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.

.

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.

References

Abdel-Satar, MA., Khalil, M.S., Mohamed, I.N., Abd-Esalam, K.A. and Verreet, J.A. (2003). Molecular phylogeny of *Fusarium* species by AFLP fingerprint. African Journal of Biotechnolology 2: 51-55.

Alves-Santos, F.M., Benito, E.P., Elsava, A.P. and Diaz-Minguez, J.M. (1999). Genetic diversity of *Fusarium oxysporum* strains from common bean fields in Spain. Applied and Environmental Microbiology 65: 3335-3340.

Appel, D.J. and Gordon, T.R. (1994). Local and regional variation in populations of *Fusarium oxysporum* from agricultural fields. Phytopathology 84: 786-791.

Appel, D.J. and Gordon, T.R. (1995). Intraspecific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. Experimental Mycology 19: 120-128.

Azevedo, Jr., J.L., Walter, M., Pereira, J.O. and Araújo, W.L. (2000). Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electronic Journal of Biotechnology 3: 40-65.

Belabid, L., Baum, M., Fortas, Z., Bouznad, Z. and Eujayl, I. (2004). Pathogenic and genetic characterization of *Fusarium oxysporum* f. sp. *lentis* by RAPD and AFLP analysis. African Journal of Biotechnology 3: 25-31.

Booth, C. (1971). "The genus Fusarium". Commonwealth Mycological Institute, Surrey England. p237.

Brown, K.B., Hyde, K.D. and Guest, D.L. (1998). Preliminary studies on endophytic fungal communities of *Musa acuminata* species complex in Hong Kong and Australia. Fungal Diversity 1: 27-51.

Carroll, G.C. and Carroll, F.E. (1978). Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. Canadian Journal of Botany 56: 3034-3043.

Carroll, G.C., (1988). Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69: 2-9.

Daneel, M., de Jager, K., van den Berg, I., Desmet, M. and De Waele, D. (2004). Occurrence of nematodes on common cultivars of banana in South Africa. Abstracts, 1st International Congress on Musa: harnessing research to improve livelihoods, Penang, Malaysia. p118.

de Graaf, J., Viljoen, A. and Govender, P. (2004). Efficacy of pseudostem and pheromone traps against *Cosmopolites sordidus* (Germar) in South Africa. Abstracts, 1st

International Congress on *Musa*: harnessing research to improve livelihoods, Penang, Malaysia. p140.

Ciegler, A., Klich, M. and Lee, L. (1982). Inhibition of expression of *Fusarium semitectum* and other fungi in immature cottonseed. Applied and Environmental Microbiology 44: 351-354.

Edel, V., Steinberg, C., Avelange, I., Laguerre, G. and Alabouvette, C. (1995). Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains. Phytopathology 85: 579-585.

Elias, K.S., Schneider, R.W. and Lear, M.M. (1991). Analysis of vegetative compatibility groups in nonpathogenic populations of *Fusarium oxysporum* isolated from symptomless tomato roots. Canadian Journal of Botany 69: 2089-2094.

Felsenstein, J. (1985). Confidence intervals of phylogenetics; an approach for using bootstrap. Evolution 39: 783-791.

Fisher, P.J. and Petrini, O. (1992). Fungal saprobes and pathogens as endophytes of rice (*Oryza sativa* L.). New Phytologist 120: 137-143.

Fisher, P.J., Petrini, O. and Lappin Scott, H.M. (1992). The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). New Phytologist 122: 299-305.

Flood, J., Whitehead, D.S. and Cooper, R.M. (1992). Vegetative compatibility and DNA polymorphisms in *Fusarium oxysporum* f. sp. *elaidis* and their relationship to isolate virulence and origin. Physiological and Molecular Plant Pathology 41: 201-215.

Ganguly, A. (1964). Wilt. In: Hughes, C.G., Abbott, E.V. and. Wismer, C.A. (Eds). Sugarcane diseases of the world, Vol 2. Elsevier Publishing Company, Amsterdam, Netherlands. pp. 131-137.

Geiser, D.M., Jimenez-Gasco, M.D., Kang, S.C., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A., and O'Donnell, K. (2004). FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. European Journal of Plant Pathology 110: 473-479.

Gold, C.S. and Dubois, T. (2005). Novel application methods for microbial control products: IITA's research against banana weevil and burrowing nematode. Biocontrol News and Information 26N: 86-89.

Gordon, T.R. and Okamoto, D. (1991). Variation within and between populations of *Fusarium oxysporum* based on vegetative compatibility and mitochondrial DNA. Canadian Journal of Botany 70: 1211-1217.

Griesbach, M. (2000). Occurrence of mutualistic fungal endophytes in bananas (*Musa* species) and their potential as biocontrol agents of the banana weevil *Cosmopolites sordidus* (Germar) (Coleoptera: Cuculionidae) in Uganda. PhD thesis, University of Bonn, Bonn, Germany. p94.

Groenewald, S., Van den Berg, N., Marasas, W.F.O. and Viljoen, A. (2006). The application of high- throughput AFLP's in assessing genetic diversity in *Fusarium oxysporum* f. sp. *cubense*. Mycological Research 110: 397-305.

Hallman, J. and Sikora, R.A. (1994). Influence of *Fusarium oxysporum*, a mutualistic fungal endophyte on *Meloidogyne incognita* infection of tomato. Journal of Plant Disease and Protection 101: 475-481.

INIBAP, (2003). Just how far are bananas from extinction? INIBAP publication http://www.futureharvest.org/pdf/Banana_final.pdf.

Karakousis, A., Gustafor, J.P., Chalmers, K.J., Barr, A.R. and Langridge, P. (2003. A consensus map of barley integrating SSR, RFLP and AFLP markers. *Australian* Journal of Agricultural Research 54: 1173-1183.

Kema, G.H.J., Goodwin, S.B., Hamza, S., Verstappen, E.C.P., Cavaletto, J.R., Van der Lee, T.A.J., de Weerdt, M., Bonants, P.J.M. and Waalwijk, C. (2002). A combined amplified fragment length polymorphism and randomly amplified polymorphism DNA genetic linkage map of *Mycosphaerella graminicola*, the Septoria Tritici leaf blotch pathogen of wheat. Genetics 161: 1497-1505.

Latch, G.C.M. (1993). Physiological interactions of endophytic fungi and their hosts. Biotic stress tolerance imparted to grasses by endophytes. Agriculture, Ecosystems and Environment 44: 143-156.

Mc Farlane, S.A. and Rutherford, R.S. (2005). *Fusarium* species isolated from sugarcane in KwaZulu-Natal and their effect on *Eldana saccharina* (Lepidoptera-Pyralidae) development *in vitro*. In: Abstracts 79th Annual South Africa Sugar Technologists Association (SASTA) congress, South Africa, 19-22 July .

Moore, N.Y., Bentley, S., Legg, K.G. and Jones, D.R. (1995). Fusarium wilt of banana. Musa Disease Fact Sheet N°. 5. INIBAP, Montpellier, France.

Mourichon, X., Carlier, J. and Fourié, E. (1997). Sigatoka leaf spot diseases. Musa Disease Fact Sheet N°. 8. INIBAP, Montpellier, France.

Myburg, A.A., Remington, D.L., O'Malley, D.M., Sederoff, R.R. and Whetten, R.W. (2001). High throughput AFLP analysis using infrared dye-labelled primers and an automated DNA sequencer. Biotechniques 30: 348-357.

Nel, B., Steinberg, C., Labuschagne, N. and Viljoen, A. (2006a). Isolation and characterization of non-pathogenic *Fusarium oxysporum* isolates from the rhizosphere of healthy banana plants. Plant Pathology 55: 207-216.

Nel, B., Steinberg, C., Labuschagne, N. and Viljoen, A. (2006b). The potential of non-pathogenic *Fusarium oxysporum* and other biological control organisms for suppressing Fusarium wilt of banana. Plant Pathology 55: 217-223.

Nelson, P.E. and Toussoun, T.A. (1986). Isolating, identifying and producing inoculum of pathogenic species of *Fusarium*. In: Hickey, K.D. (Ed). Methods of evaluating pesticides for control of plant pathogens. American Phytopathological Society Press. St Paul, MN. pp.54-59.

Nelson, P.E., Toussoun, T.A. and Marasas, W.F.O. (1983). *Fusarium* species. *An illustrated manual for identification*. The Pennyslvania State University Press, Pennyslvania. p193.

Niere, B. (2001). Significance of nonpathogenic isolates of *Fusarium oxysporum* Schlecht: Fries for the biological control of the burrowing nematode *Radopholus similis* (Cobb) Thorne on tissue cultured banana. PhD thesis. University of Bonn, Bonn, Germany. p118.

O'Donell, K. (2000). Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex**.** Mycologia 92**:** 919–938.

O'Donell, K., Kistler, H.C., Cigelnik, E. and Ploetz, R.C. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana. Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy for Sciences, USA. 95:2044-2049.

Pereira, J.O., Carneiro-Vieira, M.L. and Azavedo, J.L. (1999). Endophytic fungi from *Musa acuminata* and their reintroduction into axenic plants. World Journal of Microbiology and Biotechnolology 15: 37-40.

Petrini, O. (1986). Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema N.J. and Van Den Heuvel, J. (Eds). Microbiology of the phyllosphere. Cambridge University Press, Cambrigde. pp.175-187.

Petrini, O. (1996). Ecological and physiological aspects of host-specificity in endophytic fungi. In: Redlin, S.C. and Carris, J.M. (Eds). Endophytic fungi in grasses and woody plants. American Phytopathological Society Press. St Paul, MN. pp.87-100.

Photita, W. Lumyong, S., Lumyong, P. and Hyde, K.D. (2001). Endophytic fungi of wild banana (*Musa acuminata*) at Doi Suthep Pui National Park, Thailand. Mycological Research 105: 1508-1513.

Pocasangre, L., Sikora, R.A., Vilich, V. and Schuster, R.P. (1999). Survey of endophytic fungi from Central America and screening for biological control of the burrowing nematode (*Radopholus similis*). Acta Horticulturae 531: 283-289.

Raeder, U. and Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology 1: 17-20.

Rao, G.P., and Agnihotri, V.P. (2000). Wilt. In: Rott, P., Bailey, R., Comstock, J.C., Croft, B. and Saumtally, S. (Eds). A guide to sugarcane diseases. CIRAD/ISSCT, Montpellier, France. pp.193-197.

Rodrigues, K.F. and Samuels, G.J. (1990). Preliminary study of endophytic fungi in tropical palm. Mycological Research 94: 827-830.

Saikkonen, K., Wäli, P., Helander, M. and Faeth, S.H. (2004). Evolution of endophyte-plant symbioses. Trends in Plant Science 9: 275-280.

SAS Institute (1989). SAS/STAT User's Guide, Version 6 Fourth Edition Volume 1. SAS Institute, Cary, USA. p943.

Saubois, A., Laforet, P.E., Nepote, M.C. and Wagner, M.L. (1999). Mycological evaluation of a sorghum grain of Argentina, with emphasis on the characterization of Fusarium species. Food Microbiology 16: 435-445.

Schardl, C.L., Leuchtmann, A. and Spiering, M.J. (2004). Symbioses of grasses with seedborne fungal endophytes. Annual Review of Plant Biology 55: 315-340.

Schulz, B. and Boyle, C. (2005). The endophytic continuum. Mycological Research 109: 661-686.

Sokal, R.R. and Rohlf, F.J. (1995). Biometry. The principles and practice of statistics in biological research. 3rd ed. W.H. Freeman and Co New York. p950.

Siegel, R.W., Latch, C.G.M. and Johnson, M.C.V. (1987). Fungal endophytes of grasses. Annual Review of Phytopathology 25: 293-315.

Sikora, R., Niere, B. and Kimenju, J. (2003). Endophytic microbial diversity and plant nematode management in African Agriculture. In: Neuenschwander, P., Borgemeister, C. and Langewald, J. (Eds). Biological control in IPM systems in Africa. CAB International. Wallingford. pp.179-192.

Skovgaard, K., Bodker, L. and Rosendahl, S. (2002). Population structure and pathogenicity of members of the *Fusarium oxysporum* complex isolated from soil and root necrosis of pea (*Pisum sativum* L.). FEMS Microbiology Ecology 42: 367-374.

Stover, R.H. (1981). Fusarium diseases in the tropics. In: Nelson, P.E., Toussoun, T.A. and Cook, R.J. (Eds). Fusarium: Diseases, Biology and Taxonomy. The Pennyslvania State University Press, Pennyslvania. pp.114-128.

Surridge, A.K.J., Viljoen, A., Crous, P.W. and Wehner, F.C. (2003). Identification of the pathogen associated with sigatoka disease of banana in South Africa. Australasian Plant Pathology 32: 27-31.

Swofford, D.L. (2002). PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinaeur Associates, Sunderland, MA.

Tan, R.X. and Zou, W.X. (2001). Endophytes: a rich source of functional metabolites. Natural Products Reports 18: 448-459.

Ury, H.K. (1976). A comparison of four procedures for multiple comparisons among means (pairwise contrasts) for arbitrary sample sizes. Technometrics 18: 89-97.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.

Woo, S.L., Norviello, C. and Lorito, M. (1998). Sources of molecular variability and applications in characterization of plant pathogen *Fusarium oxysporum.* In: Bridge, P.D., Couteaudier, Y. and Clarkson, J.M. (Eds). Molecular variability of fungal pathogens CAB International. Wallingford. pp. 187-208.

Woo, S.L., Zoina, A., Del Sorbo, G., Lorito, M., Nanni, B., Scala, F. and Noviello, C. (1996). Characterization of *Fusarium oxysporum* f. sp. *phaseoli* by pathogenic races, VCGs, RFLPs and RAPD. Phytopathology 86: 966-973.

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.

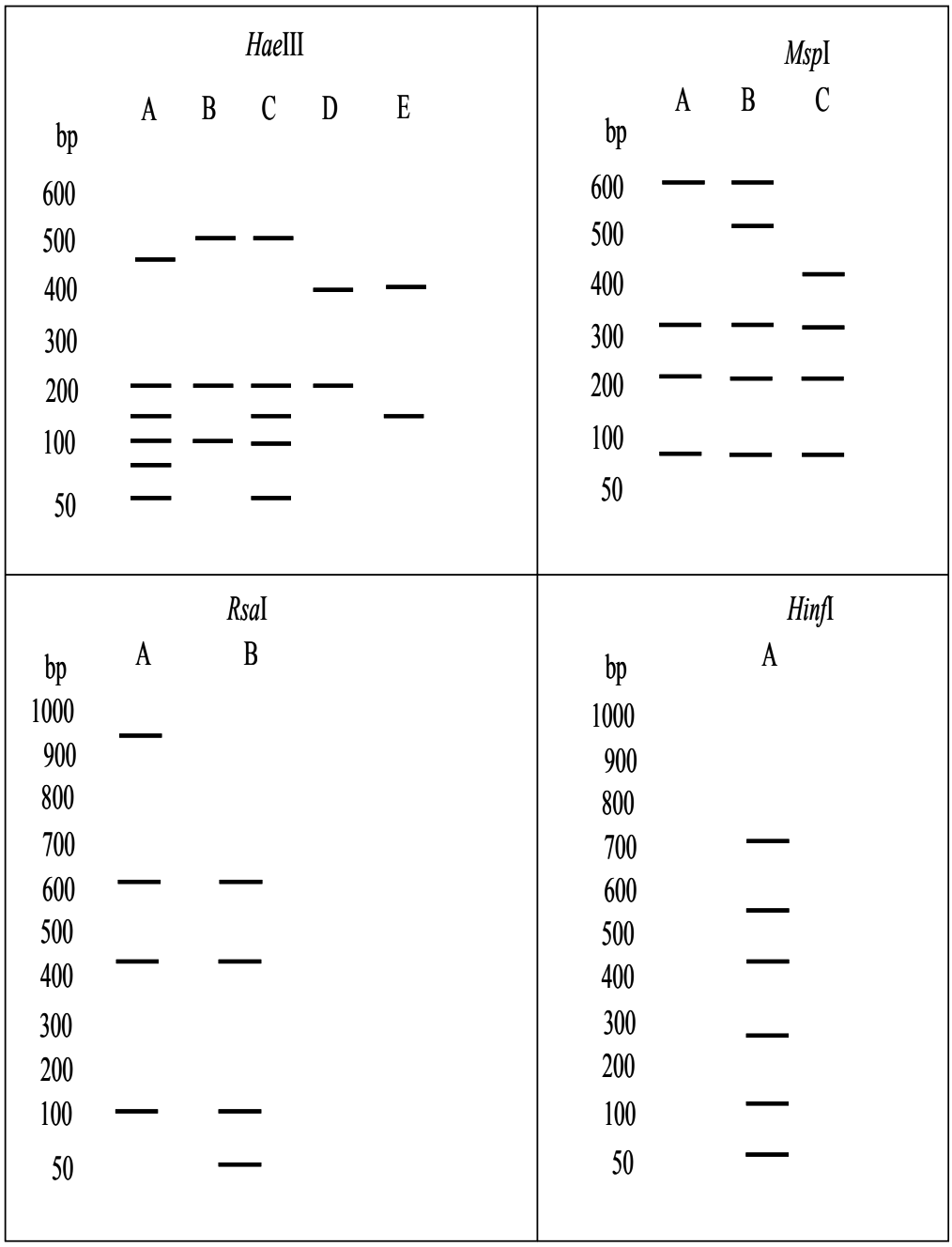

Figure 2. Phylogram inferred from amplified fragment length polymorphism analysis showing genetic relationship of 57 endophytic Fusarium isolates from banana root, rhizome and pseudostem base tissues. Distance analysis included neighbor joining as a tree building algorithm and a bootstrap of 1000 replicates.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

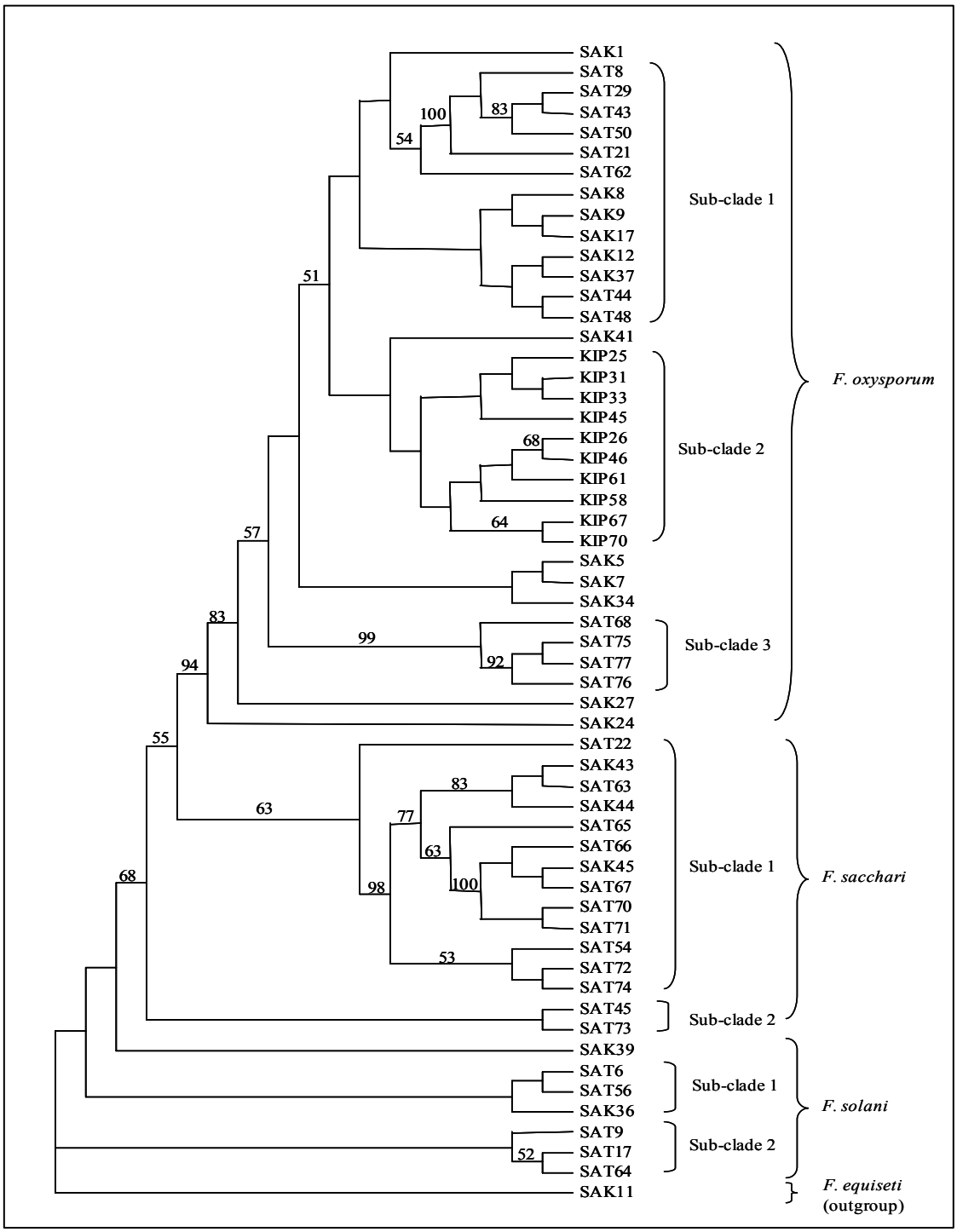

Table 1. Geographic origin of 57 endophytic isolates of *Fusarium* spp. from roots, rhizomes and pseudostem base tissues of Cavendish banana plants (*Musa* spp. AAA) in South Africa used for amplified fragment length polymorphism analysis.

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.