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.

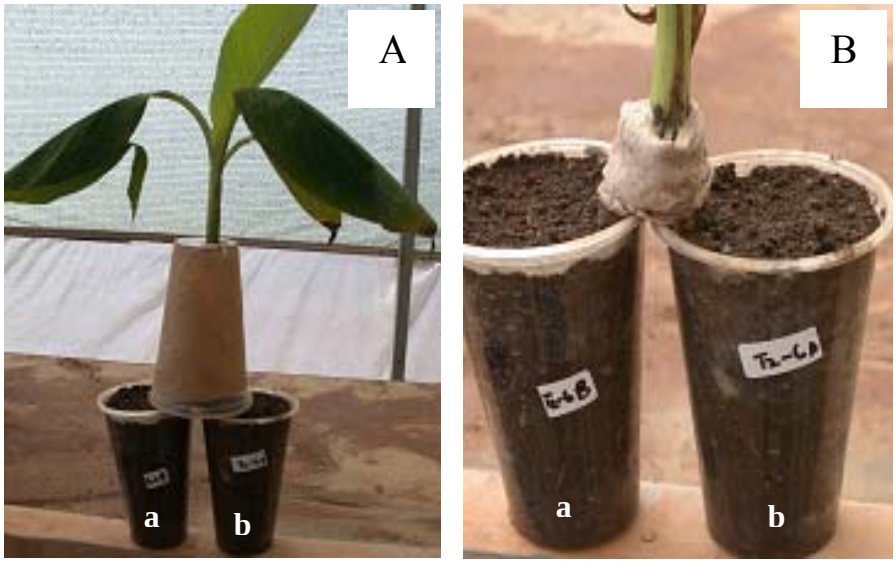


Figure 2. Protease activity of endophytic *Fusarium oxysporum* (isolate Eny1.31i) in a 65-mm-diameter Petri dish 3 days after inoculation on gelatine-amended medium. The clear zone (halo) indicates positive protease activity.

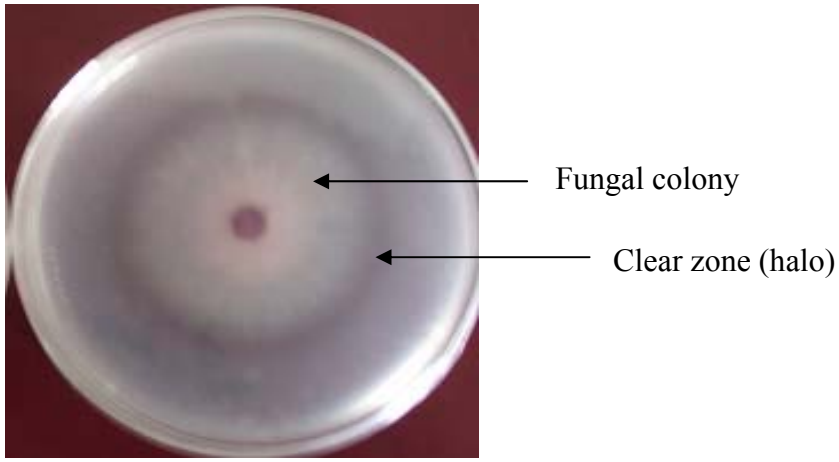
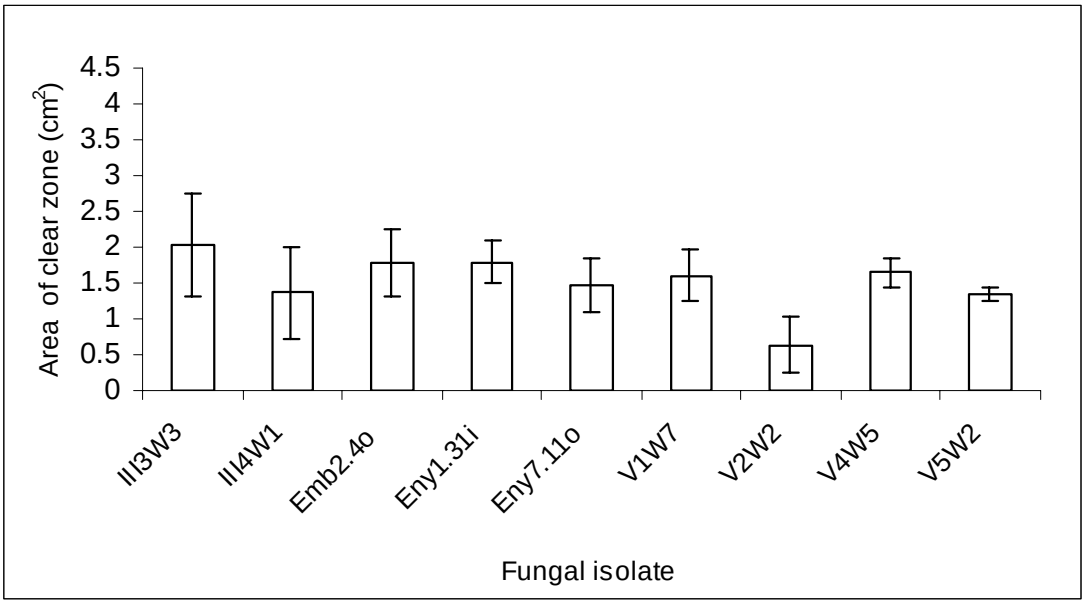
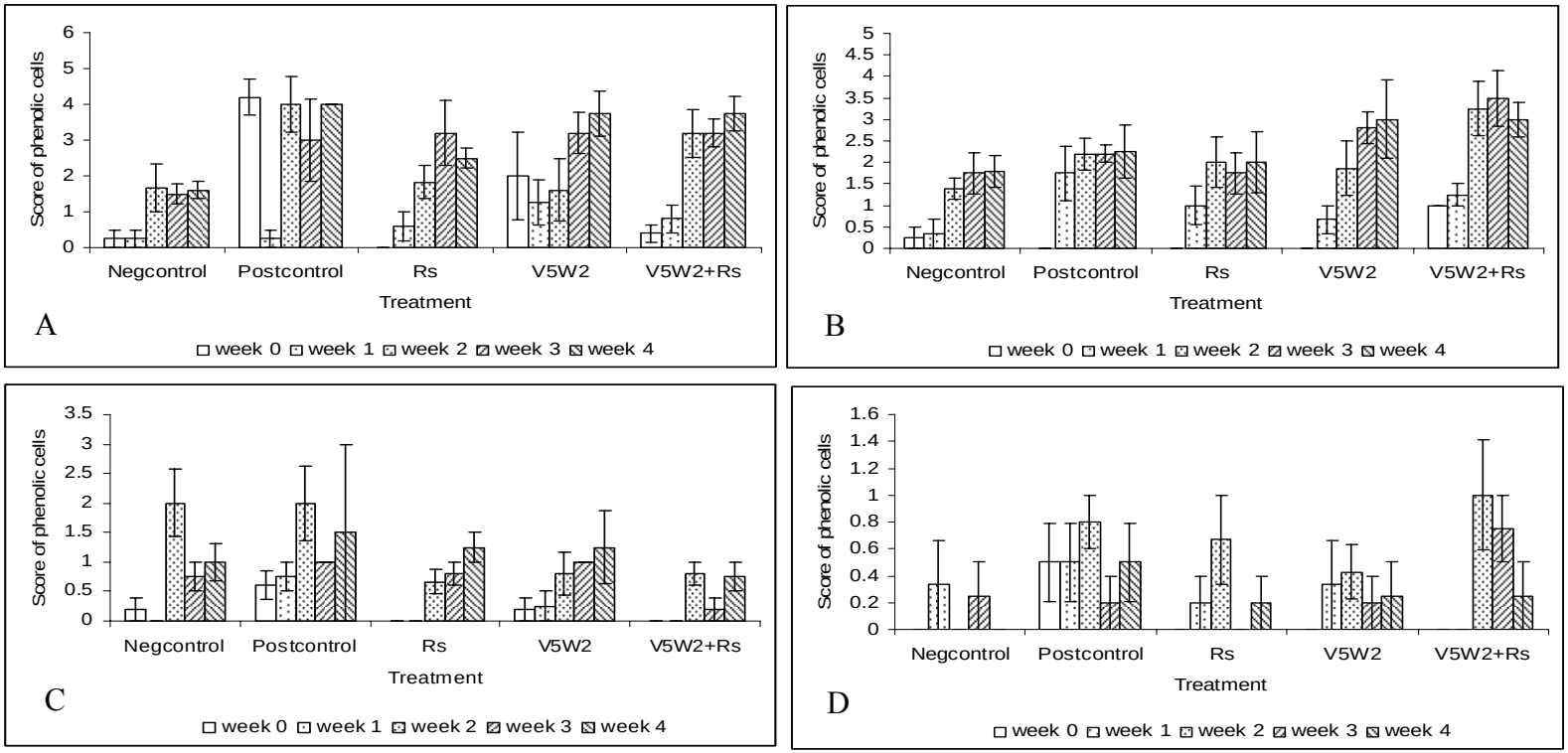


Figure 3. Level of protease activity exhibited by nine endophytic *Fusarium oxysporum* isolates 3 days after inoculation on gelatine-amended medium. The levels of protease activity were estimated using the diameter of the halo zone (cm) compared to the fungal colony diameter.



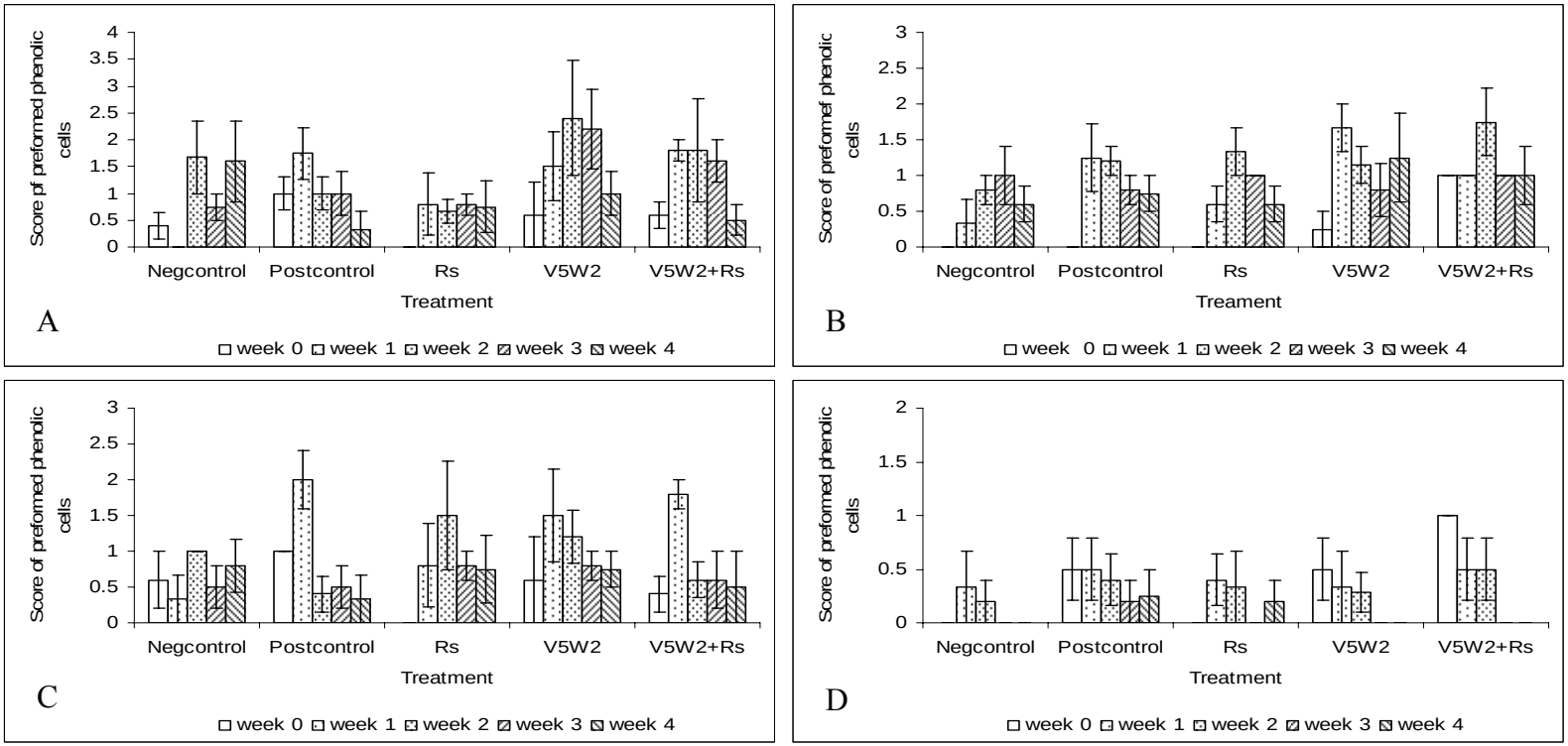
Error bars represent standard errors of the mean (n=5).

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).

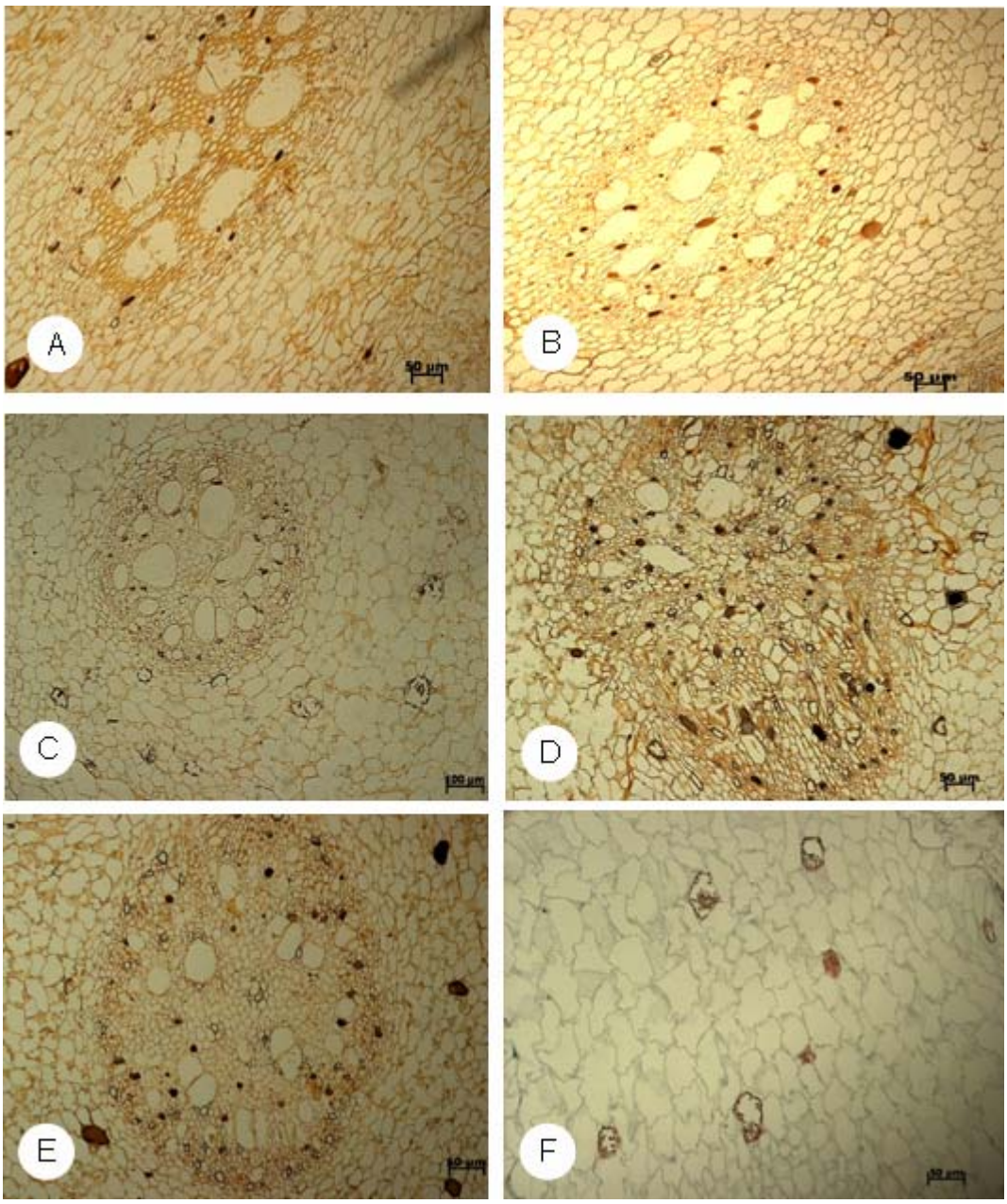
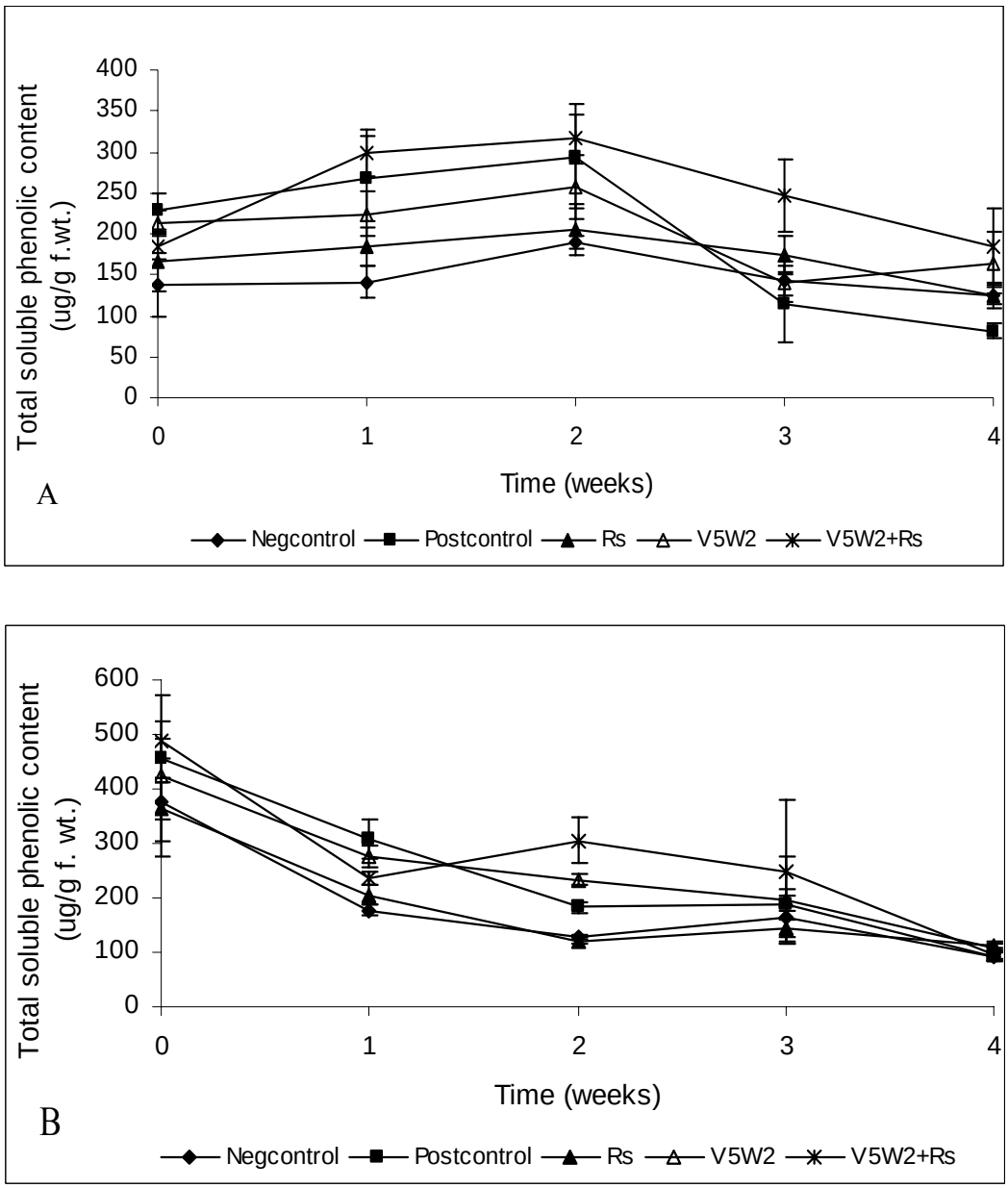


Figure 7. Total soluble phenolics (µg equivalent gallic acid/g fresh weight) in rhizomes (A) and roots (B) of banana plants treated with sterile millet seed (negative control), 50 mM K_2HPO_4 (positive control), *Radopholus similis* only, *Fusarium oxysporum* isolate *V5W2* only, and *V5W2* and *Radopholus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

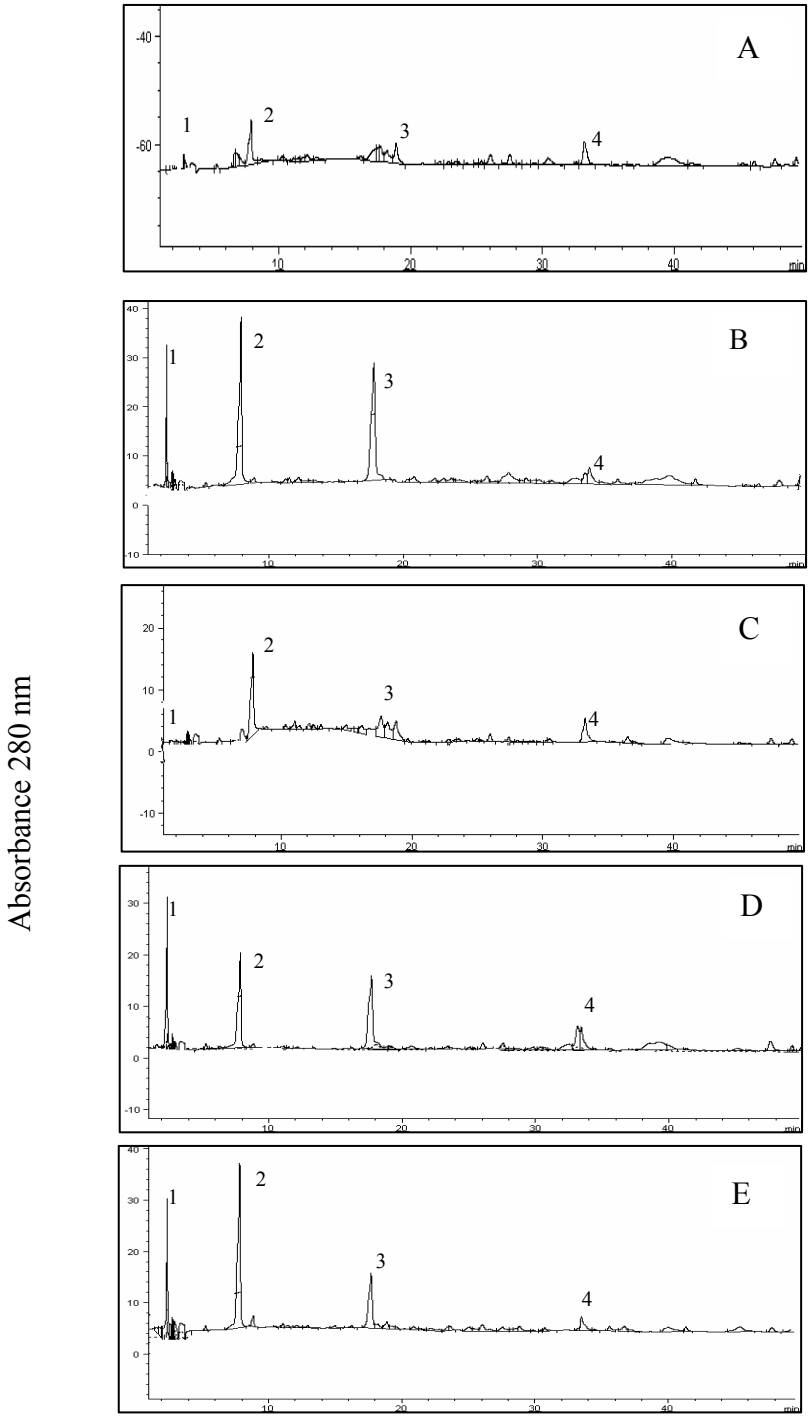
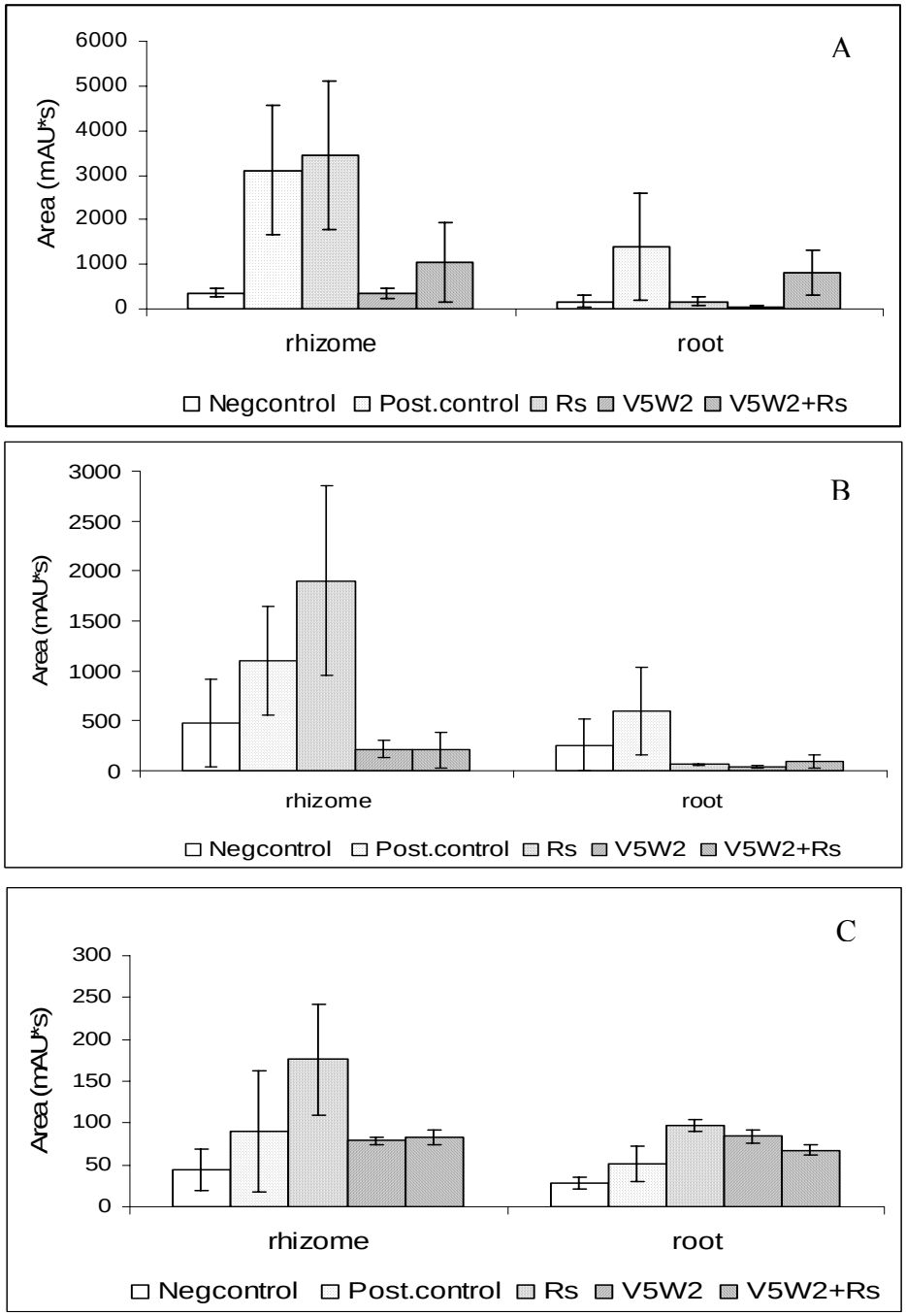


Figure 9. Three unknown compounds in rhizome and root extracts of tissue culture banana plants 2 weeks after inoculation with the endophytic *Fusarium oxysporum* isolate *V5W2*, and 1 week after inoculation with *Radopholus similis*. (A, unknown compound 2; B, unknown compound 3 and C; unknown compound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

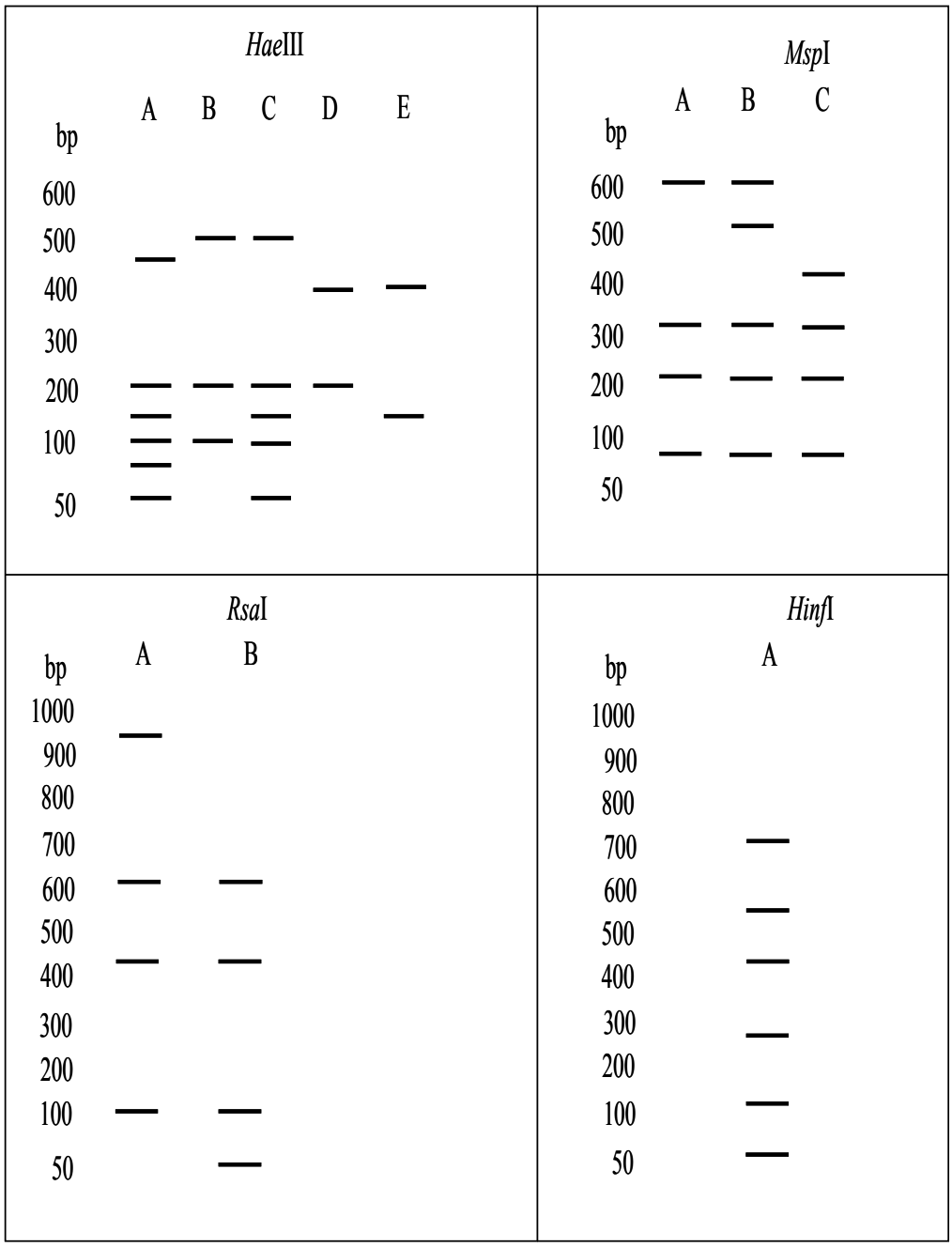

Figure 2. Phylogram inferred from amplified fragment length polymorphism analysis showing genetic relationship of 57 endophytic Fusarium isolates from banana root, rhizome and pseudostem base tissues. Distance analysis included neighbor joining as a tree building algorithm and a bootstrap of 1000 replicates. 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

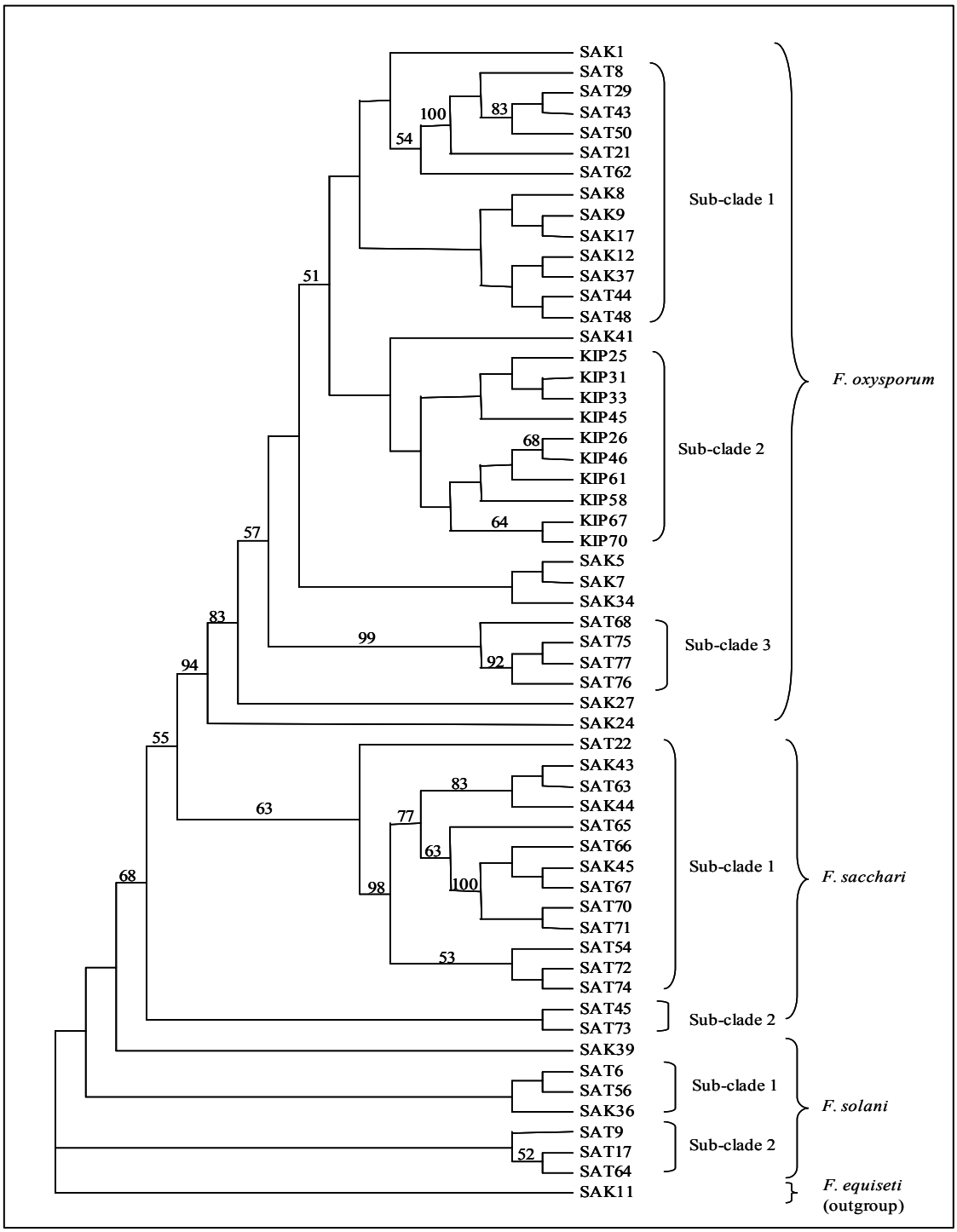

Table 1. Geographic origin of 57 endophytic isolates of *Fusarium* spp. from roots, rhizomes and pseudostem base tissues of Cavendish banana plants (*Musa* spp. AAA) in South Africa used for amplified fragment length polymorphism analysis.

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.