 IGS haplotypes.

In the current study, IGS genotypes that occurred at highest frequencies contained isolates from both collection sites, while groups that consisted solely of isolates from either of the two sites occurred at low frequencies. The greater number of IGS genotypes found in Ramsgate, compared to those in Tzaneen, despite being isolated from fewer plants, indicates a greater genetic diversity within isolates from Ramsgate. One possible explanation for this finding is that, because bananas have been cultivated in southern KZN for longer than in Tzaneen, more non-pathogens might have developed a mutualistic relationship with bananas in KZN.

IGS genotype grouping as opposed to AFLP's did not reveal a clear-cut correlation between *F. oxysporum* isolates, and their geographic origin or plant part origin. Some isolates from one site were restricted to a single IGS genotype, and others from both sites (Ramsgate and Tzaneen) shared the same IGS groups. Isolates from a particular site that occurred in the same IGS groups were not necessarily from the same plant or plant part. In fact, our results showed that the influence of plant part origin on IGS genotype grouping was very minimal. Several researchers have reported conflicting results in this regard. For example, genetic variation between isolates of *F. oxysporum* f. sp. *elaidis* have been linked to geographic origins with isolates originating from a specific site grouping together (Flood *et al*., 1992), while there was no correlation between grouping and the geographic origin of 49 strains of *F. oxysporum* isolated from pea plants (Skovgaard *et al*., 2002). Sharing of IGS genotypes by isolates obtained from different sites has previously been reported before (Appel and Gordon, 1995). Since this study was on endophytic fungi in two separated regions and from plantations that were initiated from tissue culture banana plants, we can rule out the possibility of introduction of the endophytes with planting material or long distance dispersal of soil particles and fungal spores. Absence or limited long distance dispersal of conidia may restrict gene flow, allowing for populations to evolve independently, and thus contributing to development of discrete fungal populations (Appel and Gordon, 1994). Probably, isolates from the two sites, which shared IGS genotypes, represent clonal lineages that occur irrespective of the geographic distances separating them (Appel and Gordon, 1995).

AFLP analysis proved useful in separating 57 isolates of *Fusarium* into two distinct clades according to the species, with *F. oxysporum* and *F. sacchari* clearly separated from the *F. solani* clades. Using AFLP, Abdel-Satar *et al*. (2003) were able to resolve five different *Fusarium* spp. into five distinct clusters according to each species. AFLP analysis successfully grouped the *F. oxysporum* isolates according to the plant part origin. The correlation between *F. oxysporum* isolates and the plant part origin further indicates species tissue specificity. In the current study however, AFLP analysis was not efficient in correlating the isolates of *F. oxysporum* with their geographic origin .The only exception was observed with isolates of *F. oxysporum* from Kiepersol, which formed a distinct sub-clade within the *F. oxysporum* main clade as would be expected of endophytes that co-evolve with plants over time, and that are selected to persist and form compatible long-lasting associations with bananas. In a previous investigation involving *F. oxysporum* f. sp. *lentis* no correlation was observed between 32 isolates and their geographic origin (Belabid *et al*., 2004). The findings obtained in this study are difficult to explain, but may be due to differences in plantation age in the different sites. Banana cultivation in Kiepersol has been going on for longer periods of time than in Ramsgate and Tzaneen (Altus Viljoen pers. comm.) and this may explain why isolates from this site grouped together. In well-established agronomic systems, limited genetic diversity has been observed between plants and their endophytic counterparts possibly due to a constant environment, which ensures continual persistence of the association. However, short-term establishments for annual crops, as opposed to perennial crops such as banana, may prevent the formation of stable compatible plant-endophyte combinations (Saikkonen *et al*., 2004). The *F. solani* isolates, which grouped into two distinct clades probably represent phylogenetic species within the *F. solani* species complex (O'Donnell, 2000; Geiser *et al*., 2004).

Endophytic fungi have recently gained popularity as possible biological control agents of crop pests and diseases and may act in several ways; through metabolite production, competition for nutrients and ecological niches, and/or induced resistance (Sikora *et al*., 2003; Schulz and Boyle 2005). Whether variation in endophyte genotypes can be linked to biologically important traits, such as pathogenicity or aggressiveness against pests and diseases and variations in metabolite production remains to be determined (Woo *et al*., 1998). AFLP analysis has also been useful in generating molecular markers that are linked to genes of biological interest in plants such as barley (Karakousis *et al*., 2003) and the wheat fungus, *Mycosphaerella graminicola* (Kema *et al*., 2002) might therefore be useful in

developing molecular markers for studying the dynamics and persistence of introduced agents and in tracking of the isolates in plants.

The current study has demonstrated the diversity in *Fusarium* endophytes. We have shown that different *Fusarium* spp. may occur as endophytes in crops other than those that they are known to be pathogenic to, possibly establishing mutualistic associations with the host plants. Different endophytic *Fusarium* species appear to have preferences for certain plant parts. The potential for using endophytic *Fusarium* spp. as an alternative or complementary pest and disease control option in banana is promising and requires further investigation.

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Figure 1. Schematic representation of PCR-restriction fragment length polymorphism patterns after digestion of the intergenic spacer region of the ribosomal DNA of 46 isolates of endophytic *Fusarium oxysporum* with the restriction enzymes *Hae*III, *Msp*I, *Rsa*I and *Hinf*I.

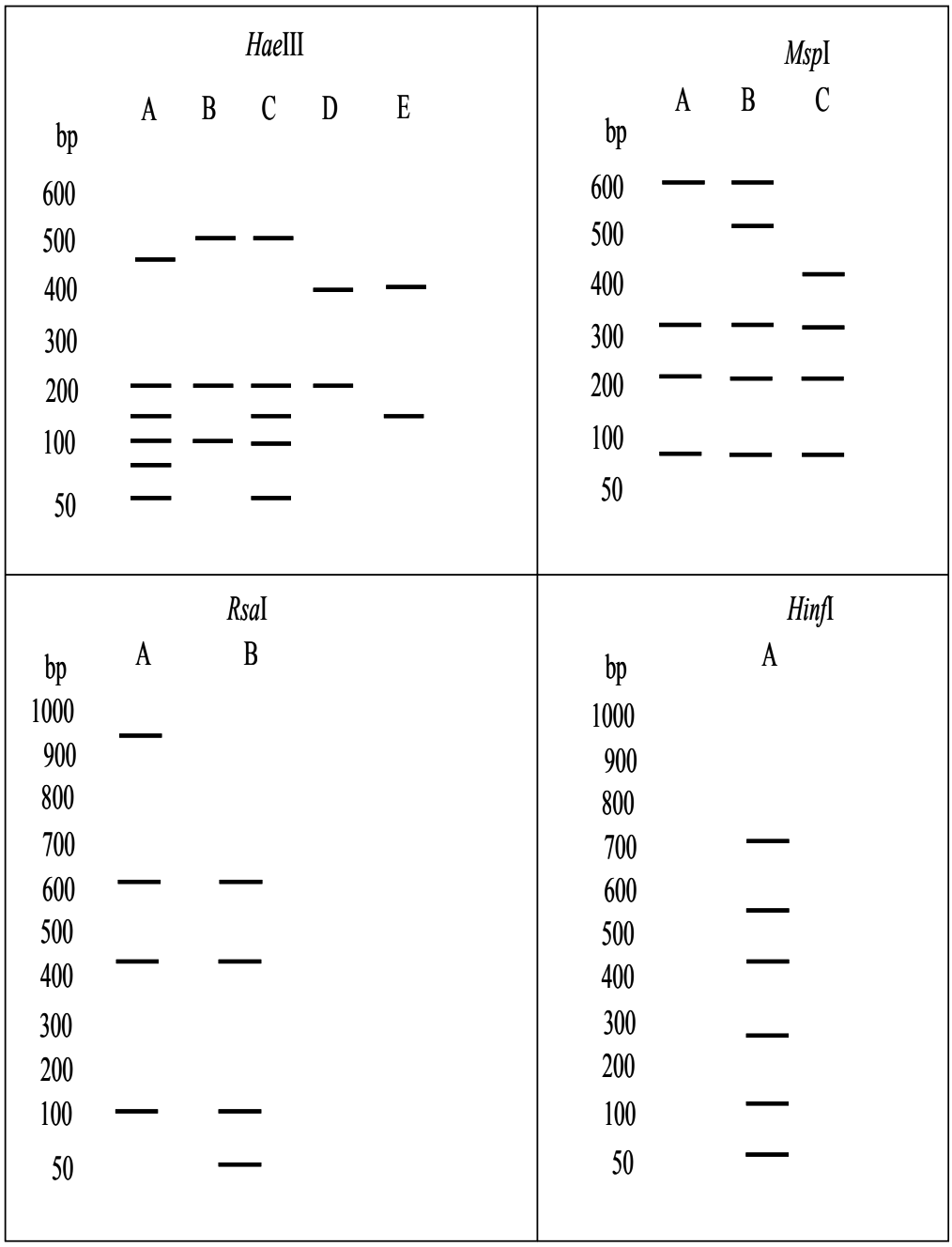

Figure 2. Phylogram inferred from amplified fragment length polymorphism analysis showing genetic relationship of 57 endophytic Fusarium isolates from banana root, rhizome and pseudostem base tissues. Distance analysis included neighbor joining as a tree building algorithm and a bootstrap of 1000 replicates. Bootstrap values are indicated above nodes, values less than 50% are not shown. The Fusarium isolates were obtained from Tzaneen, Ramsgate, and Kiepersol in South Africa and are designated with the prefix SAK, SAT, and KIP respectively

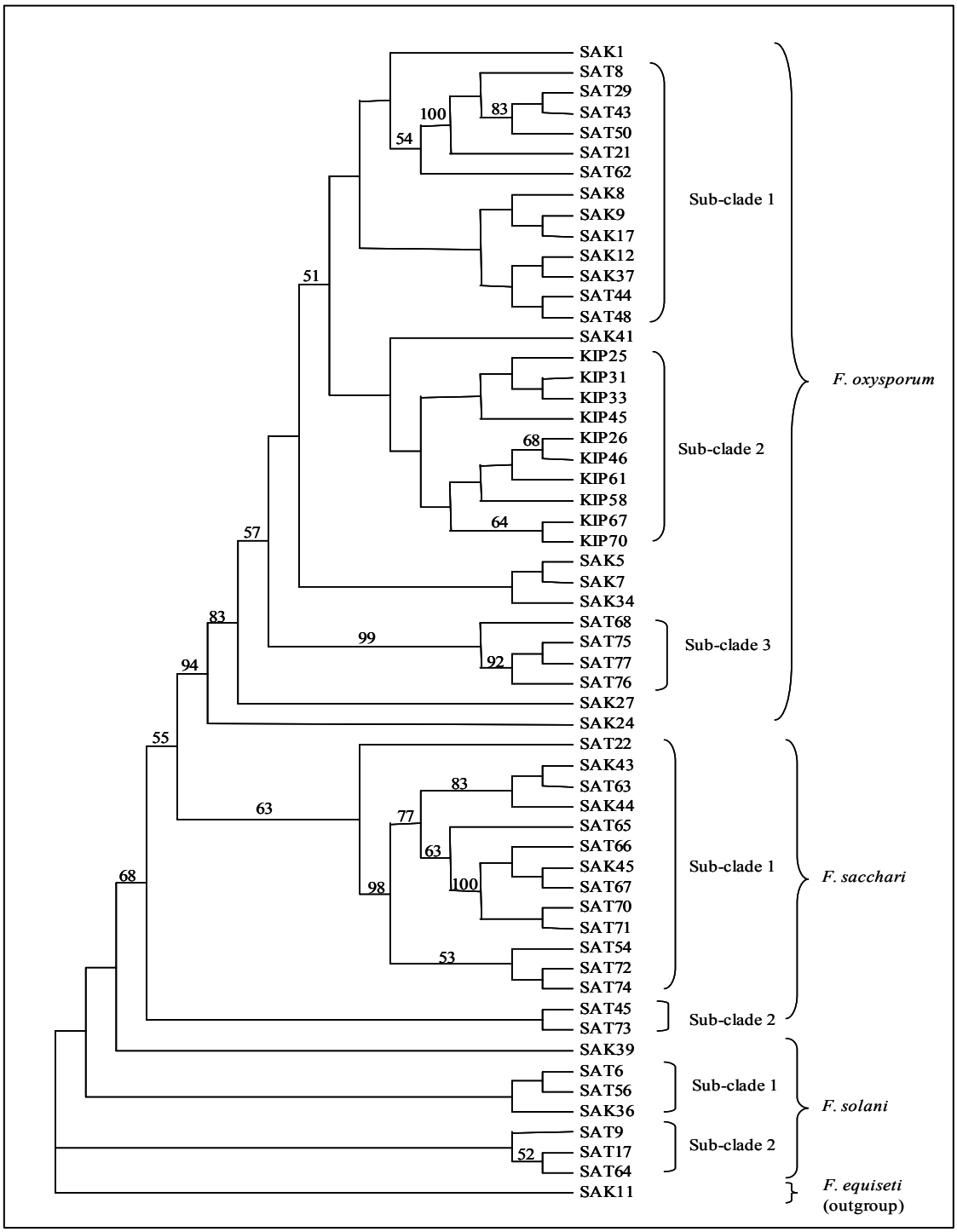

Table 1. Geographic origin of 57 endophytic isolates of *Fusarium* spp. from roots, rhizomes and pseudostem base tissues of Cavendish banana plants (*Musa* spp. AAA) in South Africa used for amplified fragment length polymorphism analysis.

Collectors no.[a]	CAV no.[b]	Species	GenBank accession number	Geographic origin[c]	Plant tissue
KIP25	CAV 542	*F. oxysporum*	DQ465926	Kiepersol	Root
KIP26	CAV 543	*F. oxysporum*		Kiepersol	Root
KIP31	CAV 546	*F. oxysporum*		Kiepersol	Root
KIP33	CAV 548	*F. oxysporum*		Kiepersol	Root
KIP45	CAV 552	*F. oxysporum*	DQ465927	Kiepersol	Root
KIP46	CAV 553	*F. oxysporum*		Kiepersol	Root
KIP58	CAV 559	*F. oxysporum*		Kiepersol	Root
KIP61	CAV 561	*F. oxysporum*		Kiepersol	Root
KIP67	CAV 563	*F. oxysporum*		Kiepersol	Root
KIP70	CAV 566	*F. oxysporum*		Kiepersol	Root
SAK1	CAV 722	*F. oxysporum*		Ramsgate	Root
SAK12		*F. oxysporum*		Ramsgate	Root
SAK17		*F. oxysporum*		Ramsgate	Root
SAK24	CAV 735	*F. oxysporum*	DQ465929	Tzaneen	Outer rhizome
SAK27		*F. oxysporum*		Ramsgate	Root
SAK34		*F. oxysporum*		Ramsgate	Root
SAK37		*F. oxysporum*		Ramsgate	Root
SAK41	CAV 754	*F. oxysporum*		Ramsgate	Root
SAK5	CAV 725	*F. oxysporum*	DQ465928	Ramsgate	Root
SAK7	CAV 727	*F. oxysporum*		Ramsgate	Root
SAK8	CAV 728	*F. oxysporum*		Ramsgate	Root
SAK9		*F. oxysporum*		Ramsgate	Root
SAT21	CAV 761	*F. oxysporum*		Tzaneen	Root
SAT29	CAV 766	*F. oxysporum*		Tzaneen	Root
SAT43	CAV 771	*F. oxysporum*		Tzaneen	Root
SAT44	CAV 772	*F. oxysporum*		Tzaneen	Root
SAT48	CAV 774	*F. oxysporum*	DQ465930	Tzaneen	Root
SAT50	CAV 776	*F. oxysporum*		Tzaneen	Root
SAT62	CAV 784	*F. oxysporum*		Tzaneen	Root
SAT68	CAV 189	*F. oxysporum*		Tzaneen	Inner rhizome
SAT75		*F. oxysporum*	DQ465931	Tzaneen	Pseudostem base
SAT76		*F. oxysporum*	DQ465932	Tzaneen	Pseudostem base
SAT77		*F. oxysporum*	DQ465933	Tzaneen	Pseudostem base
SAT8	CAV 750	*F. oxysporum*		Tzaneen	Root
SAK43		*F. sacchari*	DQ465944	Ramsgate	Inner rhizome
SAK44	CAV 1673	*F. sacchari*	DQ465943	Ramsgate	Pseudostem base
SAK45		*F. sacchari*	DQ465945	Tzaneen	Pseudostem base
SAT22	CAV 762	*F. sacchari*	DQ465934	Tzaneen	Root
SAT54	CAV 778	*F. sacchari*	DQ465935	Tzaneen	Outer rhizome
SAT63	CAV 1665	*F. sacchari*	DQ465936	Tzaneen	Inner rhizome
SAT65	CAV 1666	*F. sacchari*	DQ465937	Tzaneen	Pseudostem base
SAT66	CAV 1667	*F. sacchari*	DQ465938	Tzaneen	Pseudostem base

SAT67	CAV 1668	*F. sacchari*	DQ465939	Tzaneen	Inner rhizome
SAT71	CAV 1671	*F. sacchari*	DQ465941	Tzaneen	Inner rhizome
SAT72	CAV 1672	*F. sacchari*	DQ465942	Tzaneen	Inner rhizome
SAT73		*F. sacchari*	DQ465946	Tzaneen	Inner rhizome
SAT74		*F. sacchari*	DQ465947	Tzaneen	Pseudostem base
SAT70	CAV 1670	*F. sacchari*	DQ465940	Tzaneen	Inner rhizome
SAK36	CAV 740	*F. solani*	DQ465948	Ramsgate	Root
SAK39	CAV 742	*F. solani*	DQ465949	Ramsgate	Root
SAT17	CAV 759	*F. solani*	DQ465952	Tzaneen	Root
SAT45	CAV 773	*F. solani*		Tzaneen	Root
SAT56	CAV 780	*F. solani*	DQ465953	Tzaneen	Root
SAT6	CAV 748	*F. solani*	DQ465950	Tzaneen	Root
SAT64		*F. solani*	DQ465954	Tzaneen	Pseudostem base
SAT9	CAV 751	*F. solani*	DQ465951	Tzaneen	Root
SAK11	CAV 729	*F. equiseti*	DQ465925	Ramsgate	Outer rhizome

[a] Collectors numbers designates the isolate code given after primary isolation.

[b] CAV numbers designate codes for the fungal cultures deposited in the FABI culture collection, University of Pretoria, Pretoria, South Africa.

[c]Geographic site from which banana plants were sampled; endophytic *F. oxysporum* isolates from Kiepersol were not isolated in the current study but were obtained from the FABI culture collection.

Table 2. Incidence (%) of endophytic *Fusarium* spp. isolated from different plant parts of Cavendish bananas (*Musa* spp. AAA) in Ramsgate and Tzaneen, South Africa.

Species	Plant part									Geographic origin						Total[b]	
	Root			Rhizomes[a]			Pseudostem bases			Ramsgate			Tzaneen				
	n	%		n	%		n	%		n	%		n	%		n	%
F. oxysporum	45	32.8	a	3	2.1	a	3	2.1	a	24	17.1	a	28	20.0	c	52	37.4
F. solani	23	16.4	b	8	5.7	a	3	2.1	a	7	5.0	b	27	19.2	c	34	24.2
F. sacchari	1	0.7	c	5	3.6	a	8	5.7	a	2	1.4	b	12	8.5	ab	14	10.0
F. semitectum	0	0.0	c	10	7.1	a	5	3.6	a	5	3.5	b	10	7.1	ab	15	10.7
F. subglutinans	1	0.7	c	2	1.4	a	8	5.7	a	1	0.7	b	9	6.4	ab	10	7.1
F. dimerum	10	7.1	b	1	0.7	a	0	0.0	b	1	0.7	b	10	7.1	a	11	7.8
F. equiseti	0	0.0		3	2.1	a	0	0.0	b	2	1.4	b	1	0.7		3	2.1
F. proliferatum	0	0.0		0	0.0		1	0.7		0	0.0		1	0.7		1	0.7
Total	80	57.7		32	22.7		28	19.9		42	29.8		98	69.7		140	100

Within each plant part or site, incidence (isolation frequencies) of the different *Fusarium* spp. followed by the same letter are not statistically different at P<0.0051 after the Dunn Sidak correction.

Table 3. Intergenic spacer (IGS) region groups of 46 *Fusarium oxysporum* isolates from Cavendish banana (*Musa* spp. AAA) roots from two banana growing regions in South Africa that were subjected to PCR-restriction fragment length polymorphism analysis with four restriction enzymes *Hae*III, *Msp*I, *Rsa*I and *Hinf*I.

		Percentage[b]	Restriction enzyme			
IGS group	Isolates[a]		*Hae*III	*Msp*I	*Rsa*I	*Hinf*I
1	SAK1*, 3, 4, 16, 20, 22, 27*, 30, 35 & 37 SAT 2, 10, 16, 19, 21*, 28, 29*, 31, 34, 41, 43*, 49, 50*, 52, 61 & 62*	56.5	A	A	A	A
2	SAK 12* & 14	4.3	A	A	B	A
3	SAK 8* & SAT 8*	4.3	A	B	A	A
4	SAK 2, 5 & 15*	6.5	B	A	A	A
5	SAK 7*, 17*, 23 & 24* SAT 3, 14 & 48*	15.2	B	A	B	A
6	SAK 6 & 9*	4.3	C	B	A	A
7	SAK 41*	2.1	D	A	A	A
8	SAK 34*	2.1	D	A	B	A
9	SAT 12 & 44*	4.3	E	C	A	A

[a]Designation of isolates used in the PCR-RFLP analysis. Isolates with the prefix SAK were isolated from Ramsgate, SATfrom Tzaneen. *Fusarium oxysporum* isolates with an asterisk were selected from each IGS genotype for AFLP analysis.

[b] Percentage of total isolates in that particular IGS genotype

Summary

Radopholus similis is one of the key pests of banana in the East African highlands. Nematode damage results in large crop and yield losses. Although control of this pest had mainly relied on the use of clean planting material, re-infestation of plants in the field remains a critical concern. Alternative management options such as the use of fungal endophytes may be used to provide protection and extend plant life. In this thesis, the objective was to identify endophytic isolates of *Fusarium* spp. and especially *F. oxysporum* with potential for *R. similis* control in tissue culture banana plantlets and to study the interactions between the host plant, nematode and the endophyte. Isolates were screened both *in vitro* and *in vivo* and the most promising isolates were studied further to elucidate when, where and how the endophytes suppressed *R. similis*.

In the first phase of this research, a total of 35 endophytic *Fusarium* spp. isolates were screened for the production of secondary metabolites antagonistic to *R. similis in vitro*. All isolates tested demonstrated some level of *in vitro* antagonistic activity on the mobility of *R. similis* males, females and juveniles. The percentage of immobilized nematodes increased with increase in the length of exposure time and the concentration of the culture filtrates. An interesting finding of this research was that male *R. similis* were more sensitive to culture filtrate treatment than females. The method of using culture filtrates to identify isolates with antagonistic effects against the nematode has the advantage of being rapid, thus saving time and allows for selection of the most promising isolates for *in planta* evaluation. The actual metabolites produced by the isolates in culture were not determined in this study and warrant further investigations.

In the second part of this research, nine *F. oxysporum* isolates that showed good *in vitro* antagonistic activity against *R. similis* were screened for nematode suppression in tissue culture banana plants. A local banana cultivar susceptible to *R. similis* was used in nine screen house experiments. Endophyte treatment did not affect nematode population densities significantly although although there was a tendency for nematode populations in endophyte-treated plants to be lower than in the untreated control plants. Banana plant growth and nematode damage were also not influenced by endophyte treatment except in a few

experiments. The results obtained showed the effect of individual isolates on plant growth, nematode damage and density varied widely across repeat experiments. Despite the inconsistencies, three isolates, *V5W2*, *Eny1.13i* and *Eny7.11o* were identified as the most effective isolates. The reproducibility of the results obtained is questionable as control plants were also found to be infected by endophytic *F. oxysporum*. This research also raises a big question pertaining to the ability to monitor and track re-isolated endophytes. Thus proper endophyte marking techniques need to be developed for future experiments to compare the re-isolated endophytes with the original ones. Although the levels of nematode control afforded by endophytes may not be very high, the use of endophytes in tissue culture plants may complement the clean nature of the plants thereby boosting their ability to resist or tolerate nematode attack in the field.

The endophytes tested during the current study have shown ability to suppress *R. similis* in tissue culture banana plants. However, it is not known how, where and when the endophytes are most effective against the nematodes. In the third part of this research, the effects of three isolates, *V5W2*, *Eny1.31i* and *Eny7.11o* on the host preferences, root penetration and reproduction of *R. similis* in tissue culture banana plants were evaluated under laboratory and screen house conditions. Host preference tests provided evidence that nematodes were equally attracted to both endophyte-treated and untreated plants. Similarly, root penetration of banana plant roots by *R. similis* did not differ between endophyte-treated and untreated plants. Nematode reproduction which was assessed over a period coinciding with three nematode generations was however, influenced by endophyte treatment. Differences were observed during the second and third nematode generations with nematode reproduction rates being higher in the untreated compared to endophyte-treated plants. The results obtained demonstrate that the mechanism by which the fungal isolates control *R. similis* in the plant is mainly post-infectional, acting only after the plants have been infected and may possibly be through induced resistance in the plant against the nematode.

In the fourth part of this research, potential mechanism(s) through which endophytic isolates of *F. oxysporum* result in nematode suppression in tissue culture banana plants were studied. Nine isolates of *F. oxysporum* showed positive results for protease enzyme production but none showed chitinase and lipase activity. In split-root experiments, the densities of *R. similis* females, males, juveniles and the total nematode density were substantially lower in one half

of the split-root systems when the corresponding half was treated with one of the endophytic *F. oxysporum* isolates *V5W2*, *Eny1.131i* or *Eny7.11o*. To further investigate the role of induced resistance in nematode management by endophytes, the amounts and types of phenolic compounds were assessed in plants inoculated with isolate *V5W2*. Histological analysis showed the presence of phenolic cells in the cortex and central cylinder regions of roots and rhizomes. Although constitutive phenols occurred both in roots and corms of plants treated with isolate *V5W2* as well as untreated plants, the quantities increased over time in the endophyte-inoculated plants compared to the uninoculated plants. Higher amounts of total soluble phenolics were found in endophyte-treated than in untreated plants. Corm and root metabolite profiles of endophyte-treated and untreated plants did not differ. HPLC analysis revealed four major unidentified compounds, which were present in endophyte-treated as well as untreated plants. These results demonstrate that induction of systemic resistance by endophytic isolates of *F. oxysporum* may play a role in *R. similis* management and that treatment of plants with endophytes triggers the plant to increase synthesis of phenolic compounds. Further research needs to be conducted to identify the unknown compounds.

In the last part of this research, endophytic *Fusarium* spp. from roots, rhizomes and pseudostem bases of apparently healthy Cavendish banana plants from three sites in South Africa were assessed using morphological and PCR-RFLP and AFLP analyses. The objectives were to identify *Fusarium* endophytes in banana plants, study their relation to specific plant parts and determine the genetic diversity within and between the endophytes. From the isolations, *Fusarium oxysporum* was the predominant species isolated, followed by *Fusarium solani* and *Fusarium semitectum*. Tissue specificity was observed with *F. oxysporum* and *F. solani* being predominantly isolated from the roots whilst *F. semitectum, F. sacchari* and *F. subglutinans* were predominantly isolated from rhizomes and pseudostem bases. The *F. oxysporum* isolates from the roots were grouped into nine genotypes using PCR-RFLP analysis of the IGS region of the rDNA. Distance analysis of AFLP data of 57 *Fusarium* isolates resolved the isolates into two major clades: one consisting of the isolated *F. oxysporum* and the other of *F. sacchari*. *Fusarium oxysporum* isolates further grouped according to plant part origin while the *F. sacchari* and *F. solani* isolates grouped randomly. The results obtained demonstrated tissue specificity of endophytic *Fusarium in* Cavendish banana and also a wide inter- and intraspecific genetic variation among endophytic *Fusarium* isolates of banana in South Africa. The observed tissue specificity of various *Fusarium*

endophytes may be used in future biological control programs to target establishment of the endophytes most suited to the plants parts that require protection.