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Studies on the Characteristics of the Antagonistic Relationship between *Radopholus similis* (Cobb) Thorne and Mutualistic Endophytic Fungi in Nematode-Suppressive Banana Plants (*Musa* AAA)

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This work is dedicated to the memory of my mother,
Ľubica Haruštiakova zum Felde (2.07.1943 - † 10.09.2006).

Studies on the Characteristics of the Antagonistic Relationship between *Radopholus similis* (Cobb) Thorne and Mutualistic Endophytic Fungi in Nematode-Suppressive Banana Plants (*Musa* AAA).

The antagonistic relationship of *Radopholus similis* and seven mutualistic endophytes isolated from banana and plantain were investigated in a series of experiments. First, prior to field trials and further greenhouse experiments, the vegetative compatibility of endophytes identified as *Fusarium oxysporum* and known pathogenic *F. oxysporum* isolates was tested. Nitrate non-utilizing (*nit*) mutants were produced and crossed with complementary testers from pathogenic *F. oxysporum* f. sp. *radicis-lycopersici*, *lycopersici* and *cubense* strains. None of the endophytic *F. oxysporum* isolates formed heterokaryons with any of the pathogenic strains tested. The nematode-antagonistic endophytes are therefore not vegetatively compatible with the pathogenic strains, and can not exchange genetic information with them.

Second, the role of systemic induced resistance to nematode penetration and reproduction was tested in a series of split-root tests carried out in greenhouses in Germany and Costa Rica. The results revealed that, of the three *T. atroviride* (MT-20, ET-35 and S2) and four *F. oxysporum* (MF-25, MF-40, S9 and P12) isolates tested, only MT-20 and S2 seemed to induce systemic effects in *Musa* AAA cv. 'Valery' plants. S2 significantly and consistently reduced *R. similis* penetration, 10 days after nematode inoculation, while MT-20 significantly reduced nematode reproduction over a 9 week period. None of the tested *F. oxysporum* isolates induced systemic resistance to nematode penetration or reproduction.

Third, the possible transfer of nematode suppression from endophyte-inoculated *Musa* AAA cv. 'Valery' mother plants to daughter plants (suckers) in the field was studied. As part of a large scale field trial looking at the suppression of nematodes in the field, mother plants were inoculated with endophytes prior to field transfer, and nematode populations monitored for 7 months. At the first de-suckering, sword suckers were removed from the field and grown in the greenhouse, where they were later challenged with *R. similis*. Results from the field to greenhouse test indicated that, when nematode suppression is established in the field, this suppression is transferred from mother to daughter plants.

Finally, the effects of single and combined inoculations of two *F. oxysporum* and two *T. atroviride* on *R. similis* biocontrol in *Musa* AAA cv. 'Williams' and on plant growth of *Musa* AAA cvs. 'Grand Nain', 'Valery' and 'Williams' were investigated. Combining inoculations of mutualistic endophytes increased nematode control levels and plant growth in 'Williams' plants inoculated with both *R. similis* and fungi. Plant growth in the absence of *R. similis* was promoted by single and dual inoculations of *T. atroviride* isolates and by single inoculations of *F. oxysporum* isolates in 'Grand Nain', 'Valery' and 'Williams' plants. Dual inoculations of *F. oxysporum* and of all four endophytes did not positively affect plant growth.

Studien zur Characterizierung der Antagonistischen Beziehung zwischen *Radopholus similis* (Cobb) Thorne und Mutualistischen Endophytischen Pilzen in Nematoden-Suppressiven Bananen Pflanzen (*Musa* AAA).

Die antagonistische Beziehung von *Radopholus similis* und sieben aus Bananen und Kochbananen isolierten mutualistischen Endophyten wurden in einer Serie von Versuchen durchleuchtet. Vor weiteren Gewächshausversuchen und Feldversuchen, wurde erstens die vegetative Kompatibilität der als *Fusarium oxysporum* identifizierten Endophyten und bekannte pathogenen *F. oxysporum* Isolate getestet. Nitrate nicht verwendende (*nit*) Mutanten wurden produziert und mit komplementären Testern der pathogenen Stämme von *F. oxysporum* f. sp. *radicis-lycopersici*, *lycopersici* und *cubense* gegenüber gestellt. Keiner der getesteten endophytischen *F. oxysporum* Isolate formte einen Heterokaryon mit pathogenen Stämmen. Die Nematoden antagonistischen Endophyten sind daher nicht mit pathogenen Stämmen vegetativ kompatibel, und können keine genetische Information austauschen.

Zweitens wurde die Rolle der systemisch induzierten Resistenz zu Nematoden Penetration und Reproduktion in einer Reihe Split-Root Versuchen im Gewächshaus in Deutschland and Costa Rica nachgeprüft. Von den drei *T. atroviride* (MT-20, ET-35 und S2) und den vier *F. oxysporum* (MF-25, MF-40, S9 und P12) getesteten Pilzen, induzierten nur MT-20 und S2 eine systemische Reaktionen in *Musa* AAA cv. 'Valery' Pflanzen. S2 reduzierte in signifikanterweise und immer wieder die Penetration von *R. similis*, 10 Tagen nach Nematoden Inokulation, wehrend MT-20 in signifikanterweise die Reproduktion über eine 9-Wöchige Periode reduzierte. Keins der getesteten *F. oxysporum* Isolate induzierte eine systemische Resistenz, weder zur Penetration noch zur Reproduktion von *R. similis*.

Drittens wurde die mögliche Übertragung der durch Endophyten hervorgerufene Nematoden Suppression in *Musa* AAA cv. 'Valery' Mutterpflanzen an deren Töchter (Wurzelschössling) im Feld erforscht. Als Teil eines groß angelegten Feldversuches, wurden Mutterpflanzen mit Endophyten vor der Aussaat im Feld inokuliert, und der Nematoden Bestand über 7 Monate überwacht. Bei der ersten Schösslingsentfernung, wurden Schwertschösslinge vom Feld entnommen und ins Gewächshaus verpflanzt, wo sie später mit *R. similis* inokuliert wurden. Die Ergebnisse des Versuchs deuteten darauf hin das, dort wo sich die Nematoden Suppression im Feld etabliert, wird diese auch von Mutter- zu Tochterpflanze übertragen.

Letztlich wurden die Wirkung einzelner und kombinierter Inokulationen von zwei *F. oxysporum* und zwei *T. atroviride* auf die Biokontrolle von *R. similis* in *Musa* AAA cv. 'Williams' und auf das Wachstum von *Musa* AAA cvs. 'Grand Nain', 'Valery' und 'Williams' untersucht. In 'Williams' Pflanzen die mit *R. similis* sowie Pilzen inokuliert wurden, wurde das Niveau der Nematoden Biokontrolle und des Pflanzenwachstums durch kombinierte Inokulationen gesteigert. In der Abwesenheit von *R. similis*, wurde der Wachstum in 'Grand Nain', 'Valery' und 'Williams' Pflanzen durch die Inokulation mit einzelnen oder beiden *T. atroviride* und mit einzelnen *F. oxysporum* gesteigert. Die Inokulation mit beiden *F. oxysporum* und mit allen Pilzen förderte nicht das Wachstum der Pflanzen.

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1 GENERAL INTRODUCTION

As early as 12 thousand years ago, during the first agricultural revolution, people started to cultivate plants to meet their nutritional needs. Through selection and breeding, wild plants became crops and were adapted to growth under various conditions. Many crops are now produced in lands far removed from their centres of origin. Bananas and plantains (*Musa* spp.) are such far-flung crops and their distribution is closely linked to that of human populations in the tropics (De Langhe, 1996).

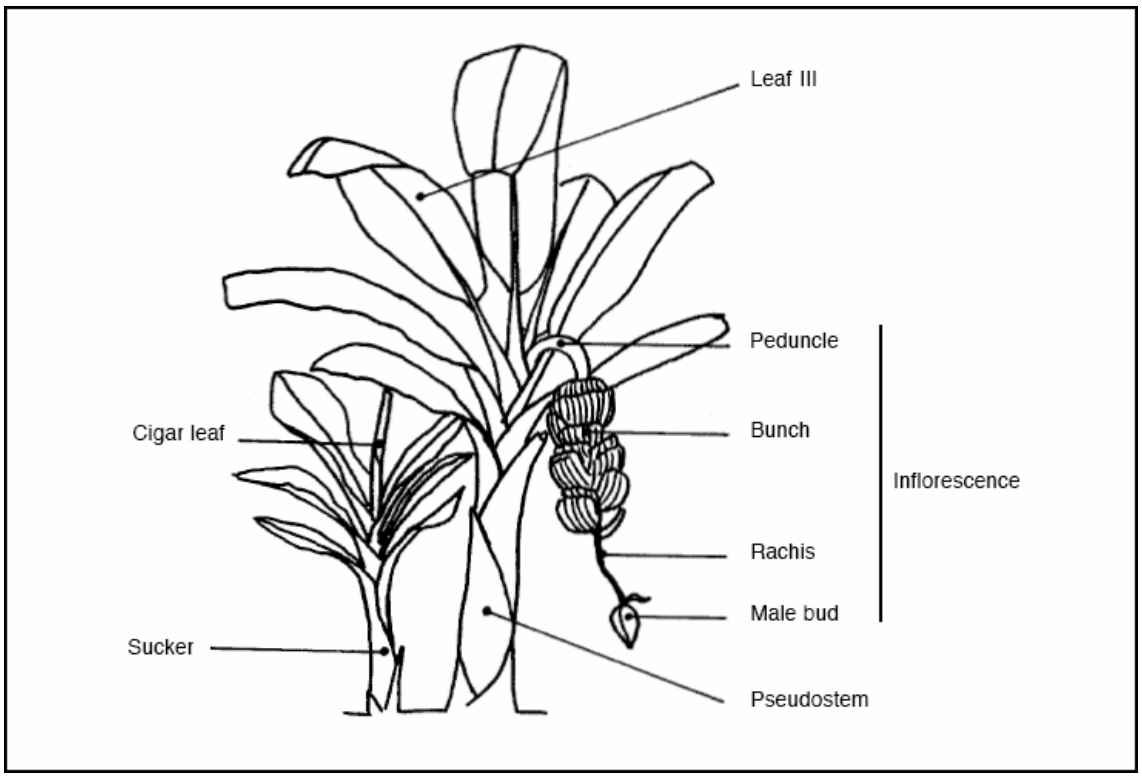
1.1 Bananas

The banana belongs to the family Musacea, genus *Musa*, and is the world's largest perennial herb, represented by almost 1000 varieties (INIBAP, 2000). The cultivated forms belong to two sections of the genus *Musa*: *Australimusa* and *Eumusa* (Rehm & Espig, 1991). The section *Eumusa* is the largest and geographically most widespread section, and contains the majority of edible bananas (Simmonds & Shepherd, 1955; Simmonds, 1966; Stover & Simmonds, 1987). Originating in the rainforests of Southeast Asia, edible fruits of *Musa* spp. most probably came about as a result of two mutation events: female sterility and parthenocarpy (Sharrock, 1998). Triploid *Musa acuminata* cultivars arose from these diploids, perhaps as a result of crosses between edible diploids and wild *M. acuminata* subspecies, resulting in a wide range of phenotypes (Sharrock, 1998). *M. acuminata* triploids, which are more vigorous and have larger fruit, have replaced the original *M. acuminata* diploids in most parts of Southeast Asia (Sharrock, 1998). Original edible diploid and triploid *M. acuminata* cultivars are believed to have first been taken by man to areas where *M. balbisiana* is native and natural hybridizations resulted in the formation of hybrid progeny with mixed genomes (Simmonds, 1962). It is thought that the subsequent dispersal of edible bananas outside Asia was brought about solely by man (Simmonds 1962). Secondary diversification within the major groups of cultivated bananas has been the result of somatic mutations rather than sexual reproduction (Sharrock, 1998).

Bananas are generally classified by a code that consists of the genus name, *Musa*, and a number of As and Bs designating their genomic composition and ploidity level (i.e. *Musa* AABB is a tetraploid *Musa* with two sets of chromosomes from *M. acuminata* and two from *M. balbisiana*). The most economically important cultivated

types used by farmers worldwide are triploids: AAA and AAB dessert bananas, AAA East African cooking and beer bananas, ABB cooking banana and AAB plantains (Ortiz, 1997). These triploid cultivars are sterile and develop seedless fruit by parthenocarpy (Simmonds, 1995). Hence, all bananas plants used for production are clones that traditionally developed from corms or suckers of so called mother plants. Today, tissue culture plants are gaining importance as planting material in commercial operations (Sarah, 2000).

Mother plants are the plants that bear fruit, and their lateral offshoots are called suckers. Banana mother plant, sucker and inflorescence are shown in Figure 1.



Source: IPGRI/INIBAP/CIRAD (1996).

Figure 1. Banana mat with mother plant, sucker and inflorescence.

There are three types of suckers produced by bananas: the maidenhead sucker - a large non-fruiting pseudostem, the sword sucker - a sucker attached to the original (mother) rhizome with narrow sword-like leaves, and the water sucker - a sucker next to but only superficially attached to the mother rhizome with broad leaves (Simmonds, 1959). Water suckers produce inferior fruit and are therefore not recommended for

propagation. The most commonly used sucker for planting is the sword sucker. In commercial operations, all suckers are periodically removed from the mother plant. Towards the beginning of flowering, the most vigorous sword sucker is chosen as follower sucker, and not removed. During harvest, the banana bunch is removed, the mother plant cut down and the follower sucker left to grow and bear fruit. The complex of mother and sucker plants, including roots and pseudostems is called a banana mat.

1.2 Bananas in Central America

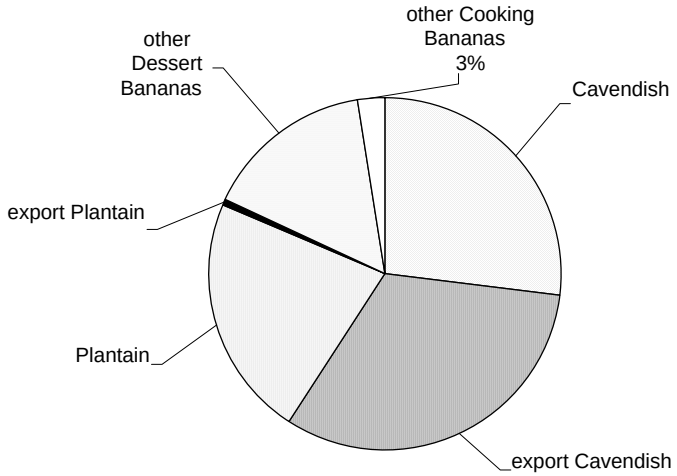
There is some evidence that bananas may have been introduced to the west coast of South America and Ecuador by Polynesian people as early as 2000 years ago, however, only the later introduction of bananas to tropical America by Spaniards and Portuguese from the 16th to 19th centuries is clearly documented (De Langhe, 1996).

The large scale commercial production of dessert bananas for export, for which Central American countries are especially renown, started in the late 1890s and early 1900s, when the interests of the Brooklyn-born Central American railroad baron Minor C. Keith merged with those of the Boston Fruit Company, to create the infamous United Fruit Company or UFCo (Chiquita, 2000). The expansion of commercial banana production was closely tied to the social, cultural, economical and political development of Central America, and the UFCo's power over and corruption of local governments and the dependence of these nations on the production and export of bananas lead to their designation as *Banana Republics*.

Over the years, the UFCo acquired both virgin land and established banana plantations throughout Latin America and the Caribbean - controlling an area the size of Switzerland by the early 1930s - and becoming the world's largest banana producer and exporter, dominating a third of the world trade (Anonymous, 1999; Bananalink, 2001). Presently, the world banana market is dominated by three U.S.-based multinational companies: Chiquita Brands International (formerly UFCo) based in Cincinnati, Ohio; Dole Food Company (formerly Standard Fruit Company), Westlake Village, California; and Fresh Del Monte Produce, Coral Gables, Florida. Together, Chiquita, Dole and Del Monte control over 65% of the world banana trade (Van de Kasteele, 1998).

In 2006, global banana production exceeded 69 million tonnes (Mt), of which over a third or 25 Mt was produced in Latin America and the Caribbean (FAOSTAT, 2008).

Production in Latin America and the Caribbean accounted for close to 83% or 10.2 Mt of world banana exports in 1998 (Lescot & Rosales, 1999; Sharrock & Frison, 1999). Of the over 31 Mt of banana and plantain produced on average per year in Latin America and the Caribbean, 22 Mt are consumed locally, that is: 7.3 Mt plantain (ABB), 6.5 Mt cooking bananas (ABB), 5.8 Mt 'Figue pomme'/'Silk', 'Pome' and 'Gros Michel' bananas and >5 Mt Cavendish bananas for local consumption, as shown in Figure 2 (Lescot & Rosales, 1999). These fruits are an integral part of the diet of most Latin Americans and are usually produced on smaller plantations, small farms and backyard plots, while most bananas produced on the large plantations run by the multinational companies are destined for the export market. The variety of cultivars grown on the large plantations is usually limited to a few Cavendish cultivars, whereas locally consumed bananas found at local markets represent a greater variety of cultivars.



Source: Lescot & Rosales (1999).

Figure 2. Diversity of *Musa* production in Latin America and the Caribbean

With the spread of the banana's popularity around the world, it's pests and pathogens were also disseminated into newly opened growing regions (Marin *et al.*, 1998). One of the most devastating of pests of bananas are nematodes. Among the nematodes attacking bananas grown in the tropics, the most important one is *Radopholus similis* (Cobb) Thorne.

1.3 Nematode Pests of Bananas in Central America

The five major nematodes that parasitize banana roots are: *Radopholus similis*, *Pratylenchus coffeae*, *P. goodeyi*, *Helicotylenchus multicinctus* and *Meloidogyne* spp. (Gowen & Quénéhervé, 1990). The migratory endoparasites *R. similis* and *Pratylenchus* spp. are the most widespread and important ones, followed by the ectoparasite *Helicotylenchus multicinctus* and the sedentary endoparasite *Meloidogyne* spp. (Sarah, 2000; Gowen, 2000a; Gowen, 2000b; De Waele, 2000). In Central America, *R. similis* is usually the most common and most damaging nematode encountered, followed by *Helicotylenchus* spp., *Meloidogyne* spp. and, not so commonly, *Pratylenchus* spp. (Araya *et al.*, 1995; Gowen, 1995; Marin *et al.*, 1998; Gowen, 2000a; Chávez & Araya, 2001; Moens *et al.*, 2001).

1.3.1 *Radopholus similis* (Cobb) Thorne

Radopholus similis (Cobb) Thorne is a migratory endoparasite that completes its life-cycle in 20-25 days in the root and corm tissues of bananas (Sarah et al., 1996). Mobile females and juveniles migrate inter- and intracellularly, feeding on cortex cell cytoplasm and thereby damaging cells. Males have an atrophied stylet and are not considered parasitic. A group of *R. similis* males and females are shown in Figure 3.

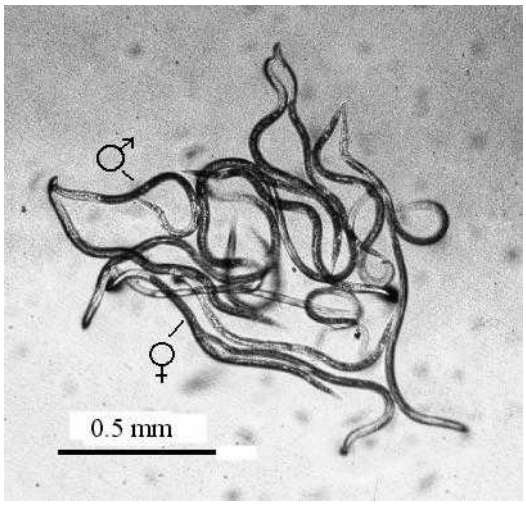


Figure 3. Group of *Radopholus similis* males and females isolated from banana roots.

Damage caused by *R. similis* begins with tunnels of necrotic tissue in roots and corms, which affect water and nutrient uptake thereby lengthening the growing period. Next, root rot develops, caused by secondary infection of damaged tissue by bacteria

and fungi, eventually leading to the Banana Toppling Disease, caused by root destruction and loss of anchorage (Gowen & Quénéhervé, 1990; Sarah *et al.*, 1996). Parasitic forms of *R. similis* can not only migrate from necrotic root tissue to adjoining fresh tissue but also through the soil, thereby gaining access to non-infested tissue such as new roots and freshly planted material (Sarah *et al.*, 1996). Substantial yield increases (20-75%) in production areas where nematicides were applied revealed the extent of production losses due to *R. similis* and nematodes in general (Broadley, 1979; McSorley & Parrado, 1986; Sarah, 1989; Gowen, 1994).

1.4 Importance of *Radopholus similis* in Central America

R. similis is considered one of the most important root pathogens attacking bananas and to be the main nematode problem in intensive, export-oriented, commercial banana production, especially of Cavendish cultivars (Sarah *et al.*, 1996). A significant correlation between *R. similis* numbers and root necrosis and damage in follower suckers has been observed in commercial banana plantations (Moens *et al.*, 2001). However, it was only through the Panama Disease induced switch from 'Gros Michel' (AAA) to Cavendish (AAA) cultivars that nematodes became a problem in banana plantations in Central America, as 'Gros Michel' cultivars are partially resistant to nematodes, while Cavendish cultivars, though resistant to Panama Disease, are susceptible to them. At present *R. similis* is considered a major limiting factor of banana production in Central America, both on commercial, export-oriented plantations and on smaller farms (Pinochet, 1986).

It is commonly believed among Central American banana producers that Panamanian and Costa Rican plantations are more heavily infested and damaged by nematodes (especially by *R. similis*) than plantations in Honduras, Guatemala and Belize. Pinochet (1988) also reported this regionally varying pathogenicity of *R. similis* populations in Central America. Other authors have studied *R. similis* populations isolated from different production areas both in Central America and in the world, and have concluded that there is a large range of intraspecific biological diversity and pathogenicity of *R. similis* (Sarah *et al.*, 1993; Fallas & Sarah, 1995; Fallas *et al.*, 1995). Pathogenicity has also been clearly linked to reproductive fitness in plant tissue (Sarah, 2000). In addition to the pathogenicity and density of nematodes in roots, choice of cultivar and abiotic and biotic factors, especially other microorganisms living in the soil

and root tissue affect the extent of damage caused by nematodes (Kaplan & Gottwald, 1992; Gowen, 1995; Spiegel & Chet, 1998; Elsen *et al.*, 2001).

1.5 Control Measures for Nematodes in Central America

The economic threshold level of a pest is an important element of Integrated Pest Management or IPM (Duncan, 1991). In general, the nematode threshold of 10000 *R. similis*/100 g functional root established by Tartré and Pinochet (1981) is used to determine the need for nematicide applications on commercial plantations (Chávez & Araya, 2001). In most commercial banana plantations, nematode populations are monitored on a monthly basis and other parameters, such as total and functional root weight and percent necrotic roots are also included in the data collected during monitoring (Moens *et al.*, 2001).

Though a variety of cultural and physical nematode control measures, such as crop rotation, fallow, flooding and soil solarization, have had some success in certain management systems, they are next to impossible or impractical in commercial plantations where bananas are grown continuously and therefore nematode control in these systems is primarily done chemically (Sarah, 1989; Gowen & Quénéhervé, 1990; Quénéhervé, 1993; Pinochet, 1996; Forgain & Gowen, 1997; Pocasangre, 2000).

A few promising microorganisms have been identified for the biological control of nematodes, but few are effective in the field as efforts have concentrated on enhancing biological control through soil inoculation with nematode-antagonistic organisms (Rodríguez-Kábana, 1991; Sarah, 2000). Notable exceptions are *Paecilomyces lilacinus* and *P. oxalicum*, which are parasites of *R. similis* eggs, juveniles and adults, and have yielded promising results in the Philippines (Sarah, 2000). Other promising microorganisms have been isolated from nematode suppressive horticultural and coffee soils in Mexico and Costa Rica and were tested on horticultural crops against two sedentary ectoparasitic nematodes, *Meloidogyne incognita* and *Rotylenchus* (Marban-Mendoza *et al.*, 1992; Zuckerman *et al.*, 1993; Dicklow *et al.*, 1993; Esnard *et al.*, 1995). However, in banana, endoparasitic nematodes such as *R. similis* and *Pratylenchus* spp. dominate and cause the most damage. Looking for antagonists of ectoparasitic nematodes in nematode suppressive soils is logical, as these parasites spend most of their life in the soil and the chances of finding their natural enemies in such soils are high. For lack of a better term, the soils of areas where endoparasitic

nematodes are suppressed have also been termed suppressive soils. However, since these nematodes spend most of their lives in plant tissue, their antagonists are probably also to be found within the plant tissue that hosts them and not in the soil. Restricting the search for antagonists of endoparasites to endophytic organisms from host plants in areas where suppression is observed is a logical avenue of investigation.

1.6 Endophytes as Biocontrol Agents of Plant Parasitic Nematodes

Based on the general definitions of endophytes by Petrini (1991) and Hawksworth *et al.* (1995), endophytic fungi can be defined as fungi that, at some point in their life cycle, live within plant tissues without causing disease symptoms and can be isolated from healthy plant tissue after surface sterilisation. The use of such fungi to increase resistance to nematodes in plants prior to field planting is seen as a novel approach to sustainable nematode management in banana (Sikora & Schuster, 1999; Niere, 2001). Sikora *et al.* (1999) coined the term *biological enhancement*, which is now used to describe the targeted application of antagonists into a plant's pathozone for effective biological control (Sikora *et al.*, 2008).

In their review of mutualistic endophytic fungi and *in-planta* suppressiveness to plant parasitic nematodes, Sikora *et al.* (2008) note that though the concept of using microorganisms to control nematodes is not new, that of using endophytes is relatively new, and published literature on the subject is therefore scarce. Studies have been carried out primarily on tomatoes and bananas, the vast majority of which included the biological enhancement of the plants with non-pathogenic *Fusarium oxysporum* and *Trichoderma* spp. isolates (Sikora *et al.*, 2008). Menjivar Banajona (2005) was the first to run a large scale field trial with endophyte inoculated banana plants. He concluded that one application of endophytes prior to field planting can eliminate a round of nematicide treatments (Menjivar Barahona, 2005). The application of endophytes can not only be limited to the pathozone, but they additionally grow in the very tissues plant parasitic nematodes feed on and live in, therefore the potential of endophytes as biological control agents (BCAs) of nematodes, especially endoparasitic ones is great.

2 RESEARCH OBJECTIVES

The endophytes used in the present study were all isolated from the roots of banana and plantain plants, growing in areas where a suppression of *R. similis* was either suspected or confirmed. Prelimiary studies included the isolation, screening, identification and selection of nematode antagonistic endophytic fungi. Endophytic fungi were isolated from banana and plantain roots from 1) commercial banana plantations in Motagua, Guatemala (zum Felde, 2002); 2) commercial plantain plantations in Sixaola, Costa Rica (Carñizares Monteros, 2003); and 3) organic banana and plantain plantations in Talamanca, Costa Rica (Menenses Hérnandez, 2003).

The isolates that best controlled *R. similis* in the greenhouse in *in planta* experiment were identified to the species level in the laboratory of Dr. H. Nierenberg, at the BBA, in Berlin, Germany. All proved to be either *Trichoderma atroviride* isolates or *Fusarium oxysporum* isolates. The seven most effective and non-pathogenic nematode-antagonistic endophytes were included in the present study.

The objectives of the present thesis work were:

1) To test the vegetative compatibility of *Radopholus similis*-antagonistic endophytic *Fusarium oxysporum* isolates with known pathogenic strains of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *lycopersici* and *cubense*.

2) To study whether or not systemic induced resistance plays a role in the *Radopholus similis* antagonistic relationship of endophytic *Trichoderma atroviride* and *Fusarium oxysporum* isolates in *Musa* AAA cultivar 'Valery'.

3) To investigate the possible transfer of nematode suppression from endophyte-inoculated *Musa* AAA cultivar 'Valery' mother plants to daughter plants (suckers) in the field.

4) To examine the effects of single and combined inoculations of mutualistic endophytic *Fusarium oxysporum* and *Trichoderma atroviride* on *Radopholus similis* biocontrol in and growth of *Musa* AAA cultivars 'Grand Nain', 'Valery' and 'Williams'.

3 TESTING THE VEGETATIVE COMPATIBILITY OF MUTUALISTIC ENDOPHYTIC ISOLATES AGAINST KNOWN PATHOGENIC ISOLATES OF *FUSARIUM OXYSPORUM* F. SP. *RADICIS-LYCOPERSICI*, *LYCOPERSICI* AND *CUBENSE*

3.1 Introduction

The most frequently articulated concern before field trials using beneficial endophytic strains of *Fusarium oxysporum* can be considered, is whether the beneficial strain is definitely non-pathogenic. Aside from classical pathogenicity tests on host plants of *Fusarium* diseases, the pathogenicity of *F. oxysporum* isolates can be tested using the vegetative compatibility of nitrate-nonutilizing (*nit*) mutants of these isolates with tester-mutants of known pathogenic *F. oxysporum* formae speciales (Katan & Katan, 1988). Vegetative compatibility has been used to differentiate fungi since the 1960s, but Puhalla (1985) was the first to use nitrate nonutilizing mutants of *F. oxysporum* to identify and differentiate various strains of this fungi. He postulated that there could be a relationship between the pathogenicity of isolates and their ability to form heterokaryontes with compatible strains. Katan and Katan (1988) studied pathogenic and non-pathogenic isolates of *F. oxysporum* from cotton. After carrying out both pathogenicity and VCG tests with these isolates, they concluded that VCG tests were as accurate as pathogenicity tests and could distinguish between pathogenic and non-pathogenic isolates in mixed *Fusarium* populations. Based on the results of these and subsequent studies with non-pathogenic *F. oxysporum* isolates (Correll *et al.*, 1987; Elias *et al.*, 1991; Gordon & Okamoto, 1991; Gordon & Okamoto 1992a, b, c; Katan *et al.*, 1994; Larkin *et al.*, 1996; Steinberg *et al.*, 1997), VCG testing is increasingly being used as an alternative to time and resource consuming pathogenicity tests, when trying to establish the non-pathogenic nature of newly isolated *F. oxysporum* strains. VCG tests were therefore carried out with 10 endophytic *F. oxysporum* isolates with known nematode antagonistic activity, a few of which had been earmarked for field trials.

3.2 Materials and Methods

Essentially, a VCG test consists of placing the mycelium or agar plugs with the mycelium of 2 or more complementary *nit* mutants (usually *nit* 1 mutants with a *nit* M tester, as *nit* 3 mutants are rarely produced) on Minimal Media (MM), at a certain distance from each other, and observing their growth at the point where the hyphae of

the two fungi meet. Nitrate nonutilizing mutants produce a thin mycelium on MM, while *F. oxysporum* strains able to utilize nitrate produce typical wild-type growth, characterized by cottony, aerial mycelium, often tinted pink to violet. If the *nit* mutants growing on MM are vegetatively compatible, heterokaryosis takes place at the junction of their hyphae, and the mycelia there produced takes on the characteristics of the wild type hyphae. A negative reaction is revealed by an absence of wild type hyphae: both fungi keep growing towards each other, mycelia growing over the same area without forming heterokaryons. No genetic information is exchanged, nitrate remains unutilized and mycelium thin and characteristic of *nit* mutants.

A list of the 10 *F. oxysporum* isolates included in this study is presented in Table 1. All endophytic isolates were subcultured on Potato Dextrose Agar (PDA) plates in preparation for the tests. Some of the isolates have been worked on for some time in Bonn (A1, Fo162 and V5W2), and were revived from cryo-pearl cultures (stored at -80ºC). Others are recent additions to the local collection (MF-25, MF-40, P3, P7, P12, S7 and S9), and were recuperated from the agar blocks in Eppendorf tubes in which they had been sent from Costa Rica.

Table 1. List of mutualistic, endophytic isolates of *Fusarium oxysporum* used in VCG tests.

Endophyte ID Code	Isolate Origin		
	Host Plant	**Origin**	**Reference**
A1	*Musa* sp.	Indonesia	Amin, 1994
Fo162	*Lycopersicon esculentum* L.	Kenya	Hallmann & Sikora, 1994
MF-25	*Musa* AAA	Guatemala	zum Felde, 2002
MF-40	*Musa* AAA	Guatemala	zum Felde, 2002
P12	*Musa* AAA	Costa Rica	Menenses. 2003
P3	*Musa* AAA	Costa Rica	Menenses. 2003
P7	*Musa* AAA	Costa Rica	Menenses. 2003
S7	*Musa* AAA	Costa Rica	Carñizares, 2003
S9	*Musa* AAA	Costa Rica	Carñizares, 2003
V5W2	*Musa* sp.	Uganda	Schuster et al., 1995

Stored testers of known pathogenic *F. oxysporum* isolates were also revived. Available testers consisted of *nit* M testers for *F. oxysporum* ff. spp. *cubense*, *radicis-lycopersici* and *lycopersici*, and *nit* 1 testers for *F. oxysporum* f. sp. *cubense* (Table 2 - for a list including isolate origin and identification code, see pp. 22-23 in Niere, 2001).

Table 2. List of VCG testers from *Fusarium oxysporum* ff. spp. *cubense*, *radicis-lycopersici* and *lycopersici* revived (from soil tube storage) and used in VCG tests in Bonn (March to July 2004).

	VCG Code		**Phenotype**	
	Fusarium oxysporum* f. sp. *cubense		***nit* M**	***nit* 1**
1)	0120		✓	✓
2)	0121		✓	✓
3)	0122		✓	✓
4)	0123		✓	✓
5)	0124		✓	✓
6)	0125		✓	
7)	0126		✓	✓
8)	0128		✓	✓
9)	0129		✓	
10)	01210		✓	✓
11)	01211		✓	✓
12)	01212		✓	✓
13)	01213		✓	✓
14)	01214		✓	✓
15)	01215		✓	✓
16)	01216		✓	
17)	01217		✓	
18)	01218		✓	✓
19)	01219		✓	✓
20)	01220		✓	
21)	01221		✓	✓
22)	01222		✓	✓
23)	01223		✓	✓
24)	01224			✓
	Fusarium oxysporum* f. sp *radicis-lycopersici			
25)	0090	subgroup I	✓	
26)	0090	subgroup II	✓	
27)	0090	subgroup III	✓	
28)	0091	subgroup I	✓	
29)	0091	subgroup II	✓	
30)	0092		✓	
31)	0093		✓	
32)	0094	subgroup I	✓	
33)	0094	“Universal”	✓	
34)	0096		✓	
35)	0098		✓	
	Fusarium oxysporum* f. sp *lycopersici			
36)	0030		✓	
37)	0032		✓	
38)	0033		✓	

The testers, which are conserved on soil tubes at 4ºC in a refrigerator in Bonn, were grown first on PDA plates, and later transferred onto MM plates for use in the VCG tests. The testers strains for *F. oxysporum* f. sp. *cubense* were originally provided by Dr. Randy Ploetz, University of Florida, USA, on dried filter paper. Testers for ff. spp. *lycopersici* and *radicis-lycopersici* were sent on agar blocks in 0.5 ml Eppendorf tubes by Dr. Talma Katan, Department of Plant Pathology, The Volcani Center, Israel. All testers were revitalized or subcultured on Synthetic Nutrient Agar (SNA) plates, and transferred to soil tubes and stored at 4ºC.

The media used in the present study are essentially those described in Puhalla and Spieth's (1983) work on heterokaryosis in *Fusarium moniliforme*, in Puhalla's later work (1985) on *F. oxysporum* VCGs and in Correll *et al.*'s (1987) work on the use of *nit* mutants in vegetative compatibility (VC) testing. The latter work was the first to identify the three now accepted nitrate nonutilizing (*nit*) mutants that *F. oxysporum* is known to produce (*nit* 1, *nit* 3 and *nit* M).

List of media used to revive and maintain fungi:

PDA - Potato Dextrose Agar (per liter):

1 l distilled H_2O
24 g Potato Dextrose Broth (Difco)
17 g Agar

SNA - Synthetic Nutrient Agar (per litre) modified by Nierenberg (1976):

1 l distilled H_2O
1 g KH_2PO_4
1 g KNO_3
0.5 g $MgSO_4 * 7 H_2O$
0.5 g KCl
0.2 g Glucose
0.2 g Sucrose
0.6 ml NaOH (1 M)
17 g Agar

Different media were used to maintain, generate, phenotype and test mutants. Both tester mutants and those generated from mutualistic endophytic *F. oxysporum* strains,

were maintained on PDA and MM plates. To generate nitrate nonutilizing mutants, two chlorate containing media were used: PDC (Potato Dextrose Agar with chlorate) and MMC (Minimal Media with chlorate) (Puhalla, 1985). To phenotype mutants, minimal media (MM), nitrite media (NM) and hypoxanthine media (HxM) were used (Correll *et al.*, 1987). Generated mutants and testers were grown on MM plates prior to testing, and MM was used for the actual tests.

Aside from PDA and PDC, all media are variations of a common basal medium, composed of the following ingredients:

Basal Media (based on Puhalla, 1985):

1 liter	distilled H_2O
30 g	Sucrose
1 g	KH_2PO_4
0.5 g	$MgSO_4 * 7\ H_2O$
0.5 g	KCl
0.01 g	$FeSO_4 * 7\ H_2O$
0.2 ml	Sterile Trace Elements Solution
20 g	Difco Agar

Trace Element Solution:

95 ml	distilled H_2O
5 g	Citric Acid
5 g	$ZnSO_4 * 7\ H_2O$
1g	$Fe(NH_4)_2(SO_4)_2 * 6\ H_2O$
0.25 g	$CuSO_4 * 5\ H_2O$
0.05 g	$MnSO_4 * H_2O$
0.05 g	H_3BO_3
0.05 g	$Na_2MoO_4 * 2\ H_2O$

The following media were used:

1) to generate mutants:

MMC – Minimal Media with Potassium Chloride (from Correll *et al.*, 1987):

1 l	Basal Medium
2 g	$NaNO_3$
15 g	$KClO_3$

PDC – Potato Dextrose Agar with Potassium Chloride (from Correll *et al.*, 1987):

1 l	PDA
15 g	$KClO_3$

2) to maintain and test mutants:

MM - Minimal Media (from Puhalla, 1985):

1 l	Basal Medium
2 g	$NaNO_3$

3) to phenotype mutants:

HxM – Hypoxanthine Media (from Correll *et al.*, 1987):

1 l	Basal Medium
0.2 g	Hypoxanthine (Merck)

NM – Nitrite Media (from Correll *et al.*, 1987):

1 l	Basal Medium
0.5 g	$NaNO_2$

PDA plates were first produced to revive the testers available in Bonn (Table 2). Under a laminar flow hood, a small quantity of the soil was removed from the soil-tubes with a flame-sterilized spatula, and sprinkled on a Petri dish containing 100% PDA. All plates were incubated in the dark at 25ºC for 1-2 weeks, until soil-free mycelium plugs could be removed from the dish. These were subcultured on MM plates until needed for VCG testing.

Since both *nit* 1 and *nit* M testers were available, and to increase chances of producing compatible mutants, both PDC and MMC plates were used for mutant production. *F. oxysporum* is known to primarily produce *nit* 1 mutants on both PDC and MMC plates, while the likelihood of producing *nit* 3 and *nit* M mutants is higher on MMC (Correll *et al.*, 1987).

Mutant Production

To produce mutants, small (4 mm^2) squares of agar with mycelium from PDA plates containing the isolates were placed in the middle of 6 cm Ø PDC and MMC plates (10 plates of each per isolate). The plates were incubated in the dark at 25ºC for 1-2 weeks, and checked for mutant production every few days. Chlorate-resistant mutant sectors are characterized by thin, fast expanding mycelium (Puhalla, 1985). Small blocks of this mycelium were subcultured on fresh MM plates. If the fungus continued to exhibit thin but normally expanding growth on MM, it was considered nitrate nonutilizing, a *nit* mutant.

Mutant Phenotyping

Additional MM, as well as NM and HxM plates were prepared to phenotype the generated mutants. Per mutant growing on MM plates, one mycelial block was subcultured on each of the three differential media plates: MM, NM, and HxM (6 cm Ø). These were then incubated at 25ºC, in the dark for 1-2 weeks. Depending on the growth form expressed on the various plates, mutants were phenotyped according to the pattern described in Table 3. Mutants that displayed growth patterns different from those presented in Table 3 were rejected, as they presumably had multiple mutations and may not have been compatible with phenotyped mutants.

Table 3. Growth patterns on differential media used to phenotype nitrate nonutilizing mutants of *Fusarium oxysporum*.

Phenotype	Differential Medium Type		
	Minimal Media	**Nitrite Media**	**Hypoxanthine Media**
nit 1	-	+	+
nit 3	-	-	+
nit M	-	+	-
Wild Type	+	+	+

-: typical mutant growth, with thin mycelium in agar; +: wild-type growth, with cottony mycelium above agar surface and often with typical *Fusarium oxysporum* coloration.

All mutants generated were first phenotyped according to the method described by Correll *et al.* (1987). Only complementary mutants from individual strains were then tested for self-compatibility, and those that proved to be compatible were included in the VC tests with tester strains of pathogenic isolates. The compatibility between a few of the endophytic strains was also tested.

Testing Self-Compatibility of Mutants

Once mutants had been phenotyped, their self-compatibility was tested. If complementary mutants (*nit* 1 and *nit* 3 or *nit* 1 and *nit* M) generated from the same isolate are unable to form heterokaryons with each other, they may be unable to form heterokaryons at all. A VC test with complementary mutants from another isolate would therefore always yield negative results. The self-compatibility tests were carried out on MM plates, and self-compatibility was demonstrated by the growth of wild-type mycelium at the junction point of two mycelia. Non-self-compatible mutants were eliminated. Complementary self-compatible *nit* 1 and *nit* M mutants from each isolate were used for VC testing with the available VCG testers.

Testing the Vegetative Compatibility of Mutants and Testers

Vegetative compatibility tests were carried out on 6 cm Ø MM plates. A total of 3 mycelium blocks were placed on each plate, at equal distances from the centre and edge of the plate and of each other. Since no *nit* 3 mutants were produced, all plates contained either one *nit* 1 Tester and two *nit* M mutants, or one *nit* M Tester and two *nit* 1 mutants.

Plates were incubated in the dark at 25ºC, and the growth of mycelium observed regularly over a 2 week period.

3.3 Results

Many mutants were generated, and phenotyping revealed a number of *nit* 1 mutants for all 10 endophytic *F. oxysporum* isolates. No *nit* M mutants could be generated from isolates MF-40 and S9 (Table 4), and no *nit* 3 mutants were generated at all.

Table 4. Number, frequency and phenotype of nitrate nonutilizing mutants recovered from *Fusarium oxysporum* isolates generated on chlorate containing media.

Endophyte ID Code	Total Number of Mutants Positively Phenotyped	Number and Frequency of *nit* 1 Mutants Recovered	Number and Frequency of *nit* M Mutants Recovered
A1	4	3 (75%)	1 (25%)
Fo162	6	3 (50%)	3 (50%)
MF-25	5	3 (60%)	2 (40%)
MF-40	2	2 (100%)	- -
P12	8	4 (50%)	4 (50%)
P3	5	2 (40%)	3 (60%)
P7	6	5 (83%)	1 (17%)
S7	9	7 (78%)	2 (22%)
S9	5	5 (100%)	- -
V5W2	14	10 (71%)	4 (29%)

* No *nit* 3 mutants were generated.

Where *nit* 1 and *nit* M mutants were generated, self-compatibility tests revealed that all isolates produced at least one pair of self-compatible mutants. These were selected for inclusion in the VC tests. As no *nit* M mutants were generated for MF-40 and S9, the *nit* 1 mutants for these isolates were tested for compatibility with *nit* M mutants from MF-25 and S7, respectively. One of the *nit* 1 mutants from MF-40 was compatible with an MF-25 *nit* M mutant, indicating that they are related, while none of the S9 *nit* 1 mutants were compatible with any of the S7 *nit* M mutants (Table 5). In the table, all combinations tested are indicate by clear boxes, while combinations not testes are shaded. Positive reactions are represented by a "+" sign, and negative ones by a "-" sign.

The results of the vegetative compatibility tests of selected self-compatible mutants with complementary testers of pathogenic *F. oxysporum* strains are presented in Tables 6 & 7. None of the *nit* 1 or *nit* M mutants generated from endophytic isolates of *F. oxysporum* were found to be vegetatively compatible with any of the VCG testers of pathogenic *F. oxysporum* strains included in the test.

Table 5. Results from the self-compatibility test with selected *nit* 1 and *nit* M mutants of nematode antagonistic endophytic *Fusarium oxysporum* isolates. (+: compatible cross, -: non-compatible cross).

		***nit* 1 Mutants**																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	***nit* M mutants**	A1-5	Fo162-1	Fo162-c	Fo162-f	MF-25-1	MF-25-2	MF-25-e	P12-1	P12-c	P12-d	P12-f	P3-a	P3-c	P3-d	P7-3	S7-3	S7-b	V5W2-3	V5W2-4	V5W2-6	V5W2-b
1	A1-1	+																				
2	A1-4	+																				
3	A1-a	+																				
4	A1-b	+																				
5	Fo162-a		+																			
6	Fo162-b		+																			
7	Fo162-d			+																		
8	Fo162-e			-																		
9	MF-25-a						+															
10	MF-25-b						+															
11	MF-25-c																					
12	MF-40-1						+															
13	MF-40-a						-															
14	P12-2																					
15	P12-a										+											
16	P12-b									+	+											
17	P12-e									+												
18	P3-1																					
19	P3-e													+								
20	P7-1															+						
21	P7-2															+						
22	P7-a															+						
23	P7-b															+						
24	P7-c															+						
25	S7-1																+	+				
26	S7-2																	+				
27	S7-a																	+				
28	S7-c																	+				
29	S7-d																	+				
30	S7-e																	+				
31	S7-f																	+				
32	S9-1																-	-				
33	S9-2																-	-				
34	S9-4																-	-				
35	S9-5																-	-				
36	S9-6																-	-				
37	S9-a																-	-				
38	S9-b																-	-				
39	V5W2-2																		+			
40	V5W2-5																					
41	V5W2-a																					+
42	V5W2-c																					+
43	V5W2-d																					-
44	V5W2-e																				+	
45	V5W2-f																				+	
46	V5W2-g																				-	
47	V5W2-h																				+	
48	V5W2-i																					+

Table 6. Vegetative compatibility of *nit* 1 mutants of *Radopholus similis*-antagonistic endophytic *Fusarium oxysporum* isolates and *nit* M testers of pathogenic *Fusarium oxysporum* f. sp. *cubense*, *radicis-lycopersici*, and *lycopersici*.

	***nit* M Testers**	***nit* 1 Endophyte Mutants**									
		A1-1	Fo162-a	MF-25-b	MF-40-a	P12-e	P3-e	P7-2	S7-1	S9-1	V5W2-2
FOC	0120	-	-	-	-	-	-	-	-	-	-
	0121	-	-	-	-	-	-	-	-	-	-
	0122	-	-	-	-	-	-	-	-	-	-
	0123	-	-	-	-	-	-	-	-	-	-
	0124	-	-	-	-	-	-	-	-	-	-
	0125	-	-	-	-	-	-	-	-	-	-
	0126	-	-	-	-	-	-	-	-	-	-
	0128	-	-	-	-	-	-	-	-	-	-
	0129	-	-	-	-	-	-	-	-	-	-
	01210	-	-	-	-	-	-	-	-	-	-
	01211	-	-	-	-	-	-	-	-	-	-
	01212	-	-	-	-	-	-	-	-	-	-
	01213	-	-	-	-	-	-	-	-	-	-
	01214	-	-	-	-	-	-	-	-	-	-
	01215	-	-	-	-	-	-	-	-	-	-
	01216	-	-	-	-	-	-	-	-	-	-
	01217	-	-	-	-	-	-	-	-	-	-
	01218	-	-	-	-	-	-	-	-	-	-
	01219	-	-	-	-	-	-	-	-	-	-
	01220	-	-	-	-	-	-	-	-	-	-
	01221	-	-	-	-	-	-	-	-	-	-
	01222	-	-	-	-	-	-	-	-	-	-
	01223	-	-	-	-	-	-	-	-	-	-
FORL	0090 subgroup I	-	-	-	-	-	-	-	-	-	-
	0090 subgroup II	-	-	-	-	-	-	-	-	-	-
	0090 subgroup III	-	-	-	-	-	-	-	-	-	-
	0091 subgroup I	-	-	-	-	-	-	-	-	-	-
	0091 subgroup II	-	-	-	-	-	-	-	-	-	-
	0092	-	-	-	-	-	-	-	-	-	-
	0093	-	-	-	-	-	-	-	-	-	-
	0094 subgroup I	-	-	-	-	-	-	-	-	-	-
	0094 “Universal”	-	-	-	-	-	-	-	-	-	-
	0096	-	-	-	-	-	-	-	-	-	-
	0098	-	-	-	-	-	-	-	-	-	-
FOL	0030	-	-	-	-	-	-	-	-	-	-
	0032	-	-	-	-	-	-	-	-	-	-

FOC: *F. oxysporum* f. sp. *cubense*, FORL: *F. oxysporum* f. sp. *radicis-lycopersici*, FOL: *F. oxysporum* f. sp. *lycopersici*; -: negative reaction; +: positive reaction.

Table 7. Vegetative compatibility of *nit* M mutants of *Radopholus similis*-antagonistic endophytic *Fusarium oxysporum* isolates and *nit* 1 testers of pathogenic *Fusarium oxysporum* f. sp. *cubense*.

	***nit* 1 Testers**	***nit* M Endophyte Mutants**							
		A1-5	Fo162-1	MF-25-2	P12-c	P3-c	P7-3	S7-3	V5W2-3
F. oxysporum f. sp. *cubense*	0120	-	-	-	-	-	-	-	-
	0121	-	-	-	-	-	-	-	-
	0123	-	-	-	-	-	-	-	-
	0124	-	-	-	-	-	-	-	-
	0125	-	-	-	-	-	-	-	-
	0126	-	-	-	-	-	-	-	-
	0128	-	-	-	-	-	-	-	-
	01210	-	-	-	-	-	-	-	-
	01211	-	-	-	-	-	-	-	-
	01212	-	-	-	-	-	-	-	-
	01213	-	-	-	-	-	-	-	-
	01214	-	-	-	-	-	-	-	-
	01215	-	-	-	-	-	-	-	-
	01218	-	-	-	-	-	-	-	-
	01219	-	-	-	-	-	-	-	-
	01221	-	-	-	-	-	-	-	-
	01222	-	-	-	-	-	-	-	-
	01223	-	-	-	-	-	-	-	-
	01224	-	-	-	-	-	-	-	-

-: negative reaction; +: positive reaction.

3.4 Discussion

The results of mutant production confirm that it is easier to produce *nit* 1 mutants than *nit* M or *nit* 3 mutants. No *nit* M mutants could be generated for isolates MF-40 and S9, and their self-compatibility could therefore not be tested. For the other 8 isolates, self-compatible mutants were found, though three sets of complementary mutants from the same isolate did not form heterokaryons. These mutants were therefore not included in the VCG tests subsequently carried out. Occasionally, such self-incompatible mutants are observed (Jacobson & Gordon, 1988; Katan, 1999). Jacobson and Gordon (1988) attribute this to the inability of an isolate to initiate or complete heterokaryon formation. However, this is not an explanation for why self-incompatibility occurs, but rather a description of what it is. Self-incompatibility may be due to an additional mutation in one or both of the crossed mutants, which render

them unable to form heterokaryons with putative complementary mutants. While self-compatibility of MF-40 mutants could not be tested, the two *nit* M mutants from MF-40 were crossed with one *nit* 1 mutant from MF-25, and formed a heterokaryon. This indicates that these two non-pathogenic isolates are related, and should Vegetative Compatibility Groups be established for non-pathogenic *F. oxysporum* isolates, these two isolates would be placed in the same VCG.

VCG tests with complementary mutants from pathogenic *F. oxysporum* strains and endophytic isolates revealed that none of the endophytic *F. oxysporum* isolates were compatible with the tested pathogenic *F. oxysporum* strains. As mentioned in the introduction, researchers working with pathogenic and non-pathogenic strains of *F. oxysporum* believe that VCG tests are a good method of distinguishing between pathogenic and non-pathogenic isolates of *F. oxysporum* in mixed populations (Katan & Katan, 1988). Due to the relative ease with which this test can be carried out in the lab, it was chosen as an alternative to a classical pathogenicity test. Such a test was necessary to address fears of a possible spread of pathogenic *F. oxysporum* strains through the inoculation of endophytic *F. oxysporum* isolates with biocontrol activity onto valuable crop plants, prior to field release. The isolates in question are being investigated for their potential as biocontrol agents against the burrowing nematode, *Radopholus similis* (Cobb) Thorne, in banana (*Musa* spp.).

This crop is susceptible to a wilt disease caused by *F. oxysporum* f. sp. *cubense* (*Foc*), also known as the Panama Disease. Three races of *Foc* are known to attack *Musa* species: race 1 attacks cultivars in the 'Gros Michel' (AAA) and 'Pome' (AAB) subgroups and the 'Silk' (AAB) and 'Pisang Awak' (ABB) clones of banana; race 2 attacks 'Bluggoe' (ABB) and close relatives; and race 4 attacks cultivars in the Cavendish subgroup (AAA) and hosts of races 1 and 2 (Ploetz, 1990; Ploetz & Pegg, 2000).

Panama disease devastated the banana industry, based on the ‘Gros Michel’ cultivar, in Central America in the 1950s and 60s. This disaster was only overcome by the adoption a new group of cultivars, resistant to race 1 of *Foc* - the Cavendish group (Stover & Malo, 1972; Stover, 1990; Rowe, 1990). The adoption of these cultivars brought with them other production constraints. For example, Cavendish bananas are more susceptible to physical damage (bruising), and therefore have to be treated with great care and shipped in boxes. The Cavendish cultivars are also more susceptible to

diseases such as Black Sigatoka, caused by the fungus *Mycosphaerella fijiensis*, as well as to nematodes, especially *R. similis* (Quénéhervé, 1993; Jeger *et al.*, 1996; Davide, 1996). Until recently, race 4 was only known to attack Cavendish cultivars under sub-tropical conditions, where cold temperatures are believed to predispose the plants to infection by the fungus (Stover & Malo, 1972; Su *et al.*, 1986; Ploetz, 1993; Pegg *et al.*, 1996). However, the continued reliance on Cavendish cultivars and the possibility of a more aggressive form of the Panama Disease developing in Central American production areas are real concerns for producers and researchers alike (Ploetz, 1990). A variant of race 4, the tropical race 4 (TR4) was recently recognized in Southeast Asia (Ploetz & Pegg 2000; Ploetz *et al.*, 2003), and the threat of another disaster like the one caused by race 1 of *F. oxysporum* f. sp. *cubense* in the mid-20th century is a real one.

The consequences of a compatibility of *F. oxysporum* isolates inoculated onto banana plantlets prior to field release and a pathogenic strain of *F. oxysporum* would be devastating and exasperate the problem. The results of this study are therefore of extreme importance to the continued use of our isolates as potential nematode biocontrol agents. Carrying it out was also a pre-requisite to investigate the biocontrol effect of the isolates under field conditions. As expected, none of the isolates proved compatible with the pathogenic *F. oxysporum* isolates tested, and their continued inclusion in the ever growing arsenal of potential biocontrol agents against *R. similis* is hereby guaranteed.

4 ROLE OF SYSTEMIC INDUCED RESISTANCE IN THE NEMATODE-ANTAGONISTIC RELATIONSHIP OF ENDOPHYTIC FUNGI

4.1 Introduction

Plants possess a number of active defence mechanisms to deal with pathogen and parasite attacks (van Loon *et. al*, 1998). When a plant nevertheless succumbs to a disease, these defence mechanisms have been breached. This may be because the pathogen encountered is very virulent and either avoids triggering or suppresses the defensive actions, evades these or is insensitive to them (van Loon *et al.*, 1998). Similar to the effects of vaccination in mammals, a plant's defence mechanisms can be activated prior to infection, resulting in decreased disease symptoms. This type of reaction is called *induced resistance*, and defined as "a state of enhanced defensive capacity developed by a plant when appropriately stimulated" (Kuc, 1982; Kuc, 1995).

As the name implies, pathogen-induced systemic acquired resistance (SAR) is the result of a local infection by a weak or incompatible form of a pathogen that provides the entire plant resistance against future attack by the same and often also other pathogens (van Loon *et al.* 1998). This type of resistance can also be triggered by chemicals involved in the plant response to pathogen infection, such as salicylic acid. In SAR, the defence response involves the triggering of the salicylic acid (SA)-dependent pathway and the production of a set of pathogen related (PR) proteins (van Loon *et al.* 1998). Induced systemic resistance (ISR), on the other hand, is the result of a non-pathogenic trigger, such as rhizospheric or endophytic bacteria and fungi, and involves the production of jasmonic acid (JA) and ethylene (Mandeel & Bakker, 1991; Fuchs *et al.*, 1997; Alabouvette *et al.*, 1998; van Loon *et al.*, 1998; Vu *et al.*, 2006).

To differentiate between SA-dependent SAR or JA- and ethylene-dependent ISR, the plant's response has to be investigated at the molecular level. However, the involvement of systemic induced resistance (SIR) to pathogens, whether it be SAR or ISR, can be investigated with more conventional methods and without delving to the molecular level, by using a split-root system. In a split-root system, pathogens and potential inducers of resistance are physically separated. Split-root systems have been successfully used to identify rhizobacteria and endophytic bacteria and fungi as inducers of systemic resistance to plant parasitic nematodes in potato, tomato and

banana (Hasky-Günter *et al.*, 1998; Munif *et al.*, 2001; Siddiqui & Shaukat, 2002; Hauschild *et al.*, 2004; Vu *et al.*, 2006).

The majority of fungal endophytes encountered in banana roots and subsequently screened for antagonistic activity against the burrowing nematode *R. similis* were *Fusarium* and *Trichoderma* spp. (Pocasangre, 2000; Niere, 2001; zum Felde, 2002; Carñizares Monteros, 2003; Meneses Hérnandez, 2003). The most effective nematode antagonists identified in screening and greenhouse studies carried out in Guatemala and Costa Rica by zum Felde (2002), Carñizares Monteros (2003) and Meneses Hérnandez (2003) were included in this study. The seven fungi selected were identified as non-pathogenic *Fusarium oxysporum* (MF-25, MF-40, S9 and P12) and *Trichoderma atroviride* (MT-20, ET-35 and S2) isolates. Due to international quarantine restrictions, the Guatemalan isolates used in Germany (MT-20, ET-35, MF-25 and MF-40) could not be transported back to Costa Rica, so experiments carried out in Costa Rica were done with a different set of endophytes isolated locally (S2, S9 and P12), in addition to the *T. atroviride* isolate MT-20 from Guatemala, which had been stored in Costa Rica.

The objective of this study was to verify whether or not the selected *R. similis* antagonistic endophytic fungi induce a systemic resistance to nematode penetration or to nematode reproduction in banana plants.

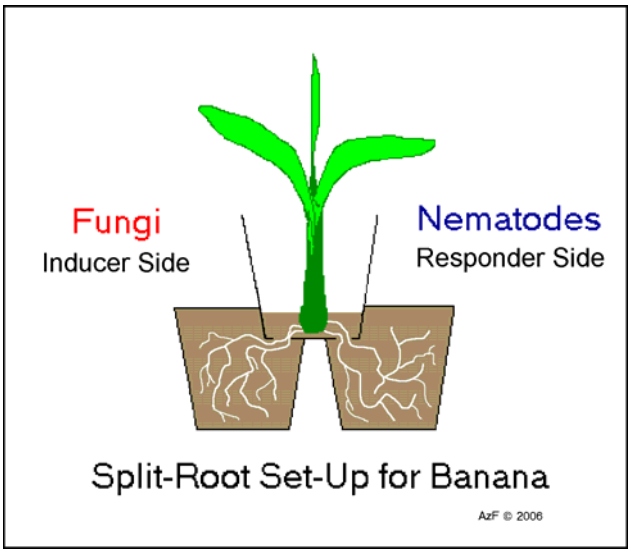
4.2 Materials and Methods

A split-root set-up was used to physically separate endophytes from nematodes, making it possible to test for the involvement of systemic induced resistance in the antagonistic relationship of these fungi and *R. similis*. Fungi were first inoculated onto the *inducer* side and nematodes later inoculated onto the *responder* side of the split-root set-up (Figure 4). A total of eight split-root tests, designated SR I to SR VIII, were carried out (Table 9). In SR I and SR II, carried out in Germany, nematode penetration was determined in plants inoculated with one of 3 endophytes, 1- and 2-weeks after nematode inoculations, respectively. In SR III and SR IV, also carried out in Germany, plants were inoculated with one of 4 endophytes, and nematode penetration evaluated 10-days after nematode inoculation. SR V to SR VIII were carried out in Costa Rica. SR V and SR VII were terminated 10 days after nematode inoculation, to test for induced systemic effects on nematode penetration, while SR VI and SR VIII were terminated 9 weeks after nematode inoculation, to test for induced systemic effects on

nematode reproduction. In all tests, tissue culture banana plants were used and the planting soil used consisted of a mixture (1:1 by volume) of sterile field soil and sand.

The original set-up, with one pot simply placed on top of the two bottom ones was unstable, with roots tending to become exposed and dry out over time. Plant senescence was frequently observed in reaction to exposed roots. To stabilize the system, the set-up was successfully modified by cutting part of the bottom pots out and partially inserting the top pot into the two bottom pots.

Figure 4. Illustration of a split-root set-up modified for use with banana plants.



Tissue Culture Banana Plants

In Germany, tissue culture 'Valery' plants were obtained from Vitropic S.A. (Saint-Mathieu-de-Tréviers, France), in agar-based rooting medium. Plants were immediately washed free of the agar and transferred to 35-cell multi-trays (75 ml capacity round cells) containing planting soil. Plants were then left to develop further in a climate chamber at 25ºC, with 16 hrs of artificial light per day, for 6-8 weeks.

In Costa Rica, tissue culture 'Valery' plants ready for transplantation into soil bags and greenhouse hardening were obtained from a commercial tissue culture laboratory (Cristal Vitro S.A., Concepción de Tres Ríos, Costa Rica), in 96 cell multi-trays. After washing seedling substrate from the roots, these plants were planted in plastic potting bags, containing planting soil. They were then left to grow over a 6 week period in the greenhouse.

After the root development period, plants were used in split-root set-ups.

Fungal Inoculum

The 7 endophytes tested and their origins are listed in Table 8. Two to four weeks before inoculation, fungi were transferred on to fresh 100% potato dextrose agar (PDA) plates, by either placing a mycelium plug from an older plate onto a new one, or by plating a cryo-bead from a -80ºC stock into the centre of the PDA plate. The PDA plates were then placed in an incubator at 25ºC and fungi left to grow and produce conidia for 2 to 4 weeks. Conidia suspensions were prepared by pouring approximately 20 ml water onto the plate surface and gently moving the water over the surface with a flame-sterilised bacteria spreader. The resulting conidia stock suspension was poured into 100-ml Erlenmeyer flasks, through 3 layers of gauze or cheese cloth, to separate conidia from mycelium. The conidia density in the stock suspensions were determined by counting conidia using a Neubauer cell counting chamber. For inoculation of *inducer* roots, suspensions containing 1 x 10^7 conidia ml^{-1} were prepared for each species.

Table 8. List of endophytes used in split-root tests carried out in Germany (2003-2004) and Costa Rica (2005-2006).

Endophyte ID	Species	Isolate Origin		
		Host Plant	Origin	Reference
MF-25	*Fusarium oxysporum*	*Musa* AAA	Guatemala	zum Felde, 2002
MF-40	*Fusarium oxysporum*	*Musa* AAA	Guatemala	zum Felde, 2002
ET-35	*Trichoderma atroviride*	*Musa* AAA	Guatemala	zum Felde, 2002
MT-20	*Trichoderma atroviride*	*Musa* AAA	Guatemala	zum Felde, 2002
S2	*Trichoderma atroviride*	*Musa* AAA	Costa Rica	Carñizares, 2003
S9	*Fusarium oxysporum*	*Musa* AAA	Costa Rica	Carñizares, 2003
P12	*Fusarium oxysporum*	*Musa* AAA	Costa Rica	Menenses, 2003

Nematode Inoculum

R. similis were reared on sterile carrot disks and kept in incubators at 30ºC (Speijer & De Waele, 1997). The *R. similis* population maintained in Germany had been originally isolated from banana roots in Uganda, and cultures were obtained for the laboratory from Prof. Dr. Dirk De Waele. The *R. similis* population in Costa Rica came from banana roots collected by CORBANA in Costa Rica. Once nematodes started

exiting the carrot disks and accumulating along the edges of the Petri dishes in which the carrot disks were kept, they were washed into a 200-ml Erlenmeyer using tap water, and counted. In SR I through SR VI, *responder* side roots were inoculated with 1000 *R. similis*, while only 500 *R. similis* were inoculated onto *responder* roots in SR VII and SR VIII.

Split-Root Set-Up, Fungal and Nematode Inoculations

The first split-root test carried out in Germany (SR I) was terminated 1 week after nematode inoculation, and the second (SR II), 2 weeks after *R. similis* inoculation (Table 9). This was done to determine how long it takes for sufficient nematodes to penetrate banana roots to observe significant differences between penetration rates. Based on the results of these first two tests, further nematode-penetration tests (SR III-V & SR VII) were terminated 10 days after nematode inoculation. The two tests carried out to determine whether there was an induced systemic effect on nematode reproduction (SR VI & VIII), were terminated 9 weeks after nematode inoculation.

In all tests, plants with well developed root systems were gently removed from the multi-tray cells or potting bags, and substrate was washed from roots, which were then separate into two equal roots parts or clusters. Each root cluster was then gently pulled through one of two large holes in the bottom of a pot (in Germany: square, 100 ml pots were used; in Costa Rica, round, 150-200 ml pots were used). This 'top pot' was lowered onto two 'bottom pots', standing side-by-side, thereby lowering one root cluster into each 'bottom pot'. In SR I - SR IV in Germany and SR VII and SR VIII in Costa Rica, both bottom pots were then filled with planting soil. For SR V and SR VII, the *inducer* roots were first dipped in a conidia suspension, before being replaced in the bottom pot, which was only then filled with the planting soil.

For SR I through SR IV, the *inducer* sides of all plants were inoculated with fungi only once, two weeks after the plants had been planted in the split-root set-up (Table 9). This was done by pipetting 5 ml of a 1 x 10^7 conidia/ml conidia suspension into three 1-cm deep holes around the area where roots entered the *inducer* side pot from the top pot, and then covering these holes with soil.

Table 9. Overview of treatments in split-root tests carried out in Germany and Costa Rica.

Test ID	Fungal isolates used	Number of conidia inoculated	Number of *R. similis* inoculated & termination date
SR I - Germany	MF-25, ET-35, MT-20	5 x 10^7 conidia, 2 weeks after planting	1000 *R. similis*, 2 weeks after fungi terminated after **1** week
SR II - Germany	MF-25, ET-35, MT-20	5 x 10^7 conidia, 2 weeks after planting	1000 *R. similis*, 2 weeks after fungi terminated after **2** weeks
SR III - Germany	MF-25, MF-40, ET-35, MT-20	5 x 10^7 conidia, 2 weeks after planting	1000 *R. similis*, 2 weeks after fungi terminated after 10 days
SR IV - Germany	MF-25, MF-40, ET-35, MT-20	5 x 10^7 conidia, 2 weeks after planting	1000 *R. similis*, 2 weeks after fungi terminated after 10 days
SR V - Costa Rica Penetration I	MT-20, S2, S9, P12	5 min dip in 1 x 10^6 conidia, at planting; 2 x 10^7 conidia, at planting; 5 x 10^6 conidia, 3 weeks after planting	1000 *R. similis*, 1 week after fungi terminated after **10** days
SR VI - Costa Rica Reproduction I	MT-20, S2, S9, P12	5 min dip in 1 x 10^6 conidia, at planting; 2 x 10^7 conidia, at planting; 5 x 10^6 conidia, 3 weeks after planting	1000 *R. similis*, 1 week after fungi terminated after **9** weeks
SR VII - Costa Rica Penetration II	MT-20, S2, S9, P12	5 x 10^7 conidia, at planting	500 *R. similis*, 2 weeks after fungi terminated after **10** days
SR VIII - Costa Rica Reproduction II	MT-20, S2, S9, P12	5 x 10^7 conidia, at planting	500 *R. similis*, 2 weeks after fungi terminated after **9** weeks

In the tests carried out in Germany (SR I to SR IV), N=7. In Costa Rica, N=6 for SR V and SR VI, and N=8 for SR VII and SR VIII.

In Costa Rica, *inducer* roots in the first two tests (SR V & VI) were dip inoculated for 5 min in 300 ml conidia suspension at planting, followed by a soil-injection-inoculation and a second soil-injection-inoculation 3 weeks after planting (Table 9). For soil-injection-inoculation, a 20-ml sterile syringe, mounted with a 5-cm long blunt-ended needle was used to inject conidia suspensions into the soil at a depth of 1-2 cm, at three sites around the roots. As dip inoculations of split-root plants proved very difficult, the second set of tests in Costa Rica (SR VII & VIII) were only inoculated

once by soil-injection-inoculation. In all experiments, control plants received the same treatment as plants inoculated with fungi, but using tap water only.

The *responder* sides of SR I to SR IV were inoculated with 1000 *R. similis*, by pipetting 3-5 ml (depending on nematode concentration) of nematode suspension into three 1-cm deep holes along the area where roots from the top pot met the *responder* pot. For SR V to SR VIII, *R. similis* were inoculated as fungi were in these tests, by using a blunt-ended needle and syringe to inject nematodes 2-3 cm into the soil in the area where roots from top pot met the *responder* side. Nematodes were inoculated 2 weeks after fungi in all tests but SR V and SR VI, where nematodes were inoculated 1 week after the 3rd fungal inoculation.

Nematode Extraction and Count

Once an experiment was terminated, *responder* and *inducer* roots were carefully separated from the top pot, and separately washed free of soil. Weight of roots from both sides, as well as shoot weight were taken. In Germany, nematodes were stained using Fuchsine Acid for ease of counting. The entire root system was stained, cut into approx. 1 cm long pieces, and macerated in 20-50 ml tap water, in a large test tube, using an Ultra Turax macerator. The macerated roots were then diluted in 100 ml tap water in graduated cylinders and nematodes in two 10-ml sub-samples counted using a Nordmeyer chamber.

In Costa Rica, the sieving maceration method of nematode extraction used was adapted from Speijer and De Waele (1997): roots were cut into approx. 1 cm long pieces and macerated in a commercial blender, 5 sec at high speed, 5 sec rest, and final 5 sec at low speed. Macerate was sieved through 3 nested sieves with 500, 150 and 25 µm opening size, respectively. Nematodes were washed from the 25 µm sieve into 250-ml pots with caps, and filled to 200 ml. Nematodes in two 2-ml sub-samples were counted. Results were averaged for each plant, and the number of nematodes that had penetrated the root system was calculated.

Statistical Analysis

All data was statistically analyzed for significant differences using the Least Significant Difference (LSD) test in the SPSS statistical program (SPSS® 13.0 for Mac). Data was not transformed for analysis.

4.3 Results

Nematode Penetration Tests in Germany - SR I & II

In the first set of tests in Germany, in which three of the four endophytes from Guatemala were tested, significant differences in nematode penetration in the *responder* roots were not seen when nematodes were extracted 7 days after being inoculated (Figure 5). Of the 1000 *R. similis* nematodes inoculated, only 13% to 20% penetrated the banana root system within the first 7 days (SR I), and treatments with MF-25, ET-35 and MT-20 reduced penetration by 35, 13 and 10%, respectively.

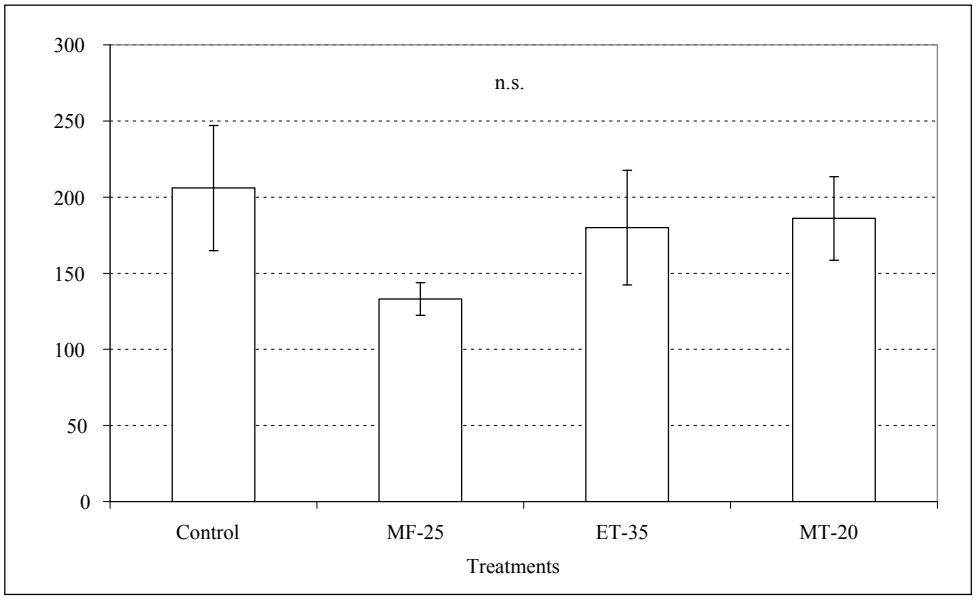


Figure 5. Number of *Radopholus similis* that penetrated the *responder* roots of banana plants in a split-root system, 7 days after nematode inoculation (SR I - Germany, 08-09.2003). Means with the same letter are not significantly different, based on LSD test ($P \leq 0.05$; N=7).

When extraction was done 14 days after nematode inoculation (SR II), results differed from 7-day data, and significant differences in penetration between the treatments and the control were observed (Figure 6). MT-20 seemed to be an especially promising SIR candidate, significantly reducing *R. similis* penetration by 44% in this test. MF-25 increased penetration non-significantly by 20% and ET-35 reduced it by 13%. However, total penetration rates were not higher than after 7 days, ranging from 9 to 20% of inoculated nematodes. A period of 10 days was chosen for further penetration tests.

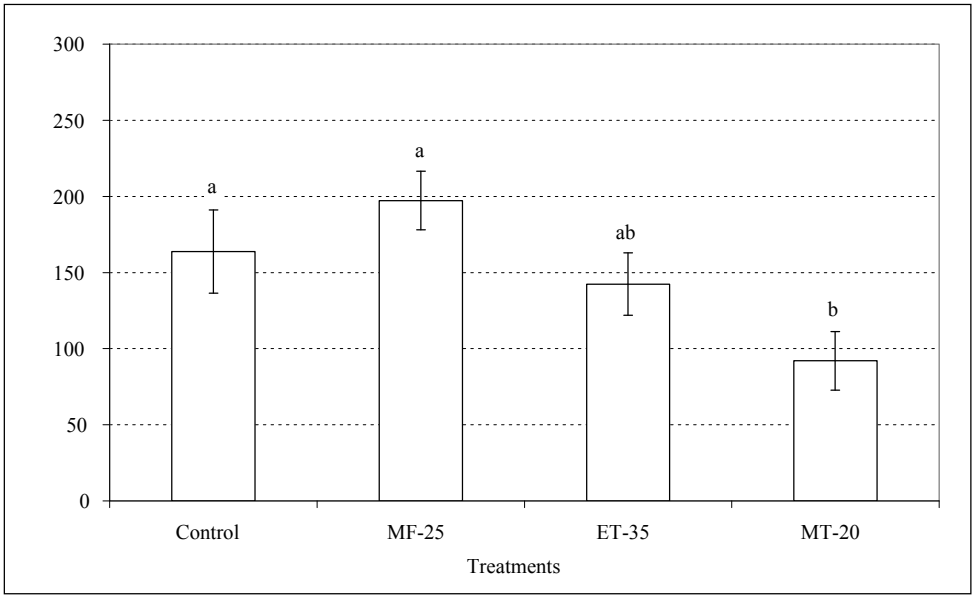


Figure 6. Number of *Radopholus similis* that penetrated the *responder* roots of banana plants in a split-root system, 14 days after nematode inoculation (SR II - Germany, 08-09.2003). Means with the same letter are not significantly different, error bars represent standard error of mean, based on LSD test (P≤0.05; N=7).

Nematode Penetration Tests in Germany - SR III & IV

In the second set of tests, where all four Guatemalan endophytes were tested, all plants were harvested 10 days after nematode inoculation. Significant differences in nematode penetration were observed between endophyte treatments, but not between treatments and the control in SR III (Figure 7). MF-25 again non-significantly increased penetration (+13%) in this test, while ET-35, MT-20 and MF-40 reduced penetration by 36, 12 and 34%, respectively.

Despite the fact that plants were kept in the same greenhouse, and tests ran parallel to each other, no significant differences were observed in SR IV (Figure 8). MF-25 again increased penetration (+15%), and ET-35 reduced it (-7%), while MT-20 and MF-40 increased penetration by 11 and 6%, respectively. As seen in the SR II, MF-25 tended to increased nematode penetration, while ET-35 decreased penetration, when compared to the control, tough not significantly.

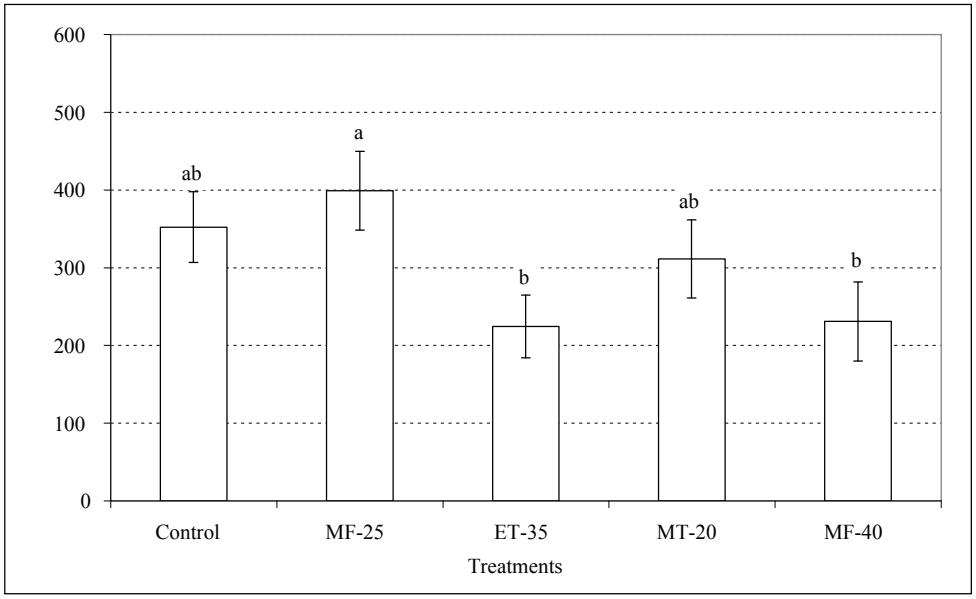


Figure 7. Number of *Radopholus similis* that penetrated the *responder* roots of banana plants in a split-root system, 10 days after nematode inoculation (SR III - Germany, 10-11.2003). Means with the same letter are not significantly different, error bars represent standard error of mean, based on LSD test (P≤0.05; N=7).

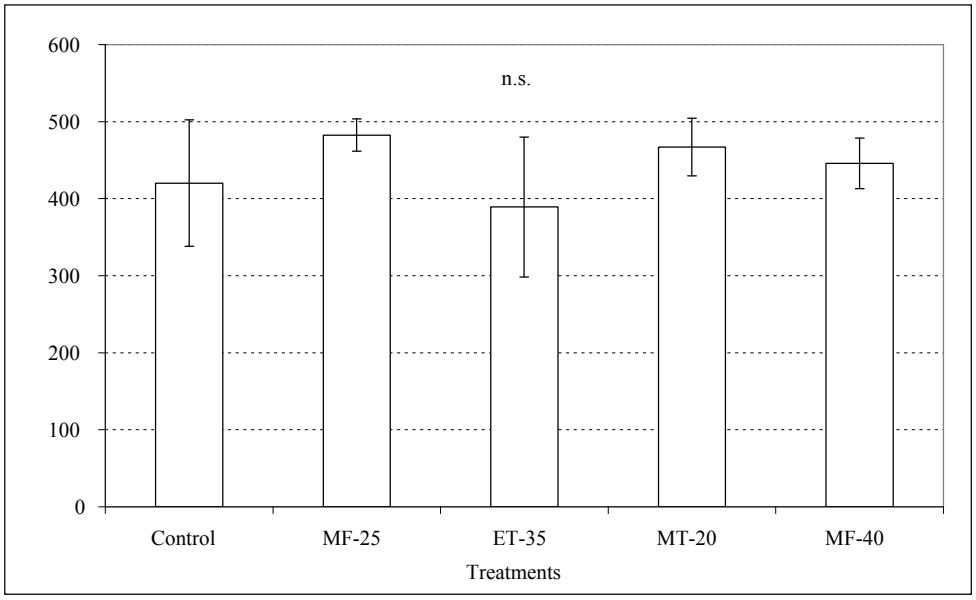


Figure 8. Number of *Radopholus similis* that penetrated the *responder* roots of banana plants in a split-root system, 10 days after nematode inoculation (SR IV - Germany, 10-11.2003). Means with the same letter are not significantly different, error bars represent standard error of mean, based on LSD test (P≤0.05; N=7).

Nematode Penetration Tests in Costa Rica - SR V & VII

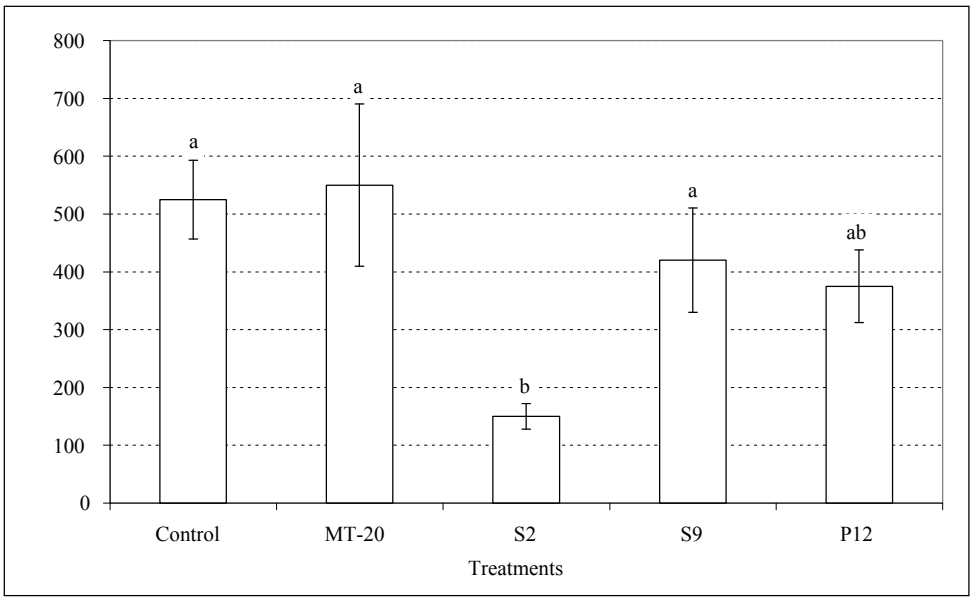


Figure 9. Number of *Radopholus similis* that penetrated the *responder* roots of banana plants in a split-root system, 10 days after nematode inoculation (SR V - Costa Rica, 02-03.2005). Means with the same letter are not significantly different, error bars represent standard error of mean, based on LSD test (P≤0.05; N=6).

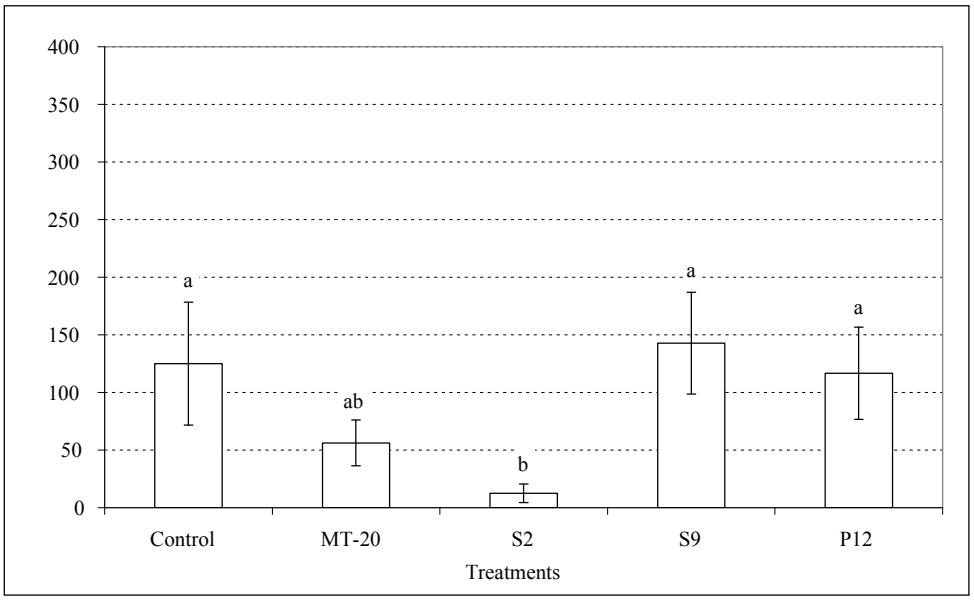


Figure 10. Number of *Radopholus similis* that penetrated the *responder* roots of banana plants in a split-root system, 10 days after nematode inoculation (SR VII - Costa Rica, 11-12.2005). Means with the same letter are not significantly different, error bars represent standard error of mean, based on LSD test (P≤0.05; N=7).

In both SR V (Figure 9) and SR VII (Figure 10), a similar trend is discernable with regard to SIR for the isolates S2 and P12. S2 significantly reduced nematode penetration by 71 and 90% compared to the control in SR V and SR VII, respectively. P12 tended to reduce penetration, but not significantly compared to the control (29 and 7% reduction in SR V and SR VII, respectively). S9 did not significantly affect penetration, reducing it by 20% in SR V and increasing it by 14% in SR VII. MT-20 once again gave contradictory effects, increasing penetration non-significantly in SR V by 5%, and reducing it non-significantly by 55% in SR VII.

Nematode Reproduction Tests in Costa Rica - SR VI & VIII

Plants in the first test looking at systemically induced effects on reproduction of *R. similis* in banana roots in Cost Rica (SR VI) were exposed to extreme temperatures in the greenhouse (>60ºC). This is most likely why nematodes did not reproduce in the roots over the 9 weeks the experiment ran. No significant differences between nematode numbers in roots could be observed and results are inconclusive (data not shown).

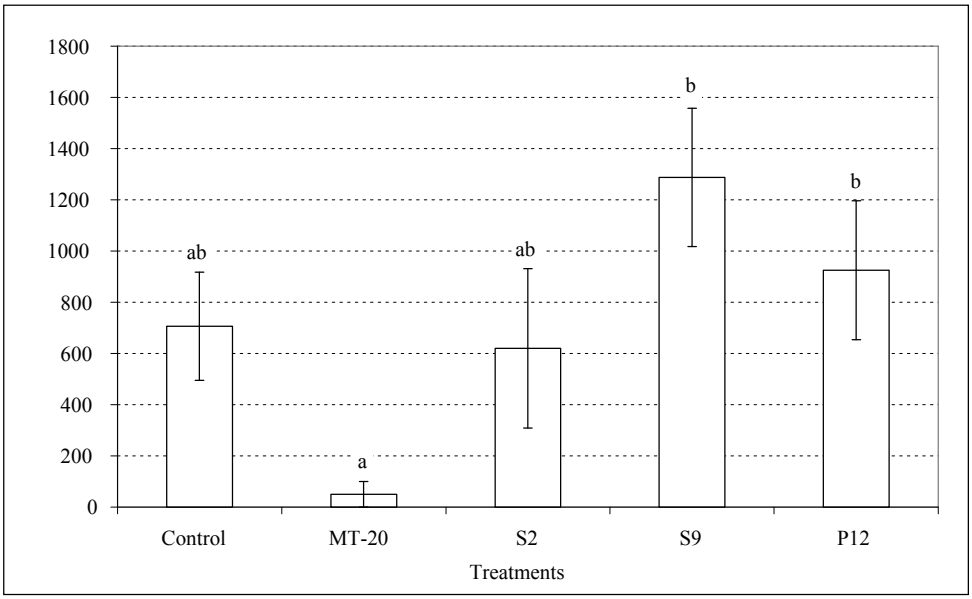


Figure 11. Number of *Radopholus similis* that penetrated the *responder* roots of banana plants in a split-root system, 9 weeks after nematode inoculation (SR VIII - Costa Rica, 11.2005-01.2006). Means with the same letter are not significantly different, error bars represent standard error of mean, based on LSD test (P≤0.05; N=7).

When this test was repeated at lower and more stable temperatures (SR VIII), significant effects on nematode reproduction were detected between endophyte

treatments, but not between treatments and the control (Figure 11). MT-20 reduced nematode reproduction by 93%, while S9 and P12 increased it by 82 and 31%, respectively, though not significantly, when compared to the control after 9 weeks. S2 decreased reproduction non-significantly by 12%.

4.4 Discussion

Four effective nematode-antagonistic fungi from Guatemala, as determined by greenhouse tests in Costa Rica (zum Felde, 2000; zum Felde *et al.*, 2005), were tested in the first set of split-root tests in Germany (SR I - SR IV). The results were variable, with every repetition of the test giving apparent contradictory information. There is no clear and consistent evidence that any of the fungi tested induce systemic resistance to nematode penetration. Nematode penetration was generally low, even in control plants. In SR I and SR II, where nematode penetration was evaluated 1- and 2-weeks after inoculation, results indicate that nematodes initially penetrated roots to similar degrees, irrespective of treatment, and later apparently exited roots, in treatments with the *T. atroviride* isolates ET-35 and MT-20. Nematodes in MF-25 treated plants continued to penetrate, reaching levels slightly greater than those in control plants. The plant may need a certain amount of time after nematode attack to fully activate its defences, despite a potential induction of resistance due to prior fungal infection. The fungi alone may not be enough to trigger a full-fledged defensive response, while the interplay of fungi and nematode penetration may do so. The time between nematode inoculation and extraction may play a role in the success of detecting SIR in banana plants.

In the second set of split-root tests (SR III and SR IV), when nematodes were extracted 10 days after inoculation, the *F. oxysporum* isolate MF-25 tended to increase penetration, while the *T. atroviride* isolate ET-35 tended to decreased it, as they did in test SR II. The *T. atroviride* isolate MT-20 gave mixed results, as did the *F. oxysporum* isolate MF-40. Only in the MT-20 treatment in test SR II, where nematodes were extracted 14-days after nematode inoculation was a significant difference in nematode penetration between the control and a fungal treatment observed. Apparently the only systemic effect of the *F. oxysporum* isolate MF-25 is to attract *R. similis* to banana roots. However, it is a proven nematode antagonist (zum Felde *et al.*, 2005). It's mode of action may be restricted to direct interaction with the nematode once it has penetrated the root, and only observable over time. Tests run to detect nematode antagonism

concentrated on nematode reproduction over 9 weeks, and not on nematode penetration (zum Felde *et al.*, 2005). In treatments with the *T. atroviride* isolates, nematode numbers decreased over time. This may be due to the stimulation of the plant's natural defences, after the combined trigger effect of fungal inoculation and nematode penetration, or it may be the effect of fungal metabolites reaching distant parts of the plant, in response to nematode attack. However, only ET-35 consistently, if only slightly, reduced nematode numbers in roots over 10 to 14 days, while nematode numbers in plants treated with MT-20 fluctuated.

Systemic resistance to *R. similis* penetration was induced by a Ugandan *F. oxysporum* isolate (V5W2) in a study by Vu *et al.* (2006). They inoculated fungi twice, once one week after planting, and again 2 weeks later. In the present study, fungi were only inoculated once, 2 weeks after planting, though with five times more conidia per plant. Potentially, the two week period between the fungal and the nematode inoculations was not enough to allow for the expected systemic effect to manifest itself. In Vu *et al.*'s (2006) experiments, fungi and plants had four weeks of interaction before the nematode-challenge inoculation took place. Also, nematode penetration rates in control plants in Vu *et al.*'s (2006) experiments ranged from 50 to 100% of inoculated nematodes, while penetration in endophyte treated plants ranged from 35 to 100% of inoculated nematodes. These high penetration rates positively influenced the results obtained, while the extreme low penetration rates in the present study (max. ≤50%) may be partially responsible for a lack of significant results and may have hindered the visualisation of a potential induced systemic effect. Nevertheless, the only conclusion that can be drawn from the results remains that none of the four Guatemalan fungi studied induce systemic resistance to *R. similis* penetration after 2 weeks of interaction with the banana plant under greenhouse conditions in Germany.

In Costa Rica four endophytes with proven efficiency in controlling *R. similis* in greenhouse trials (zum Felde, 2002; Carñizares Monteros, 2003; Menenses Hérnandez, 2003) were chosen for split-root tests (SR V - SR VIII). These fungi consisted of the Guatemalan *F. oxysporum* isolate MT-20, which was also tested in Germany, and 3 new Costa Rican endophytes: *T. atroviride* isolate S2 and two *F. oxysporum* isolates S9 and P12.

In SR V and SR VII, MT-20 continued to give variable results, non-significantly increasing nematode penetration in one test and decreasing it in the second. The two *F.*

oxysporum isolates, S9 and P12 never significantly increased or decreased nematode penetration, indicating that they do not induce systemic resistance to nematode penetration. However, the *T. atroviride* isolate S2 consistently and significantly reduced nematode penetration 10-days after nematode inoculation. It was the only endophyte tested that induced systemic resistance to penetration.

Penetration rates in the SR V were higher than in SR VII, ranging from 5 to 15% in S2 treated plants and 26 to 55% in control plants, respectively. In SR V, the period between planting, fungal inoculations and nematode inoculation was much larger than in SR VII. Plants were inoculated at planting and 3 weeks later in SR V, allowing fungi and plants to interact for 4 weeks before being challenged with nematodes. In SR VII, fungi were inoculated at planting and nematodes 2 weeks later. This difference in time between planting and nematode inoculation may partially explain why overall nematode penetration rates were different between the two tests: the longer the roots developed in the soil, the more root exudates enter the soil, allowing nematodes to better orient themselves and find roots to penetrate. This phenomenon would not have been observed in experiments carried out in Germany, as all plants were challenged with nematodes at the same time, namely 2 weeks after fungal inoculation and a total of 4 weeks after planting. By the time nematodes were inoculated in Vu *et al.*'s (2006) experiments, plants had been in the soil for over 5 weeks, and colonized by the endophytic fungi for 4 weeks. Banana roots grow very fast and small pots were used. In one week, the roots could colonize the soil in the pot more extensively, increasing not only contact area with nematodes, but also releasing more exudates into the soil. Fungi could modify or add to these root exudates, thereby either increasing or decreasing nematode attraction to roots, and consequently affecting nematode penetration rates. However, the longer the fungi reside in the plant's roots, the greater the potential that the effect seen is not only due to the activation of the plant's inherent defence mechanism, but also to fungal metabolites that may be carried systemically through the plant. However, whether or not fungal metabolites are capable of moving through a plant in a way similar to certain systemic fungicides has never been specifically studied. Nevertheless, the toxins produced by some pathogenic fungi are known to move through plants (Van Alfen, 1989), eventually blocking xylem vessels and causing the plant to wilt. These toxins are fungal metabolites, so there is evidence that at least some movement of fungal metabolites is possible within a plant.

In the long-term experiment, where effects on nematode reproduction were studied, nematode reproduction was affected by systemic activity. Whether this can be attributed to the activation of the plant's inherent resistance to nematode reproduction, or to systemically transported fungal metabolites could not be verified. Generally, treatments with *T. atroviride* tended to limit nematode reproduction to that seen in the control or reduce it below control levels, while *F. oxysporum* treatments tended to increase nematode reproduction.

In conclusion, none of the *F. oxysporum* isolates tested (MF-25, MF-40, S9 and P12) induced systemic resistance to nematode penetration or reproduction. Of the two *T. atroviride* isolates, MT-20 either did not affect nematode penetration significantly or decreased it slightly, once significantly. It does however seem to have a significant negative effect on nematode reproduction, reducing *R. similis* reproduction by more than 90% over 9 weeks. Isolate S2 consistently and significantly reduced nematode penetration compared to the control, and does seem to induce systemic resistance to nematode penetration. However, S2 has no effect on nematode reproduction.

To better understand the type of systemic resistance involved (SAR vs. ISR), the molecules produced by the plant and the genes involved in the response to both endophyte colonization and nematode attack have to be studied. Without this information, one can only confirm that systemic induction occurs, but not which specific plant defence pathways are involved. Another unknown factor is role of fungal metabolites and their potential systemic transport through plant tissues. Studies looking at the plant's responses to endophyte colonization and nematode attack and the fate of fungal metabolites in plant tissues at the molecular level would help identify the type of systemic resistance involved in the antagonistic system observed and to clarify whether or not fungal metabolites can be systemically transported to distal tissues within plants.

5 TRANSFER OF SUPPRESSION IN SUCCESSIVE GENERATIONS OF BANANA PLANTS FROM THE FIELD

5.1 Introduction

Nematode suppressive fields have been reported for both sedentary and migratory nematodes in various regions of the world (Bird & Brisbane, 1988; Crump & Kerry, 1987; Kerry & Crump, 1977; Kluepfel *et al.*, 1993; Westphal & Becker, 1999). A previous study (zum Felde, 2003), confirmed the existence of such an area in the Motagua Valley of Guatemala, where *Radopholus similis* is suppressed in commercial banana plantations. The fact that the observed suppression was transferred from one farm district (Motagua A) to another (Panchoy) by transplanting corms, lead to the conclusion that the suppressive agents must reside within the plant, as opposed to in the soil. The banana cultivar used (*Musa* AAA cv. 'Valery') is not resistant to nematodes and care was taken during transplantation to not transfer soil along with corms. In fact, corms were not only pared prior to being planted in the new field, but also hot water treated, so neither soil nor nematodes were transferred into the new field.

Endophytic fungi were subsequently isolated from banana roots from both districts, screened for biocontrol activity in green house tests, and promising biocontrol candidates identified. The results confirmed that the suppression observed in Guatemala is at least partially due to endophytic fungi. This type of suppression was termed *in-planta* suppression.

Nematode *in-planta* suppression in the Motagua Valley is long-term, if not permanent, and newly planted bananas take on the suppressive character of their neighbours. Therefore the endophytes responsible for suppression are presumably disseminated, either through the soil or via run-off water or through direct transferred from mother to daughter plant.

This study was undertaken to determine whether the suppression of nematodes caused by individual endophytic fungi inoculated onto tissue culture plants prior to field planting is transferred to successive generations of banana plants over suckers in the field.

5.2 Materials & Methods

This study constituted part of a large scale field trial run by INBAP and the Del Monte Fresh Fruit Company, on Del Monte land in Costa Rica. The field trial's objectives were to evaluate the nematode biocontrol and plant growth promoting effects of four individual endophytic fungal isolates in the field. Four farms were chosen for the experiment: Carmen-2, Bananita, Duacari-2 and Formosa. On each farm, an area of approx. 2 ha was cleared by injecting the systemic herbicide Roundup into existing banana mats. Rows were cleared in the litter and planted to 1850 plants/ha. Tissue culture cv. 'Valery' banana plants (*Musa* AAA) were used. The six treatments tested were as follows: four treatments inoculated with one of the four endophytes listed in Table 10, a nematicide treatment and an absolute control. The field trial and the transfer of suppression experiment were repeated twice, once in the rainy season (planting September-October 2004) and once in the dry season (planting June-July 2006).

Table 10. List of endophytes used to inoculate field trial plants.

Endophyte Inoculated	Endophyte Identity and Origin
MT-20	*Trichoderma atroviride* / Motagua, Guatemala
S2	*Trichoderma atroviride* / Sixaola, Costa Rica
S9	*Fusarium oxysporum* / Sixaola, Costa Rica
P12	*Fusarium oxysporum* / Talamaca, Costa Rica

The endophytes used for inoculation were reared on 100% PDA plates in the laboratory at CATIE. For inoculation, suspensions of 1.5 x 10^6 spores/ml were prepared and multi-trays containing 96 tissue culture plants were dipped into trays containing the spore suspension for 5 minutes. The inoculated plants were then planted into 1.5 L plastic bags containing commercial potting soil and left to harden in a commercial screenhouse for 6 weeks. Over 13000 plants were thus inoculated, approx. 3250 per endophyte treatment. An additional 7000 plants were planted into bags and later served as control and nematicide treatment plants. On each farm, 500 plants were planted per treatment.

Nematicides were applied around the planting holes of plants in the nematicide treatment blocks according to the following application schedule: 23.3 g Nemacur 15G, 15 days after planting; followed by an application of a different nematicide every three

months: first 23.3 g of Mocap 15G, then 20.0 g of Counter 15G and finally 23.3 g Rugby 10G.

Two months after planting, monthly monitoring of nematode populations and collection of plant growth parameters started. Sampling was done according to the method described by Speijer and Gold (1996). Composite root samples were made up of the excavated roots of ten plants per treatment block per farm. Nematode data was collected for a period of 7 months, after which monitoring ceased as plants started to flower so as not to negatively affect yields.

Transfer of suppression was evaluated as follows: suckers were collected from the field trial 12-14 weeks after field planting, coinciding with the first desuckering of the trial plots. Twenty sword suckers, with corms approx. 10 cm in diameter were collected per treatment per farm. Suckers were subsequently washed free of soil and the corms pared before being transported to the greenhouse for planting and resprouting.

In the greenhouse, pared sucker corms were planted in 1.5 L plastic bags containing sterile potting mix (sand:soil, 1:1 v/v) and regularly watered. Eight to ten weeks after planting, sufficient corms (6-10) had sprouted per treatment per farm. Sets of corms from the same farm from each treatment were then moved to a new table, arranged in a random block design, and inoculated with 1000 *R. similis* nematodes. Nematodes were left to develop in the banana roots for 9 weeks before the experiment was terminated, at which point the roots were washed free of soil, weighed and root structure analysed using WinRhizo.

In the laboratory, nematodes were extracted from the roots using the maceration-sieving technique adapted from the one described by Speijer and De Waele (1997): the entire root system was cut into 1 cm pieces, and macerated in a commercial blender in 200 ml tap water for 5 sec at low, and 5 sec at high speed. The suspension was sieved in nested sieves of 1000 mm, 150 mm and 45 mm apertures. The content of the last sieve was washed into a 250 ml capacity capped plastic jar and filled with tap water to 200 ml.

Nematodes in two 2 ml sub-samples were counted. The number of nematodes per root system and per g root were then calculated. Data were analysed using the Tukey test ($P \leq 0.05$) in the SPSS statistical program (SPSS® 13.0 for Mac). All nematode data were ln (x+1) transformed for analysis.

5.3 Results

Though the collected sucker appeared healthy at the time of collection and after paring, not all sprouted after being planted in the greenhouse. From the rainy season trial, sufficient corms from Bananita, Duacari-2 and Formosa were recovered and used in the transfer of suppression test. Corms from Carmen-2 did not sprout. From the dry season trial, only the pared suckers from Bananita sprouted, while those from Carmen-2, Duacari-2 and Formosa rotted in the planting bags. Results presented are therefore restricted to those from the corms of three farms in the rainy season (Bananita, Duacari-2 and Formosa) and from Bananita only in the dry season.

Bananita - Rainy Season

The overall nematode population in the roots of resprouted corms was lower in endophyte treated plants than in the control and nematicide treated plants (Figure 12). Field applications of nematicide did not protect the removed suckers from future nematode attack while endophyte inoculation of mother plants did.

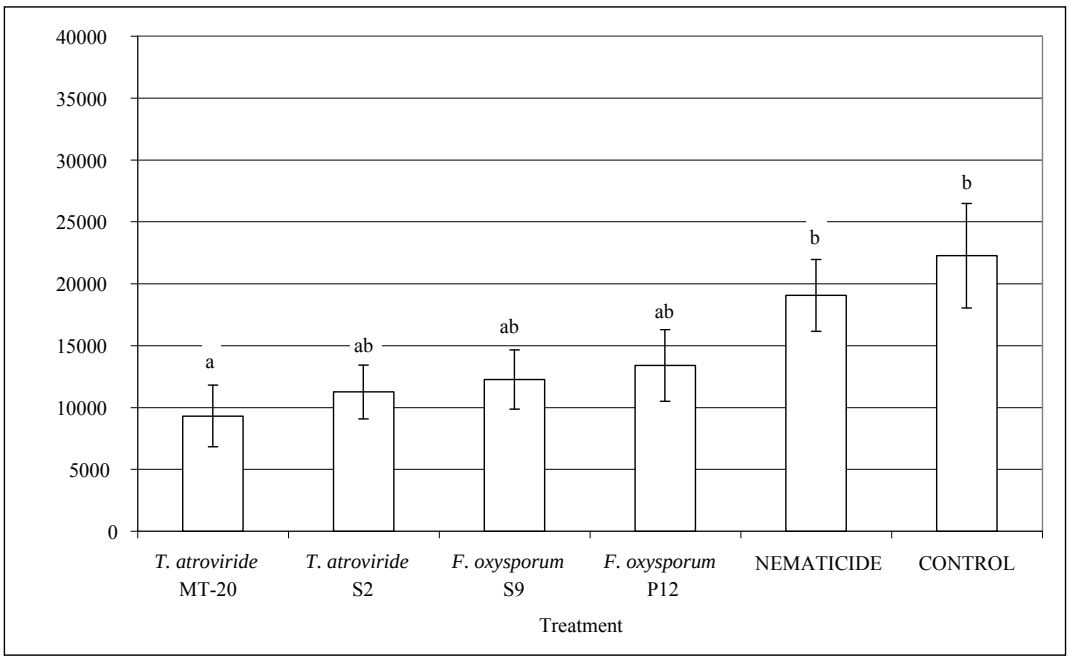


Figure 12. *Radopholus similis* per root system of banana suckers from Bananita during the rainy season, 9 weeks after inoculation. (N=10) Columns with the same letter are not significantly different from each other (P≤0.05, Tukey Test). Bars represent standard error of means.

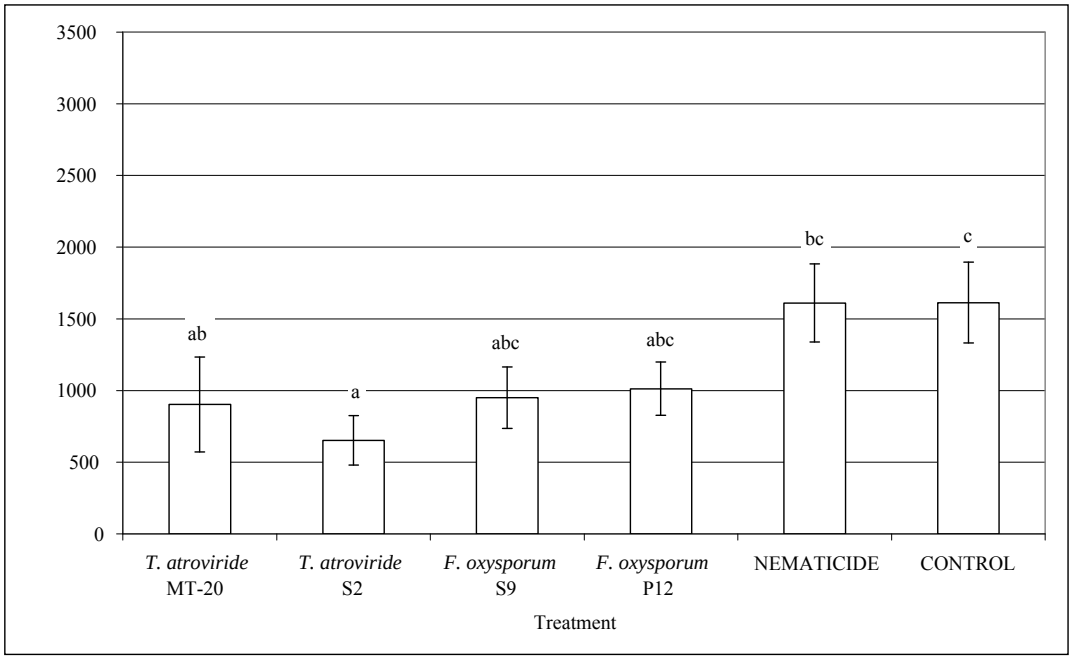


Figure 13. *Radopholus similis* density per g root of banana suckers from Bananita during the rainy season, 9 weeks after inoculation. (N=10) Columns with the same letter are not significantly different from each other (P≤0.05, Tukey Test). Bars represent standard error of means.

Nematode density was affected in endophyte treated plants (Figure 13), with the two *T. atroviride* isolates (MT-20 and S2) having the greatest nematode density reducing effect. No residual effect of field nematicide applications could be observed.

No clear nematode suppression in endophyte treatments was seen in nematode populations in the field trial at the time of sucker removal (Table 11). Nematicide treatment in Bananita at that time of sucker removal and over time was efficient, and over the 7-month run of the experiment, it became obvious that the endophytes did suppress *R. similis* in this farm (Menjivar Barahona, 2005).

Results indicated that especially the *T. atroviride* isolate S2 has nematode biocontrol activity, comparable in efficiency to the nematicide treatment. The nematode density results from endophyte treatments and control corms are comparable to the 7-month average field trial results, with S2 controlling nematodes the best, followed by the other three endophyte treatments with intermediate results. Results for the nematicide treatment are of course different, as no nematicides were used in the greenhouse and nematode populations in corms from nematicide-treated blocks increased as they did in the control corms.

Table 11. *Radopholus similis* population in roots of field trail plants from Bananita during the rainy season, at sucker removal (Jan. 2005) and on average, over 7 months of monitoring (Dec. 2004-June 2005). (N=10, P≤0.05, Tukey test) Columns with the same letter are not significantly different from each other.

Treatment	# *R.similis*/100g root at sucker removal		# *R.similis*/100g root over 7 months	
Trichoderma atroviride isolate MT-20	16475	ab	21662	bc
Trichoderma atroviride isolate S2	24950	b	16079	a
Fusarium oxysporum isolate S9	13600	ab	24832	bc
Fusarium oxysporum isolate P12	12650	ab	19083	bc
Nematicide	8683	a	17196	b
Control	20553	ab	35876	c

Figure 14 shows the dynamics of the field nematode population. Nematode populations in the control, nematicide treated and S9 treated plots increased steadily over time, while those in MT-20, S2 and P12 treated plots initially increased, then either levelled off or decreased, before ending below the level of both control and nematicide treated plots. It appears that nematode suppression by endophytes took a few months to establish.

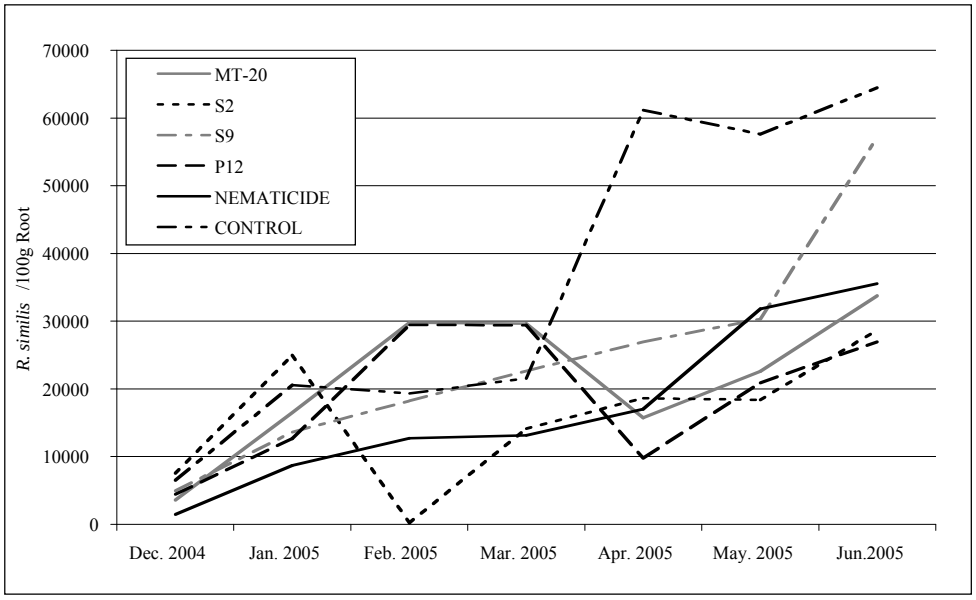


Figure 14. Dynamics of the field population of *Radopholus similis* during the rainy season in Bananita farm (N=10) (adapted from Menjivar Barahona, 2005).

Duacari-2 - Rainy Season

Corms from Duacari-2 did not sprout as readily as did Bananita corms. Only 4 corms from the control, 5 from the nematicide treatment, 7 from the P12 and 6 from the MT-20, S2 and S9 treatments sprouted. This highly variable replica number affected both results and the statistical analysis, so that no significant differences in nematode population (Figure 15) nor nematode density (Figure 16) could be observed in the roots of re-sprouted corms from Duacari-2.

MT-20 treated plants had the lowest nematode density and population, indicating suppression may have been transferred, but not enough suckers sprouted to reveal statistically relevant differences. Nematode density and population in nematicide treated corms was also quite low, though once again replica numbers were insufficient to reveal significant differences.

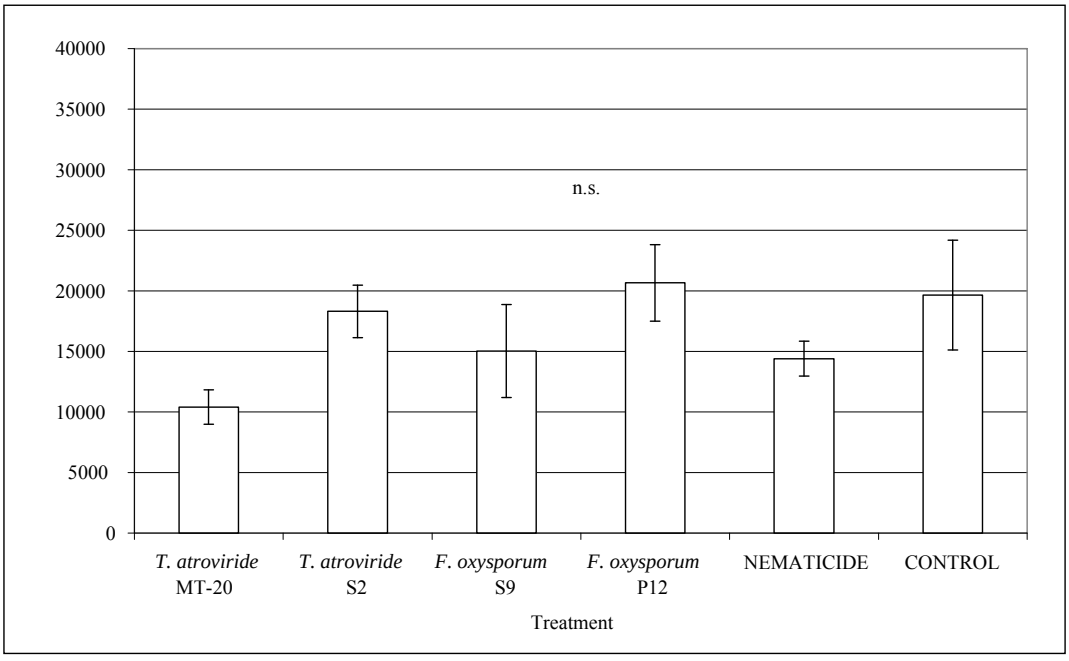


Figure 15. *Radopholus similis* per root system of banana plants from Duacari-2 during the rainy season, 9 weeks after inoculation. (N=4-7) No significant differences were found (P≤0.05, Tukey Test). Bars represent standard error of means.

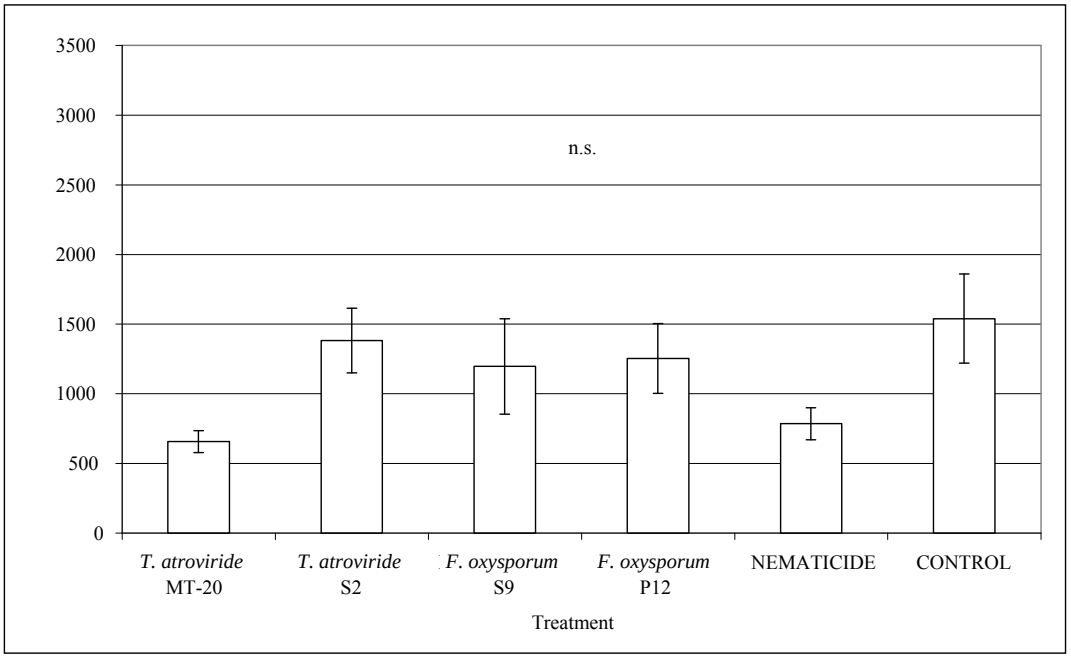


Figure 16. *Radopholus similis* density per g root of banana plants from Duacari-2 during the rainy season, 9 weeks after inoculation. (N=4-7) No significant differences were found (P≤0.05, Tukey Test). Bars represent standard error of means.

The field trial nematode populations for Duacari-2 reveal a strange phenomenon (Table 12), the nematode population in the control block was extremely low at the beginning of the experiment, increased over time, and closely followed the curve of the nematode population in the nematicide treated block (Figure 17).

Table 12. *Radopholus similis* population in roots of field trail plants from Duacari-2 in the rainy season, at sucker removal (Jan. 2005) and on average, over 7 months of monitoring (Dec. 2004-June 2005). (N=10, P≤0.05, Tukey test) Columns with the same letter are not significantly different from each other.

Treatment	# *R.similis*/100g root at sucker removal		# *R.similis*/100g root over 7 months	
Trichoderma atroviride isolate MT-20	13650	c	14275	d
Trichoderma atroviride isolate S2	8400	c	12355	cd
Fusarium oxysporum isolate S9	8300	c	12249	cd
Fusarium oxysporum isolate P12	5175	c	7339	bc
Nematicide	650	b	9182	b
Control	100	a	7380	a

In general, the nematode population in Duacari-2 was not as great as in the other farms included in the field trial (Figure 17).

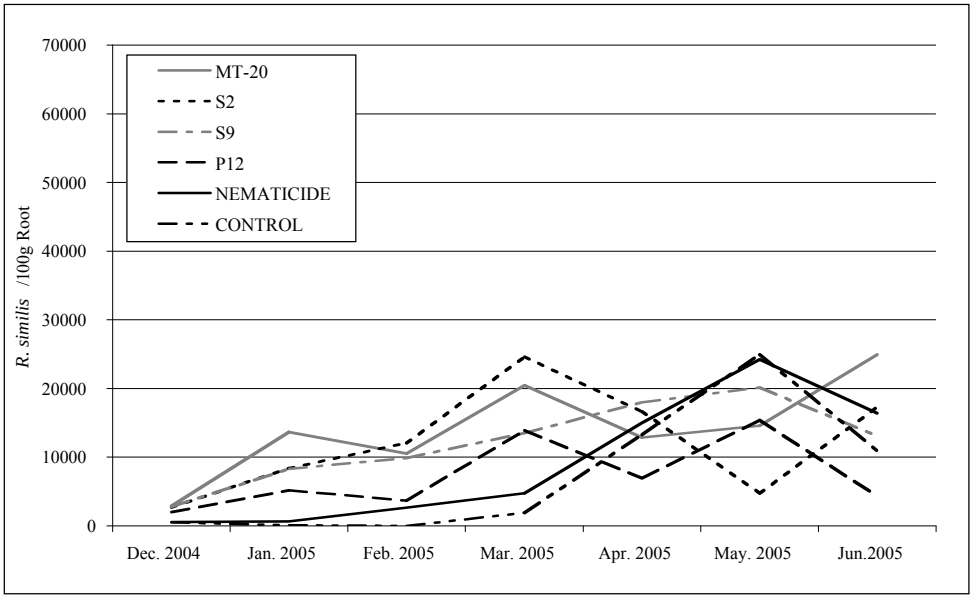


Figure 17. Dynamics of the field population of *Radopholus similis* during the rainy season in Duacari-2 farm (N=10) (adapted from Menjivar Barahona, 2005).

Formosa - Rainy Season

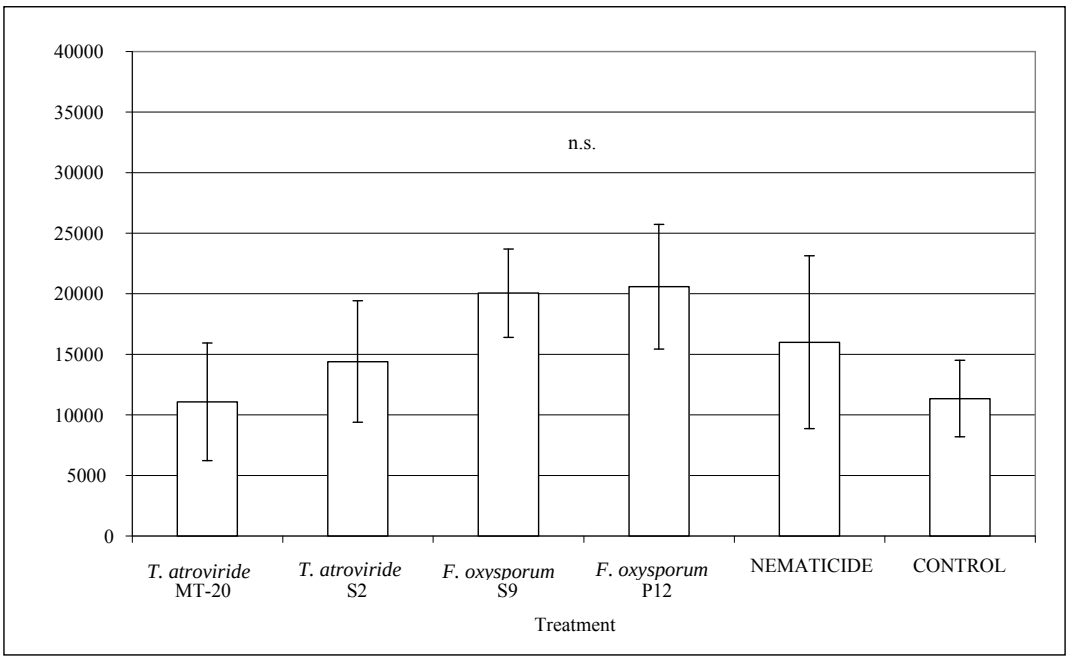


Figure 18. *Radopholus similis* per root system of banana plants from Formosa during the rainy season, 9 weeks after inoculation. (N=5-6) No significant differences were found ($P \leq 0.05$, Tukey Test). Bars represent standard error of means.

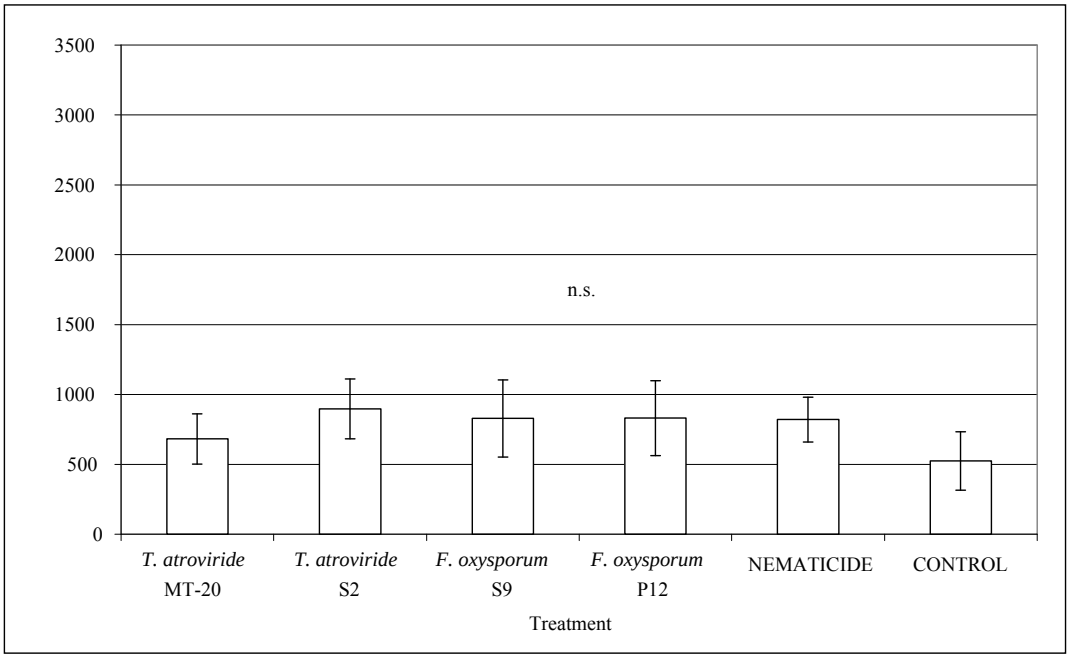


Figure 19. *Radopholus similis* density per g root of banana plants from Formosa during the rainy season, 9 weeks after inoculation. (N=5-6) No significant differences were found (P≤0.05, Tukey Test). Bars represent standard error of means.

More suckers from Formosa were recovered than from Duacari-2, but neither nematode densities nor population in corms differed significantly (Figure 19 & Figure 20). Field nematode population at sucker removal indicted some biocontrol effect in treatments MT-20 and S2, and this observation held true over the 7 month run of the field trial. In Formosa, nematicide applications did not control nematode populations at sucker removal nor over time (Table 13).

Table 13. *Radopholus similis* population in roots of field trail plants from Formosa during the rainy season, at sucker removal (Jan. 2005) and on average, over 7 months of monitoring (Dec. 2004-June 2005). (N=10, P≤0.05, Tukey test) Columns with the same letter are not significantly different from each other.

Treatment	# *R.similis*/100g root at sucker removal		# *R.similis*/100g root over 7 months	
Trichoderma atroviride isolate MT-20	4430	ab	18234	b
Trichoderma atroviride isolate S2	5760	a	13468	a
Fusarium oxysporum isolate S9	8020	b	25251	c
Fusarium oxysporum isolate P12	8230	b	15918	bc
Nematicide	6400	b	28113	c
Control	4540	b	28677	c

Nematode population dynamics also reveal the non-efficacy of nematicide treatment in Formosa farm (Figure 20). Despite fluctuations in nematode densities in the field, the MT-20, S2 and P12 endophyte treatments controlled nematode populations to a certain degree, especially over time.

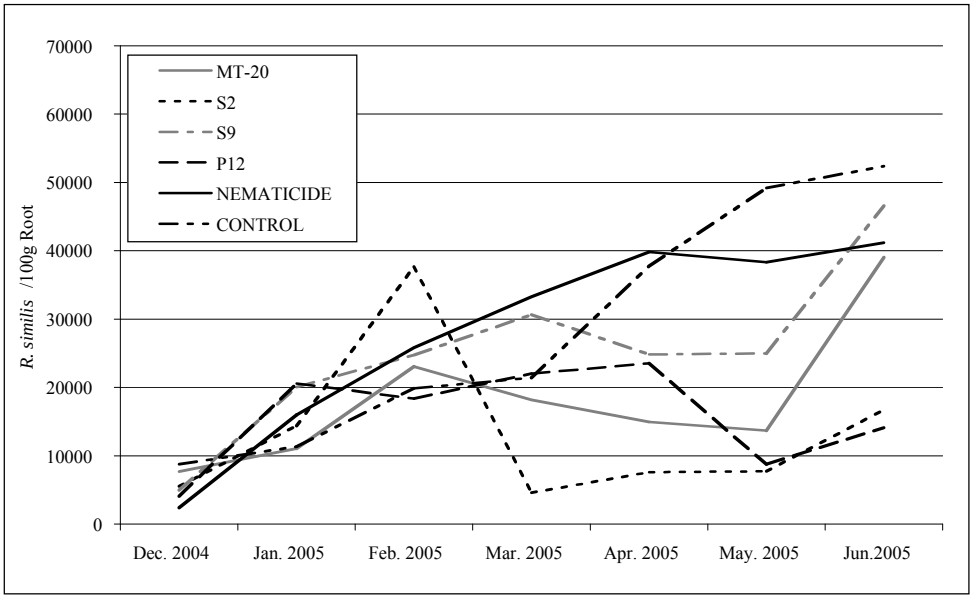


Figure 20. Dynamics of the field population of *Radopholus similis* during the rainy season in Formosa farm (N=10) (adapted from Menjivar Barahona, 2005).

Bananita - Dry Season

Corms taken from Bananita during the dry season sprouted well, though nematode penetration was very poor. No significant differences in nematode population or density could be observed in the corms, 9 weeks after nematode inoculation (Figures 19 & 20).

While no significant differences were seen between nematode densities in treatments at the time of sucker removal, fewer nematodes were encountered in endophyte treated plants in the field compared to those treated with nematicides. Oddly, the fewest nematodes were encountered in the control (Table 14). Over the 4-month course of the trial, the two *T. atroviride* treatments (MT-20 and S2) reduced the number of *R. similis* in 100 g roots significantly, as compared to the control, while neither the nematicide treatment nor the treatment with *F. oxysporum* isolate S9 had an effect. A non-significant reduction was observed in plants treated with *F. oxysporum* isolate P12.

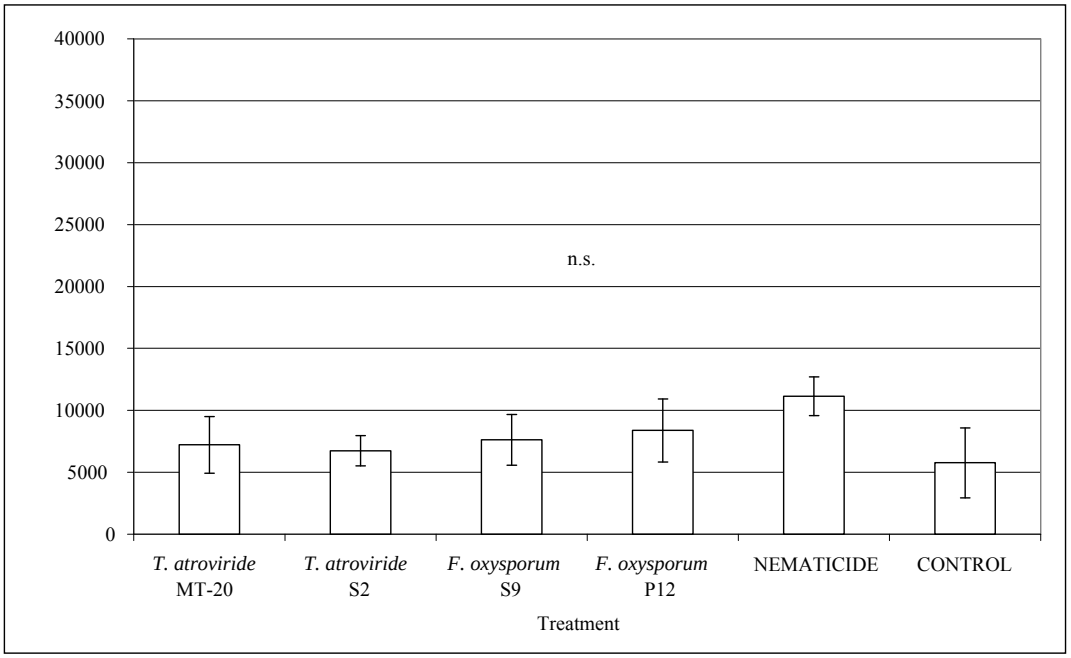


Figure 21. *Radopholus similis* per root system of banana plants from Bananita during the dry season, 9 weeks after inoculation. (N=9) No significant differences were found (P≤0.05, Tukey Test). Bars represent standard error of means.

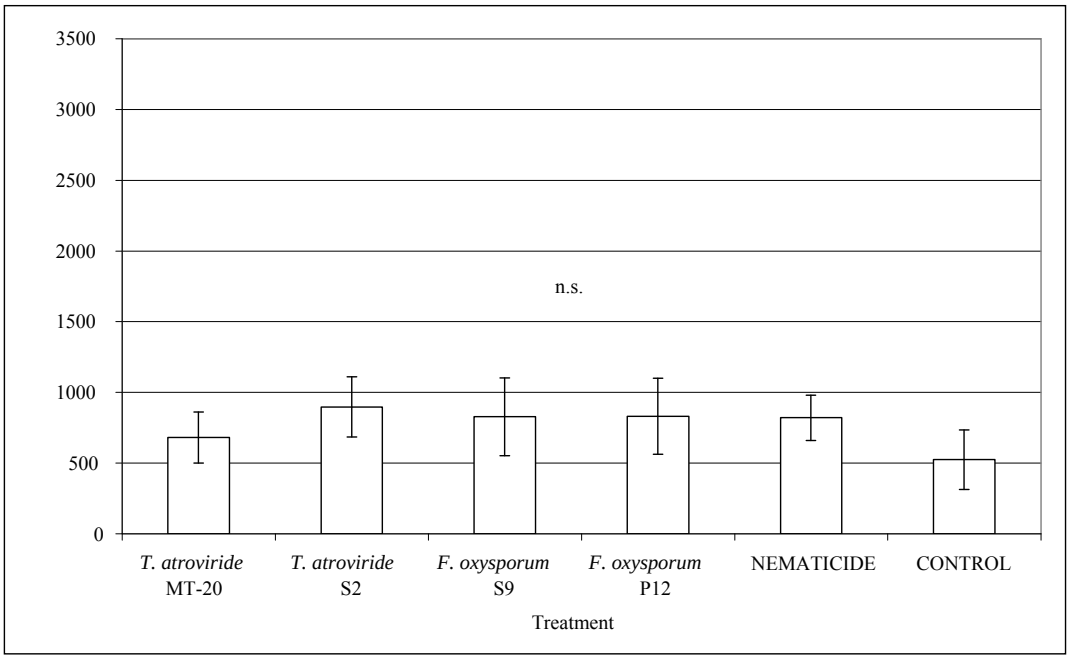


Figure 22. *Radopholus similis* density per g root of banana plants from Bananita during the dry season, 9 weeks after inoculation. (N=9) No significant differences were found (P≤0.05, Tukey Test). Bars represent standard error of means.

Table 14. *Radopholus similis* population in roots of field trail plants from Bananita, at sucker removal (Oct. 2005) and on average, over 4 months of monitoring (Oct. 2005-Jan. 2006). (N=10, P≤0.05, Tukey test) Columns with the same letter are not significantly different from each other.

Treatment	# *R.similis*/100g root at sucker removal	# *R.similis*/100g root over 7 months
Trichoderma atroviride isolate MT-20	2300 a	7294 b
Trichoderma atroviride isolate S2	4275 a	5387 a
Fusarium oxysporum isolate S9	4050 a	10100 c
Fusarium oxysporum isolate P12	2525 a	6367 bc
Nematicide	7425 a	11245 c
Control	1900 a	11471 c

Nematode population dynamics (Figure 23) reveal an initial gradual to steep increase in nematode numbers over the first 3 months of monitoring in all treatments, followed by a drop in all treatments but S2 thereafter, which is the most effective nematode-antagonist in the rainy-season farms. Nevertheless, the average 4-month nematode density in S2 treated plants was significantly lower than that in control and nematicide treated plants (Table 14).

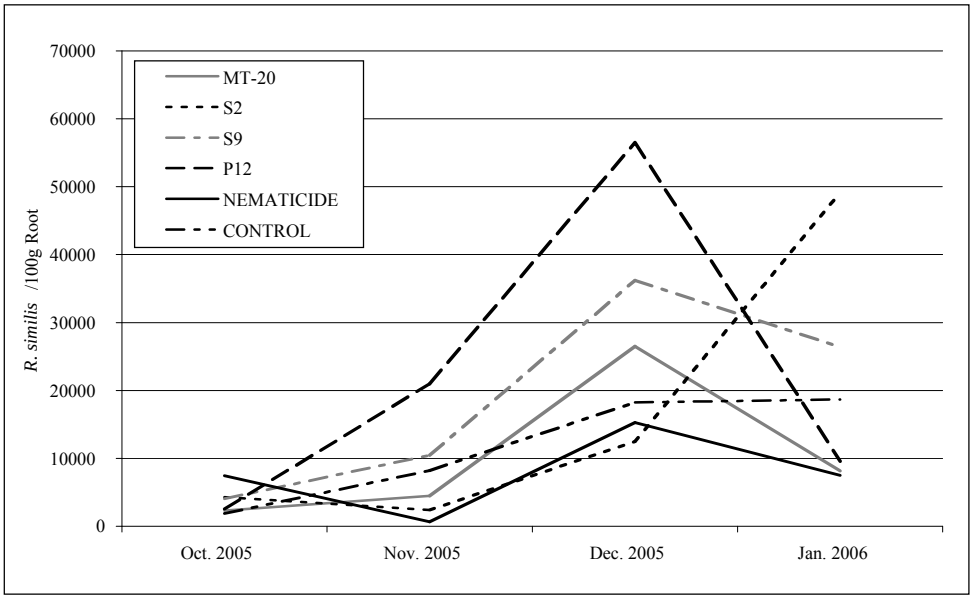


Figure 23. Dynamics of the field population of *Radopholus similis* during the dry season in Bananita farm (N=10) (data from C. Castillo and L. Pocasangre, data not published).

5.4 Discussion

The cause of suppression in nematode suppressive soils has been related to a variety of soilborne microorganisms, including a range of fungi that act either as nematode endoparasites (Sayer, 1980; Seinhorst, 1966; Eayre *et al.*, 1987; Jaffee, 1986), cyst endoparasites (Velvis & Kamp, 1996), egg pathogens (Kerry *et al.*, 1982; Kerry, 1990) or nematode trapping/predating fungi (Balan & Gerber, 1972; Gaspard & Mankau, 1987; Gray, 1988; Mankau & Wu, 1985). Recently, nematode antagonistic endophytic fungi, especially *Trichoderma* and *Fusarium* spp. have been isolated from roots of tomatoes (Hallmann & Sikora, 1994), bananas (Pocasangre, 2000; zum Felde, 2002; Menenses Hérnandez, 2003; Carñizares Monteros, 2003) and rice (Le, 2006). Notably some of these isolates were recovered from areas with so-called *nematode suppressive soils.* However, as the identified causal agents of the observed suppression were endophytes, the designation of the soils as suppressive is misleading, and the term *in-planta suppression* was coined (zum Felde *et al*., 2005). This phenomenon is especially evident in perennial and vegetatively disseminated crops such as bananas, where the naturally occurring, endophyte associated suppression not only persists in the field over decades, but can also be transferred from one field to another by transplanting suppressive suckers (zum Felde *et al.*, 2005). Exactly what set of endophytes are responsible for naturally occurring suppressive banana plants has not yet been discovered, though a range of endophytes has been isolated from such plants and screened for nematode antagonistic activity (Pocasangre, 2000; zum Felde, 2002).

With suppressive soils, small portions of such soil can be used as an inoculum to transfer suppression to a conducive soil (Westphal, 2005). However, using suppressive soils to inoculate non-suppressive soils on the large scale is hardly an economically viable way of controlling plant parasitic nematodes. In order for endophyte mediated nematode suppression to persist in a field with suppressive plants, it is the endophytes that must be transferred from one plant to the next. Theoretically, this can occur directly from mother to daughter plant, during the period in which both plants share the same corm and root system - an *in situ* transfer. Alternatively, as the identified endophytes responsible for nematode suppression are not obligate endophytes, such as mycorrhizal fungi, and can survive saprophytically in the soil, transfer may occur by infection of new roots from endophyte spores or hyphae present in the soil. In this study, an indirect way of investigating the transfer of endophytes was used. As inoculated endophytes

could not be readily differentiated from other *Fusarium* and *Trichoderma* spp., the transfer of nematode suppression as opposed to and as an indicator of endophyte transfer was studied.

Transfer of suppression could only be observed in one of the four sets of suckers tested, those from Bananita in the rainy season. Suckers from this farm had the highest regeneration rates, and nematodes were clearly suppressed in the field, especially by the endophyte S2. Bananita was flooded for part of the month of January 2005, but sustained no lasting damage. This unusual situation may have aided the inoculated endophytes in establishing themselves and colonizing greater parts of the root system, as competitors were hindered from doing so while anoxic conditions reigned.

Neither transfer of suppression, nor field suppression of nematodes could be observed for Duacari-2. In general, the nematode population in this farm was not as great as in the other farms included in the field trial. Possibly indigenous nematode-antagonists played a role in keeping field populations of nematodes low in this farm. This putative local suppression was not transferred onto the suckers though, as nematode numbers in the corms were not notably different from those in corms from the other farms. The low regeneration rates of corms from this farm affected the statistical significance of the results.

While nematodes were suppressed in endophyte treatments in the field in Formosa, no transfer of suppression could be observed. As nematicides had no effect on the nematode population in the field, it can be assumed that the nematicides were rapidly biodegraded in this farm. The highly active microbial population present in soils where enhanced biodegradation of nematicides is observed (Moens et al., 2004), and presumably also in the roots of plants growing therein, may have affected the inoculated endophytes, eventually hindering their transfer to suckers via the soil or displacing them in sucker tissues.

In the only dry season suckers to regenerate, no transfer of suppression could be observed, despite nematode suppression in the field in endophyte-treated plants. Nematode penetration was very poor in these suckers, maybe due to high temperatures in the greenhouse or a less viable nematode inoculum.

Since the suppression of nematodes in naturally-occurring suppressive plants, as seem in the Motagua Valley of Guatemala, can persist at a very high level and for

decades, natural transfer of endophytes from one generation to the next must take place. In this study, establishment and transfer of suppression due to endophytes was positively affected by high soil moisture. Potentially the slower root growth under wet soil conditions allows the endophytes to colonize greater portions of the root system. Once endophytes colonize banana roots and protect plants from nematode attack, this protection was transferred onto at least one following generation, eventually more. This is the first time a transfer of suppression could be proven for a set of individual endophytes under field and greenhouse conditions.

6 EFFECT OF SINGLE AND COMBINED INOCULATIONS OF MUTUALISTIC ENDOPHYTIC FUNGI ON NEMATODE CONTROL AND PLANT GROWTH

6.1 Introduction

A wide range of microorganisms from both the rhizosphere and the endorhiza have been identified as potential biocontrol agents (BCAs) of plant-parasitic nematodes (Hoffmann-Hergarten *et al.*, 1998; Kerry, 2000; Meyer *et al.*, 2002; Hallmann *et al.*, 2001; Chen & Dickson, 2004a, b). Among these potential BCAs are a selection of endophytic fungi that have been isolated from diverse crops such as rice, maize, tomato and banana (Hallmann & Sikora, 1994; Schuster *et al.*, 1995; Pocasangre, 2000; Pocasangre *et al.*, 2000; Niere, 2001; zum Felde, 2002; Menenses Hérnandez, 2003; Carñizares Monteros, 2003; Vu, 2005; zum Felde *et al.*, 2005; Le, 2006). Root endophytes are very interesting for biological control of nematodes, as they often colonize the very same tissues attacked by nematodes. In addition to the nematode antagonistic effects of certain endophytes, a number of fungi isolated from banana have exhibited plant growth promoting effects in greenhouse and field experiments (Pocasangre *et al.*, 2000; zum Felde, 2002; Menenses Hérnandez, 2003; Carñizares Monteros, 2003; Menjivar Barahona, 2005).

Despite ever increasing interest in alternative control measures, one of the critiques often aimed at biocontrol systems, is the inconsistency of the control levels attained. Where endophytes are concerned, this inconsistency may be due to both abiotic and biotic factors affecting an antagonist's ability to colonize and grow in the endorhiza and to improper inoculum application technology (Dababat, 2006).

In natural nematode suppressive soils, a range of natural enemies of nematodes are present, attacking their host at different stages of its life cycle, i.e. eggs, juveniles, sedentary females, etc. (Kerry, 1990). Kerry (1990) states that in such systems, each antagonist may kill relatively few nematodes, and that it is in fact only the combined effects of several nematode antagonists that result in the suppressive character of the soil. In an effort to emulate the conditions of naturally nematode suppressive systems in the greenhouse, and to attain more consistent and potent nematode biocontrol and plant growth promoting effects, plants in this study were inoculated with multiple nematode-antagonistic endophytes, as opposed to a single isolate. The objectives of this study

were to evaluate the effect of single and combined inoculations of fungal endophytes on biocontrol of *Radopholus similis* in banana roots and on plant health and growth.

To do so, two test were carried out, one with *Musa* AAA cv. 'Williams' plants inoculated with both fungi and nematodes, and a second with *Musa* AAA cvs. 'Grand Nain', 'Valery' and 'Williams' plants inoculated only with fungi. The aim was to differentiate the purely plant growth promoting effects of the fungi alone, from the plant growth 'permitting' effect seen in plants inoculated with both nematodes and nematode-antagonistic endophytes. The three most popular banana cultivars presently used on commercial plantations in Latin America were included in the second test to verify whether growth promoting effects could be observed across a variety of popular cultivars.

6.2 Materials and Methods

6.2.1 Nematode Biocontrol and Plant Growth in Endophyte Treated *Musa* AAA cv. 'Williams' Plants

The endophytes used in this study were selected from those previously screened and tested by a group of Masters students in the nematology laboratory at CATIE, in Costa Rica (zum Felde, 2002; Menenses Hérnandez, 2003; Carñizares Monteros, 2003). The selected isolates were the four most effective nematode antagonists discovered: two *Trichoderma atroviride* isolates (MT-20 and S2) and two non-pathogenic *Fusarium oxysporum* isolates (S9 and P12). Eight treatments were included in the first test: four *single inoculations* where plants were inoculated with the conidia of one of the four endophytes included in the study and 500 *R. similis*; two *dual inoculations*, where plants were inoculated with the conidia of either both *T. atroviride* or both *F. oxysporum* isolates and 500 *R. similis*; one *combined inoculation* where plants were inoculated with conidia of all four endophytes and 500 *R. similis*; a control treatment, inoculated only with 500 *R. similis* and an absolute control, which was not inoculated.

The treatment codes presented in Table 15 will be used from here on to refer to the eight treatments used, so as to make presentation of results clearer and avoid cluttered figures.

Table 15. Treatments used to test the effect of inoculations with one, two or four endophytes on the biocontrol of *Radopholus similis* and on the growth of *Musa* AAA cv. 'Williams' plants.

Treatment Codes	Endophytes Inoculated	Endophyte Identity and Origin
Ta-1	MT-20	*Trichoderma atroviride*, Motagua, Guatemala
Ta-2	S2	*Trichoderma atroviride*, Sixaola, Costa Rica
Fo-1	S9	*Fusarium oxysporum*, Sixaola, Costa Rica
Fo-2	P12	*Fusarium oxysporum*, Talamaca, Costa Rica
Ta-Dual	MT-20 & S2	*Trichoderma atroviride*, Guatemala & Costa Rica
Fo-Dual	S9 & P12	*Fusarium oxysporum*, Costa Rica
Combined	MT-20, S2, S9 & P12	*T. atroviride* & *F. oxysporum*, Guatemala & Costa Rica
Nematode Control[1]	-	-
Absolute Control[2]	-	-

[1] Only *R. similis* inoculated, but no fungi; [2] Neither *R. similis*, nor fungi inoculated.

The isolates were grown on 100% PDA plates for 2 weeks, until they produced enough conidia to prepare suspensions. Conidia were washed off media surface using sterile tap water and a sterile bacterial spreader, and counted using a Neubauer cell counting chamber. Conidial suspensions of 1 x 10^6 conidia/ml were prepared for each isolate. Dip-inoculations were performed on approximately 15 cm high tissue culture plants taken from multi-trays. The cone of rooting substrate adhering to the roots was not washed off prior to inoculation. The entire root system with adhering substrate of 11 plants was simultaneously dipped into a conidia suspension for 5 minutes. For multiple inoculations, root sytems were successively dipped in conidial suspensions of individual endophytes for 5 minutes.

After inoculation, plants were planted in 500 ml pots containing a sterile mix of sand and soil (1:1). Two weeks after planting, all plants inoculated with fungi and the control plants were inoculated with 500 mixed stages of *R. similis* nematodes taken from sterile carrot disk cultures, as described by Speijer and De Waele (1997). The absolute control was left uninoculated. Plants were watered daily, but never fertilized.

Two months after nematode inoculation, soil was washed from the roots of plants, and the following data was collected: number of leaves and roots, pseudostem diameter, root and shoot weight. Nematodes were then extracted from roots using a maceration and sieving method (Speijer & De Waele, 1997) as follows: 1) the entire root system was cut into 1 cm pieces, and macerated in a commercial blender in 200 ml tap water

for 5 sec at low, and 5 sec at high speed; 2) the suspension was sieved in nested sieves of 1000 mm, 150 mm and 45 mm apertures; 3) the content of the last sieve was washed into a 250 ml capacity plastic beaker and filled up to 200 ml. For each sample, nematodes in two 2-ml sub-samples were counted. The total number of nematodes per root system (nematode population) and the number of nematodes per g root (nematode density) were calculated.

The experimental design was a completely randomized block design, with nine treatments and eleven repetitions. Data was analysed using the Tukey test in the SPSS statistical program (SPSS® 13.0 for Mac). Nematode data was ln (x+1) transformed for analysis in SPSS. In addition, orthogonal contrasts were carried out using the SAS statistical program (SAS/STAT® Software, SAS Institute Inc.) with $\sqrt{(x+0.5)}$ transformed nematode data. Plant morphological data was not transformed for analysis.

6.2.2 Plant Growth Promotion in Endophyte Treated *Musa* AAA cvs. 'Grand Nain', 'Valery' and 'Williams' Plants in the Absence of Nematodes

Endophyte treatments in this test were the same as those in the previous test. However, as no nematodes were inoculated onto any plants in this test, only one control was used. In addition, three *Musa* AAA cultivars: 'Valery', 'Williams' and 'Grand Nain' were tested. Endophyte inoculum was obtained as described in section 6.2.1.

The inoculation method was slightly modified: as opposed to successive 5 minute dip inoculations into single isolate conidia suspensions as used in the previous test, in this test, roots of micropropagated banana plants were dipped once into a conidia suspension, containing conidia from either 1, 2 or 4 endophytes, for 5 minutes. The suspensions for single inoculations contained 1.5 x 10^6 conidia/ml of one isolate (see Table 16 for treatment codes and isolates used), dual isolate inoculations contained 1.5 x 10^6 conidia/ml of each isolate used, therefore had a final conidia concentration of 3 x 10^6 conidia/ml, while the combined inoculum, contained a final concentration of 6 x 10^6 conidia/ml. Plant were then planted in 1L potting bags, containing a sterile soil:sand mix (1:1). Plants were watered daily, but never fertilized. Over the next 12 weeks, plant height, total number of leaves and pseudostem diameter were taken very two weeks. After 12 weeks, plants were harvested, roots washed free of soil, and shoot and root weights, as well as the number of roots, leaves and plant height were noted.

Additionally, root length, average root diameter, root volume, and root lengths in root diameter classes were analysed using the WinRHIZO® program (WinRHIZO® 2004c, Regent Instruments Inc.).

The experimental design was a completely randomized block design, with eight treatments and twelve repetitions. Data was analysed using the Tukey test in the SPSS statistical program (SPSS® 13.0 for Mac). Data was ln (x+1) transformed for analysis. Once again, orthogonal contrasts were carried out using the SAS program (SAS/STAT® Software, SAS Institute Inc.), with non-transformed data.

Table 16. Treatments used to test the effect of single and combined inoculations of endophytic fungi on the growth of *Musa* AAA cultivars 'Grand Nain', 'Valery' and 'Williams'.

Treatment Codes	Endophytes Inoculated	Endophyte Identity and Origin
Ta-1	MT-20	*Trichoderma atroviride*, Motagua, Guatemala
Ta-2	S2	*Trichoderma atroviride*, Sixaola, Costa Rica
Fo-1	S9	*Fusarium oxysporum*, Sixaola, Costa Rica
Fo-2	P12	*Fusarium oxysporum*, Talamaca, Costa Rica
Ta-Dual	MT-20 & S2	*Trichoderma atroviride*, Guatemala & Costa Rica
Fo-Dual	S9 & P12	*Fusarium oxysporum*, Costa Rica
Combined	MT-20, S2, S9 & P12	*T. atroviride* & *F. oxysporum*, Guatemala & Costa Rica
Control	-	-

6.3 Results

6.3.1 Nematode Biocontrol and Plant Growth in Endophyte Treated *Musa* AAA cv. 'Williams' Plants

Two months after inoculation with *R. similis*, the total number of nematodes in roots (Figure 24) and their density (Figure 25) were both significantly lower in plants inoculated with endophytes than in the control plants. Single inoculations significantly reduced both the total number and the density of *R. similis* in banana roots. Dual inoculations controlled *R. similis* better than single inoculations, while the combined inoculation with four fungi yielded the best nematode control results overall.

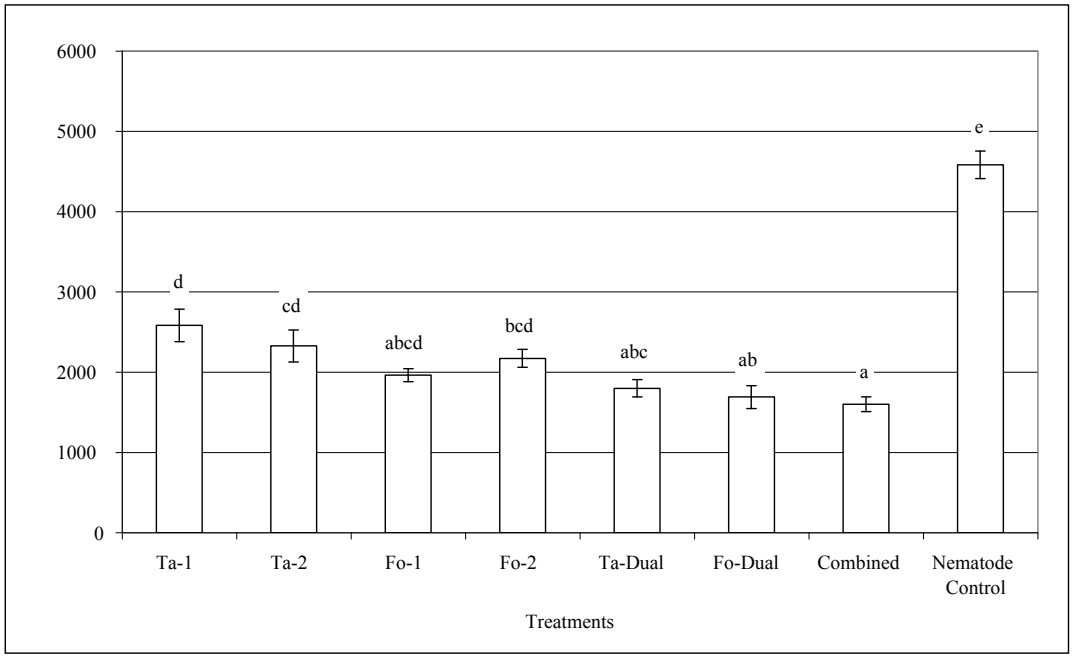


Figure 24. Effects of inoculations with one, two or four endophytic fungi on the number of *Radopholus similis* in the root system of *Musa* AAA cv. 'Williams' plants, 2 months after nematode inoculation. (N=11) Columns with the same letter are not significantly different from each other (P≤0.05, Tukey Test). Bars represent standard error of means.

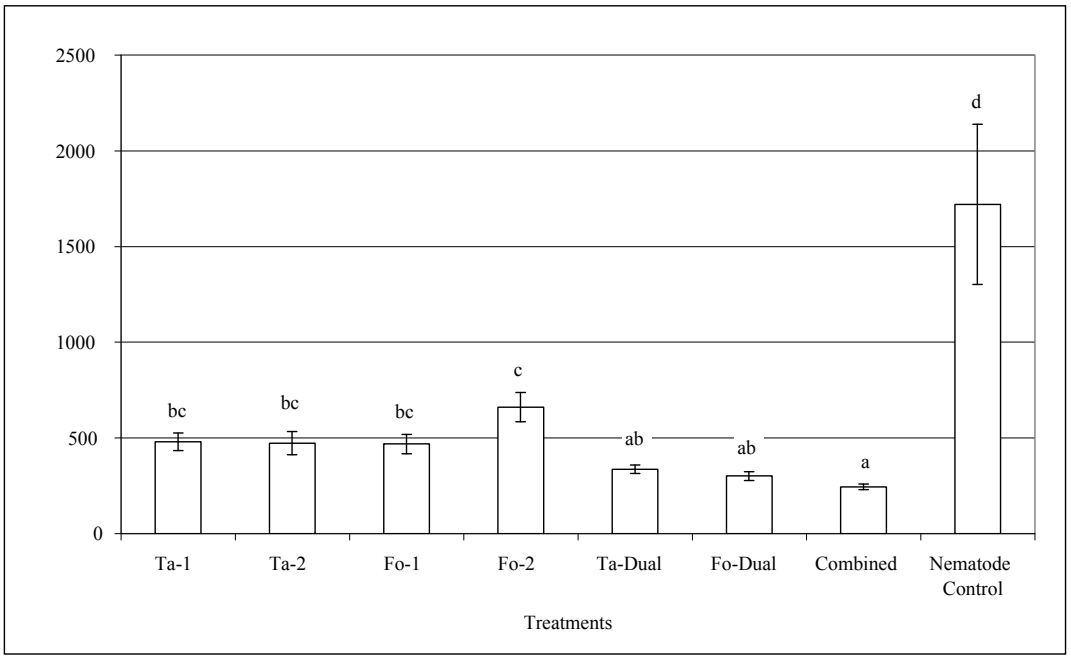


Figure 25. Effects of inoculations with one, two or four endophytic fungi on the number of *Radopholus similis* per g root of *Musa* AAA cv. 'Williams' plants, 2 months after nematode inoculation. (N=11) Columns with the same letter are not significantly different from each other (P≤0.05, Tukey Test). Bars represent standard error of means.

Single inoculations with *F. oxysporum* isolates tended to control nematodes better than single inoculations with *T. atroviride*, though the effect of Fo-2 on the density of nematodes was poorest among the simple inoculations (Table 17). Fo-Dual inoculations with both *F. oxysporum* isolates controlled nematodes slightly better than the Ta-Dual inoculations with both *T. atroviride* isolates, though not significantly so.

Table 17. Effects of inoculations with one, two or four endophytic fungi on nematode population and density in the roots of *Musa* AAA cv. 'Williams' plants, 8 weeks after nematode inoculation.

Treatment	*R. similis*/root system		% reduction	*R. similis*/g root		% reduction
Nematode Control	4582	e	-	1721	d	-
Ta-1	2582	d	44	480	bc	72
Ta-2	2327	cd	49	472	bc	73
Fo-1	1964	abcd	53	469	bc	62
Fo-2	2173	bcd	57	661	c	73
Ta-Dual	1800	abc	61	337	ab	80
Fo-Dual	1691	ab	63	301	ab	83
Combined	1600	a	65	245	a	86

Means in columns followed by different letters are significantly different at P≤0.05, Tukey Test, N=11. Data was ln (x+1) transformed for statistical analysis.

Orthogonal contrasts revealed highly significant differences between the number of *R. similis* per root system and per g root in endophyte inoculated and control plants, as well as between combined and dual inoculations (Table 18). Only the *R. similis* population in Ta-Dual and Fo-Dual root systems differed significantly, with Fo-Dual containing less nematodes than Ta-Dual, while nematode density was not significantly different. The effect of single and dual inoculations of *F. oxysporum* on *R. similis* differed significantly from each other, with Fo-Dual reducing nematode population and density more than Fo-1 or Fo-2. The same can be said when comparing single and dual inoculations of *T. atroviride*: the dual inoculation increased nematode control. Nematode control was not significantly different between plants inoculated with only one endophyte when comparing effects of inoculations with fungi of the same species (Ta-1 vs. Ta-2 and Fo-1 vs. Fo-2).

For all morphological characteristics studied, orthogonal contrasts revealed highly significant differences between inoculated and non-inoculated plants, with inoculated plants consistently outperforming control plants (Table 18).

Table 18. Orthogonal contrasts carried out on morphological and nematode data collected from *Musa* AAA cv. 'Williams' plants inoculated with one, two or four endophytes and nematodes, 2 months after nematode inoculation (N=11).

	Nematode Data		**Morphological Data**					
Contrasts	***R. similis*/ root system**	***R. similis*/ *g* root**	**Plant Height**	**Pseudostem Diameter**	**Nr. of Leaves**	**Shoot Weight**	**Nr. of Roots**	**Root Weight**
Controls[1] vs. Treatments with Fungi	**	**	**	**	**	**	**	**
Nematode[2] vs. Absolute[3] Control	-	-	n.s.	n.s.	n.s.	n.s.	*	n.s.
Combined vs. Dual Inoculations	**	**	**	**	*	**	**	**
Ta-Dual vs. Fo-Dual	*	n.s.	**	**	n.s.	**	n.s.	*
Fo-Dual vs. Fo-1 and Fo-2	*	**	**	n.s.	n.s.	**	**	**
Fo-1 vs. Fo-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ta-Dual vs. Ta-1 and Ta-2	**	n.s.	**	n.s.	n.s.	**	n.s.	n.s.
Ta-1 vs. Ta-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.

[1] Nematode Control and Absolute Control; [2] Nematode Control: inoculated with *R. similis*, but not with endophytic fungi; [3] Absolute Control: not inoculated with *R. similis*, nor with endophytic fungi; *: significant difference (P≤0.05); **: highly significant difference (P≤0.01); n.s.: no significant difference according to SAS Orthogonal Contrasts test.

R. similis did not significantly affect plant height, pseudostem diameter, number of leaves, or shoot and root weight. Only the number of roots was significantly reduced in plants inoculated with nematodes, compared to those of the absolute control (Table 19). Significant differences were revealed between dual and combined inoculations, with the combined inoculation yielding larger and heavier plants, with more roots and leaves (Table 19). The dual inoculations with *T. atroviride* (Ta-Dual) produced plants with significantly higher, wider and heavier shoots than the dual inoculation with *F. oxysporum* (Fo-Dual), while *F. oxysporum* inoculated plants had greater root weight and number of roots. Not many significant differences were noted between treatments Fo-1 and Fo-2, nor among treatments Ta-Dual, Ta-1 and Ta-2.

With the exception of plants in treatment Fo-2, all inoculations of individual endophytic fungi positively influenced plant growth, as revealed by greater plant height, pseudostem diameter, number of leaves and roots, and shoot and root weight, when compared to the absolute control and nematode control plants (Table 19).

Table 19. Effects of inoculations with one, two or four endophytic fungi and 500 *Radopholus similis* on growth of *Musa* AAA cv. 'Williams' plants.

Treatment	Plant Height (cm)	Pseudostem Diameter (cm)	Number of Leaves	Shoot Weight (g)	Number of Roots	Root Weight (g)
Control	12.04 ab	1.27 ab	6 a	25.59 abc	21 a	8.34 ab
Ta-1	10.73 a	1.14 ab	5 a	24.46 abc	21 a	15.74 d
Ta-2	12.32 ab	1.30 b	6 a	21.43 ab	21 a	14.38 cd
Fo-1	11.99 ab	1.30 b	6 a	31.93 c	23 a	18.08 d
Fo-2	12.37 ab	1.22 ab	5 a	31.36 bc	22 a	15.20 cd
Ta-Dual	11.93 ab	1.09 ab	5 a	25.21 abc	21 a	10.43 bc
Fo-Dual	11.73 ab	1.05 a	5 a	19.82 a	21 a	6.26 a
Combined	12.53 b	1.18 ab	5 a	20.15 a	24 a	6.88 ab

Means in columns followed by different letters are significantly different at $P \leq 0.05$, Tukey Test, N=11.

In general, single inoculations with *Trichoderma* isolates yielded heavier plants than single inoculations with *Fusarium* isolates, and dual inoculations yielded better results than single inoculations. All Ta-Dual and Fo-Dual plants outperformed absolute control plants. The combined inoculation consistently produced the best results, yielding greatest pseudostem diameter, number of leaves and roots, and root and shoot weights than any other treatment. Ta-1, Ta-2 and Ta-Dual plants had greater root and shoot weights than Fo-1, Fo-2 and Fo-Dual plants.

Of the four endophytes inoculated, Fo-2 had the least growth promoting effects and was not as effective in controlling nematodes as the other three isolates inoculated singly. All inoculations with either one or both *T. atroviride* isolates (Ta-1, Ta-2, Ta-Dual or Combined) and the inoculation with both *F. oxysporum* isolates (Fo-Dual) compensated the plant growth reducing effects of *R. similis*, significantly increasing root growth beyond that of the absolute control plants and effectively controlling nematodes.

6.3.2 Plant Growth in Endophyte-Treated *Musa* AAA cv. 'Grand Nain', 'Valery' and 'Williams' Plants

For the three cultivars studied, changes in plant height, pseudostem diameter and total number of leaves followed similar patterns over the 12 weeks of data collection and, for the most part, no significant differences could be found between the sets of data

from the three cultivars (data not shown). Plant height increased steadily over time in all treatments and cultivars, though treatment Ta-2 depressed height increase in 'Grand Nain' from week 6 to 12.

Pseudostem diameter also increased steadily over the 12-week run of the experiment, with the exception of a slight stagnation of increase towards week 10 in 'Grand Nain' and 'Valery' plants in the combined treatment. For all plants and in all treatments, the number of leaves increased over the first 6 weeks after planting, decreasing thereafter in week 8 and then increasing again to week 10 and finally decreasing or stagnating till the end of data collection at week 12. Treatment Fo-2 positively affected the number of leaves in cv. 'Williams' over the 12 weeks of data collection, though not for the two other cultivars. Leaf numbers in plants in treatment Fo-Dual were slightly depressed on all cultivars, especially over the first 6 weeks, and towards the end of data collection in all cultivars in treatments Ta-Dual and Ta-2. The drop in total leaf numbers between weeks 6 and 8 and between weeks 10 and 12 was due to senescence and removal of older leaves.

Effect of single, double and multiple inoculations of endophytes on the growth of Musa *AAA cv. 'Grand Nain' plants*

After 12 weeks, neither the height nor the diameter of the pseudostem of 'Grand Nain' plants, nor the number of leaves or roots produced were significantly affected by the endophyte treatments, when compared to the control (Table 20). However, the pseudostem diameter of Fo-Dual inoculated plants was noticeably smaller than in other treatments. Shoot weights in treatments Fo-Dual and combined were significantly lower than those in treatments Fo-1 and Fo-2, but shoot weights did not differ significantly between treatments with endophytes and the control. All single inoculations significantly increased root weight, when compared to the control. Root weights in the two double and the combined treatments did not significantly differ from those of the control, and roots weights were lower than those in single treatments.

Orthogonal contrasts revealed significant differences in root weight, length, diameter and volume between control and endophyte inoculated plants (Table 21). Root weight is lower in control plants than in all inoculated plants except for Fo-Dual and combined plants, where root weight is even lower than in the control plants (Table 20).

When comparing combine inoculated plants with the other endophyte inoculated plants, significant differences are additionally seen in pseudostem diameter, number of leaves and shoot weight.

Table 20. Effect of inoculations with one, two or four endophytic fungi on growth of *Musa* AAA cv. 'Grand Nain' plants, 12 weeks after planting in the greenhouse.

Treatment	Plant Height (cm)	Pseudostem Diameter (cm)	Number of Leaves	Shoot Weight (g)	Number of Roots	Root Weight (g)
Control	12.04 ab	1.27 ab	6 a	25.59 abc	21 a	8.34 ab
Ta-1	10.73 a	1.14 ab	5 a	24.46 abc	21 a	15.74 d
Ta-2	12.32 ab	1.30 b	6 a	21.43 ab	21 a	14.38 cd
Fo-1	11.99 ab	1.30 b	6 a	31.93 c	23 a	18.08 d
Fo-2	12.37 ab	1.22 ab	5 a	31.36 bc	22 a	15.20 cd
Ta-Dual	11.93 ab	1.09 ab	5 a	25.21 abc	21 a	10.43 bc
Fo-Dual	11.73 ab	1.05 a	5 a	19.82 a	21 a	6.26 a
Combined	12.53 b	1.18 ab	5 a	20.15 a	24 a	6.88 ab

Means in columns followed by different letters are significantly different at P≤0.05, Tukey Test, N=12.

Table 21. Orthogonal contrasts carried out on morphological data collected from *Musa* AAA cv. 'Grand Nain' plants inoculated with one, two or four endophytes, 12 weeks after planting (N=12).

	Morphological Data						WinRHIZO Data		
Contrasts	Plant Height	Pseudostem Diameter	Nr. of Leaves	Shoot Weight	Nr. of Roots	Root Weight	Root Length	Root Diameter	Root Volume
Control vs. Treatments	n.s	n.s	n.s	n.s	n.s	**	**	**	**
Combined vs. other Inoculations	n.s	**	*	**	n.s	**	**	**	**
Dual vs. Single Inoculations	n.s	*	*	**	n.s	**	**	**	**
Ta-Dual vs. Fo-Dual	n.s	n.s	n.s	*	n.s	**	**	n.s	**
Fo-1 and Fo-2 vs. Ta-1 and Ta-2	n.s	*	n.s	**	n.s	*	*	**	**
Ta-1 vs. Ta-2	*	n.s	n.s	n.s	n.s	n.s	n.s	**	*
Fo-1 vs. Fo-2	n.s	n.s	n.s	n.s	n.s	n.s	n.s	*	**

*: significant difference (P≤0.05); **: highly significant difference (P≤0.01); n.s.: no significant difference according to SAS Orthogonal Contrasts test.

Combine inoculated plants generally had a greater shoot height, average pseudostem diameter, low shoot and root weight (Table 20), shorter roots with a greater average diameter and of lesser volume (Table 22) than other endophyte inoculated plants, with the exception of Fo-Dual plants. Dual inoculations were not as effective in promoting plant growth as were single inoculations, though the Ta-Dual inoculation had a greater positive effect on plant growth than the Fo-Dual inoculation. Inoculations with *T. atroviride* had greater root growth promoting effects than *F. oxysporum* inoculations, with Ta-2 especially increasing root length and Ta-1 increasing root volume and weight.

No great differences were revealed when comparing inoculations with isolates from the same species (Ta-1 vs. Ta-2 and Fo-1 vs. Fo-2).

Table 22. Effect of inoculations with one, two or four endophytic fungi on root length, average root diameter and root volume of *Musa* AAA cv. 'Grand Nain' plants, 12 weeks after planting in the greenhouse.

Treatment	Root Length (cm)		Root Diameter (mm)		Root Volume (cm^3)	
Ta-1	1928.75	c	1.00	a	20.33	cd
Ta-2	2021.83	c	1.00	a	17.08	c
Fo-1	1823.08	c	1.00	a	24.92	d
Fo-2	1814.58	c	1.00	a	20.00	c
Ta-Dual	836.42	b	1.00	a	11.58	b
Fo-Dual	456.33	a	1.00	a	6.25	a
Combined	559.75	ab	1.17	ab	8.08	ab
Control	587.33	ab	1.33	b	9.75	ab

Means in columns followed by different letters are significantly different at $P \leq 0.05$, Tukey Test, N=12.

Effect of single, double and multiple inoculations of endophytes on the growth of Musa *cv. 'Valery' plants*

The height, pseudostem diameter, number of roots, and shoot weight of 'Valery' plants was not significantly affected by the endophyte treatments (Table 23). Of the plants inoculated with a single endophyte, only those in treatment Fo-2 had significantly less leaves than the control, while the number of leaves in dual and combined treatments was non-significantly lower than the control. All single inoculation treatments increased root weight, but only treatment Fo-1 was significantly higher than the control (Table 23). Root weights in the dual and combined treatments did not significantly differ from those of the control, and in the Fo-Dual and the combined

treatment, root weights were lower than those of single treatments. Shoot weight of 'Valery' plants was not significantly affected by endophyte treatments, though single inoculations slightly increased and multiple inoculations slightly decreased it. This was similar to the effect seen in *Musa* AAA cv. 'Grand Nain' plants.

Table 23. Effect of simple and combined inoculations of endophytic fungi on growth of *Musa* AAA cv. 'Valery' plants, 12 weeks after planting in the greenhouse.

Treatment	Plant Height (cm)	Pseudostem Diameter (cm)	Number of Leaves	Shoot Weight (g)	Number of Roots	Root Weight (g)
Control	13.33 a	1.30 a	6 b	29.85 a	18 a	11.51 ab
Ta-1	13.69 a	1.34 a	7 b	33.36 a	19 a	18.57 bc
Ta-2	13.37 a	1.33 a	6 b	35.36 a	20 a	17.93 bc
Fo-1	12.98 a	1.37 a	7 b	34.28 a	21 a	19.74 c
Fo-2	12.35 a	1.24 a	4 a	34.99 a	18 a	15.95 abc
Ta-Dual	13.38 a	1.25 a	5 ab	28.55 a	19 a	13.46 abc
Fo-Dual	13.08 a	1.22 a	5 ab	30.67 a	17 a	6.07 a
Combined	13.32 a	1.32 a	5 ab	26.36 a	19 a	8.60 ab

Means in columns followed by different letters are significantly different at $P \leq 0.05$, Tukey Test, N=12.

Orthogonal contrasts revealed few or no significant differences between treatments with regard to plant height, pseudostem diameter, number of leaves and roots, and shoot weight. As was the case for cv. 'Grand Nain', significant differences were revealed for root weight, length, average diameter and volume for cv. 'Valery' plants (Table 24). Control roots generally weighed less (Table 23), were shorter, wider and less voluminous (Table 25) than inoculated roots, with the exception of root from plants inoculated with both *F. oxysporum* (Fo-Dual) or all four isolates.

Combined inoculations did not positively affect root growth. The Ta-Dual inoculation had a significantly greater positive effect on root growth than the Fo-Dual inoculation, yielding heavier, longer and more voluminous roots. No significant differences were observed between any of the single inoculations, nor between single inoculations with *T. atroviride*. Fo-2 did not promote root growth as well as Fo-1 did.

As was the case for cv. 'Grand Nain' plants, inoculation with Fo-2 did not greatly increase growth of cv. 'Valery' plants, and Dual-Fo and combined inoculations had a neutral to negative effect on plant growth.

Table 24. Orthogonal contrasts carried out on morphological data collected from *Musa* AAA cv. 'Valery' plants inoculated with one, two or four endophytes, 12 weeks after planting (N=12).

	Morphological Data						WinRHIZO Data		
Contrasts	**Plant Height**	**Pseudostem Diameter**	**Nr. of Leaves**	**Shoot Weight**	**Nr. of Roots**	**Root Weight**	**Root Length**	**Root Diameter**	**Root Volume**
Control vs. Treatments	n.s.	n.s.	n.s.	n.s.	n.s.	**	**	**	**
Combined vs. other Inoculations	n.s.	n.s.	n.s.	n.s.	n.s.	**	**	**	**
Dual vs. Single Inoculations	n.s.	*	**	n.s.	*	**	**	n.s.	**
Ta-Dual vs. Fo-Dual	n.s.	n.s.	n.s.	n.s.	n.s.	**	**	**	**
Fo-1 and Fo-2 vs. Ta-1 and Ta-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ta-1 vs. Ta-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fo-1 vs. Fo-2	n.s.	n.s.	n.s.	n.s.	*	**	n.s.	**	**

*: significant difference (P≤0.05); **: highly significant difference (P≤0.01); n.s.: no significant difference according to SAS Orthogonal Contrasts test.

Table 25. Effect of inoculations with one, two or four endophytic fungi on root length, average root diameter and root volume of *Musa* AAA cv. 'Valery' plants, 12 weeks after planting in the greenhouse.

Treatment	Root Length (cm)		Root Diameter (mm)		Root Volume (cm^3)	
Ta-1	1946.17	bc	1.00	a	20.42	cd
Ta-2	2045.18	c	1.00	a	21.09	cd
Fo-1	1941.50	bc	1.00	a	23.50	d
Fo-2	1873.27	bc	1.00	a	18.55	c
Ta-Dual	1674.82	b	1.00	a	17.18	c
Fo-Dual	577.25	a	1.00	a	7.17	a
Combined	785.00	a	1.08	a	9.83	ab
Control	860.45	a	1.18	a	12.27	b

Means in columns followed by different letters are significantly different at P≤0.05, Tukey Test, N=12.

Effect of single, double and multiple inoculations of endophytes on the growth of Musa *cv. 'Williams' plants*

Neither plant height, pseudostem diameter, number of leaves nor shoot height of *Musa* AAA cv. 'Williams' plants was significantly affected by endophyte treatments, when compared to the control (Table 26). Pseudostem diameter only significantly

differed between two treatments (Fo-1 > Ta-Dual). There were significantly less roots produced by cv. 'Williams' plants in the Fo-2 treatment than in the control, Ta-1, Ta-2, and combined treatments (Table 26). Root weights in single and Ta-Dual treatments were non-significantly greater than the weight of control roots. In Fo-Dual treatment, the root weight was significantly lower than in the control, and non-significantly lower than in the combined treatment.

Shoot weight was not significantly affected by the treatments, though, as in cv. 'Grand Nain' and cv. 'Valery' plants, single inoculations tended to increase shoot weight. Ta-Dual had positive effect on root weight while Fo-Dual decreased root weight significantly, compared to the control and all other treatments but the combined one.

Table 26. Effect of inoculations with one, two or four endophytic fungi on growth of *Musa* AAA cv. 'Williams' plants, 12 weeks after planting in the greenhouse.

Treatment	Plant Height (cm)	Pseudostem Diameter (cm)	Number of Leaves	Shoot Weight (g)	Number of Roots	Root Weight (g)
Control	12.62 a	1.28 ab	7 a	25.48 a	20 b	10.29 bc
Ta-1	12.33 a	1.29 ab	7 a	27.12 a	19 b	15.08 c
Ta-2	12.38 a	1.33 ab	7 a	30.28 a	21 b	16.53 c
Fo-1	12.10 a	1.36 b	7 a	28.08 a	19 ab	16.96 c
Fo-2	12.83 a	1.33 ab	6 a	25.55 a	16 a	14.90 bc
Ta-Dual	12.60 a	1.18 a	6 a	25.31 a	19 ab	13.53 bc
Fo-Dual	12.62 a	1.28 ab	6 a	20.32 a	18 ab	5.51 a
Combined	13.27 a	1.24 ab	6 a	23.07 a	20 b	8.13 ab

Means in columns followed by different letters are significantly different at $P \leq 0.05$, Tukey Test, N=12.

Once again, orthogonal contrasts revealed few significant differences with regard to plant height, pseudostem diameter, number of leaves and roots, and shoot weight (Table 27). Root weight, length, average diameter and volume was significantly different between control and endophyte inoculated cv. 'Williams' plants. Control roots weighed less (Table 26), were shorter and less voluminous (Table 28) than inoculated plants, with the exception of Fo-Dual and combine inoculated plants. Roots of combine inoculated plants were also smaller than other inoculated plants, with the exception of Fo-Dual inoculated roots. Effects of Dual inoculations were significantly different from one another, with Fo-Dual inoculations not having any root growth promoting effects

on cv. 'Williams' plants, while Ta-Dual increased root weight, length and volume, and decreased root average diameter compared to Fo-Dual inoculated plants. No significant differences were observed between the single inoculations, or between inoculations with one or the other isolate from the same species (Fo-1 vs. Fo-2 or Ta-1 vs. Ta-2).

Table 27. Orthogonal contrasts carried out on morphological data collected from *Musa* AAA cv. 'Williams' plants inoculated with one, two or four endophytes, 12 weeks after planting (N=12).

	Morphological Data						WinRHIZO Data		
Contrasts	**Plant Height**	**Pseudostem Diameter**	**Nr. of Leaves**	**Shoot Weight**	**Nr. of Roots**	**Root Weight**	**Root Length**	**Root Diameter**	**Root Volume**
Control vs. Treatments	*	n.s.	*	n.s.	n.s.	**	**	**	**
Combined vs. other Inoculations	n.s.	n.s.	n.s.	*	n.s.	**	**	*	**
Dual vs. Single Inoculations	n.s.	n.s.	**	**	n.s.	**	**	*	**
Ta-Dual vs. Fo-Dual	n.s.	*	n.s.	*	n.s.	**	**	**	**
Fo-1 and Fo-2 vs. Ta-1 and Ta-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ta-1 vs. Ta-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fo-1 vs. Fo-2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

*: significant difference (P≤0.05); **: highly significant difference (P≤0.01); n.s.: no significant difference according to SAS Orthogonal Contrasts test.

Table 28. Effect of inoculations with one, two or four endophytic fungi on root length, average root diameter and root volume of *Musa* AAA cv. 'Williams' plants, 12 weeks after planting in the greenhouse.

Treatment	Root Length (cm)		Root Diameter (mm)		Root Volume (cm^3)	
Ta-1	1927.50	d	1.00	a	19.33	def
Ta-2	1923.25	d	1.00	a	20.58	ef
Fo-1	1889.00	d	1.00	a	22.55	f
Fo-2	1698.73	cd	1.00	a	17.18	de
Ta-Dual	1477.25	c	1.00	a	15.17	cd
Fo-Dual	471.83	a	1.25	b	6.50	a
Combined	698.42	ab	1.00	a	8.92	ab
Control	822.25	b	1.00	a	11.50	bc

Means in columns followed by different letters are significantly different at P≤0.05, Tukey Test, N=12.

6.4 Discussion

In 2002, Meyer and Roberts reviewed studies looking at the effects of combinations of biocontrol agents, and concluded that such combinations are often done in an attempt to increase the stability, intensity and/or reliability of biocontrol performance. Nevertheless, they are not always beneficial, as antagonism can occur between biocontrol organisms, and lead to unchanged control levels (Zaki & Maqbool 1991; Viaene & Abanoi 2000) or even to decreased control (Esnard *et al.*, 1998; Chen *et al.*, 2000), when compared to individual applications of biocontrol agents. However, many combinations studied have resulted in increased biocontrol levels (Guetsky *et al.*, 2001; Guetsky *et al.*, 2002; Meyer & Roberts, 2002). Combinations tested against nematodes include fungi with fungi (Khan *et al.*, 1997; Duponnois *et al.*, 1998; Hojat Jalali *et al.*, 1998; Chen *et al.*, 2000) and fungi with bacteria (Maheswari & Mani, 1988; de Leij *et al.*, 1992; Siddiqui & Mahmood, 1993; Perveen *et al.*, 1998; Chen *et al.*, 2000), with most combinations involving two organisms, but few combinations of three or more organisms (Esnard *et al.*, 1998). The majority of biocontrol agents tested against nematodes were isolated from the rhizosphere or rhizoplane and tested on *Meloidogyne* spp. (Meyer & Roberts, 2002). Diedhiou *et al.* (2003) tested an arbuscular mycorrhizal fungi (AMF), *Glomus coronatum*, and a non-pathogenic endophytic *Fusarium* oxysporum against *Meloidogyne incognita* on tomato, with interesting results regarding the interaction of the two fungi within the plant, but observed no increased nematode control related to combined inoculation. Sikora and Reimann, (2004) studied the effects of combining AMF and bacteria. To the best of our knowledge, combinations of two or more endophytic fungi have never been studied. All fungi included in this study are proven endophytes, having been repeatedly re-isolated from roots of inoculated banana plants, occasionally also from corms and pseudostems (Pocasangre *et al.*, 2004).

Individual application of each of the four tested endophytic fungi significantly reduced the total number and density of *R. similis* nematodes in banana roots. Dual inoculations of either both *F. oxysporum* isolates (Fo-Dual) or both *T. atroviride* isolates (Ta-Dual) increased the positive effects on nematode control. This indicates that these isolates of the same species act together, which is especially evident for the combination of the *Fusarium* isolates. Both single *F. oxysporum* (Fo-1 and Fo-2) inoculations significantly reduced the number and density of *R. similis* in roots, while the Fo-Dual inoculation yielded even better biocontrol results. The increased biocontrol

effect is partially additive, but not synergistic, as the degree of control does not equal that of both individual endophytes, nor does it exceed this ideal additive degree of biocontrol, as would be the case in a synergistic relationship, where the combined effects are more than the sum of the partial effects.

The effects of single and double inoculations of the *Trichoderma* isolates on biocontrol were similar, with the Ta-1 and Ta-2 inoculations significantly reducing numbers and densities of *R. similis* in roots, and the Ta-Dual inoculation yielding even better results. Inoculating all four endophytes yielded even better nematode biocontrol results than single or double inoculations. Once again, the effect was only partially additive.

As was the case for nematode biocontrol, plant growth was promoted in the first test where both nematodes and fungi were inoculated. With positive effects increasing as the number of fungi inoculated increased. Fo-2 had no significant positive effect on plant growth on its own, while Fo-1 had a marked positive effect on plant growth, and the effect of the Fo-Dual inoculation greatly surpassed that of individual *Fusarium* inoculations, indicating a synergistic effect of these *Fusarium* isolates on plant growth. The plant growth promotion effects of *Trichoderma* isolates differed from those of *Fusarium* isolates, in so far as both individual inoculations (Ta-1 and Ta-2) had marked positive effects on plant growth, while the Ta-Dual inoculation only increased shoot growth, with root weight and numbers not as high as for individual inoculations. Inoculating all four endophytes yielded even better results than single or double inoculations for all morphological and biocontrol parameters examined. The results suggested that combining compatible biocontrol agents stabilizes biocontrol and intensifies the protection gained by the plant over the parasite and adds a plant growth promoting effect. These effects were neither strictly additive nor synergistic, but simply greater than that of individual inoculations.

However, in the second test, where only fungi were inoculated, combinations of endophytes did not additionally increase plant growth, neither compared to the controls nor to the single inoculations. In fact, root and shoot weights were always lower in plants inoculated with 2 or more fungi than in plants inoculated with only one endophyte. This was especially so in plants in the Fo-Dual and combined inoculations. However, the combined endophyte inoculations were not entirely responsible for this effect. The soil that was used for potting had been autoclaved in large autoclave bags.

Approximately half a bag of was used per treatment. The control plants were the first to be planted, followed by the single inoculated plants, the dual inoculated plants and finally the combine inoculated plants. Fo-Dual and combined inoculated plants were planted in soil from the same bag of autoclaved soil. This soil was much finer than that used for the first 6 treatments, causing the pores at the bottom of planting bags to get clogged with silt. The soil in these bags was therefore very wet for the entire run of the experiment. This was only noticed when plants were harvested. Water-logging and the associated lack of oxygen is well known to stunt the growth of banana roots and cause root necrosis (Turner, 2005). Roots from plants in treatments Fo-Dual and combined for all three cultivars were more greatly affected by excess water than by the multiple inoculations and the data collected from these plants can be discarded. It does not however, explain why plants inoculated with the two *T. atroviride* isolates (Ta-Dual) did not have heavier roots than those plants inoculated with only one of *T. atroviride* isolate, which was the case in both tests. The inoculation method, i.e. successive dips in single-isolate conidia suspensions vs. a single dip in a mixed-isolate conidia suspensions, may have affected the results, though this seems unlikely, as the isolates did not inhibit each other's growth on Petri dishes.

Generally, greater growth promoting effects from endophyte inoculation were seen in cv. 'Grand Nain' than in cvs. 'Valery' or 'Williams', especially where single inoculations were concerned. 'Valery' and 'Williams' plants did not responded well to Fo-2 inoculation alone, and this inoculation was also the least effective in controlling *R. similis*. Fo-1 had the best growth promoting effects, while Ta-1 and Ta-2 had overall positive effects on growth. Ta-Dual inoculations had a positive effect on root growth in all cultivars.

As the results for Fo-Dual and combined inoculations did not reveal the effects of the inoculations, but rather those of excessive water, this test should be repeated to discover the real effects if dual and combined inoculations of endophytes on banana growth in the absence of nematodes. Additionally, it would be interesting to test whether or not the presence of *R. similis* triggers additional plant growth promotion by endophytes, as appears to have been the case in the first test where both endophytes and nematodes were inoculated.

Guetsky *et al.* (2002) demonstrated that the use of a combination of biocontrol agents improved biocontrol efficacy and consistency. This was also demonstrated in this

study. Meyer and Roberts (2002) suggest that more effective disease suppression of some combinations of biocontrol agents is due to additive or synergistic effects of their combined mechanisms of disease suppression against the pathogen. It is possible that such effects play a role in the present study. While the exact modes of action of the isolates are not yet known, there are indications that they may in fact be different for each isolate, or at least each genus. Both *Trichoderma* isolates scored high in parasitism tests conducted prior to *in planta* testing (zum Felde, 2002; Carñizares Monteros, 2003), while the metabolites of the two *Fusarium* isolates had *in vitro* nematistatic and nematicidal effects on *R. similis* (Carñizares Monteros, 2003; Menenses Hérnandez, 2003).

Guetsky *et al.* (2001) postulate that as long as biocontrol agents have different ecological requirements, their combined use will increase reliability and decrease variability of biocontrol, and Meyer and Roberts (2002) conclude that negative effects of combinations of biocontrol agents result from their mechanism(s) of control being directed not only at the plant pathogen, but also at the companion biocontrol agent within the combination. In fact, *Trichoderma* and *Fusarium* spp. have both been successfully used to suppress *Fusarium* wilt (Park *et al.*, 1988; Mao *et al.*, 1998). However, though all four tested biocontrol agents were isolated from internal tissues of banana roots and presumably occupy the same or at least similar ecological niches, they nevertheless do not compete with each other, complementing each other instead, and making them ideal candidates for a stable and effective biocontrol strategy, when used in combination.

As to growth promotion, it seems that single inoculations are more consistent than combined inoculations. However, if a combination of nematode biocontrol and growth promotion are desired, combined inoculations are the better choice, as the increase in biocontrol is significant in multiple inoculations and plants inoculated with both nematodes and multiple endophytes grow at least as well as, if not better, than plants without any endophytes or with only nematodes. The fact that the plants in the first test, inoculated with both fungi and nematodes were bigger and heavier than both the control and absolute control plants is important, as inoculations with endophytes not only allowed plants to compensate for loss of mass usually associated to nematode attack, but increase their mass above and beyond that. The results for single inoculations and

dual inoculations with *T. atroviride* in the second test confirmed these positive effects on plant growth in the three *Musa* AAA cultivars tested.

Niere (2001) postulated that the variation in *R. similis* control he encountered may be cultivar related. Whether biocontrol is equally effective in 'Grand Nain', 'Valery' and 'Williams' plants has not been studied as such, though both greenhouse and field trials have been carried out with the tested endophytes and cultivars, all with positive results (Menenses Hérnandez, 2003; Carñizares Monteros, 2003; Menjivar Barahona, 2005). This is not surprising, as all the cultivars are from the Cavendish group, and the endophytes were isolated for Cavendish, esp. cv. 'Valery', plants.

Further research is needed to verify the plant growth promotion effect of combined inoculations, both in the presence and absence of nematodes, to explore the antagonistic effects of combined applications of endophytes on nematodes under field conditions, and to reveal the mechanisms by which the fungal endophytes promote plant growth.

7 GENERAL CONCLUSIONS

The conclusions of the present thesis work are:

1) The 10 *Radopholus similis*-antagonistic endophytic *Fusarium oxysporum* isolates tested were not vegetatively compatible with known pathogenic strains of *F. oxysporum* f. sp. *radicis-lycopersici*, *lycopersici* or *cubense*.

2) Systemic induced resistance to nematode penetration probably plays a role in antagonistic relationship of the endophytic *Trichoderma atroviride* isolate S2 and *R. similis* in *Musa* AAA cultivar 'Valery' plants. SIR to nematode reproduction may play a role the antagonistic relationship of *T. atroviride* isolate MT-20 and *R. similis* in 'Valery' plants. No SIR to either *R. similis* penetration or reproduction was observed for the four *F. oxysporum* isolates tested: MF-25, MF-40, S9 and P12 in 'Valery' plants.

3) When nematode suppression was established in the field by inoculation of mother plants with endophytes prior to field transfer, this suppression was transferred from mother to daughter *Musa* AAA cv. 'Valery' plants.

4) Combining inoculations of mutualistic endophytes increased nematode control levels and plant growth promoting effects in *Musa* AAA cv. 'Williams' in the presence of *Radopholus similis*. Plant growth in the absence of *R. similis* was promoted by single and dual inoculations of *T. atroviride* isolates and by single inoculations of *F. oxysporum* isolates in *Musa* AAA cv. 'Grand Nain', 'Valery' and 'Williams'. Dual inoculations of *F. oxysporum* and of all four endophytes did not positively affect plant growth.

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