Plant defence responses against *Radopolus similis* in East African Highland bananas (EAHB- AAA) inoculated with endophytic non-pathogenic *Fusarium oxysporum*

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DEDICATION

To my family

DECLARATION

I hereby certify that this research, unless specifically indicated to the contrary in the text, is the result of my own investigation and that no part of this thesis has been submitted to any other university.

Pamela Paparu

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PREFACE

Bananas and plantains (*Musa* spp.) are considered the fourth most important staple food crop in the world. Their production is, however, constrained by biotic and abiotic factors. The biotic constraints include the banana weevil (*Cosmopolites sordidus* (Germar)), a complex of plant-parasitic nematodes, most importantly *Radopholus similis* (Thorn) Cobb, black Sigatoka (caused by *Mycosphaerella fijiensis* Morelet), Fusarium wilt (caused by *Fusarium oxysporum* f.sp. *cubense* Snyder and Hans - *Foc*), banana streak and banana bunchy top viruses, and more recently, banana bacterial wilt (*Xanthomonas vasicola* pv. *musacearum* Yirgou and Bradbury). The main abiotic constraints include poor soil management, soil nutrient deficiency, drought and socio-economic factors.

In the Lake Victoria Basin region of Uganda the most economically important pests of bananas are the banana weevil and *R. similis.* No single control strategy has proved effective against these two pests. An integrated pest management strategy for weevils and nematodes currently involves the use of clean planting material, such as tissue culture banana seedlings and suckers treated with hot water, the use of cultural practices, and field sanitation. Biological control strategies using non-pathogenic *F. oxysporum* and the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin is being explored. Non-pathogenic *F. oxysporum* endophytes of banana, previously isolated from plants in farmers' fields, have been successfully re-introduced into tissue culture plants. These endophytes have colonized both root and rhizome tissues extensively, and have shown potential to reduce pest populations and damage *in vivo*. Despite this success, certain hindrances still need to be overcome before the interaction between non-pathogenic *F. oxysporum* and their banana host is fully understood.

Determining the extent of plant colonization by the endophytes is important in selecting potential biocontrol agents. However, it is often difficult to do this as experimental plants often get contaminated by *F. oxysporum* strains other than the *F. oxysporum* endophytes introduced onto banana roots in the screen house. If two or more endophytes need to be inoculated into a single banana plant simultaneously, it is important that such isolates be effectively marked for identification upon re-isolation. While it is now known that the mode of protection with banana fungal endophytes involves induced resistance, little is known

about the induction and persistence of defence-related responses in the host plant that lead to the expression of a resistant phenotype.

In this thesis, **Chapter 1** reviews literature available on the protection of host plants by fungal endophytes against pests and diseases, with particular focus on the interaction between non-pathogenic *F. oxysporum* endophytes and the banana plant. Banana production and its major constraints is first discussed. The review then focuses on the use of fungal endophytes to protect plants against pests and diseases, by discussing their modes of action. As induced resistance has been identified as one of the modes of action of how *F. oxysporum* endophytes protect bananas against *R. similis*, the review discusses the molecular basis of induced resistance in detail.

Putative defence-related genes have been identified in Cavendish banana plants infected with *Foc*. In **Chapter 2** the involvement of eight of these defence-related genes is investigated following colonization of *R. similis*-susceptible and -tolerant East African highland banana (EAHB) cultivars by endophytic non-pathogenic *F. oxysporum* and challenge with *R. similis*.

Chapter 3 identifies novel genes up-regulated in *R. similis*-susceptible and -tolerant EAHB cultivars following colonization by endophytic non-pathogenic *F. oxysporum*. cDNA-AFLP, which allows for gene identification without prior sequence knowledge, was used to study differential gene transcription. Polymorphic bands identified from cDNA-AFLP gels were cut out, re-amplified, sequenced and subjected to BLASTX searches. The expression profiles of four defence-related genes were further investigated by quantitative real-time PCR following endophyte colonization and *R. similis* challenge.

Phenylpropanoid pathway enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) are involved in plant defence pathways leading to lignification, synthesis of secondary metabolites including salicylic acid and phytoalexins, wound healing and the oxidative burst. In **Chapter 4,** expression of PAL, POX and PPO, which are all enzymes involved in the phenylpropanoid pathway, was analysed in *R. similis*-susceptible and -tolerant banana cultivars following colonization by endophytic non-pathogenic *F. oxysporum* and challenge with *R. similis*. Enzyme assays were conducted up to 60 days after nematode challenge of 8-wk-old plants previously inoculated with the endophytic isolate V5w2.

In **Chapter 5**, endophytic isolates with the potential to control *R. similis* and the banana weevil were marked with benomyl- and chlorate resistance, and transformed with green- and red fluorescent proteins. They were then tested for their ability to colonize roots and rhizomes in comparison with their wild types cultures. The marked isolates have been developed to overcome hindrances associated with determining actual colonization percentages and *in vivo* tracking of introduced endophytes.

As a result of the involvement of different modes of action, it is believed that dual or multiple inoculations of biological control agents promotes pathogen and pest control. In **Chapter 6**, plant colonization by dually-inoculated isolates of non-pathogenic *F. oxysporum* endophytes with the ability to reduce nematode and weevil damage in banana is investigated. The effect of dual endophyte inoculations on pest control and plant growth is also determined.

ABBREVIATIONS AND SYMBOLS

α	Alpha
AFLP	Amplified Length Polymorphism
AMV	Avian Myeloblastosis Virus
β	Beta
BLAST	Basic Local Alignment Search Tool
BLASTX	BLAST algorithm to compare the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.
bp	Base pairs
BR	Benomyl resistant
BTH	Benzothiadiazole
CaCl	Calcium chloride
Ca^{2+}	Calcium (II) ions
°C	Degrees Celsius
CDPK	Calcium-dependant protein kinase
cDNA	Complementary deoxyribonucleic acid
CHR	Chlorate resistant
cm	centimetres
COI1	Coronatine insensitive 1
cv	cultivar
dai	Days after inoculation
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dpnc	Days post nematode challenge
ds-cDNA	Double stranded complementary deoxyribonucleic acid
DsRed	Red fluorescent protein
E	Endophyte
EAHB	East African highland banana
EDTA	Ethylenediamine tetraacetic acid
EtOH	Ethanol

FABI	Forestry and Agricultural Biotechnology Institute
f.sp.	Formae speciales
Foc	*Fusarium oxysporum* f.sp. *cubense*
g	Gram
GFP	Green fluorescent protein
h	Hours
HCl	Hydrochloric acid
hyg	Hygromycin
H_2O_2	Hydrogen peroxide
ICIPE	International Centre for Insect Physiology and Ecology
ID	Identity
IITA	International Institute for Tropical Agriculture
INIBAP	International Network for Improvement of Bananas and Plantain
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISR	Induced systemic resistance
JA	Jasmonic acid
l^{-1}	Per litre
LB	Luria Bertani
LOX	Lipoxygenase
M	Molarity
min	Minutes
mg	Milligrams
$MgCl_2$	Magnesium Chloride
ml	Millilitres
ml^{-1}	Per millilitre
mm	Millimetres
MM	Minimal media
mg	Milligrams
mM	Millimolar
mRNA	Messenger ribonucleic acid
n	Sample size
NaCl	Sodium chloride

NaOCl	Sodium hypochlorite
NARO	National Agricultural Research Organization
ηg	Nanogram
nm	Nanometre
nmol	Nanomoles
PAE	Pectinaseacetly esterase
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
pH	Log hydrogen ion concentration
PEG	Polyethylene glycerol
POX	Peroxidase
ppm	Parts per milli
PPO	Polyphenol oxidase
PR	Pathogenesis-related
PVPP	Polyvinypolypyrrolidone
RDA	Representational difference analysis
RH	Relative humidity
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RS	*Radopholus similis*
RT	Reverse transcriptase
s	Seconds
SAR	Systemic acquired resistance
SDW	Sterile distilled water
spp	species
SSH	Suppression Subtractive Hybridisation
TDF	Transcript derived fragments
Tm	Melting temperature
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol chloride
U	Units

µg	Micrograms
µl	Microlitre
µM	Micromolar
UT	Unsubtracted “tester”
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
χ^2	Chi-square
%	Percentage
ΔOD	Change in optical density

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CHAPTER 1

LITERATURE REVIEW

The use of fungal endophytes, especially non-pathogenic *Fusarium oxysporum*, in protecting banana plants against diseaeses and pests

INTRODUCTION

Bananas and plantains (*Musa* spp.) are regarded as the fourth most important staple food crop in the world. This can be attributed to their cultivation mainly by the resource poor in gardens and small-farmer holdings in the tropics and sub-tropics (Jones, 2000). For small-holder farmers, banana contributes to food security and provides a steady supply of income when traded as a cash crop (NARO, 2001). The highest consumption in the world is in Uganda, where the average person eats approximately 250 kg of East African Highland bananas (EAHB) (AAA-EA) per annum (Simmonds, 1966). Bananas provide the most economical source of carbohydrates in terms of cost per hectare, per ton and per calorie (Swennen, 1984). They are grown with no renewal of plantations for decades (Sarah, 1989; Speijer *et al.*, 1999), provide soil cover and are a source of mulching material (INIBAP, 1986). Banana is increasingly becoming an important export crop, with world exports projected to reach 15 million tons by 2010, an increment of 28% compared with the period 1998-2000 (FAO, 2002). Countries that lead in world banana exports include Ecuador, the Philippines, Costa Rica and Columbia (FAO, 2002).

Despite the significance of banana in the world, the production of the crop has declined in recent years due to biotic and abiotic constraints (Gold *et al.*, 1993; Tushemereirwe *et al.*, 1996; 2003). For instance, in 2001 productivity in central and southwestern Uganda was estimated to be 6 and 17 tons per hectare, respectively, which is much lower than the potential of 60 tons per hectare attainable (Tushemereirwe *et al.*, 2001). The major biotic constraints to sustainable banana production include the banana weevil (*Cosmopolites sordidus* (Germar)), a complex of plant-parasitic nematodes, most importantly *Radopholus similis* (Cobb) Thorne, black Sigatoka (caused by *Mycosphaerella fijiensis* Morelet), Fusarium wilt (caused by *Fusarium oxysporum* f.sp. *cubense* (E.F. Sm) Snyder and Hans), banana streak (BSV) and banana bunchy top viruses (BBTV), and more recently, banana bacterial wilt caused by *Xanthomonas vasicola* pv. *musacearum* (Gold *et al.*, 1993; 1994; Tushemereirwe *et al.*, 1996; 2003; 2004). The main abiotic constraints include poor soil management, soil nutrient deficiency, drought and socio-economic factors (INIBAP, 1986).

In recent years, research on the use of fungal endophytes as plant bio-enhancers and bio-protectors has become of great significance. The endophytic bacterium *Pseudomonas fluorescens* (Flügge) Migula has been widely used to protect crop plants from viral, bacterial

and fungal diseases (Alstrom, 1991; Maurhofer *et al.*, 1994; 1998; Ramamoorthy *et al.*, 2001), and endophytic fungi such as *Fusarium oxysporum* Schlecht.: Fries and *Trichoderma* spp. have been used to enhance plants for protection against pathogenic fungi such as *F. oxysporum* (Belgrove, 2007) and plant pathogenic nematodes such as *R. similis* (Athman, 2006; Sikora *et al*., 2008) and *Meloidogyne incognita* (Kofoid and White) Chitwood (Hallman and Sikora (1994a, 1994b). The objective of the current literature review is to provide an overview of beneficial interactions between plants and their fungal endophytes, and will in the end review the current knowledge on the interaction between EAHB and endophytic non-pathogenic *F. oxysporum* endophytes.

BANANAS

The banana plant is a large perennial monocot that is divided into an underground stem (rhizome), a pseudostem and leaves (Vasquez, 2003). The underground stem has buds from which a cluster of shoots arise, and these are called suckers. The plant has a typical monocotyledonous root system where all roots are similar; hence their capacity to obtain water and nutrients and to serve under adverse conditions is similar for all roots (Vasquez, 2003). The roots arise from a cambium-like apical meristem of the central cylinder (Stover and Simmonds, 1987). From each rhizome a terminal inflorescence arises which extends throughout the pseudostem and matures into a fruit (the bunch) (Stover and Simmonds, 1987).

The centre of diversity of wild *Musa* spp. L. is Indochina and South East Asia, where the earliest domestication of banana is thought to have happened (Simmonds, 1962; Price, 1994). All bananas originate from the interspecific hybridization of two species, *M. acuminata* Colla and *M. balbisiana* Colla, to produce diploid, triploid and tetraploid varieties (Simmonds, 1962). Cultivation results in the production of seedless bananas that can be subdivided in cooking bananas, dessert bananas and beer bananas. Cooking bananas include the EAHB and the plantains (AAB). Dessert bananas consist mainly of AAA types (cvs Cavendish, Lacatan and Gros Michel), and beer bananas of ABB (cvs Bluggoe and Pisang Awak) and AB dessert and juice types (cv Ndiizi) (Karamura *et al.*, 1998). Today, bananas are widely cultivated in tropical and subtropical regions around the world, including, Asia, Africa, South and Central America, Oceania and the Caribbean (Dale, 1999).

It is believed that bananas were introduced into Africa via the Indian Ocean islands close to the eastern African coast (Verin, 1981). Since the islands were inhabited by Indonesian migrants in the 5th century AD, they could have carried material from Southeast Asia (AA and AAA bananas) and the Indian sub-continent (ABB and AAB bananas) (Price, 1994). From there it was taken to Central and South America where bananas are produced for export to the American and European markets. Export bananas are mainly dessert bananas and consist primarily of the Cavendish subgroup (AAA), and include cultivars such as Williams, Grand Naine and Valery. They make up 43% of world banana production. In Africa, dessert bananas grown include Dwarf Cavendish, Lacatan, Red Banana, and Gros Michel. However, the most widely grown bananas are the EAHB in eastern and central Africa, and the plantains in western Africa (Jones, 2000). These bananas are primarily used for cooking. Cavendish dessert bananas are grown on large commercial farms in the low-lying (below 800 m above sea level) coastal regions in South Africa, Somalia and Ethiopia, while dessert bananas belonging to Gros Michel are found around Lake Victoria region at a slightly higher altitude where they form an important staple crop in the area (Karamura *et al.*, 1998). Beer bananas (ABB) are widely used and grown in Uganda, Rwanda, Burundi and Tanzania (Karamura *et al*., 1998).

Banana production systems can be divided into the backyard garden system, the subsistence system and the commercial plantation system (Karamura *et al*., 1998). The different systems vary according to the intensity and level of their management, planting materials used, irrigation, cropping system, pest and disease control options, and associated end uses (Karamura *et al.*, 1998). The backyard garden system, for example, is low input, peri-urban and priority in this system is often given to crops other than banana. Banana subsistence system accounts for 87% of global banana/plantain production (INIBAP, 1996), and is mostly established for food security, but bunches may also be sold in the local markets for income. This system is characterized by the production of several cultivars on the same farm. In Uganda, Karamura *et al*. (1996) reported farmers growing 12 different cultivars on a single farm. Commercial banana plantation systems account for 12% of global banana production (Robinson, 1996), and are characterized by single cultivars and management uniformity. All stages of production such as selection and treatment of planting material, crop establishment, pest and disease control and marketing are intensive (Robinson, 1996).

Most cultivated banana cultivars are triploid, seedless or sterile, and are propagated vegetatively (Gübbük and Pekmezci, 2004). Conventional propagation materials include rhizomes, young and mature suckers, and sword suckers (Arias, 1992). These materials are often infested with pests and pathogens (Arias, 1992; Sagi *et al.*, 1998) and are slow in multiplication (Vuylsteke, 1998). As a consequence an alternative propagation method by means of *in vitro* propagation has been developed (Cronauer and Krikorian, 1984; Vulysteke, 1998; Kalimuthu *et al.*, 2007). Tissue culture banana production through shoot-tip culture was introduced in countries such as Israel, Thailand, Canary Islands and South Africa as early as 1985. The technology has become very popular among banana growers because plants produced through tissue culture have superior qualities such as physiological uniformity, high multiplication rate and are free from field pests such as nematodes and weevils (Pocasangre, 2006). However, tissue culture plants have a disadvantage of being highly susceptible to pests such as nematodes and weevils in the field, since they are propagated under sterile conditions which equally get rid of all beneficial organisms in the starting material (Pocasangre, 2006).

PESTS AND DISEASES OF BANANA

Throughout history, the banana plant has been attacked by pathogens and pests that were introduced with their host into new geographic regions. Fusarium wilt, for instance, destroyed the banana export industry based on Gros Michel in Central America (Stover, 1962), while black Sigatoka is considered as the most devastating disease of bananas in the world today (Ploetz, 2001). More recently, banana bacterial wilt devastated EAHB in central and eastern Africa (Tushemereirwe *et al.*, 2003), while nematodes and the banana weevil are still considered the primary concern to sustainable banana production in the region (Gold *et al.*, 1993; 1994). Viral diseases such as banana bunchy top and banana leaf streak are also causing damage to banana plantations in many parts of the world.

Radopholus similis

Radopholus similis occurs in most banana growing regions of the world (Gowen, 2005). The nematode is a migratory endoparasite, with all growth stages occurring within the root. It may survive for up to 6 weeks in soil in the absence of an alternative host (Marin *et al.*, 1998). Within plant roots, optimum temperature for *R. similis* reproduction is 30^{o}C and the full life cycle can be completed in 20-25 days (Sarah *et al.*, 1996). The most infective stages of *R. similis* are the females and juveniles, with males presumed non-infective (Gowen and

Queneherve, 1990). Most feeding occurs in root- and rhizome cortex, resulting in the formation of extensive red-brown lesions (Gowen, 1995). The nematode spreads within and between fields through infested planting material, soil movement, root contact and in run-off water. Current control strategies involve an integrated pest management approach with components such as habitat management (field sanitation), use of clean planting materials (tissue culture plants and use of pared and hot water-treated suckers) and chemical control (Furadan) (Speijer *et al.*, 1994). Biological control strategies include the use non-pathogenic *F. oxysporum* endophytes (Schuster *et al*., 1995; Niere, 2001; Athman, 2006).

Cosmopolites sordidus

The banana weevil is an important pest of banana in most countries where banana is cultivated (Gold, 1998). The adult weevil is free-living but may occasionally be found in leaf sheaths, crop residues or in soils at the base of the plant. The preferred sites of oviposition are the pseudostem base and rhizome surface, and the most favourable growth stage for oviposition is at flowering (Koppenhofer, 1993). The eggs are deposited singly in holes perforated using the rostrum of the ovipositing female. Emerging larvae feed in the rhizome where they pass through five to eight instars (Gold *et al.*, 1999). Most of the damage to banana is caused by the feeding larvae, and heavily infested plants become prone to drought and secondary infection by pathogens, and may topple before maturity (Sikora *et al.*, 1989). Yield losses due to weevil damage may reach 100% in severely damaged fields (Gold *et al.*, 2004). The pest is spread mainly through the dissemination of infested planting material and by the weevil walking into neighbouring fields (Gold and Bagabe, 1997). The banana weevil can be managed by field trapping of adult weevils, the use of tissue culture banana plants and the application of organophosphates and carbamates around infected plants (Gold *et al.*, 2001). Biological control strategies include use of the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin (Nankinga, 1995) and non-pathogenic *F. oxysporum* endophytes (Griesbach, 2000).

Fusarium wilt (Panama disease)

Fusarium wilt of bananas is caused by *F. oxysporum* f.sp. *cubense* (*Foc*) (Stover and Waite, 1954) and is an important disease of banana in almost all banana-growing regions of the world. The disease was first reported in Central America (from where it got its name ‘Panama disease’) in the 1800’s. By 1854, Panama disease was reported in Australia, and in early 1900, the disease was observed in Asia, Central America and West Africa; and by 1950, it

had spread to almost all banana-growing regions of the world (Jeger *et al.*, 1996). Originally, *Foc* race 1 attacked only cv Gros Michel. In efforts to maintain banana production, Gros Michel was replaced by Cavendish cvs in affected areas. However, Cavendish is now susceptible to *Foc* race 4 which is present in countries like Australia, Canary Islands, Philippines, Malaysia and South Africa (Gowen, 1995; Bentley *et al.*, 1998). *Foc* enters roots through wounds and intact surfaces of primary roots, from where it invades the vascular system and produces conidia that clogg the water transport system, thus interfering with water movement to the shoot (Jeger *et al.*, 1995). External symptoms of Panama disease include yellowing of margins of older leaves, and their collapse downwards from the stalk to form a skirt around the plant (Moore *et al.*, 1995). Eventually, the apical meristem may die to leave a pseudostem which soon collapses as the disease progresses (Stover, 1962). The most visible internal disease symptom is the discolouration of the vascular tissues. The only effective control strategy for Panama disease is the use of resistant varieties, though prospects of microbial control look feasible. For example, field control of *Foc* in cv Rasthali was achieved using *Trichoderma harzianum* Rifai, applied to the rhizosphere as a powdered formulation (Thangavelu *et al.*, 2004). Similarly, enhancement of banana plants with endophytic *F. oxysporum* for protection against *Foc* is being studied (Belgrove, 2007).

Black leaf streak disease (black Sigatoka)

Black Sigatoka, caused by the fungal pathogen ***M. fijiensis,*** is an economically important disease of bananas and plantains, and is considered the most important disease constraint to banana production in tropical areas world-wide (Stover, 1983). Black Sigatoka affects leaf photosynthetic area and may cause up to 50% yield losses if not controlled (Mobambo *et al.*, 1993). Infection occurs through ascospores and conidia, and is most favourable in conditions of high humidity. Inoculum may be moved downward from the upper leaves on a plant or from one plant to another by wind (Foure, 1987). On plantains, the incubation period in wet season is about 14 days, while it is longer in dry season (an average of 23 days) (Mobambo *et al.*, 1996). Disease symptoms, mostly on younger leaves include black leaf streak on the lower leaf surface, which enlarge to form necrotic lesions with yellow haloes and light grey centres (Mourichon *et al.*, 1997). There is a general lack of resistance to black Sigatoka in the African gene pool, with important cvs such as Williams being highly susceptible. One cv that reportedly shows some resistance to the disease is Yangambi (Km-5) (Vuylesteke *et al.*, 1993). Control of black Sigatoka currently is achieved through use of fungicides such as triazole, morpholine, trifloxystrobin and azoxystrobin (Pérez *et al.*, 2002). Cultural practices

such as intercropping with plants having leafy canopies, and wider spacing are also used to contain the disease (Emebiri and Obiefuna, 1992).

Xanthomonas wilt of banana

Banana bacterial wilt caused by *X. vasicola* pv. *musacearum* has been present on enset in Ethiopia for years, but was reported for the first time in central Uganda in 2001 (Tushemereirwe *et al.*, 2003; 2004). Currently, the disease is present in Rwanda, eastern Democratic Republic of Congo (Ndugu *et al.*, 2005), Tanzania (Mgenzi *et al.*, 2006) and Kenya (Mbaka *et al.*, 2007). All banana cvs grown in the areas where the disease is present are susceptible; attaining losses of 100% in severely affected fields (Eden-Green, 2005). The disease is spread through use of infected planting material, farm tools and vectors such as bees. Symptoms of banana bacterial wilt include wilting of young plants, pre-mature ripening of fingers and characteristic yellow bacterial ooze from cut pseudostems (Tripathi *et al.*, 2004). Currently, control strategies for the disease include complete destruction of infected mats, removal of male buds and bagging of inflorescences to stop vector transmission (Blomme *et al.*, 2007).

Major viral diseases of banana

The two most important viral diseases of banana are banana bunchy top disease (BBTD) and banana streak disease (BSD).

BBTD is caused by banana bunchy top virus (BBTV) and is the most important viral disease of bananas and plantains (Kavino *et al.*, 2007). BBTV is transmitted by the vector *Pentalonia nigronervosa* Coq. (banana aphid) and its symptoms include dark-green streaks of variable length in leaf veins, midribs and petioles; dwarfing of plants, leaf chloris, and crowded and bunched leaves that give a 'rosette' appearance (Iskra-Caruana, 1990). Control of BBTD is based on vector control using chemicals, use of disease-free planting material and biological control using endophytic bacteria such as *P. fluorescens* (Kavino *et al.*, 2007; Harish *et al.*, 2008).

BSV belongs to the family Caulimoviridae and the genus *Badnavirus.* The virus causes banana streak disease in both bananas and plantains, and has a world-wide distribution (Tushemereirwe *et al.*, 1996). Yield losses due to BSD infection range from 7-90% (Dahal *et al.*, 2000; Daniells *et al.*, 2001) and vary according to cultivars grown and cultural practices

used in plantation management. BSV is transmitted by the citrus mealybug *Planococcus citri* (Risso) (Jones and Lockhart, 1993) and the pink sugarcane mealybug (*Saccharicoccus sacchari* (Cockerell)) (Kubiriba *et al*., 2001). In Uganda, badnaviruses are also transmitted by the pineapple mealybug *Dysmicoccus brevipes* (Cockerell) (Kubiriba *et al*., 2001). BSD symptoms include continuous chlorotic/necrotic streaks on leaves and pseudostems, and stunting of plants (Harper *et al*., 2004). Control strategies for the disease include eradication of infected plants, use of BSV-free plants and vector control (Helliot *et al*., 2002).

FUNGAL ENDOPHYTES

Fungal endophytes are organisms which, at some stage in their life cycle, colonize plant tissues without causing any visible symptoms (Petrini, 1991). The interaction between an endophyte and its host is believed to be adjusted along the demands of the host and the invader, but is often beneficial to the host. Should the interaction become unbalanced, the outcome is either disease or exclusion of the invader by host defence mechanisms (Kogel *et al*., 2006). The balanced state of this interaction is represented by mutualism and commensalism. In a mutualistic relationship, the host plants provide nutrients, protection and aid in endophyte transmission to the next generation of hosts (Clay and Schardl, 2002). Beneficial effects of fungal endophytes to the host plant include increased resistance to pests and diseases through production of toxic fungal alkaloids and induced resistance, increased plant growth, and drought tolerance through changes in root morphology (Richardson *et al*., 1990). In commensalism, the endophyte benefits from the host but neither harms nor benefits its host (Saikkonen *et al*., 1998; Faeth *et al*., 2004).

Classification of fungal endophytes

Fungal endophytes can be separated into three major groups. The first group of endophytes belong to the Clavipitaceae family within the phylum Ascomycota, and include *Balansia* spp., *Epichloë* spp. and *Neotyphodium* spp. These endophytes are highly specialized, form systemic life-long infections and occur commonly in grasses (Saikkonen *et al*., 1998; Clay and Schardl, 2002). In systemic infections, fungal mycelial biomass occurs throughout the plant (Welty *et al*., 1994; Schulthess and Faeth, 1998), but infection sometimes may not be complete, as tillers emerging from infected grasses have been reported not to be infected (Schulthess and Faeth, 1998). Endophytes representing the Clavipitaceae form strong mutualistic relationships

with the host and depend on the latter for dispersal to the next generation of hosts (Saikkonen *et al.*, 1998; 2004).

A second group of fungal endophytes form specialized intrinsic relationships with plant roots, termed mycorrhiza. Two types of mycorrhizae are recognized, depending on their position on the root and lack of or penetration of root cortical cells (Allen, 1991). Ectomycorrhizal fungi do not penetrate root cells, while endomycorrhizal (arbuscular mycorrhizal) fungi (AMF) penetrate root epidermis and cortical cells to obtain carbon from host plants. The fungi also develop a network of hyphae on the root surface which absorb and translocate phosphorus and other mineral nutrients from the soil to the roots (Harrison, 1997). Arbuscular mycorrhizae are the more common of the two mycorrhizal associations and the fungi in this group belong to the order Glomales within the phylum Ascomycota and are obligate symbionts that colonize the roots of almost all higher plants, including most cultivated plant species. The Glomales comprise of over 130 species in seven genera (Abbott and Robson, 1984; Sikora *et al.*, 2003).

The final group of fungal endophytes are loosely associated with their hosts. They form localized infections and often have a saprophytic stage in the rhizosphere. This group mostly comprises of hyphomyceteous fungi. Some of these endophytes may represent latent pathogens or dormant saprophytes. Localized endophyte infection has been reported for woody plants, where infection is present only in the leaves, petioles or the bark (Carroll, 1988). Endophytic non-pathogenic *F. oxysporum* falls in this group. These endophytes are horizontally transmitted through sexual spores, and as a consequence never form close mutualistic relationships with their hosts (Saikkonen *et al.*, 1998; 2004).

Ecology of fungal endophytes

The importance of fungal endophytes was first noticed due to the detrimental effect they had on grazing livestock (cattle and sheep) (Bacon *et al.* 1977). It has been established that endophytes in cultivated and natural pastures have an impact on the growth and reproduction of their hosts, herbivores and pathogens of grasses, and even on natural enermies of the herbivore (Clay, 1990; Kimmons *et al.* 1990; White *et al.*, 1993; Breen, 1994). Fungal endophytes are symbionts of 20-30% of all grass species (Leuchtmann, 1992). Their symbiotic interaction is characterized by processes such as host penetration, biochemical or genetic recognition between the host and the symbiont, and extra- and intracellular

colonization of the host (Redman *et al.*, 2001). Most studies on plant-endophyte interactions have been conducted on two agronomically important grasses: *Festuca arundinacea* Schreb (tall fescue) and *Lolium perenne* L. (perennial rye grass), and their interactions with the leaf endophyte *Neotyphodium* spp. and its sexual stage *Epichloë* spp. (Saikkonen *et al*., 1998). However, fungal endophytes have now been isolated from a number of agricultural crops such as banana (Schuster *et al*., 1995; Griesbach, 2000), orange (*Citrus sinensis* O.) (Araújo *et al*., 2001), rice (*Oryza sativa* L.) (Tian *et al*., 2004), cocoa (*Theobroma cacoa* L.) (Arnold and Herre, 2003), barley (*Hordeum vulgare* L.) (Schulz *et al*., 2002) and lemon (*Citrus limon* L.) (Durán *et al*., 2005).

Protection of plants against pathogens and pests

Fungal endophytes have been reported to protect plants against pathogens and pests.

Protection against pathogens

Fungal endophytes have the ability to reduce the damage caused by pathogens in plants. Endophyte-induced defence responses against Fusarium wilt diseases have been reported for tomato, barley, *Asparagus sp.* and bananas (Fuchs *et al*., 1997; Duijff *et al*., 1998; He *et al*., 2002; Waller *et al*., 2005; Belgrove, 2007). Endophytic *F. oxysporum* Fo47, for instance, reduced Fusarium wilt in tomato by increasing the activity of chitinases by 26%, β-1,3-glucanases by 220% and β-1,4-glycosidase by 68% (Fuchs *et al*., 1997; Duijff *et al*., 1998). *Piriformospora indica* Sav. Verma also reduced damage caused by *Fusarium culmorum* (WM.G.SM) Sacc. in barley by reducing loss in root and shoot fresh weight by more than 12 times when compared with endophyte-free control plants (Waller *et al*., 2005). The treatment of *Asparagus officinalis* L. plants with non-pathogenic *F. oxysporum* and challenge with *F. oxysporum* f. sp. *asparagi* S.I. Cohen reduced root lesions from 50 to 25% (He *et al*., 2002). The endophyte increased the activities of defence enzymes such as peroxidase (POX) and phenylalanine ammonia-lyase (PAL), relative to endophyte non-inoculated controls. A 12-fold increase in lignin deposition was also observed in endophyte-treated and pathogen-challenged plants.

Non-pathogenic endophytic *Colletotrichum magma* (path-1) was used to control anthracnose caused by wild type *C. magma* (L2.5) on water melon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai) and wilt caused by *F. oxysporum* f.sp. *niveum* (E.F.Sm) Snyder and Hans in

cucumber (Redman *et al.*, 1999). In endophyte-inoculated plants, between 0 and 30% necrosis and plant death were observed on stems and roots of challenged plants, respectively, which was significantly lower than percentages observed in endophyte-free plants. Disease control was associated with a rapid increase in POX activity and lignification. Non-pathogenic binucleate *Rhizoctonia* isolates protected bean seedlings against damping-off, root rot and stem canker caused by *R. solani* Kühn and *Pythium* spp. by increasing activities of chitinases, POX and β-1,3-glucanases (Xue *et al.*, 1998). Similarly, AMF protected tomato plants against the root pathogen *Phytophthora parasitica* Dast (Cordier *et al.*, 1998; Pozo *et al.*, 2002).

Protection against nematodes

Endophytes affect nematodes by means of their direct interaction with the host rather than by their nematicidal effects (Sikora *et al.*, 2008). Interactions between endophytes and their hosts include toxin/metabolite production *in planta* by the endophyte (Schulz *et al.*, 1999; 2002), changes in host physiology that promote growth (Clay, 1987; Assuero *et al.*, 2000; Morse *et al.*, 2002), and induced resistance in plants upon endophyte colonization (Baldrige *et al.*, 1998; He *et al.*, 2002; Bargabus *et al.*, 2004; Athman, 2006).

Negative effects of endophytes on nematodes have been reported for crops such as tomato, banana, soy bean (*Glycine max* (L.) Merr., tall fescue and perennial rye grass. Hallman and Sikora (1994a) reported that endophytic *F. oxysporum* reduced the incidence of galling in tomato roots caused by *M. incognita* by 52-75%. In a related study, Hallman and Sikora (1996) observed that secondary metabolites of *F. oxysporum* endophytes from tomato reduced nematode hatching and juvenile mortality *in vitro* and repelled 56% of the root-knot juveniles in tomato roots inoculated with endophytic *F. oxysporum* (Dababat and Sikora, 2007). The repellent effect was attributed to toxic metabolites produced by the endophytes in roots, since root extracts from endophyte-inoculated plants were equally repelling to the nematodes. When EAHB was inoculated with endophytic *F. oxysporum*, *R. similis* population densities were reduced by 42-79% (Niere *et al.*, 1998), a process most likely attributed to induced resistance (Athman, 2006; Vu *et al.*, 2006). Other than induced resistance, endophyte-infected roots have been reported to have a repelling effect on *R. similis* (Vu, 2005). The repellent effect was attributed to toxic metabolites, as root extracts were equally repelling to the nematodes.

The endomycorrhizal fungus *Glomus mossea* (Nicole and Gerd) and endophytic *Trichoderma pseudokoningii* Rifai reduced the number of *M. incognita* in soya bean roots colonized by *M. incognita* (Oyekanmi *et al*., 2007). Endophyte-infected plants also enhanced root growth and osmotic adjustment in growing points of tall fescue, thus reducing nematode parasitism (Elmi *et al*., 2000). The fungal endophyte *Neotyphodium lolii* (Latch, Christensen and Samuels) Glenn, Bacon and Hanlin suppressed the number of female *M. naasi* Franklin and consequent root galls of perennial ryegrass (Stewart *et al*., 1993). In tall fescue, another pasture grass, *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon and Hanlin reduced *M. marylandi* Jepson and Golden and *M. graminis* Sledge and Golden populations (Kimmons *et al*., 1989; 1990; Elmi *et al*., 1999; 2000).

Protection against insects

The first incidence where an endophytic fungus was used to protect plants against insects was demonstrated by Webber (1981) when the endophyte *Phomopsis oblonga* (Desm.) Traverso was shown to protect elm trees (*Ulmus* spp.) against the beetle *Physocnemum brevilineum* (Say). Since then, several studies have reported the detrimental effects of fungal endophytes on insect pests. The antagonistic effect of endophytes against insects is best known in clavicipitaceous grasses. For example, in tall fescue and perennial rye grasses, endophytes were reported to reduce attack by herbivores by 66 and 71%, respectively (Saikkonen *et al*., 1998). Insect populations are primarily reduced due to the ability of endophytes to produce toxic alkaloids (Siegel *et al*., 1990; Clement *et al*., 1997; Wilkinson *et al*., 2000; Bultman *et al*., 2004). For instance, population of the aphid *Schizaphis graminum* Rondani was reduced by the presence of loline alkaloids in tall fescue infected with *Epichloë festucae* Leuchtman, Schardl and Siegel. Apart from having an insecticidal activity after ingestion of sap from the phloem, the alkaloids acted as feeding deterrence to the aphids (Wilkinson *et al*., 2000). Jallow *et al*. (2004) further demonstrated that the endophyte *Coenophialum strictum* L. reduced the growth rate, prolonged the development periods, suppressed the moulting and resulted in high mortality of the larvae of *Helicoperva armigera* Hübner that fed on leaves of tomato plants. Vidal (1996) also demonstrated that tomato plants infected with the endophyte *C. strictum* were less suitable for the development of whitefly (*Trialeurodes vaporariorum* West.) off-springs compared to non-infected plants.

Modes of action

Production of secondary metabolites

Endophytes produce toxic secondary metabolites to overcome host defence mechanisms when penetrating and colonizing their host plants. Schulz *et al.* (2002) termed such a relationship "balanced antagonism". Endophytic secondary metabolites (alkaloids) were first recognised in grasses because of their detrimental effect to livestock. In 1977, Bacon *et al.* (1977) established that the endophytic fungus *Epichloë typhina* (Fr.) Tul produced alkaloids in tall fescue that were toxic to domestic mammals. Perennial rye grass infected with endophytes was later observed to cause a nervous disorder called "rye grass staggers" in cattle, sheep and deer (Gallagher *et al.*, 1981, Rowan and Gaynor, 1986). It is now well known that toxicity in pasture grasses upon endophyte infection is associated with the ability of the endophyte to produce the alkaloid Lolitrem in the host (Kemp *et al.*, 2007). Endophytes also influence the interaction of their host with other organisms by producing alkaloids that are toxic to herbivores (Siegel *et al.*, 1990; Clement *et al.*, 1997; Wilkinson *et al.*, 2000; Bultman *et al.*, 2004).

Four major classes of alkaloids are produced in the grass-endophyte symbiosis. These include pyrrolizidines (lolines), ergot alkaloids (ergovaline), indole diterpenes (lolitrem A, paxilline) and pyrrolopyzine (Bush *et al.*, 1997). Lolines act as metabolic toxins and feeding deterrents to insects (Bush *et al.*, 1997). They are not widely distributed among endophyte-infected grasses, but show the highest concentration in substantially infected grasses (Clay and Schardl, 2002). Lolines affect insect pests by causing reduction in growth rates or inhibiting reproduction (Siegel *et al.*, 1990; Riedell *et al.*, 1991). Ergovaline is toxic to invertebrate- and vertebrate herbivores such as cattle and has been isolated from tall fescue infected with *N. coenophialum* (Arechavaleta *et al.*, 1992). Lolitrems occur in perennial rye grass infected with *Neotyphodium* endophytes. They have neurotoxic effect on sheep that feed on infected grass (Clay and Schardl, 2002). Pyrrolopyzine is represented by alkaloids such as peramine which is known to be toxic to insects such as the Argentine stem weevil (*Listronotus bonariensis* (Kuschel) (Rowan and Latch, 1994). The alkaloid is produced in planta by *N. coenophialum*, *N. lolii* and *E. festucae* in stems and leaves of tall fescue and rye grass.

The alkaloids also include flavonoids such as tricin and quinines such as rugulosin. Ju *et al.* (1998) reported the isolation of 7-*O*-(*â*-D-glucopyranosyl) tricin and isoorientin from the

seed, leaves and stroma of blue grass (*Poa ampla* Merr.) infected with the endophyte *N. typhnium*. These alkaloids were found to be toxic against larvae of the mosquito *Culex pipiens* L. In balsam fir, rugulosin from the endophytic fungus *Hormonema dematiodes* Lagerb. showed insecticidal activity against spruce budworm (genus *Choristoneura* Lederer) (Calhoun *et al.*, 1992). A metabolite called colletotric acid, produced by the endophytic fungus *Colletotrichum gloeosporioides* (Penz.) Sacc., and isolated from stems of *Artemisia mongolica* (Besser) Fisch., was reported to have antibacterial effects against *Bacillus subtilis* (Ehrenberg) Cohn, *Staphylococcus aureus* Rosenbach and *Sarcina lutea* (Schroeter) Lehmann and Neumann (Zou *et al.*, 2000). Isolation of rhizoctonic acid, monomethylsulochrin, ergosterol and trihydroxyergosta-7,22-diene was reported from *Cynodon dactylon* (L.) Pers. infected with a *Rhizoctonia* sp. endophyte (*Helicobacter pyroli* (Marshall) Goodwin) *in vitro* (Ma *et al.*, 2004). Albeit in very minute quantities, the metabolite rotenone was produced by a *Penicillium* sp. endophyte colonizing *Derris ellipitica* Benth. The metabolite was shown to possess anti-feeding activity against third instar larvae of *Plutella xylostella* L. in laboratory experiments.

Growth promotion

Endophytes may promote, have no effect, or influence plant growth negatively. Promotion of plant growth and vigour by fungal endophytes indirectly provides plants with improved resistance to pests and diseases. Plant growth promotion is speculated to result from increased nutrient uptake and/or through production of plant growth regulators such as abscisic acid and indole acetic acid (IAA) (De Battista *et al.*, 1990). *Trichoderma* spp., the most common saprophytic fungi in the rhizosphere, have been shown to penetrate intact root surfaces and colonize plants internally (Yedidia *et al.*, 2001). Endophyptic colonization by *T. harzianum* resulted in plant growth promotion in cucumber plants grown hydroponically. Growth promotion by endophytic *Trichoderma* spp. was attributed to mechanisms such as control of rhizosphere pathogens, enhanced plant hormone production and a direct plant-fungus interaction (Inbar *et al.*, 1994; Yedidia *et al.*, 2001). Redman *et al.* (2001) reported a two-fold height increment in tomato plants inoculated with non-pathogenic strains of *C. magma* as compared to non-inoculated plants. Similarly, Latch *et al.* (1985) reported an increased growth rate through an increase in leaf area and tillering for perennial rye grass infected with *N. lolii*. *Neotyphodium* sp. colonization also promoted growth of tall fescue (De Battista *et al.*, 1990), *Cladorrhinum foecundissimum* Saccardo and Marchal colonization promoted growth of cotton (*Gossypium hirsutum* Ichielevich-Auster) (Gasoni and De Gurfinke, 1997), *F.*

oxysporum endophytes promoted growth of EAHB (Niere, 2001), *Neotyphodium* spp. endophytes promoted growth of *Lolium multiforum* Lam and *L. perenne* (Hesse *et al.* 2004; Vila-Aiub *et al.*, 2005), and *P. indica* promoted growth in barley (Waller *et al.*, 2005).

Endophyte infection has an unpredictable effect on plant growth under conditions of drought, nutrient deficiency, limited light and salt stress. Growth advantage during water stress is believed to be the result of an adjustment of plant osmotic potential or increased concentrations of sugars (Latch, 1998). For example, Morse *et al.* (2002) demonstrated that infection by *N. coenophialum* was beneficial to the growth of Arizona fescue (*Festuca arizonica* Vasey) under low water availability. In another example, endophyte-infected tall fescue plants exhibited a higher net growth rate during water deficiency and showed greater drought tolerance than endophyte-free plants (West *et al.*, 1988; 1993; Asseuro *et al.*, 2000). Endophyte infection is also reported to affect community structure in arid areas because of growth advantages to infected grasses. In a survey by Piano *et al.* (2005) in Sardinia, Italy (arid area with annual rainfall of between 400-1100 mm), 58 out of 60 tall fescue accessions collected were infected with the endophytes *N. coenophialum* and *F. arundinacea.* The two accessions that did not have endophytes were collected from a mountainous region where rainfall is not limited. This is an example of adaptive advantage offered by endophytes to the host. *Piriformospora indica*, an endophyte in barley, was reported to benefit the plant during salt stress. Waller *et al.* (2005) observed reduced necrosis due to increased concentration of sodium chloride in endophyte-infected barley plants.

Contrary to the above reports, reduced growth of endophyte-infected hosts has been observed under conditions of drought, nutrient deficiency and limited light. Under these conditions the cost of a plant harbouring an endophyte will outweigh the benefits. For example, Ahlholm *et al.* (2002) reported growth advantages for endophyte-infected *Festuca pratensis* Huds. plants under suitable plant growth conditions, but observed reduced tillering and growth rate in poor soil and water-stressed conditions. Similarly, Cheplick *et al.* (2000) reported reduced tillering in perennial ryegrass infected with *N. lolii* under drought conditions. Yet in another study, tall fescue and perennial ryegrass seedlings harbouring endophytes showed reduced biomass in nutrient-deficient soils, compared to endophyte-free seedlings (Cheplick *et al.*, 1989). Similarly, Pinto *et al.* (2000) reported reduced photosynthetic rates at low light in banana plants inoculated with endophytic non-pathogenic *Colletotricum musae* (Berk. and M.A. Curtis) Arx as compared to non-inoculated plants. Since reduction in chlorophyll was not

significant, the reduced photosynthetic rate was believed to be due to an inhibitory effect on electron transport. They speculated that the fungus might have produced a toxic compound which uncoupled electron transport without affecting chlorophyll content. In maize, Pinto *et al*. (2000) reported reduced chlorophyll content and a large reduction in carbon assimilation in plants inoculated with non-pathogenic *Fusarium moniliforme* Sheldon that resulted in reduced photosynthetic rates.

In some cases the effect of fungal endophyte colonization on plant growth was found to be neutral (neither positive nor negative). For example, *E. festuca* infection of *Festuca rubra* L. did not affect biomass accumulation (Zabalgogeazcoa *et al*., 2006). De Souza *et al*. (2008) also reported no increment in growth of cocoa plants infected with *Trichoderma stromaticum* Samuels and Pardo-Schultheiss, compared with endophyte-free plants.

Induced resistance

Induced resistance is the activation of plant defence pathways following elicitation by biotic/abiotic elicitors (Kloepper *et al*., 1992; Sticher *et al*., 1997; Benhamou and Garand, 2001). Plants expressing induced resistance become resistant to attack by a wide range of pathogens, insects and viruses (Sticher *et al*., 1997; Van Loon *et al*., 1998). Resistance may be expressed locally at the site of elicitation or in plant parts that are distant from the point of elicitation (systemically). Induced resistance is expressed in two forms, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Vallad and Goodman, 2004).

SAR follows exposure of roots or leaves to biotic or abiotic elicitors, and often results in the activation of defence responses after an initial attack by a pathogen or wounding. It is effective against most pathogenic fungi, bacteria and viruses (Ryals *et al*., 1994; 1996; Maleck and Dietrich, 1999). SAR is associated with both local and systemic responses, such as the hypersensitive response, which limits spread of the pathogen (De Wit, 1992), cell wall lignification and deposition of callose (Benhamou and Nicole, 1999), phytoalexin synthesis (Binks *et al*., 1996; Benhamou and Nicole, 1999) and production of pathogenesis related (PR) proteins (Benhamou *et al*., 1989; Ward *et al*., 1991; Rahimi *et al*., 1996; Van Pelt-Heerschap and Smit-Bakker, 1999). PR proteins, which are considered to be markers of SAR, contain four families of chitinases (PR-3, PR-4, PR-8 and PR-11), one family of β-1,3-glucanases (PR-2), and one of proteinase inhibitors (PR-6). Chitinases and β-1,3-glucanases are often co-

expressed in pathogenesis-related responses and function as cell wall hydrolases to weaken cell walls of pathogens (Van Loon, 1997). Proteinase inhibitors are toxic to herbivorous insects by inhibiting gut protein digestion, leading to abnormal growth (Urwin *et al.*, 1997; Vain *et al.*, 1998). No enzymatic activity has been found for PR-1 and PR-5. However, there is a lot of support for the defensive role of especially PR-1 (Silvar *et al.*, 2008). In tobacco, PR-1 enhances tolerance to *Phytophthora nicotianae* Breda de Haan, and the bacterial pathogens *Pseudomonas syringae* Van Hall and *Ralstonia solanacearum* (Smith) Yabuuuchi (Sarowar *et al.*, 2005). PR-5 is structurally related to maize trypsin and proteinase inhibitors (Payne *et al.*, 1988; Richardson *et al.*, 1987). Signalling of SAR is dependent on the accumulation of salicylic acid (SA) (Malamy *et al.*, 1990; Durrant and Dong, 2004), as was demonstrated by the development of SAR following the exogenous application of SA (Van Loon, 1997). Plants with an impaired ability to produce SA reportedly showed an increased susceptibility to pathogens and loss of the ability to develop SAR (Gaffney *et al.*, 1993; Nawrath and Métraux, 1999).

ISR refers to resistance induced in plants after root colonization by plant growth-promoting rhizobacteria (PGPR) (Pieterse *et al.*, 2002). ISR is phenotypically similar to SAR, but is independent of SA. It requires jasmonic acid (JA) and ethylene (ET) responsiveness to develop (Pieterse *et al.*, 1996). The onset of ISR is reportedly not correlated with the accumulation of PR proteins (Hoffland *et al.*, 1995; Van Loon, 1997; Van Wees *et al.*, 1997; Pieterse *et al.*, 1996; 1998; 2002; Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001; Verhagen *et al.*, 2004). Genes involved in JA signaling include *lipoxygenases* (*LOX*), *allen oxidase synthase* (*AOS*) and *coronatine insensitive 1* (*COI1*) (Turner *et al.*, 2002). *LOX* is the first enzyme in JA biosynthesis (Melan *et al.*, 1993). *COI1* gene codes for an F-box protein essential for assembling SCF^{COI1} complexes during JA-signaled processes such as defence and pollen development (Li *et al.*, 2004). *AOS* is involved in wound-induced defence signaling and pollen development (Kubigsteltig *et al.*, 1999). *Constitutive tripple response 1 (CTR1)* and *ethylene response 1* (*ETR1)* genes are negative regulators of ethylene response in plants, and their down-regulation indicates activation of the ET signaling pathway (Clark *et al.*, 1999). The activation of the ET pathway was reported in cucumber for *T. asperellum* Samuels, Hieckfeldt and Nirenberg strain T203-induced resistance against *P. syringae* pv. *lachrymans* (Smith and Bryan) Young, Dye and Wilkie (Shoresh *et al.*, 2005).

After elicitor application, defence responses in the host plant may be activated and further enhanced after pathogen challenge, activated and enhanced along with new defence responses after pathogen challenge, and activated only after pathogen challenge (Walters and Boyle, 2005). The last scenario is termed "priming", and involves a temporal delay in resistance induction in the absence of pests or pathogens. It is characterized by no or a limited expression of resistance markers upon an initial elicitation, but a bigger expression of resistance upon pest or pathogen challenge (Conrath *et al*., 2002; 2006; Heil and Bostock, 2002). Priming of plant defences was first reported in carnation (*Dianthus caryophyllus* L.*)* following colonization by the rhizobacterium *P. fluorescens* WCS417r (Van Peer *et al*., 1991), where increased phytoalexin production was observed in plants challenged with *F. oxysporum* f.sp. *dianthi* (Prill. and Detacr.) Raillo. In another example, *Paenibacillus alvei* (Cheshire and Cheyne) Ash, Priest and Collins (K165) primed *Arabidopsis thaliana* L. for stronger expression of *PR-2* and *PR-5* genes upon challenge with *Verticillium dahliae* Kleb. (Tjamos *et al*., 2005). Pre-treatment of cucumber with benzothiadiazole (BTH) resulted in a faint expression of *PR-1* and *POX* genes, but challenge with *Colletotrichum orbiculare* (Berk. and Mont.) Arx further enhanced expression of these genes. The expression of *PAL1* was also observed for the first time after pathogen challenge (Cools and Ishii, 2002).

Priming is believed to have evolved in plants to circumvent energy costs associated with induced resistance in enemy-free environments. It has often been observed that well-defended plants have their growth and reproductive capacities affected, because nutrients may be diverted to meet energy costs of resistance induction. For example, induced resistance in spring wheat (*Triticum aestivum* L., cv Hanno) by BTH in the absence of a pathogen resulted in reduced biomass and number of ears and grains (Heil *et al*., 2000). Prior to Heil *et al*. (2000), Smedegaard-Pertersen and Stolen (1981) had observed that inoculation of a resistant barley variety with an avirulent powdery mildew pathogen resulted in reduced grain yield, size and protein content. It was later shown by Walters (1985) that the interaction between barley and an avirulent powdery mildew pathogen is characterized by increased host respiration, indicating costs due to resistance induction. The observed disadvantages of resistance induction are reportedly aggravated by environmental constraints such as nitrogen deficiency (Heil *et al*., 2000). Similarly, in *Arabidopsis* BTH induced low levels of chitinase and POX under low nitrogen levels, and induced plants had significantly lower protein content compared with non-induced plants receiving a similar nitrogen treatment (Dietrich *et*

al., 2004). Contrary to the above findings, Iriti and Faoro (2003) reported no negative effect of BTH-induced resistance on yield in beans in the absence of a pathogen.

Indicators of induced resistance in plants

Cell wall modifications: One of the major changes in plants expressing induced resistance is the reinforcement of mechanical properties of plant cell walls by deposition of newly formed molecules such as callose and lignin (He *et al*., 2002; Jeun *et al*., 2004; Saravanan *et al*., 2004). It is speculated that the accumulation of structural substances increases the strength of host cell walls, thus inhibiting host colonization by pathogens. Without biochemical analysis, reinforcement of cell walls following resistance induction can be inferred from the limitation of colonizing fungi to the epidermal cells (Yedidia *et al*., 1999; Schulz and Boyle, 2005; Paparu *et al*., 2006; Chatterton *et al*., 2008).

Lignification is strongly correlated to resistance in plants because it has been observed in plants after infection by both pathogenic and non-pathogenic microorganisms (Carver *et al*., 1994; He *et al*., 2002). Lignin is a non-constitutive barrier, deposited in plants only upon resistance induction (Vance *et al*., 1980). Increased lignification is believed to play a role in blocking pathogens from invading the plant through physical exclusion (Nicholson and Hammerschmidt, 1992; Hammond-Kosack and Jones, 1996). Attempted hyphal penetration into cell walls often results in wall reinforcements at such sites. Shiraishi *et al.* (1995) reported activation of PAL activity at potential sites of entry by the pathogenic fungus *Erysiphe graminis* f.sp. *hordei* (EM. Marchal) race 1 and the non-pathogen *Erisiphe pisi* DC. in roots of barley. PAL is the first enzyme in the lignin synthesis pathway.

Deposition of callose in cell walls is another mechanism of cell wall reinforcement in induced plants. Benhamou and Garand (2001) reported callose deposition in pea (***Pisum sativum*** L.) roots inoculated with non-pathogenic *F. oxysporum* (Fo47). Cell wall reinforcements limited proliferation of the fungus to epidermal cells. β-1,3-glucan is a molecule involved in callose synthesis, and its accumulation has been observed following endophyte colonization. Deposition of β-1,3-glucan in cell walls of pea and tobacco roots during mycorrhizae colonization was reported by Gianinazzi-Pearson (1995). Similarly, callose deposition was observed in cells adjacent to infected host cells in roots and leaves of cucumber plants inoculated with *T. harzianum*, strengthening epidermal and cortical cells (Yedidia *et al*., 1999).

POX is involved in cell wall strengthening by cross-linking extensin molecules and polymerizing hydroxycinnamyl alcohols to form lignin (Irving and Kuc, 1990; Dalisay and Kuc, 1995). Activation of POX has been observed where plant defence responses were induced. For example, pathogenic *C. magma* challenge of plants previously inoculated with *C. orbiculare* (non-pathogen) resulted in increased POX activity and lignification in cucumber and water melon roots (Redman *et al.*, 1999). This occurrence resulted in complete exclusion of the pathogen from induced roots. Similarly POX activity was up-regulated in *T. asperellum* T203-inoculated plants following challenge by *P. syringae* pv. *lachrymans* (Shoresh *et al.*, 2005).

Biochemical changes: Biochemical changes in plants following resistance induction include the accumulation of secondary metabolites such as phenolics. Accumulation of phenolic compounds within host cells is believed to be the first indication of resistance expression in plants (Nicholson and Hammerschmidt, 1992). These compounds are induced by wounding (Evensen *et al.*, 2000), pathogen infection (De Ascensao and Dubery, 2003) and colonization of host plants by non-pathogenic microorganism. For example, Benhamou *et al.* (1996; 1998) and Benhamou and Garand (2001) observed defence-related reactions in studies involving cytochemical analysis of pea roots inoculated with non-pathogenic *F. oxysporum* Fo47. They reported accumulation of phenolic-like compounds in host cell walls and intercellular spaces. In a related study, accumulation of hydroxycinnamic acid amide was reported in barley during the early stages of colonization by *Glomus intraradices* Schenk and Smith (Peipp *et al.*, 1997). Recently, Athman (2006) observed phenolic compounds in banana roots inoculated with non-pathogenic *F. oxysporum* isolate V5w2. The evidence for involvement of phytoalexins in plant disease resistance was put forward by Thomzik *et al.* (1997), who demonstrated resistance of tomato plants transformed with a gene encoding a phytoalexin (stilbene synthase) against *Phytophthora infestans* (Mont.) de Bary.

Defence gene expression: Production of PR proteins such as chitinases and β-1,3-glucanases has been reported following resistance induction (Baldrige *et al.*, 1998; Duijff *et al.*, 1998; Xue *et al.*, 1998; Bargabus *et al.*, 2004). Activities of enzymes such as PAL, POX, and polyphenol oxidase (PPO) are also known to be up-regulated during resistance induction. Defence gene expression has been reported for a number of host-endophyte interactions. For example, the early stage of mycorrhizal establishment in plant roots is characterized by weak

and transient expression of defence-related genes, with the expressions becoming stronger during the arbuscule stage (when haustoria are inside plant cells). *POX* and *catalase* were reported to be transiently expressed during appresoria formation in the interaction between the mycorrhizal fungus *G. mossea* and *N. tabacum* (Blilou *et al*., 2000). Defence genes reported to be strongly expressed during the arbuscule stage include *callose* and *PR-1* in pea roots (Gallote *et al*., 1993), *PAL* in *Medicago truncatula* Gaerth (Harrison and Dixon, 1993), *β-1,3-glucanase* in *Phaseolus vulgaris* L. (Blee and Anderson, 1996; Lambias and Mehdy, 1996), *chitinases* in *P. vulgaris* (Lambias and Mehdy, 1996) and senescence- and stress-related genes such as *glutathione-s-transferase* in potato (*Solanum tuberosum* L.) (Franken *et al*., 2000). Waller *et al.* (2008) observed systemic up-regulation of a PR gene *HvPr*17*b* in barley following colonization by the endophytic fungus *P. indica*, and further after challenge with the leaf pathogen *Blumeria graminis* f.sp. *hordei* L. Christensen *et al.* (2002) suggested the involvement of *HvPr*17*b* in recognition and signaling in cell walls through the ability to release elicitors from pathogen cell walls, since the protein showed no protease activity *in vitro*.

Production of cell wall lytic enzymes, such as chitinases and β-1,3-glucanases, has been reported following resistance induction upon pathogen infection, or following colonization by non-pathogenic microorganisms. In the pathogenic interaction, β-1,3-glucanases were induced in susceptible and resistant tomato varieties following infection by the vascular wilt fungus *F. oxysporum*, in potato leaves following infection of roots by the cyst nematode *Globodera pallida* (Stone) Behrens (Rahimi *et al*., 1996), and in intracellular fluids of carnation after leaf treatment with avirulent race 1 and virulent race 8 of *F. oxysporum* f.sp. *dianthi* (Van Pelt-Heerschap and Smit-Bakker, 1999).

In beans, inoculation of hypocotyls with non-pathogenic *R. solani* and challenge with pathogenic *R. solani* and *Colletotrichum lindemuthianum* (Sacc. and Magnus) Briosi and Cavara significantly increased activity of chitinases and β-1,3-glucanases, compared with pathogen-challenged only plants and plants inoculated with the non-pathogen. This increased activity was positively correlated with root rot and anthracnose resistance (Xue *et al*., 1998). Similarly, colonization of roots of chickpea (*Cicer arietinum* L.) by an incompatible race 0 of *F. oxysporum* f.sp. *ciceris* Matuo and Sato and non-host *F. oxysporum* resulted in activation of chitinases and β-1,3-glucanases (Cachinero *et al*., 2002). Similar to the transient expression of certain genes during mycorrhizal establishment, chitinase enzyme activity was transiently

up-regulated in cucumber plants inoculated with endophytic *T. harzianum* T203 (Yedidia *et al*., 2000).

Biochemistry and molecular genetics of fungal endophyte-induced resistance

Events leading to resistance induction

Rapid defence responses by a host depend on the ability of the host to recognize an invader or specific elicitor molecules (Garcia-Garrido and Ocampo, 2002). The latter may be secreted by the invader (exogenous elicitor) or may be components of the host cell wall released during penetration (endogenous elicitor) (Heath *et al*., 1996). Morphological recognition between an endophyte and its host starts when specific exudates are released by the host that initiate hyphal proliferation (Gianinazzi-Pearson *et al*., 1990). In mycorrhiza, a *gus*-derived diffusible signal molecule, called β-glucuronidase fusion, is involved in attracting fungal hyphae to the host (Parniske, 2004).

Recognition at the site of infection initiates cellular signalling processes that activate defence responses locally and systemically. Elicitor recognition is followed by several biochemical changes within the host. One of the earliest reactions is the change in plasma membrane permeability, subsequently leading to Ca^{2+} ion and proton influx (Ebel and Scheel, 1997). These ions are necessary for adequate induction of the oxidative burst, phytoalexin production and defence gene activation (Jabs *et al*., 1997; Pugin *et al*., 1997). Ca^{2+} signaling is very essential for the activation of defence responses in higher plants and Ca^{2+} ion influx is required for the activation of molecules involved in signal transduction, such as calmodulin and protein kinases (Heo *et al*., 1999).

Penetration of intact epidermal cells by fungal endophytes is a pre-requisite for the induction of resistance in host plants, as was demonstrated with the biocontrol non-pathogenic *F. oxysporum*-induced resistance in cucumber against the Fusarium wilt pathogen *F. oxysporum* f. sp. *cucumerinum* Owen (Qaher, 2006). Upon penetration of host cells, an oxidative burst (generation of reactive oxygen species (ROS)) results. It occurs in affected cells within seconds or minutes of plant cell wall contact with elicitor molecules (Bradley *et al*., 1992) and reaches maximum activity soon after induction. The oxidative burst is a common feature of all plant-fungal interactions (Grayer and Kokubun, 2001). ROS produced by plants include superoxides, hydrogen peroxide (H_2O_2) and singlet oxygen (Wojtaszek, 1997). ROS may

have direct anti-microbial activity (Peng and Kuc, 1992; Mellersh *et al.*, 2002; Vranova *et al.*, 2002; Wang *et al.*, 2008), act as messengers for activating defence responses (Chen *et al.*, 1995; Jabs *et al.*, 1997; Chamnongpol *et al.*, 1998), may be involved in cell wall modification through POX-catalysed cell wall cross linking of protein polymers (Brisson *et al.*, 1994), and may trigger the hypersensitive response (Levine *et al.*, 1994). The hypersensitive response (cell death) during the oxidative burst results from a combined activity of ROS, sulphur and nitrogen intermediates which inactivate and destroy proteins, lipids and nucleic acids (Sedlarova *et al.*, 2007). To counteract this effect, plants usually deploy enzymatic (POX and catalases) and non-enzymatic (phenolic compounds) antioxidants to avert damage to host cells (Vranová *et al.*, 2002; Herbbete *et al.*, 2003).

A third early defence response is the activation of protein kinase activity. Protein kinases are downstream elicitor recognition plasma membrane-bound enzymes (Hammond-Kosack and Jones, 1996) that are involved in signal transduction and enhancement of the transcription of target genes. Some resistance genes in plants encode receptor-like protein kinases that are involved in downstream signaling. For example, the *Pto* gene of tomato which confers resistance to bacterial speck disease encodes a serine/threonine kinase that interacts with other proteins to produce PR proteins (Zhou *et al.*, 1997). As an early event in the establishment of mycorrhizae, receptor kinase-mediated transmembrane signalling (which also work in pathogen recognition) interprets the intruder as 'friendly' (Zipfel and Felix, 2005). This can lead to the activation of Ca^{2+} and calmodulin-dependent protein kinases (Levy *et al.*, 2004; Parniske, 2004).

Genes implicated in endophyte-induced resistance in plants

Endophyte colonization of a host plant may result in direct up-regulation, a down-regulation or suppression, transient up-regulation, and priming for up-regulation of defence-related genes after pathogen attack. Direct up-regulation following endophyte inoculation is most likely to occur for defence genes involved in signal transduction. For example, Shoresh *et al.* (2005) reported the up-regulation of *LOX*, *CTR1* and *ETR1* after *T. asperellum* T203 colonized cucumber roots. Suppression of certain defence-related genes is considered to be necessary for the establishment of host-endophyte symbiosis. For instance, *PAL* activity was suppressed in *Medicago sativa* L. following root colonization by *Glomus intraradices* Schenck and Smith (Kapulnik *et al.*, 1996), and *PR-3* (*chitinase*) activity was suppressed in *N. tabaccum* following *G. intraradices* colonization (Lambias and Mehdy, 1993). Transient

expression of *POX* was observed in *N. tabaccum* following colonization by *G. mossea* (Blilou *et al.*, 2000). Transient defence gene expression seems to occur as a universal response to mycelial penetration of intact cell walls. Priming of gene activity seems to be the most common occurrence for endophyte-induced resistance. For example, Johnson *et al.* (2003) reported priming of *PR-10* activity following *N. coenophialum* infection of tall fescue. Similarly, *chitinase* and *POX* activities were primed by *T. asperellum* in cucumber (Shoresh *et al.*, 2005), and *LOX* activity was primed by *Pseudomonas putida* Trevisan in *P. vulgaris* (Ongena *et al.*, 2004).

Phenylalanine ammonia lyase (PAL): Plant colonization by both pathogenic and non-pathogenic microorganisms is known to induce PAL production (Diallinas and Kanellis, 1994). PAL is a catalyst in the first step of the phenylpropanoid pathway (involved in deamination of phenylalanine to cinamic acid), which leads to the formation of lignin. PAL is also a precursor for phenylpropanoid-derived secondary plant products such as SA and isoflavonoid phytoalexins that are involved in defence (Ward *et al.*, 1991). Increased PAL activity was observed by He *et al.* (2002) in *A. officinalis* roots inoculated with non-pathogenic strains of *F. oxysporum*, and in cucumber roots and leaves after inoculation with *T. asperellum* (Shoresh *et al.*, 2005). In *M. sativa* inoculated with *G. intraradices*, hyphal penetration during root colonization was associated with systemic induction of PAL (Volpin *et al.*, 1994). In beans, root colonization by *G. intraradices* did not affect *PAL* transcript levels, but subsequent challenge with *R. solani* resulted in significant up-regulation of *PAL* transcripts in mycorrhizal plants (Guillon *et al.*, 2002).

Peroxidase (POX): POX is involved in plant defence responses such as cross-linking of cell wall polymers leading to increased wall strength (Bradley *et al.*, 1992), suberization and wound-healing (Sherf *et al.*, 1993), lignification (Walter, 1992) and production of antimicrobial radicals (Peng and Kuc, 1992).

Lipoxygenase (LOX): LOXs are involved in JA synthesis (Octadecanoid pathway) by introducing molecular oxygen to linolenic acid's 9- and 31-hydroperoxides. They play a regulatory role in the production of defence-related compounds (Pena-Cortes *et al.*, 1991; Royo *et al.*, 1996). LOXs are reportedly associated with wound-induced activation of proteinase inhibitors (PIs) (Royo *et al.*, 1996; Heitz *et al.*, 1997). In tomato, activation of LOX preceded that of PI in the wound-signaling pathway (Heitz *et al.*, 1997), confirming

their regulatory role in the production of defence genes. A similar situation was reported by Shoresh *et al.* (2005) following inoculation of cucumber with the biocontrol fungus *T. asperellum* T203. Up-regulation of *LOX* following root colonization by *T. harzianum* preceded that of *CTR1*, which is a key enzyme in the ET response pathway.

Chitinase: Chitinases are cell wall lytic enzymes capable of degrading cell walls of pathogens and pests. Other than having a direct effect on cell walls of pathogens and pests, they also indirectly release oligosaccharide signaling molecules that activate a variety of plant defences (Ryan, 1988; Ham *et al*., 1991). *VCH3*, an isoform of chitinase, was up-regulated in grapevine (*Vitis amurensis* Rupr.) following inoculation with the mycorrhizal fungus *Glomus vesiforme* (Karsten) and Berch. The activity of the gene was further activated following challenge with the root-knot nematode, resulting in resistance against the nematode (Li *et al*., 2006). Similarly, *P. syringae* pv. *lachrymans* challenge of cucumber roots previously inoculated with *T. asperellum* T203 resulted in increased chitinase activity in roots (Shoresh *et al*., 2005). Another chitinase gene *Mchitinase III-4* was reportedly up-regulated in *M. truncatula* during mycorrhizal establishment (Salzer *et al*., 2000).

PR-1: *PR-1* is widely known as a marker gene for SAR in plants (Van Loon, 1997), particularly during pathogenic interactions. The gene is known to be expressed in resistant crop varieties following pathogen attack (Faize *et al*., 2004; Van den Berg *et al*., 2007). In the endomycorrhizal symbiosis with *N. tabaccum*, a slight increase in PR-1 activity was observed during the arbuscule stage (Gianinazzi-Pearson *et al*., 1992).

THE INTERACTION BETWEEN BANANA AND NON-PATHOGENIC *FUSARIUM OXYSPORUM* ENDOPHYTES

In EAHB, successful colonization of tissue culture plants by non-pathogenic *F. oxysporum* endophytes has been demonstrated (Schuster *et al*., 1995; Griesbach, 2000, Niere, 2001; Paparu *et al*., 2006). All studies have looked at colonization at sites of inoculation (roots and rhizomes), thus there is no knowledge of systemic colonization of bananas by endophytic *F. oxysporum*. In histological studies where the endophyte was stained *in planta*, Paparu *et al*. (2006) reported limitation of endophytic mycelia to the epidermis and outer cortex. This occurrence is speculated to result from host responses to hyphal penetration of host cells, resulting in cell wall strengthening in non-infected cells.

Fusarium oxysporum endophytes have been shown to protect banana plants against nematodes (Niere, 2001; Athman, 2006; Vu *et al*., 2006), weevils (Griesbach, 2000; Kapindu, personal communication) and *Foc* (Belgrove, 2007) in greenhouse and screenhouse experiments. Induced resistance seems to be the most likely mode of action for *F. oxysporum* endophyte control of banana pests, particularly *R. similis* and Fusarium wilt disease. In split-root experiments, where the pest and endophyte were introduced onto roots that were spatially separated, reduced root penetration and nematode multiplication was observed in roots distant from the ones that were inoculated with the endophytic isolate V5w2 (Athman, 2006; Vu *et al*., 2006), demonstrating induced resistance. Similarly, production of secondary metabolites (phenolic compounds), which can be considered a marker for induced resistance has been reported in banana following endophytic *F. oxysporum* colonization and pest challenge of endophyte-inoculated plants. For example, Athman (2006) reported increased production of phenolic compounds in roots inoculated with the endophyte and later challenged with *R. similis*. In an experiment to study the mode of action of *F. oxysporum* endophytes against *Foc*, Belgrove (2007) ruled out competition between the endophyte and the pathogen, as this was not observed. However, she observed an increase in production of total and cell wall bound phenolic compounds in endophyte-inoculated roots, implicating induced resistance.

CONCLUSIONS

Fungal endophytes have been used successfully to protect agricultural crops against a variety of diseases and pests. In banana, such protection has been demonstrated mostly in controlled environments. In one study, however, Pocasangre *et al*. (2007) reported endophytic protection of banana plants in the field. In Panamá, endophytic *Trichoderma atroviride* P. Karst. (formerly *T. harzianum*) isolate MT-20 protected tissue culture banana plants from *R. similis* better than two applications of Mocap® and Counter® nematicides (Pocasangre *et al*., 2007). While protection by endophytic *F. oxysporum* in banana was shown to involve antibiosis to weevil larvae and eggs, the primary mode of protection against *R. similis* appears to involve induced resistance. The benefit of using endophytes to protect banana plants against pests and diseases is that endophytes can be established on sterile plantlets produced in tissue culture. These plants can then be supplied to farmers for planting, without additional applications of endophytes in the field or other expensive costs related to disease and pest management.

However, a number of challenges remain to be adressed in the effort to develop endophyte-enhanced plantlets.

One of the greatest challenges in using endophyte-enhanced plants for pest and disease control is to establish and sustain endophytes or their activity in banana plants over several plant cycles. The ability of fungal endophytes to colonize and survive in banana plants, therefore, appears to be important when selecting such endophytes. The ability of different endophytic isolates to colonize and establish in banana roots and rhizomes can be assessed through *in vivo* plant colonization studies. It is also important that the resistance provided by endophytes be sustained, and that endophyte-enhanced plants not become more susceptible to new pests and diseases. The ideal situation, therefore, would be the colonization of banana plants with endophytes, or combinations of endopytes, that would protect the plant against more than one pest and/or pathogen.

The identification of endophytes *in vivo* is of great importance when strains are compared for plant colonization abilities. Currently there is no marker to identify endophytes *in vivo.* Marked isolates will also be important in studying the potential of dual/multiple endophyte inoculations. Dual inoculation of microbial control organisms is believed to offer better protection, because of the different modes of action involved (Ramamoorthy *et al.*, 2001). In preliminary studies at IITA-Uganda (Kapindu, personal communication), the mode of action of endophytic *F. oxysporum* isolate Emb2.4o against banana weevil eggs and larvae was observed to be antibiosis, while Athman (2006) demonstrated induced resistance as mode of action of isolate V5w2 against *R. similis.* In the first report of dual inoculation of *F. oxysporum* endophytes of banana, Dubois *et al.* (2004) reported increased root weight and reduced *R. similis* necrosis for certain combinations of dually inoculated isolates, though these were not consistent across replicates. As a consequence, more research is needed to investigate the effect of dual endophyte inoculation on control of banana pests. Nematodes and banana weevils often occur in the same field, yet available research has shown that fungal endophytes differ in their potential to control the two pests. Thus the possibility of inoculating into a single plant an endophyte isolate effective against the banana weevil, and another effective against *R. similis* will be a significant stride towards field application of endophyte-enhanced banana plants.

Research on the molecular basis of endophyte-induced resistance in banana is required to understand plant responses to endophyte colonization, and pathogen and pest attack of endophyte-inoculated plants. Plant responses to endophyte colonization and pathogen challenge may take one or more of the following routes: i) Defence activation in the host plant and further enhancement after pathogen challenge, ii) defence activation and further enhancement along with new defence responses after pathogen challenge, and iii) defences activated only after pathogen challenge (priming) (Walters and Boyle, 2005). The event of priming is of particular significance, as the economics of bio-enhancement of plants for resistance should be considered as a pre-requisite when implementing the technology of plant bio-enhancement. There is need to identify endophytes that prime banana plants for response to pests and pathogens, and not ones that dissipate the plants resources by continuously activating plant defence responses. Direct activation of plant defence responses by an endophyte in the absence of pests or pathogens may negatively affect developmental processes such as growth (Heil *et al.*, 2000). Since bio-enhancement of banana plants occurs at the plantlet stage, and under the controlled environments of a screenhouse/greenhouse, activation of defence responses at this stage would be wasteful, hence the need to identify endophytic isolates capable of priming plant defences.

The interaction between endophyte isolate and banana cultivar is also important. Griesbach (2000) and Niere (2001) observed differences among banana cultivars in root and rhizome colonization, and pest control by endophytic *F. oxysporum* isolates. Similarly, Athman (2006) observed differences in protease activity among endophytic isolates, with isolates Eny1.31i, III3w3, Emb2.4o and V5w4 showing higher activity compared with isolates Eny7.11o, V1w7 and V5w2. Therefore, it will be interesting to elucidate defence responses in banana cvs susceptible and tolerant to pests. Genes induced in banana following inoculation with endophytic non-pathogenic *F. oxysporum*, and challenge with *R. similis*, can be used to screen endophytes for their potential to induce resistance upon plant colonization, and banana cultivars for their ability to respond to such endophytes.

The use of tissue-cultured plants for establishment of new banana plantations by commercial and subsistence farmers has increased in recent years, because of the advantages offered by these plants. At propagation, tissue-cultured plants are free from pests and pathogens, easy and quick to multiply, and show uniform growth in the field (Vuylsteke, 1989; Pocasangre, 2006). However, tissue-cultured plants soon get infested with pests and pathogens after field

establishment. To overcome this constraint, bio-enhancement of tissue-cultured banana plants with beneficial endophytes such as fungi and bacteria is being promoted to extend protection offered by the use of clean planting material. The potential of supplying bio-enhanced bananas to farmers is huge, because i) endophytes occur inside plants and are (or their effects are) most likely to come into contact with target organisms, ii) are less exposed to environmental conditions which adversely affect their efficacy, iii) once applied there is no need for field re-application, and iv) they offer a suitable alternative to the use of chemicals which are being discouraged because of their detrimental effects on the environment.

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CHAPTER 2

Defence-related gene expression in susceptible and tolerant bananas (*Musa* spp.) following inoculation with non-pathogenic *Fusarium oxysporum* endophyte V5w2 and challenge with *Radopholus similis*

Paparu, P., Dubois, T., Coyne, D. and Viljoen, A. (2007). Defence-related gene expression in susceptible and tolerant bananas (*Musa* spp.) following inoculation with non-pathogenic *Fusarium oxysporum* endophytes and challenge with *Radopholus similis*. *Physiological and Molecular Plant Pathology* 71: 149-157.

ABSTRACT

Radopholus similis is a major pest of East African highland cooking bananas (*Musa* spp.) in Uganda. Non-pathogenic *Fusarium oxysporum* endophytes, isolated from bananas in farmers' fields, have shown potential to reduce *R. similis* numbers in tissue culture banana. The mechanism through which endophytes confer resistance to nematodes has previously been demonstrated to involve induced resistance. In this study, the expression of eight defence-related genes in banana was investigated using quantitative real-time reverse transcription PCR. Plants of susceptible (cv Nabusa, AAA-EA) and tolerant (cv Kayinja, ABB) banana cultivars were inoculated with endophytic *F. oxysporum* strain V5w2. Gene expression levels were analysed following endophyte inoculation and nematode challenge. Endophyte colonization of roots of the tolerant cv induced transient expression of *POX* and suppressed expression of *PR-3*, *lectin*, *PAE*, *PAL* and *PIR7A*. *Catalase* and *PR-1* activities were up-regulated in the tolerant cv 33 days after endophyte colonization of roots, but their expressions were further up-regulated following nematode challenge. Apart from *POX* and *lectin*, the other genes analysed were not responsive to endophyte colonization or *R. similis* challenge in the susceptible cv Nabusa. This is the first report of endophyte-induced defence-related gene expression in banana.

INTRODUCTION

Fungal endophytes occur in all plants, and often infect their hosts without causing any observable disease symptoms (Petrini, 1986; Schulz *et al*., 1999). Apart from grasses, in which they have been intensively studied, endophytes have been isolated from agricultural crops such as banana (*Musa* spp.) (Schuster *et al*., 1995; Griesbach, 2000), rice (*Oryza sativa* L.) (Tian *et al*., 2004), cocoa (*Theobroma cacoa* L.) (Arnold and Herre, 2003) and barley (*Hordeum vulgare* L.) (Schulz *et al*., 2002). While in the plant, endophytes may be mutualists, commensalists and even parasitic (Saikkonen *et al*., 1998; Faeth *et al*., 2004). In a mutualistic association between an endophyte and its host, the latter provides nutrients and protection, and aids in transmission to the next generation of hosts for vertically transmitted endophytes. The endophyte in return, is believed to offer increased resistance or tolerance to biotic (pests and diseases) and abiotic (drought and salinity) stresses. The commensalists benefit from the host, but neither harms nor benefits their hosts. Parasites are detrimental to their hosts (Redman *et al*., 2001).

The use of pest management practices in Sub-Saharan Africa is severely hampered by economical and environmental constraints. For example, the use of synthetic pesticides is discouraged due to their persistence in soils and their negative effect on the environment and human health. These pesticides are also expensive, often unavailable and poorly understood by farmers (Sikora *et al*., 1989). For many crops, breeding for host resistance is slow due to the lack of knowledge of resistance mechanisms, resistance markers and genetics of resistance (Kiggundu *et al*., 1999). In banana, durable resistance against major pests such as the burrowing nematode *Radopholus similis* (Cobb) Thorne is yet to be successfully bred into economically important cultivars. Problems associated with the use of traditional pest management practices have led to many crop protectionists turning to control strategies that are naturally occurring and hence environmentally friendly. These include the use of microbial control agents such as entomopathogenic fungi, rhizobacteria and fungal endophytes. Fungal endophytes are particularly attractive for control of pests such as the burrowing nematode because they occur inside the plant where the destructive stages of this pest exist.

Fungal endophytes are beneficial to host plants through various mechanisms, including the production of secondary metabolites, which are used in direct antagonism against pests and diseases (Siegel *et al.*, 1990; Sikora *et al.*, 1993; Hallman and Sikora, 1994; Wilkinson *et al.*, 2000; Bultaman *et al.*, 2004), changes in host physiology, which lead to increased plant growth (Asseuro *et al.*, 2000; Morse *et al.*, 2002), and induction of pest and disease resistance in plants (Duijff *et al.*, 1998; Xue *et al.*, 1998; He *et al.*, 2002; Bargabus *et al.*, 2004). Two types of induced resistance exist. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) can be differentiated on the basis of signal molecules and genes up-regulated following resistance induction, but not their phenotype. SAR is dependent on the salicylic acid (SA) pathway and its onset is characterized by expression of genes that encode pathogenesis-related (PR) proteins such as β-1,3-glucanases (PR-1 family), endo-chitinases (PR-3 family) and thaumatin-like proteins (PR-5 family) (Ward *et al.*, 1991; Uknes *et al.*, 1992; Rahimi *et al.*, 1996; Van Pelt-Heerschap and Smit-Bakker, 1999). SAR is widely known to be associated with pathogen attack or in response to the exogenous application of chemicals such as SA and benzothiadiazole. ISR, conversely, is reported due to root colonization by plant growth-promoting rhizobacteria and its onset is not characterized by accumulation of PR proteins (Hoffland *et al.*, 1992; Pieterse *et al.*, 1996; 1998; 2002; Van Loon, 1997; Van Loon *et al.*, 1998; Van Wees *et al.*, 1999; Verhagen *et al.*, 2004).

Endophyte-induced defences in plants are expressed through structural and biochemical mechanisms. Structural mechanisms include the reinforcement of plant cell walls by deposition of newly formed molecules of callose, lignin and phenolic compounds (He *et al.*, 2002; Jeun *et al.*, 2004). Other physical mechanisms of resistance include the occlusion of colonized vessels by gels, gums and tyloses (Gordon and Martyn, 1997; Olivain and Alabouvette, 1999). Physical barriers are formed by the plant to prevent further ingress of the invading organisms (Schmelzer, 2002). Major biochemical changes following resistance induction include accumulation of secondary metabolites such as phytoalexins (Kuc and Rush, 1985; Baldridge *et al.*, 1998), and production of PR proteins such as β-1,3-glucanases and chitinases (Duijff *et al.*, 1998; Xue *et al.*, 1998; Bargabus *et al.*, 2004). β-1,3-glucanase is reported to release elicitors for phytoalexin synthesis (Keen and Yoshikawa, 1983). Chitinase and peroxidase enzymes are known to be induced during endophyte colonization (Yedidia *et al.*, 1999).

There is evidence that non-pathogenic *F. oxysporum* endophytes can act against *R. similis* through induced resistance. In plant colonization studies, Paparu *et al.* (2006a) reported limited *F. oxysporum* endophyte colonization in the root epidermis, speculating that physical barriers prevented further ingress into the root cortex. In screenhouse split-root experiments where the pest and endophyte were inoculated separately onto spatially separated roots, reduced penetration and multiplication of nematodes was observed, demonstrating induced resistance (Athman, 2006). Also, an increase in production of phenolic compounds was observed in roots inoculated with endophytes and later challenged with *R. similis* (Athman, 2006).

The current study was designed to compare expression of defence-related genes in roots of a susceptible East African Highland banana (EAHB) cultivar (cv Nabusa, AAA-EA) and a tolerant banana cultivar (cv Kayinja, ABB), following inoculation with endophytic *F. oxysporum* and challenge with *R. similis*. While the biochemical and structural responses of endophyte colonization of plant roots have previously been investigated (Benhamou and Garand, 2001; Athman, 2006), little is known regarding the endophyte-induced resistance pathway and the molecules involved.

MATERIALS AND METHODS

Fungal inoculum and nematode preparation

A non-pathogenic endophytic *F. oxysporum* strain V5w2 was isolated from EAHB plants by Griesbach (2000) and is currently stored at the facilities of the International Institute of Tropical Agriculture (IITA) in Kampala, Uganda in soil, on filter paper and in 15% glycerol (Leslie and Summerell, 2006). Strain V5w2 was chosen because of its demonstrated ability to suppress *R. similis* in screenhouse experiments (Athman, 2006). From filter paper, the fungus was sub-cultured on half strength potato dextrose agar (PDA) (19 g PDA and 19 g agar l^{-1} distilled water) and a fungal spore suspension prepared 7 days after growth at ± 25^{o}C. Spore densities were subsequently determined under a light microscope (100× magnification) using a haemocytometer. Spore concentrations were adjusted to 1.5 x 10^{6} spores ml^{-1} with sterile distilled water (SDW).

Radopholus similis nematodes were cultured on carrot discs according to Speijer and De Waele (1997). The nematodes were originally isolated from banana roots and maintained at 27^{o}C on sterile carrot discs at IITA, Kampala, Uganda. A nematode suspension was prepared by rinsing nematodes from the carrot discs and from the edge of the Petri dishes using sterile distilled water. A final volume of 110 ml sterile distilled water containing 178 female and juvenile *R. similis* ml^{-1} was obtained.

Plant material

EAHB cv Nabusa (highly susceptible to *R. similis*) and cv Kayinja (tolerant to *R. similis*) were inoculated with the non-pathogenic endophytic *F. oxysporum* strain V5w2. Tissue culture banana plants were propagated using a standard shoot-tip culture protocol (Vuylsteke, 1998). Four weeks after rooting, plants were removed from the rooting medium and their roots and rhizomes rinsed in tap water. The plants were then replanted to 250 ml plastic cups, with their roots suspended in a nutrient solution [40 ml Micromix® (Magnesium 2%, Sulfur 16%, Manganese 7.5% and Zinc 18%) (Fleuron, Braamfontein, 2017, South Africa), 24 g $Ca(NO_3)2.H_20$ and 36 g Agrasol® (Fleuron) l^{-1} SDW], and their stems secured by the lid of the plastic cups (Fig.1A). To enhance root growth, the plants were maintained in a plant growth room set to a photoperiod of 18/6 h light/dark routine, an average temperature of 23^{o}C and 70% relative humidity for 4 weeks.

Inoculation of tissue culture plants

Plants were removed from the nutrient solution and their roots washed in distilled water. They were then treated as follows: 1) roots of both cultivars were harvested immediately (0 h) from endophyte-free plants to determine constitutive expression of genes, 2) both cultivars were endophyte-inoculated, and roots harvested 2 days later, 3) both cultivars were endophyte-inoculated, and roots harvested 33 days later, 4) both cultivars were endophyte-inoculated, challenged with *R. similis* at 30 days, and roots harvested 3 days later, and 5) endophyte-free plants of both cultivars were challenged with *R. similis* at 30 days and roots harvested 3 days later. Each treatment had three biological replicates, each consisting of three plants pooled together at harvest.

Plants were inoculated with the endophytic *F. oxysporum* isolate by dipping their roots in spore suspensions for 4 h. Non-inoculated plants were dipped in SDW for the same duration. Plants were then planted in pots (120 mm wide and 90 mm deep) containing sterile soil

(autoclaved at 121ºC and 1 bar for 1 h) for the duration of the experiment. Plants were challenged with ***R. similis*** by excavating the soil at the base of the plant and pipetting 2 ml inoculum (containing approximately 350 nematodes) directly on the roots. The excavated soil was then replaced. Non-challenged plants also had the soil around their roots excavated, but replaced without nematode application. The experiment was conducted in a plant growth room with a photoperiod of 12/12 h light/dark routine and an average temperature of 25^{o}C, and plants were watered twice weekly (Fig.1B). At harvest, root samples were collected for RNA extraction, endophyte re-isolation and nematode extraction.

Endophyte re-isolation and nematode extraction

Endophyte re-isolations from roots were undertaken according to Paparu *et al*. (2006b). Re-isolation of inoculated isolates was carried out in a laminar flow cabinet, where the roots and rhizomes were sterilized in 5% NaOCl for 1 min and in 75% EtOH for 1 min. They were then rinsed thrice in sterile distilled water and placed on sterile paper. From each root and rhizome, eight pieces were cut and inserted halfway in PDA supplemented with antibiotics (0.1 g penicillin G, 0.2 g streptomycin sulfate and 0.05 g chlortetracycline l^{-1}) in 90-mm diameter Petri dishes and incubated in the laboratory (25^{o}C and a photoperiod of 12/12 h light/dark routine) for 7 days. Fungi growing from plated root and rhizome pieces were viewed under a compound microscope (100× and 400× magnification) and identified as *Fusarium oxysporum* by looking at its characteristic macroconidia (sickle-shaped with attenuated apical cell), short phialides and chlamydospores (Nelson *et al*., 1983). Roots for nematode extraction were washed with tap water and cut into 0.5-cm pieces and crushed using a pestle and mortar. Nematodes were extracted from the crushed roots over 24 h using the modified Baermann funnel method (Hooper *et al.*, 2005).

RNA extraction and cDNA synthesis

Total RNA was extracted from banana roots using the RNeasy Plant Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. RNA yield was determined by measuring absorbency at 260 nm, using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Montchanin, USA). One µg RNA was DNAseI treated (Fermentas Life Sciences, Hanover, USA) and first-strand cDNA synthesised by random hexamer priming using Power Script TM Reverse Transcriptase (BD, Biosciences, Belgium) according to the method by Lacomme *et al.* (Lacomme *et al.*, 2003). The cDNAs were assayed for genomic

DNA contamination by PCR using the specific primer set actinF (5'ACCGAAGCCCCTCTTAACCC-3') and actinR (5'-GTATGGCTGACACCATCACC-3'), and PCR products separated by electrophoresis through a 2% agarose gel containing ethidium bromide.

Quantitative real-time (qRT)-PCR primers

The expression profile of eight banana defence-related genes was analysed in cDNA samples obtained from banana roots (Table 1). Three of these genes, *PR-1*, *catalase* and *pectin acetylesterase* (*PAE*) were previously reported to be up-regulated in a Cavendish selection (AAA) tolerant to *F. oxysporum* f.sp. *cubense* (*Foc*), (the causal agent of Fusarium wilt of banana) (Van den Berg *et al*., 2007). *PAL*, *POX*, *PR-3*, *lectin* and *PIR17* (a *peroxidase*) were found to be up-regulated following *Foc* challenge of cv Lady Finger (genomic group AAB) and cv Cavendish plants previously inoculated with non-pathogenic *F. oxysporum* (Forsyth, 2006). Primer sequences for these genes were identified by Van den Berg *et al*. (2007) and Forsyth (2006). An endogenous gene, *Musa* 25S rRNA (AY651067) (5'ACATTGTCAGGTGGGGAGTT3'; 5'CCTTTTGTTCCACACGAGATT3') (Van den Berg *et al*., 2007), was used as a control gene since its expression remains relatively constant. All primers were synthesized by Operon Biotechnologies (Cologne, Germany) (Table 1).

Gene expression analysis using q RT-PCR

Quantitative real-time reverse transcription-PCR was performed using a LightCycler version 1.2 instrument (Roche Diagnostics). The LightCycler FastStart DNA Master^{PLUS} SYBR Green I system (Roche Diagnostics) was used for real-time PCR, using the first strand cDNAs as template. Dilution series and standard curves were performed to examine the linearity of amplification over the dynamic range. A serial dilution (1:10, 1:100 and 1:1000) was performed and used to draw standard curves for all genes. A 10-µl reaction for PCR amplification contained 5 µl FastStart DNA Master^{PLUS} SYBR Green I master mix, 2 µl of forward and reverse primer (10 µM), 1 µl cDNA template and 2 µl PCR grade water (Roche). Control treatments contained water instead of cDNA template. All PCR reactions were performed in triplicate. The cycling conditions were as follows: pre-incubation for 10 min at 95°C, followed by 55 cycles, each consisting of 10 s denaturing at 95°C, 10 s annealing at 65°C, 10 s primer extension at 72°C, and data acquisition at 95°C. For PCR amplification of all experimental samples, 1:10 cDNA template dilutions were used.

Data analysis

Percentage colonization for each experiment was analyzed using logistic regression and alpha-levels for pairwise mean comparisons conducted using Dunn-Sidak corrected 95% confidence intervals. For all parameters in which a t-test statistic was used to compare differences between sample means, a Statterthwaite's approximation t-value was used when the variances were unequal (Sokal and Rohlf, 1995; SAS Institute, 1989).

Standard regression curves were calculated from amplification data from the serial dilutions (Ginzinger, 2002). Expression data was normalized using the amplification data for the specific target gene and the endogenous control gene, *Musa* 25S rRNA as previously described (Applied Biosystems, 2001). Lightcycler data was subjected to analysis of variance and multiple mean comparisons performed using Tukey's studentized range test. Comparisons between treatments were made using a pooled t-test in SAS (SAS Institute, 1989).

RESULTS

Re-isolation of *F. oxysporum* from roots at the different sampling times yielded varying percentages, indicating successful endophyte colonization of roots. For the cv Nabusa, percentage re-isolation of *F. oxysporum* ranged from 37.2% to 78.4%, and for the cv Kayinja it was between 13.3 % and 54.7%. For both cultivars percentage colonization was highest 2 days after endophyte inoculation (dai) and *F. oxysporum* was re-isolated from roots of non-inoculated plants at zero hour and 3 days after nematode challenge (Table 2). Nematodes successfully entered the roots for all treatments that involved *R. similis* challenge (Table 2).

High quality RNA (Fig. 2A) was isolated from all banana root tissues and amplification of first strand cDNA with Actin gene primers indicated high quality cDNA with no genomic DNA contamination (Fig. 2B).

The expression profiles of defence-related genes produced constitutively (at 0 h) by cvs Kayinja and Nabusa differed significantly, with the exception of *catalase* and *PR-1*, where no significant differences in gene expression levels were found. In the tolerant cv Kayinja, activities of *PR-3* ($t = 15.59$, $P < 0.0001$), *lectin* ($t = 15.77$, $P < 0.0001$), *PAE* ($t = 3.41$, $P = 0.0024$), *PAL* ($t = 2.30$, $P = 0.029$) and *PIR7A* ($t = 6.27$, $P < 0.0001$) were significantly higher

than in cv Nabusa. The constitutive expression of *POX*, however, was significantly higher in cv Nabusa compared with the tolerant cv Kayinja (t = 3.62, *P* = 0.0014) (Fig.3).

When inoculated with the endophyte, gene expression in roots of the tolerant cv Kayinja and susceptible cv Nabusa changed dramatically over time. *POX* activity was significantly up-regulated in the tolerant cv Kayinja 2 dai (t = 6.55, *P* < 0. 0001) and was reduced significantly 33 dai (Fig. 3A). In the susceptible cv Nabusa, the expression of *POX* was significantly reduced (t = 3.15, *P* = 0.0045) 2 dai, but increased again significantly after 33 days. The activities of *PR-3* (t = 12.47, *P* < 0.0001), *lectin* (t = 15.79, *P* < 0.0001), *PAE* (t = 3.47, *P* = 0.0021), *PAL* (t = 2.70, *P* = 0.012) and *PIR7A* (t = 5.35, *P* < 0.0001) were all significantly reduced in the tolerant cv Kayinja 2 dai (Fig. 3B, C, D, E and F, respectively), but did not change significantly in the susceptible cv Nabusa. No significant induction of *catalase* (Fig. 3G) and *PR-1* (Fig. 3H) expression occurred in either of the cultivars 2 dai. After 33 days, *lectin* was significantly down-regulated in both cultivars (t = 6.38, *P* < 0.0001 and t = 3.13, *P* = 0.0045 for cvs Nabusa and Kayinja, respectively) and *PAL* activity significantly up-regulated in the tolerant cv Kayinja at the same time (t = 3.88, *P* = 0.0007). *PAL* activity did not change for the susceptible cv Nabusa. Similarly, activities of *PR-3*, *PAE*, *PIR7A*, *catalase* and *PR-1* did not change significantly for any of the cultivars.

Apart from *POX*, all the genes tested in this study showed no significant differences in expression levels between the tolerant and susceptible banana cultivars 2 dai. *POX* was highly expressed in the tolerant cv Kayinja compared with its expression in the susceptible cv Nabusa (t = 5.83, *P* < 0.0001). After 33 days, the activities of *PAL* (t = 5.02, *P* < 0.0001), *PIR7A* (t = 3.37, *P* = 0.0043), *PR-1* (t = 3.36, *P* = 0.0026) and *PAE* (t = 2.19, *P* = 0.039) were significantly higher in the tolerant cv Kayinja, while that of *POX* was significantly higher in the susceptible cv Nabusa (t = 3.52, *P* = 0.0018). The expression levels of *lectin*, *PR-3* and *catalase* were not different between the two cultivars 33 dai.

The effect of endophyte inoculation or nematode challenge on gene expression differed for both cultivars. In the susceptible cv Nabusa, endophyte inoculation resulted in higher expression of *lectin*, compared with nematode challenge (t = 5.51, *P* < 0.0001) (Fig. 3C). For *POX* the reverse was true. A higher expression was observed following nematode challenge than endophyte inoculation (t = 2.98, *P* = 0.0067) (Fig. 3A). Endophyte inoculation and nematode challenge had similar effects on the expression of the other genes in the susceptible

cultivar. For the tolerant cv Kayinja, the expression of *POX* was higher 2 dai compared with its expression after nematode challenge (t = 6.68, *P* < 0.0001). However, nematode challenge in the same cultivar resulted in higher expression of *PAL*, compared with endophyte inoculation (t = 2.60, *P* = 0.016) (Fig.1E). Endophyte inoculation and nematode challenge had similar effects on the expression of other genes in the tolerant cultivar.

The tolerant cv Kayinja inoculated with endophytic non-pathogenic *F. oxysporum* and challenged with *R. similis* resulted in the up-regulation of some genes. Although insignificant, the activities of *PR-3* (Fig.3B), *PAE* (Fig. 3D), *catalase* (Fig. 3G) and *PR-1* (Fig. 3H) were increased by 53, 26, 17 and 14%, respectively, when compared with endophyte-inoculated and nematode non-challenged plants. In contrast, the expression level of *POX* was reduced significantly by 55% (t = 2.23, *P* = 0.035) in the susceptible cv Nabusa. Gene expression between the tolerant and susceptible cultivars was also different after nematode challenge of endophyte-inoculated plants. The expression of *lectin* (t = 2.36, *P* = 0.027), *PAE* (t = 3.35, *P* = 0.0028), *PAL* (t = 4.80, *P* < 0.0001), *PIR7A* (t = 2.43, *P* = 0.024), *catalase* (t = 2.80, *P* = 0.010) and *PR-1* (t = 4.07, *P* = 0.0004) was significantly higher in the tolerant cv Kayinja, compared with the susceptible cv. Nabusa, while expression levels of *PR-3* increased insignificantly. Expression of *POX* was non-significantly lower in cv Kayinja than in the susceptible cv Nabusa. When endophyte-inoculated cv Kayinja plants were compared to non endophyte-inoculated plants challenged with *R. similis* after 30 days, the activities of *catalase* (t = 2.81, *P* = 0.010), *PR-1* (t = 2.27, *P* = 0.032), *PAE* and *PAL* were 172, 42, 30 and 26% higher, respectively.

DISCUSSION

The constitutive expression of *PR-3*, *lectin*, *PAL*, *PAE* and *PIR7A* was higher in the tolerant cv Kayinja, compared with the susceptible cv Nabusa. Interestingly, *POX* expression constitutively was higher in the susceptible cultivar. Unlike the above genes, there was no significant difference in the constitutive expression of *catalase* and *PR-1* between the tolerant and susceptible cultivars. The differences in constitutive gene expression might explain why cv Kayinja is more tolerant to *R. similis* than cv Nabusa, as *PAL* and *PAE* both contribute directly or indirectly to cell wall strengthening (Yalpani and Raskin, 1993; Savary *et al*.,

2003) and the PR-proteins are known to be associated with plant defence against biotic stresses such as pathogen attack (Faize *et al*., 2004).

Constitutive expression of *PR-3*, *lectin*, *PAE*, *PAL* and *PIR7A* were significantly down-regulated in the tolerant cv Kayinja, and that of *POX* in the susceptible cv Nabusa, 2 days after root infection by the endophytic non-pathogenic *F. oxysporum* strain. The reasons for these down-regulations are unclear, but one can presume that the down-regulated genes are not required for the mutualistic relationship between the endophyte and banana, or their strong down-regulation is necessary for the establishment and development of an endophyte-banana symbiosis. *PAL* and *POX* are enzymes involved in the phenylpropanoid pathway that leads to the synthesis of defence-related phenolics such as lignin and phytoalexins. An increase in their activity is associated with wounding and abiotic and biotic stresses (Yalpani and Raskin, 1993). *Lectins* are unspecific defence proteins produced to act against herbivorous higher animals and phytophagous invertebrates such as plant nematodes with the ability to bind to foreign glycans (Peumans and Van Damme, 1995). Suppression of defence genes has previously been reported in similar symbiotic plant-fungi interactions such as mycorrhiza. For example, Kapulnik *et al.* (1996) reported suppression of phenylpropanoid pathway enzymes during establishment of the mycorrhizal fungus *Glomus intraradices* (Schenck and Smith) in alfalfa (*Medicago sativa* L.) and tobacco (*Nicotiana tabacum* L.) roots. In a related study, inoculation of common beans (*Phaseolus vulgaris* L.) with *G. intraradices* reduced *PR-3* activity in roots of mycorrhizal plants compared with non-mycorrhizal plants (Lambias and Mehdy, 1993).

Transient expression of *POX* was observed following colonization of the tolerant cv Kayinja by non-pathogenic *F. oxysporum* in our study, but the increased level had decreased significantly by 33 dai. Transient expression of *POX* and *catalase* activity was previously reported for the interaction between the mycorrhizal fungus *G. mossea* (Nicol. and Gerd.) and *N. tabacum* (Blilou, 2000). In tobacco, expression of these two genes was up-regulated during appresoria formation, but the increased expressions were later reduced to levels similar to that in non-inoculated plants, similar to our findings. This indicates that the initial plant reaction towards colonization by fungal endophytes is a defence response, as POX is known as a key enzyme in the early oxidative response of plants to pathogens (Blilou, 2000). Whether this response is sustained or not depends on the subsequent activity of the fungus within the plant.

It should, however, be noted that a reduced expression of the *POX* gene may occur while the activity of peroxidase remains high in the plant.

The significant up-regulation of *PAL* and non-significant up-regulation of *catalase, PR1* and *PAE* in the nematode-tolerant banana cv Kayinja 33 dai indicates that these genes might be involved in the protection of banana plants against pathogen attack. An increased *catalase* activity after endophyte infection points to its involvement in signal transduction during plant-defence (Chen *et al*., 1993). *Catalase* is a tetrameric iron porphyrin necessary for plants to control fluctuating levels of reactive oxygen species under stressful conditions (Vandenabeele *et al*., 2004). As a consequence of the role it plays, *catalase* is a well-known signal molecule leading to SAR in plants (Bagnoli *et al*., 2004). Lignin is formed in the phenylpropanoid pathway, and the first step in this pathway is the deamination of phenylalanine to cinamic acid and is catalysed by the enzyme PAL. Other than lignin, PAL is a precursor for phenylpropanoid-derived secondary plant products such as SA and isoflavonoid phytoalexins that are involved in defence (Ward et al., 1991). Pectin cell walls have high contents of C_2 and C_3 acetyl esters, which impart physical, chemical and functional properties. PAE hydrolyzes acetyl esters in the homogalacturonan regions of pectin, thereby modifying cell walls especially during root development and pathogen attack (Savary *et al*., 2003). Increased *PAE* activity has been reported in *Arabidopsis thaliana* (L.), following infection by root-knot and cyst nematodes (Vercauteren *et al*., 2002). Increased expression of *catalase* and *PAE* was reported in response to *Fusarium* infection for the incompatible reaction between a Fusarium wilt-tolerant Cavendish banana selection (GCTCV-218) and *Foc* (Van den Berg *et al*., 2007). Similarly, increased *catalase* activity was reported for the interaction between chickpeas (*Cicer arietinum* L.) and *F. oxysporum* f.sp. *ciceris* (García-Limones *et al*., 2002). The early expression of *PR-1* in tolerant crop cultivars following pathogen attack is well documented. For example, expression of *PR-1* was induced in pear (*Pyrus pyrifolia* (Nakai) against infection by Japanese pear scab (*Venturia nashicola* Tanaka and Yamamoto) (Faize *et al*., 2004), and in the tolerant banana cv GCTCV-218 upon *Foc* challenge (Van den Berg *et al*., 2007). *PR-1* proteins, as with other PR proteins, are well known markers of SAR. Although their specific function is not clear, *PR-1* has been associated with antifungal properties such as the hydrolysis of fungal cell walls (Van Loon, 1997).

For three genes, *R. similis* challenge of endophyte-inoculated plants resulted in a further up-regulation of expression levels observed 33 dai. A non-significant up-regulation was observed

for *PAE, catalase* and *PR-1* following *R. similis* challenge of endophyte-inoculated plants of the nematode-tolerant cv Kayinja, compared with endophyte-inoculated only plants of the same cv at 33 dai. For *catalase* and *PR-1*, this expression was significantly higher than that for non-inoculated plants challenged with *R. similis.* This means that the endophyte-induced state in the plant enabled it to respond more effectively to *R. similis* challenge. This phenomenon has been reported for other inducers of plant defence such as SA and the biocontrol fungus *Trichoderma asperellum* strain T203. Treatment of parsley (*Petroselinum crispum* L.) cells with SA elicited COA ligase (4CL) a major gene encoding key enzymes in the phenylpropanoid pathway. A further up-regulation in gene activity was observed following infection with the pathogen *Phytophthora megasperma* f. sp. *glycinea* (Thulke and Conrath, 1998). Similarly, β-1,3-glucanase activity was up-regulated in cucumber (*Cucumis sativus* L.) 48 h after inoculation with *T. asperellum*. Challenge of *T. asperellum* inoculated plants with *Pseudomonas syringae* pv. *lachrymans* resulted in a significant up-regulation of β-1,3-glucanase activity compared with *T. asperellum* inoculated only and non-inoculated-pathogen infested plants (Shoresh *et al.*, 2005). The observation made for *PAE,* where gene activity was down-regulated following colonization by non-pathogenic *F. oxysporum* and was up-regulated after pest challenge has been reported. The activity of *PR-10* in tall fescue (*Lolium arundinaceum* Schreb.) was down-regulated following infection with the fungal endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams), but challenge with the grey leaf spot fungus *Pyricularia grisea* (Cooke) Sacc. resulted in its up-regulation (Johnson *et al.*, 2003).

The up-regulation of defence-related genes in plants inoculated with non-pathogenic *F. oxysporum* and challenged with *R. similis* was observed only in the tolerant banana cv. Kayinja, and not in cv Nabusa. *Fusarium oxysporum* isolate V5w2, however, has been reported to suppress *R. similis* in the susceptible banana cv Nabusa in screenhouse pot trials (Athman, 2006). It is therefore possible that other genes are induced by isolate V5w2 in the susceptible cultivar, which still requires identification. Another possibility is that up-regulation of the genes screened in the current study occurs much later than 3 days post nematode challenge, as was reported in okra (*Abelmoschus esculentus* L. Moench.) where *PAL* activity increased only 15 days after nematode challenge following spraying with salicylic acid (Nandi *et al.*, 2003).

Our study provides the first report of endophyte-induced defence-related gene expression in banana. Though there is indirect evidence that non-pathogenic *F. oxysporum* endophytes can act against *R. similis* through induced resistance, little is known about the resistance pathway and molecules involved. We report endophyte potentiation of the activities of two well known defence-related genes (*catalase* and *PR-1*), for greater expression upon *R. similis* challenge in the tolerant cv Kayinja. Of much interest also was the significant early down-regulation of certain defence-related genes (*POX, PR-3, PAE, PAL* and *lectin*) upon inoculation with a non-pathogenic *F. oxysporum* endophyte.

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Table 1. Primer sequences of defence-related genes studied in roots of banana cultivars susceptible (Nabusa, AAA-EA) and tolerant (Kayinja, ABB) to *Radopholus similis* by reverse transcription (RT)-PCR following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with *Radopholus similis*

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product size (bp)
POX[1]	CGGTAGGATCCAAAGAAAGC	TTCAGAGCATCGGATCAAGG	150
PR-3[1]	GTCACCACCAACATCATCAA	CCAGCAAGTCGCAGTACCTC	150
PAL[1]	CCATCGGCAAACTCATGTTC	GTCCAAGCTCGGGTTTCTTC	150
Lectin[1]	CCACGAGGTTTGCATCACTAC	CCCTTCATTCCCACCAGATAC	150
PIR7A[1]	ACCTTCGATCTCCTCCACTTC	GGTCGGTGAGAAGGGTGTT	150
PR-1[2]	TCCGGCCTTATTTCACATTC	GCCATCTTCATCATCTGCAA	126
Catalase[2]	AAGCATCTTGTCGTCGGAGTA	CGCAACATCGACAACTTCTTC	96
PAE[2]	GGCTCTCCTTTCTGGATGTTC	TCAGCAAGGCACTTGACTTTT	105
Musa 25S rRNA [2]	GTAAACGGCGGGAGTCACTA	TCCCTTTGGTCTGTGGTTTC	106

[1]Primer sequences previously identified by Forsyth (2006).

[2]Primer sequences previously identified by Van den Berg *et al*. (2007).

POX = peroxidase, *PAL* = phenylalanine ammonia lyase, *PR* = pathogenesis-related and *PAE* = pectin acetylesterase.

Table 2. Percentage colonization of roots of tissue culture plants of cv Nabusa (AAA-EA) and cv Kayinja (ABB), and total *Radopholus similis* number in a gram of root 3 days post nematode challenge (dpnc)

Cultivar	Isolate	Treatment[1]	% root colonization by *F. oxysporum*	Total *R. similis* in a gram of root 3 dpnc[2]
Nabusa	None	Zero hour	5.0 f	n/a
Nabusa	V5w2	2 DAI	78.4 a	n/a
Nabusa	V5w2	Non-challenged	37.2 b	n/a
Nabusa	V5w2	*R. similis* challenged	37.7 b	5.9 ± 0.1 c
Nabusa	None	*R. similis* challenged	7.2 ef	5.3 ± 1.0 c
Kayinja	None	Zero hour	14.4 de	n/a
Kayinja	V5w2	2 DAI	54.7 ab	n/a
Kayinja	V5w2	Non-challenged	13.3 de	n/a
Kayinja	V5w2	*R. similis* challenged	28.1 bd	14.6 ± 3.0 b
Kayinja	None	*R. similis* challenged	17.1 de	87.4 ± 17..2 a

[1]dai = Days after inoculation.

[2]n/a = Not applicable

Means in a column followed by the same letter (superscript) are not statistically different ($P \leq 0.05$, Tukey's studentized range test).

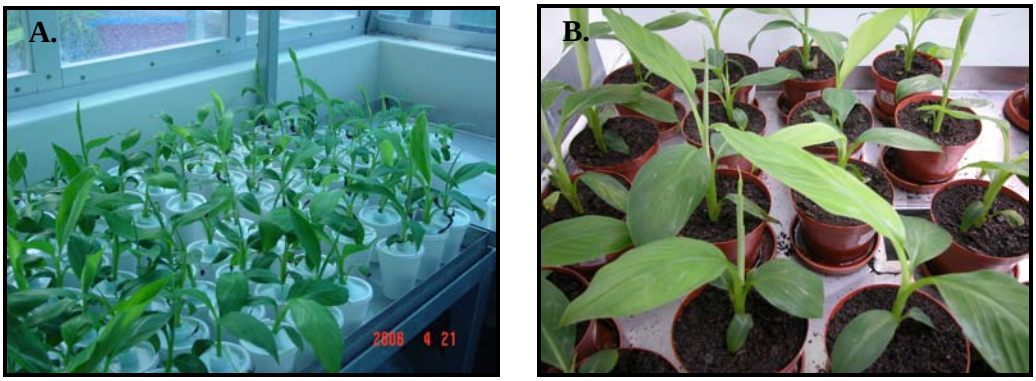


Figure 1. Banana plants of cv Nabusa (AAA-EA) in hydroponic cup system (A) and plants of cv Kayinja (ABB) in soil (B).

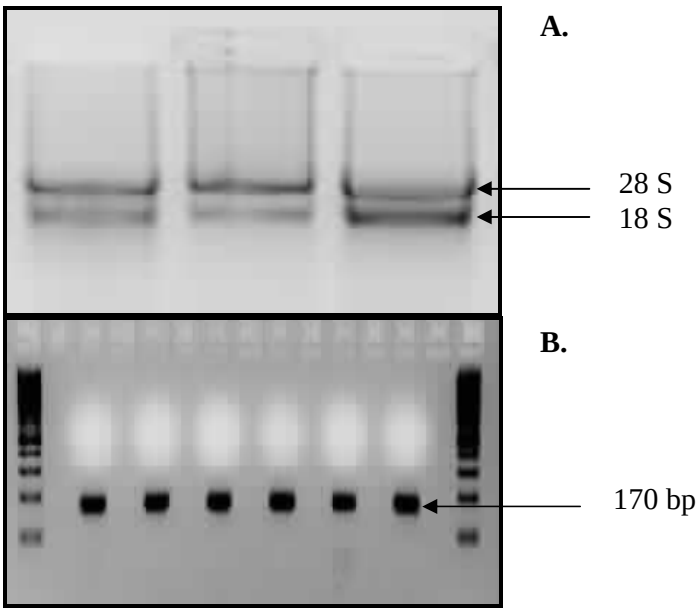
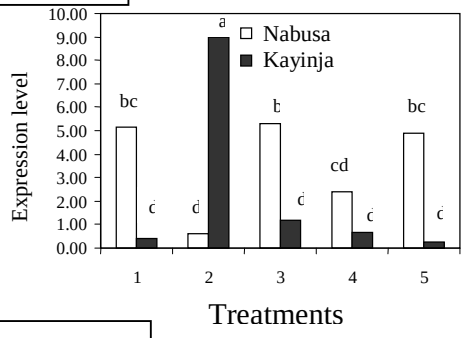
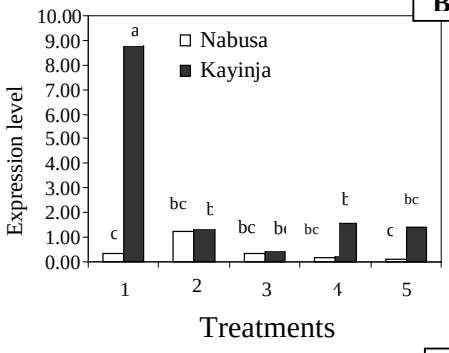


Figure 2. Total RNA from banana roots assayed by electrophoresis on 2% (w/v) agarose gel (A). Actin-based control for monitoring contamination of cDNA with genomic DNA (B), 100-bp molecular marker (Roche Diagnostics) (Lane 1) and PCR products from first strand cDNA synthesis assayed by electrophoresis on 2% (w/v) agarose gel.

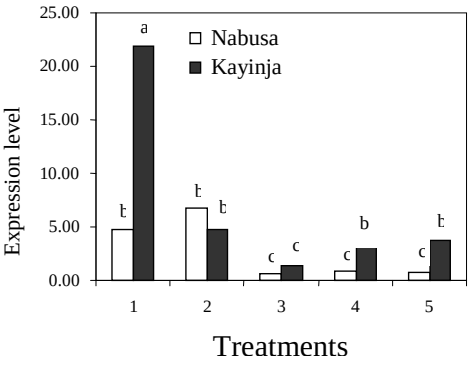
A - *POX*



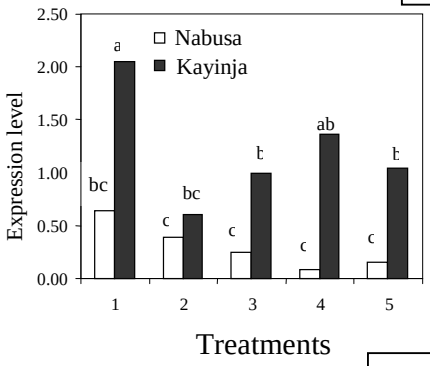
B – *PR-3*



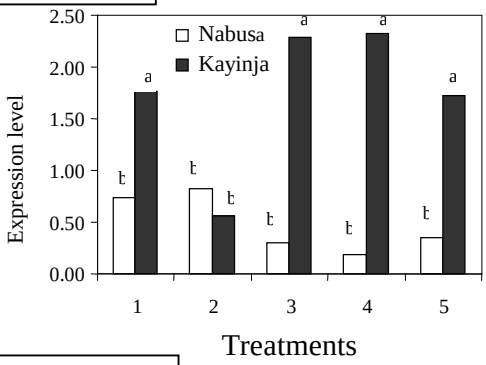
C - *Lectin*



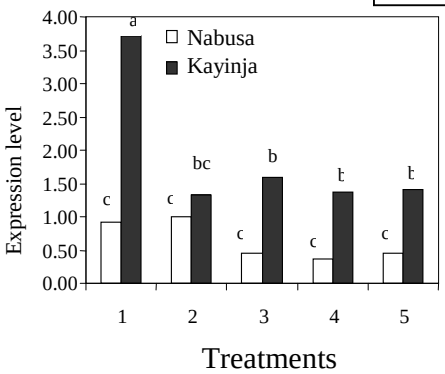
D - *PAE*



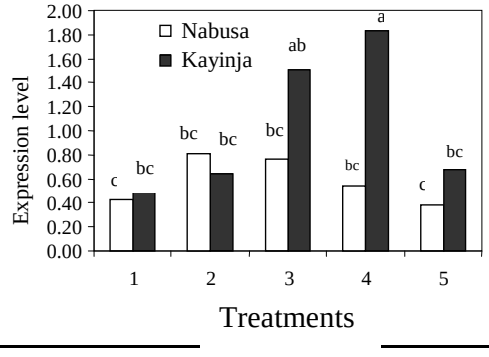
E - *PAL*



F – *PIR7A*



G - *Catalase*



H – *PR-1*

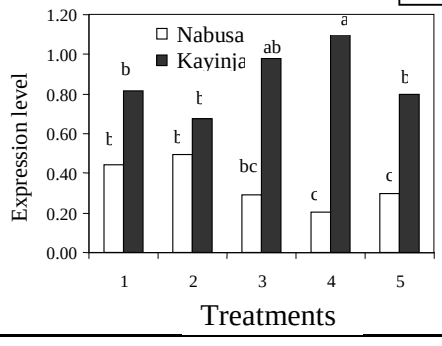


Figure 3. Expression of defence-related genes in roots of banana cultivars susceptible (Nabusa, AAA-EA) and tolerant (Kayinja, ABB) to *Radopholus similis*. Treatment 1 = non-inoculated plants (0 h), 2 = plants inoculated with non-pathogenic *Fusarium oxysporum* isolate V5w2 at 2 days after inoculation (dai), 3 = plants inoculated with isolate V5w2 at 33 dai, 4 = plants inoculated with isolate V5w2 and challenged with *R. similis* 30 dai and harvested 3 days after nematode challenge, and 5 = endophyte-free plants challenged with *R. similis* on day 30 and harvested 3 days later. (A) *peroxidase (POX)*, (B) *endochitinase (PR-3)*, (C) *lectin*, (D) *pectin acetylesterase*, (E) *PAL*, (F) *PIR7A* (*peroxidase*), (G) *catalase* and (H) *PR-1* genes. Bars carrying different letters are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

CHAPTER 3

Differential gene expression in nematode-susceptible and -tolerant East African Highland bananas (*Musa* spp.) following inoculation with non-pathogenic *Fusarium oxysporum* endophytes

ABSTRACT

The plant-parasitic nematode *Radopholus similis* and the banana weevil *Cosmopolites sordidus* are major pests of banana (*Musa* spp.) in East Africa. Endophytic non-pathogenic *Fusarium oxysporum* isolates have been shown to significantly reduce banana weevil damage and *R. similis* populations, respectively, in screenhouse pot trials. Studies on the mode of action of two endophytes, particularly against the nematode, have implicated induced resistance. cDNA-AFLPs were used to identify genes induced by Emb2.4o and V5w2 in banana plants susceptible (cv Nabusa, AAA-EA) and tolerant (cv Kayinja, ABB) to *R. similis.* Plants of both cultivars were inoculated with Emb2.4o and V5w2, and roots collected for RNA extraction 2, 7 and 30 days after endophyte inoculation. Nematode challenge of plants was carried out 30 days after endophyte inoculation and roots harvested for RNA extraction 3 days later. Fifty-five up-regulated genes were assigned putative identities, and included those involved in signal transduction, cell wall strengthening, the jasmonic acid (JA) pathway and transport of defence molecules. The expression profiles of TDFs with similarity to *ABC transporter*, *coronatine insensitive 1* (*COI1*), *lipoxygenase* (*LOX)* and *β 1,3-glucan synthase* genes were confirmed using quantitative real-time PCR. Challenge of endophyte-inoculated plants with *R. similis* resulted in further up-regulation of the activities of *β-1,3-glucan synthase* and *COI1* in the susceptible cv Nabusa, and that of *COI1* and *LOX* in the tolerant cv Kayinja. Our results confirmed induced resistance as a mode of action for *F. oxysporum* endophyte control of *R. similis* in banana. This investigation represents the first report of the isolation and identification of genes involved in the interaction between endophytic *F. oxysporum* and banana.

INTRODUCTION

Bananas (*Musa* spp.) are considered the most important staple food crop in the East African Highlands of Uganda (INIBAP, 1986), where the predominant types grown are the East African Highland cooking bananas (AAA-EA). For small-holder farmers in this region, banana contributes to food security and provides a source of income to farmers throughout the year (NARO, 2001). Agronomically, banana provides good ground cover, resulting in reduced soil erosion on steep slopes, and is a principal source of mulch for maintaining and improving soil fertility (INIBAP, 1986). However, banana production in the East African Highland region has declined in recent years due to biotic and abiotic constraints (Gold *et al.*, 1993). The major biotic constraints include the banana weevil (*Cosmopolites sordidus* Germa), a complex of banana parasitic nematodes, leaf diseases (caused by several fungal pathogens), fusarium wilt (caused by *Fusarium oxysporum* f. sp. *cubense* (E.F. Sm) Snyder and Hans), the banana streak virus (Tushemereirwe *et al.,* 1996) and, more recently, banana bacterial wilt caused by *Xanthomonas vasicola* pv. *musacearum* Yirgou and Bradbury (Tushemereirwe *et al.*, 2003). The most widespread abiotic constraints to banana production in the region include declining soil fertility due to intensive land use (Okech *et al.,* 1996), reduction of farm inputs such as mulches, land shortage, inadequate labour and poor marketing strategies (ICIPE, 1992).

The economically most important pests of banana in Uganda are the banana weevil and *Radopholus similis* (Cobb) Thorne (Gold *et al*., 1993; 1994). An integrated pest management approach that includes habitat management, biological control, host plant resistance, use of clean planting materials and chemical control is currently used to control the two pests (Speijer *et al.,* 1994; Gold *et al.*, 2001). However, some disadvantages of using these control methods include infestation of suckers in the field where clean planting materials are used (Gold *et al.*, 2001) and high cost of chemical pesticides (Sikora *et al*., 1989). Biological control strategies for the banana weevil and plant parasitic nematodes include the use of entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin (Nankinga, 1995) and non-pathogenic *Fusarium oxysporum* Schlect.: Fries endophytes of banana (Schuster *et al*., 1995; Griesbach, 2000; Niere, 2001). Of these, non-pathogenic *F. oxysporum* can be established within plants, while *B. bassiana* has to be applied as a soil treatment in weevil-infested plantations. Use of fungal endophytes to control the banana weevil and banana parasitic nematodes is an attractive option because the endophytes occur within the plant where the

destructive stages of the two pests are found. Similarly, the use of microbial antagonists that occur within the plant might offer a better control option than others because they are less exposed to environmental influences (Sikora, 1997).

Fungal endophytes are organisms which, at some stage in their life cycle, colonize living plant tissues without causing any visible symptoms (Petrini, 1991). They have been isolated from almost every plant species studied (Schulz *et al*., 1998), including banana (Schuster *et al*., 1995; Griesbach, 2000). Interactions between an endophyte and its host may be neutral, parasitic or mutually beneficial. Mutualistic plant-endophyte interactions involve defending host plants against pests and diseases, increasing plant resistance to abiotic stresses, and producing growth-promoting substances such as auxins and gibberellins in plants (Azevedo, 1998; Saikkonen *et al.*, 1998; Schardl *et al.*, 2004). In return the plant provides the fungus with nutrition and a suitable environment to survive (Petrini, 1986).

The potential of using non-pathogenic *F. oxysporum* endophytes as antagonists against *R. similis* and the banana weevil (*C. sordidus*) is considerable. These endophytes have been shown to kill *R. similis* juveniles and banana weevil eggs *in vitro* (Schuster *et al*., 1995; Griesbach, 2000; Niere, 2001). They have also been successfully introduced into tissue culture plants (Schuster *et al*., 1995; Griesbach, 2000; Niere, 2001; Paparu *et al*., 2004), and are reported to reduce pest populations in *in vivo* pot trials (Griesbach, 2000; Niere, 2001). Recently, Athman (2006) demonstrated induced resistance as the most likely mode of action of *F. oxysporum* endophytes against *R. similis*. She demonstrated an increased phenolic deposition in endophyte-inoculated and *R. similis*-challenged roots of banana.

Fungal endophytes trigger defence reactions within intact cell walls during penetration that lead to their reinforcement (Yedidia *et al*., 1999; Benhamou and Garand, 2001). This assumption is deduced from the limited ingress of endophytic fungi into the cortex and vascular bundle of plants (Olivain and Alabouvette, 1999; Bao and Lazarovits, 2001; Paparu *et al*., 2006a). Plant defence responses induced at the point of infection can also spread systemically throughout the plant and protect parts that have not been inoculated (Duijff *et al*., 1998; Athman, 2006). Biochemical changes that are associated with induced resistance include the accumulation of secondary metabolites such as phytoalexins (Kuc and Rush, 1985; Baldrige *et al*., 1998; Athman, 2006), production of PR proteins such as chitinases and β-1,3-glucanases (Baldrige *et al*., 1998; Duijff *et al*., 1998; Xue *et al*., 1998; Benhamou and

Garand, 2001; Bargabus *et al.*, 2004) and an increased activity of enzymes involved in the phenylpropanoid pathway (lignin synthesis).

The molecular genetics of induced resistance in plants can be studied by using molecular techniques such as complementary (c)DNA-amplified fragment length polymorphisms (AFLPs) (Bachem *et al.*, 1996), suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999), representational difference analysis (RDA) (Lisitsyn and Wigler, 1993), gene macro- and microarray analyses, and oligonucleotide chips (Lockhart and Winzeler, 2000). Of these, cDNA-AFLP analysis allows high-throughput transcript profiling in gene expression systems without the need for prior knowledge of gene sequences. The technique generates transcript profiles from RNA samples by assaying the abundance of transcript-derived cDNA fragments (TDFs) using polyacrylamide gel electrophoresis. TDFs with interesting patterns are then identified by fragment isolation, sequencing and homology searches. cDNA-AFLPs have an advantage over techniques such as microarray transcript profiling since it represents an open gene discovery system with an essentially unlimited number of primer-enzyme combinations to be assayed (Breyne *et al.*, 2003). In contrast, microarray profiling is a closed system where the transcripts not represented on the array are not quantified. The partial isolation and identification of cDNA-AFLP fragments allows for the characterization of novel genes underlying plant responses to biotic and abiotic signals.

The phenotypic and biochemical response of banana plants to fungal endophyte infection has been shown to vary among banana cultivars and endophyte isolates (Athman, 2006). Little, however, is known about the genetic responses of EAHB following infection with endophytic *F. oxysporum.* In the current study, cDNA-AFLP analysis was used to investigate differential gene expression in EAHB cultivars highly susceptible (Nabusa, AAA-EA) and tolerant (Kayinja, ABB) to *R. similis* following inoculation with endophytic *F. oxysporum*.

MATERIALS AND METHODS

Fungal inoculum and nematode preparation

Two non-pathogenic *F. oxysporum* endophytes Emb2.4o and V5w2, isolated from EAHB plants by Schuster *et al.* (1995) and Griesbach (2000), respectively, were used in this study. Isolate V5w2 was chosen because of its demonstrated ability to suppress *R. similis* in screenhouse experiments (Athman, 2006) and Emb2.4o for its ability to reduce weevil

damage in screenhouse pot trials (Kapindu, personal communication). Both isolates are maintained in soil, on filter paper and in 15% glycerol (Leslie and Summerel, 2006) at the facilities of the International Institute of Tropical Agriculture (IITA) in Kampala, Uganda. Spore suspensions of both isolates were prepared after culturing on half strength potato dextrose agar (PDA) (19 g PDA and 19 g agar l^{-1} distilled water) at ± 25°C for 7 days. The Petri dishes were then flooded with sterile distilled water (SDW) and the colony surface scrapped to obtain a spore suspension. The suspension was sieved through a sterile cheese cloth and the spore concentrations adjusted to 1.5×10^6 spores ml^{-1} with SDW using a haemocytometer.

Radopholus similis population was initiated using nematodes obtained from infested roots at IITA banana fields in Namulonge, Uganda, and cultured according to the method described by Speijer and De Waele (1997). For nematode isolation, infested banana roots were first macerated in a blender (Waring, Connecticut, USA) at low speed for 15 s and extracted overnight using the modified Baermann method (Hooper *et al*., 2005). Female *R. similis* were then hand-picked from the nematode suspension and surface sterilized with a 600-ppm streptomycin sulphate solution. Sterile females were inoculated on surface-disinfected (dipped in absolute ethanol and flamed) 0.5-cm-diameter carrot (*Daucus carota* L.) discs in 30-mm-diameter Petri dishes, sealed with parafilm, and incubated at 27°C for 3-4 weeks, after which the nematodes were harvested for experimentation. A nematode suspension was prepared by rinsing nematodes from the carrot discs and from the edge of the Petri dishes into 110 ml SDW to give a total of 178 female and juvenile *R. similis* ml^{-1}.

Production and inoculation of banana plants

Tissue culture banana plants of the EAHB cv Nabusa (highly susceptible to *R. similis*) and cv Kayinja (tolerant to *R. similis*) were propagated using a standard shoot-tip culture protocol (Vuylsteke, 1998). Four weeks after rooting, plants were removed from the rooting medium and their roots and rhizomes rinsed in tap water. The plants were then replanted in 250-ml plastic cups, with their roots suspended in a nutrient solution [40 ml Micromix® (Magnesium 2%, Sulfur 16%, Manganese 7.5% and Zinc 18%) (Fleuron, Braamfontein, South Africa), 24 g $Ca(NO_3)2{\cdot}H_20$ and 36 g Agrasol® (Fleuron) l^{-1} sterile tap water], and their stems secured by the lid of the plastic cups. To enhance root growth, the plants were maintained in a plant

growth room set to a photoperiod of 18/6 h light/dark routine, an average temperature of 23^{o}C and 70% relative humidity (RH) for 4 weeks.

The banana plants were inoculated with endophytic *F. oxysporum* isolates by dipping their roots and rhizome in spore suspensions for 4 h. Non-inoculated plants were dipped in SDW for the same duration. Plants for cDNA-AFLP analysis were planted in cups as described above, and those for qRT-PCR were planted in sterile soil in pots (120 mm wide and 90 mm deep). Root challenge by *R. similis* was done by excavating the soil at the base of the plant and pipetting 2 ml of the nematode inoculum (containing approximately 350 nematodes) directly onto the roots. The excavated soil was then replaced. Non-challenged plants also had the soil around their roots excavated, but replaced without nematode application. All experiments were conducted in a plant growth room with a photoperiod of 12/12 h light/dark routine and a temperature of approximately 25^{o}C, and plants in soil were watered twice per week. At harvest, root samples were collected for RNA extraction, endophyte re-isolation and nematode extraction. Endophyte re-isolations from roots were undertaken according to Paparu *et al.* (2006b), and nematodes were extracted using the modified Baermann funnel method (Hooper *et al*., 2005).

RNA extraction and cDNA synthesis

Plants were removed from the nutrient solution and their roots washed in distilled water. Total RNA was extracted from banana roots and rhizomes using the RNeasy Plant Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. RNA yield was determined by measuring absorbency at 260 nm, using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Montchanin, USA).

Total RNA was digested with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) for 30 min at 37^{o}C, and column-purified using the QIAGEN RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions. Poly A^+ RNA was isolated using the Oligotex mRNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Double-stranded (ds)-cDNA synthesis was carried out from 150 ηg of purified poly A^+ RNA using the cDNA Synthesis kit from Roche. The first strand was synthesized in a reaction volume of 21 µl containing 2 µl oligo dT_{15} primer (200 µM), 9.5 µl RNase-free water, and 9.5 µl poly A^+ RNA. Samples were then incubated at 70^{o}C for 10 min and placed on ice, followed by the addition of 8 µl 5 x Reverse Transcriptase buffer, 1 µl Avian Myeloblastis

Virus (AMV), 4 µl 0.1 M dithiothreitol (DTT), 1 µl RNase inhibitor (25 U/µl) and 4 µl 10 mM dNTP-mix. The samples were incubated at 42^{o}C for 60 min, and the reaction stopped by placing it on ice.

The second strand was synthesized from the first by adding 30 µl 5 x second strand buffer, 1.5 µl 10 mM dNTP-mix, 6.5 µl second strand enzyme blend (DNA polymerase 1, *Escherichia coli* ligase and RNase H) and 72 µl SDW to the tube containing first strand cDNA. The mixture was incubated at 16^{o}C for 2 h, after which 20 µl (20 U) of T4 DNA polymerase was added and the mixture incubated for 5 more min. The reaction was stopped by the addition of 17 µl 0.2 M EDTA (pH 8.0). The ds-cDNA was purified using the High Pure Product Purification Kit from Roche. All cDNAs were assayed for genomic DNA contamination by PCR using the actin-specific primer set actinF (5'ACCGAAGCCCCTCTTAACCC-3') and actinR (5'-GTATGGCTGACACCATCACC- 3'), and PCR products separated by electrophoresis through a 2% (w/v) agarose gel containing ethidium bromide.

Isolation and identification of defence-related genes in EAHB

cDNA-AFLP analysis: Differential gene expression in susceptible (Nabusa) and tolerant (Kayinja) EAHB cultivars upon endophyte and nematode infections was determined by cDNA-AFLPs. Roots were harvested from both cultivars immediately before endophyte inoculation (0 h), and 2, 7 and 30 days after inoculation (dai), separately, with isolates Emb2.4o and V5w2. cDNA-AFLP analysis was performed according to the protocol described by Bachem *et al*. (1996), using the AFLP Expression Analysis Kit of LI-COR (LI-COR Biosciences, Lincoln, NE). Ds-cDNA was used as template in the generation of TaqI+0 / MseI+0 pre-amplification PCR products, which involved three steps: restriction digestion of cDNA, adapter ligation, and pre-amplification PCR.

Restriction digestion and adapter ligation: Restriction digestion was done in two steps. In the first step, a *Taq1* restriction digestion reaction mixture of 20 µl was prepared that consisted of 10 ng of ds-cDNA, 4 µl of 5x RL buffer, 0.5 µl *Taq1* enzyme and 5.5 µl of SDW. The mixture was incubated at 62^{o}C for 2 h, and placed on ice. This was immediately followed by *Mse1* restriction digestion. This step commenced by adding 1 µl 5 x RL buffer, 0.5 µl *Mse*1 enzyme and 3.5 µl SDW to the *Taq1* mixture. The mixture was incubated in two steps: first at 37^{o}C for 2 h and then at 80^{o}C for 20 min. Adaptor ligation mix (4.5 µl) and T4 DNA ligase (0.5 µl)

were added to the 25 µl *Taq1*/*Mse1* restriction digest mix, gently mixed and incubated at 20^{o}C for 2 h.

Pre-amplification: The ligation mixture was diluted with 1 x TE buffer at a ratio of 1:10. One µl of the diluted ligation mixture was then mixed with 10 µl of a pre-amp primer mix (containing EcoR 1 and Mse 1 primers), 1.25 µl 10 x amplification buffer, and 0.25 µl Taq DNA (Roche Diagnostics). This was followed by 25 cycles at 94^{o}C for 30 s, 56^{o}C for 1 min, and 72^{o}C for 1 min. Pre-amplification products were assayed for quality and quantity by electrophoresis on 1% agarose gels.

Selective amplification: The diluted pre-amplification products (1:300 in SABAX water) provided a template for selective amplification of all 64 +2/+2 primer combinations afforded by the eight *Taq*I+2 primers and eight *Mse*I+2 primers (+GA, +GT, +TC, +TG, +CT, +CA, +AG and +AC on both adaptor primers) available in the AFLP Expression Analysis Kit. The amplification reaction mixture consisted of 3 µl *Taq* DNA polymerase working mix, 1 µl diluted pre-amplification DNA, 1 µl *Mse1* primer containing dNTP's and 0.25 µl IRDyeTM 700-labelled *Taq1* primer. A touchdown PCR was used that consisted of one cycle at 94^{o}C for 30 s, 65^{o}C for 30 s and 72^{o}C for 1 min; 12 cycles where the annealing temperature was lowered by 0.7^{o}C per cycle, denaturation maintained at 94^{o}C for 30 s and amplification at 72^{o}C for 1 min; 23 cycles at 94^{o}C for 30 s, 56^{o}C for 30 s and 72^{o}C for 1 min and held at 4^{o}C. Infrared dye (IRDye700, LI-COR)-labeled *Taq*I+2 primers in this kit are fluorescent and aid in fragment visualization. Selective PCR products were resolved on 8% denaturing polyacrylamide gels in a model 4200S LI-COR DNA Analyzers (Myburg *et al.*, 2001), and cDNA-AFLP images were saved in 16-bit TIFF format for image analysis.

Image analysis and TDF quantification: LI-COR TIFF images were cropped using Quantity One 1-D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) before cDNA-AFLP band sizes and intensities were determined using the AFLP-QuantarPro software (KeyGene products B.V., Wageningen, The Netherlands). Lane finding, band finding and sizing were performed as described in the AFLP-QuantarPro user manual, with band finding and scoring parameters previously described for LI-COR gels (Myburg *et al.*, 2001). Based on visual analysis, differentially expressed TDFs were quantified for all primer combinations. In this study a TDF is used to refer to a polymorphic band (up-regulated in endophyte-inoculated plants, but not in control).

TDF isolation and identification: Polyacrylamide gels containing fragments of interest (TDFs up-regulated relative to non-inoculated plants) after partial electrophoresis on LI-COR DNA Analyzers were scanned using the Odyssey Infrared Imager (LI-COR). TDFs ranging in size from 140 to 469 bp were excised using a scalpel blade, and the PCR products suspended in 20 µl SDW. Elution was achieved by 10-15 cycles of freezing (-70°C) and thawing at room temperature. Eluted PCR products were re-amplified with the same primer combinations used in the final amplification reaction. The final amplification step, however, included an elongation step of 20 min. Re-amplified fragments were confirmed by visualization on a 2% (w/v) agarose gel.

Re-amplified TDFs were cloned into competent *Eschericia coli* cells using the InsTAclone Cloning Kit (MBI Fermentas, Hanover, MD) according to the manufacturer's instructions. The transformed cells were then plated on Luria-Bertani (LB) agar containing 250 µg ml^{-1} ampicillin, 60 µg ml^{-1} X-gal and 60 µg ml^{-1} isopropanol-β-thiogalactopyranoside (IPTG), and the plates incubated overnight at 37^{o}C. Transformed cells developed white colonies, while non-transformed ones were blue. Each transformant was separately transferred to 700 µl LB broth amended with 100 µg ml^{-1} ampicillin, incubated overnight 37^{o}C, and shaken at 200 rpm. Glycerol (300 µl) was added to 1.5-ml Eppendorf tubes containing the transformants, and the latter stored at -80^{o}C until sequencing.

The presence or absence of TDF inserts was determined with a colony PCR using M13F-pUC(-40) (5'-GTTTTCCCAGTCACGAC-3') and M13R-pUC(-26) (5'-CAGGAAACAGCTATGAC-3') universal primers. The reaction mixture contained 2 µl of transformed bacterial cells grown overnight in broth as template DNA (broth was put in 1.5 ml Eppendorf tubes and placed in boiling water for 10 min before adding to PCR mix), 1.5 µl $MgCl_2$, 2.5 µl NH_4^+ buffer, 2 µl 2.5 mM dNTP's, 0.4 µl of each primer (10 µM), 0.5 U ***Taq*** polymerase and SDW. PCR conditions were as follows: denaturation at 94^{o}C for 2 min, 30 cycles of 30 s at 94^{o}C, 30 s at 55^{o}C and 1 min at 72^{o}C, and a final elongation step of 7 min at 72^{o}C. PCR products were separated on 2% (w/v) agarose gel. TDF inserts were sequenced using ABI Bigdye terminator chemistry on ABI3100 instruments with the universal M13-pUC vector primers at Macrogen Corp. (Rockville, USA).

Gene regulation analysis using qRT-PCR

The regulation of defence-related genes in EAHB was determined by quantitative real-time reverse transcription (qRT)-PCR on a LightCycler version 1.2 instrument (Roche Diagnostic). After assigning putative identities to TDFs, four TDFs were chosen for regulation analysis, based on 1) similarity to defence-related genes, 2) percentage identity of more than 50% and 3) significance of the alignment (e-value). The TDFs selected for qRT-PCR included those containing fragments with similarity to *ABC transporter*, *coronatine insensitive* 1 (*COI*1), *lipoxygenase* (*LOX*) and *β 1,3-glucan synthase*. Primer pairs were designed as balanced pairs between 55.1 and 64.1°C Tm for the four TDFs with the objective to amplify fragments of between 150 and 283 bp using DNAMAN (Lynnon Biosoft, Quebec, Canada) (Table 1). An endogenous gene, *Musa* 25S rRNA (AY651067) (5'-ACATTGTCAGGTGGGGAGTT-3'; 5'-CCTTTTGTTCCACACGAGATT-3') (Van den Berg *et al*., 2007), was used as a control gene since its expression remains relatively constant. All primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd (Hatfield, South Africa).

For gene regulation studies, only isolate V5w2 was used as endophyte, and plant roots of both cultivars were harvested as follows: 1) immediately before inoculation (0 h), 2) 2 and 33 dai, 3) 3 days after endophyte-inoculated plants (inoculated for 30 days) were challenged with *R. similis* and 4) 3 days after endophyte-free plants were challenged with *R. similis*. Total RNA was extracted from banana roots using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. One µg RNA was DNAseI treated (Fermentas Life Sciences, Hanover, USA) and first-strand cDNA synthesised by random hexamer priming using Power Script TM Reverse Transcriptase (BD Biosciences, Erembodegem, Belgium) according to the method by Lacomme *et al.* (2003). The cDNAs were assayed for genomic DNA contamination by using the actin-specific primer set actinF (5'-ACCGAAGCCCCTCTTAACCC-3') and actinR (5'-GTATGGCTGACACCATCACC-3'), and PCR products separated by electrophoresis through a 2% agarose gel.

Dilution series and standard curves were performed to examine the linearity of amplification over the dynamic range. A serial dilution (1:10, 1:100 and 1:1000) was performed and used to draw standard curves for all genes. A 10-µl reaction for PCR amplification contained 5 µl FastStart DNA Master^{PLUS} SYBR Green I master mix, 2 µl of forward and reverse primer (10 µM), 1 µl cDNA template and 2 µl PCR grade water (Roche). Control treatments contained

water instead of the cDNA template. All PCR reactions were performed in triplicate. The cycling conditions were as follows: pre-incubation for 10 min at 95°C, followed by 55 cycles, each consisting of 10 s denaturing at 95°C, 10 s annealing at 63°C, 10 s primer extension at 72°C, and data acquisition at 95°C. For PCR amplification of all experimental samples, 1:10 cDNA template dilutions were used.

Data analysis

For RNA isolation, each treatment had three biological replicates, each consisting of three plants pooled together at harvest. TDF sequences were assigned putative identities by translating BLAST (BLASTX) (Altschul *et al.*, 1990) against the non-redundant protein database in GenBank. The degree of sequence similarity between a TDF and a known sequence was represented by the E-value. For gene expression analysis, standard regression curves were calculated using crossing-points from amplification data from the serial dilutions (Ginzinger, 2002). Expression data was normalized using the amplification data for the specific target gene and the endogenous control gene, *Musa* 25S rRNA, as previously described (Applied Biosystems, 2001). Lightcycler data was subjected to analysis of variance and multiple mean comparisons performed using Tukey's studentized range test. Comparisons between treatments were made using a pooled t-test in SAS Institute (1989).

RESULTS

Isolation and identification of defence-related genes in EAHB

A total of 62 TDFs were successfully isolated from roots and rhizomes of endophyte-inoculated EAHB plants. In this study, the word TDF is used to refer to fragments of the same length produced by the same primer combination. Of the 62 TDFs, 55 were successfully assigned putative identities (Table 2), while seven had no significant similarity to any protein in the non-redundant protein database in Genbank. TDFs were broadly classified into seven functional categories: defence/stress-related, primary metabolism, transport, signal transduction, cell wall biosynthesis, cell differentiation and development, and regulation (Fig. 1). Among TDFs with putative identities, those involved in cell differentiation and development, primary metabolism and defence/stress were most abundant, making 22, 21 and 15%, respectively, of the total number of TDFs. TDFs with similarities to proteins of unknown function made up 18% of total TDFs (Fig.1).

Of the 62 TDFs, 67.7% were up-regulated in the cv Nabusa, 6.5% in cv Kayinja and 25.8% in both cultivars (Table 2). The majority of TDFs related to cell differentiation and development (75%), primary metabolism (75%) and defence/stress (62.5%) were up-regulated in the susceptible cv Nabusa. Only one TDF related to cell differentiation and development, and one related to primary metabolism, was up-regulated in the tolerant cv Kayinja. TDFs related to cell differentiation and development, primary metabolism and defence/stress up-regulated in both cultivars were 9.2, 9.2 and 37.5%, respectively. Among TDFs associated with cell differentiation and development, primary metabolism and defence/stress, only *COI*1 and a senescence protein were up-regulated in the rhizome of cv Nabusa.

Seven TDFs with similarity to defence-related genes, *glycolate oxidase*, *COI*1, *cathepsin B-like protease*, *beta-N-acetyl hexosaminidase*, two *calmodulin-*Ca^{2+} and *LOX*, were up-regulated in the susceptible cv Nabusa (Table 2). Only three of these, *COI*1, *calmodulin-*Ca^{2+} and *LOX*, were also up-regulated in the tolerant cv Kayinja. TDFs with similarity to *beta-N-acetyl hexosaminidase*, *calmodulin-*Ca^{2+}, *COI*1 and *LOX* were up-regulated 2 dai with isolates Emb2.4o and V5w2, while *glycolate oxidase* was up-regulated 2 dai with isolate Emb2.4o (Table 2). With the exception of *glycolate oxidase*, these TDFs remained up-regulated until 30 dai. *Cathepsin B-like protease* was up-regulated at 7 and 30 dai. Two TDFs with similarity to proteins involved in cell wall strengthening (*cellulose synthase* and *β 1,3-glucan synthase*) were up-regulated in cv Nabusa alone (Table 2). *β 1,3-glucan synthase* was up-regulated early (2 dai) and stayed up-regulated until 30 dai, while *cellulose synthase* was up-regulated at 7 dai and remained up-regulated until 30 dai. Differences were also observed between roots and rhizomes in TDF up-regulation. TDFs isolated from roots accounted for 86% of the total number of TDFs, compared to the 6 and 8% of TDFs obtained from the rhizome alone and from both the roots and rhizomes, respectively. Of the defence-related genes, only *COI1* was up-regulated in both the roots and rhizomes of cv Nabusa, while none of the cell wall-strengthening genes was up-regulated in the rhizome.

Both non-pathogenic *F. oxysporum* isolates used as endophytes, Emb2.4o and V5w2, were able to up-regulate TDFs in EAHB (Table 2). When cvs Nabusa and Kayinja were inoculated with Emb2.4o, 40.3% of the TDFs were up-regulated, while 11.3% were up-regulated after inoculation of plants with V5w2. The rest of the TDFs were up-regulated following inoculation of EAHB with either Emb2.4o or V5w2. There was no difference in the timing of gene regulation between the two isolates, with both isolates up-regulating gene expression at

2, 7 and 30 dai. All the defence-related TDFs were up-regulated by isolate Emb2.4o, while only *calmodulin-Ca*$^{2+}$, *COI*1, *LOX* and *β 1,3-glucan synthase* were up-regulated after inoculation with isolate V5w2. In banana roots, 45.8% of the total TDFs were up-regulated by Emb2.4o alone, some of which include TDFs with similarity to *cathepsin B-like protease, cellulose synthase, glycolate oxidase* and two *protein kinases* (Table 2). Only 2.1% of the total TDFs were up-regulated by isolate V5w2 in banana roots, while those up-regulated by both isolates made up 52.1% of total number of TDFs. In the rhizome, five TDFs were up-regulated by both isolates, none by Emb2.4o and a TDF with similarity to an unknown *Oryza sativa* L. protein by V5w2.

Gene regulation analysis using qRT-PCR

Expression of the *ABC transporter* gene was significantly up-regulated in both the susceptible (cv Nabusa) (t = 25.6) and the tolerant banana (cv Kayinja) (t = 28.1, $P < 0.0001$) 2 dai with endophytic *F. oxysporum* isolate V5w2 (Fig. 2A). Gene expression, however, reduced significantly 33 dai in both cultivars (t = 38.44 for cv Nabusa, and t = 59.04 for cv Kayinja; $P < 0.0001$), and was significantly lower than at 0 h (t = 12.83 for cv Nabusa and t = 30.97 for cv Kayinja; $P < 0.0001$). Interestingly, when endophyte-inoculated plants were challenged with *R similis*, the *ABC transporter* gene was again up-regulated significantly in both cvs compared with nematode non-challenged plants or endophyte-free plants challenged with nematodes. In both cvs, *R similis* challenge of non-inoculated plants resulted in reduced expression compared with the expression at 0 hr (t = 9.13 for cv Nabusa, and t = 31.29 for cv Kayinja; $P < 0.0001$) (Fig. 2A).

Endophyte colonization resulted in a significant up-regulation of *β 1,3-glucan synthase* in cvs Nabusa and Kayinja 2 dai (t = 8.31 and t = 19.13, respectively; $P < 0.0001$), and remained high in both cvs at 33 dai (Fig. 2B). When inoculated with the endophyte and challenged with *R. similis*, cv Nabusa showed a significant up-regulation of *β 1,3-glucan synthase* (68.2%), compared with endophyte-treated plants at 33 dai (t = 6.64, $P < 0.0001$). However, *R. similis* challenge of endophyte-inoculated plants of the tolerant cv Kayinja resulted in reduced expression of *β 1,3-glucan synthase* (35.7%), compared with endophyte-inoculated and *R. similis* non-challenged plants (t = 6.32, $P < 0.0001$) of the same cv. In the susceptible cv Nabusa, *R. similis* challenge of non-inoculated plants resulted in higher expression of *β 1,3-glucan synthase,* compared with plants at 0 hr (t = 9.22, $P < 0.0001$).

Two dai of the susceptible cv Nabusa, the expression of *COI1* gene was increased from 0.01 to 0.23 ηg (t = 3.98, *P* = 0.014). *COI1* expression did not change significantly up to 33 dai (Fig. 2C). However, when endophyte-inoculated plants were challenged with *R. similis*, *COI1* gene was significantly up-regulated compared with endophyte non-inoculated and *R. similis* challenged plants (t = 3.79, *P* = 0.0026). *COI1* expression was significantly up-regulated in the tolerant cv Kayinja only at 33 dai (t = 10.89), and even more significantly following *R. similis* challenge of plants previously inoculated with the endophyte (t = 22.04, *P* < 0.0001). *Radopholus similis* challenge of neither the endophyte non-inoculated cv Nabusa nor cv Kayinja plants resulted in the up-regulation of the *COI1* gene.

The *LOX* gene was not significantly up-regulated in either of the EAHB cvs 2 dai inoculation with V5w2 (Fig.2D). Thirty-three dai, however, the expression of *LOX* in cv Nabusa was significantly increased (t = 10.30, *P* < 0.0001), but not in cv Kayinja. The observed expression at 33 dai in cv Nabusa was significantly reduced in endophyte-inoculated plants following *R. similis* challenge (t = 8.79, *P* < 0.0001). When challenged with *R. similis*, endophyte-inoculated cv Kayinja plants showed 88.8% up-regulation of *LOX* activity compared with endophyte-inoculated and *R. similis* non-challenged plants (t = 26.48, *P* < 0.0001), and a 77.8% up-regulation compared with endophyte non-inoculated and *R. similis*-challenged plants (t = 22.53, *P* < 0.0001). In the susceptible cv Nabusa, there was no significant difference in the expression of *LOX* following *R. similis* challenge of endophyte non-inoculated plants, compared with plants at 0 h. On the contrary, *R. similis* challenge of endophyte non-inoculated plants of the tolerant cultivar resulted in a significant up-regulation of *LOX*, compared with non-inoculated plants at 0 h (t = 8.17, *P* < 0.0001).

Non-pathogenic *F. oxysporum* endophytes and *R. similis* were recovered readily from the infected banana roots at harvest, thereby validating the results obtained in the inoculation experiment. The first strand cDNA synthesized from banana root RNA was of high quality, and its amplification with actin gene primers indicated no genomic DNA contamination (results not shown).

DISCUSSION

The endophytic *F. oxysporum* isolates Emb2.4o and V5w2 were able to transcribe genes in banana plants involved in plant defence/stress, primary metabolism, transport, signal transduction, cell wall biosynthesis, cell differentiation and development, and regulation. This indicates that banana plants respond by means of a diverse range of regulatory processes following endophyte colonization. A number of unknown genes were also observed that may have a novel functional role in the endophyte-banana interaction. Our study presents the first report of defence gene expression in susceptible and tolerant banana cvs following colonization by microbial biocontrol agents, and of plant gene identification in an endophyte-banana interaction.

The greatest number of genes induced by endophytic *F. oxysporum*, surprisingly, was found in the roots of the *R. similis*-susceptible cv Nabusa, and not in the tolerant cv Kayinja. Seven TDFs with similarity to defence-related genes were up-regulated in cv Nabusa, compared with three in cv Kayinja. Similarly, TDFs related to cell wall strengthening were up-regulated in only cv Nabusa following endophyte colonization. For example, *glycolate oxidase* and *cathepsin B-like protease* were up-regulated in roots of both endophyte-inoculated and non-inoculated plants of the tolerant cv Kayinja, but not in non-inoculated plants of the susceptible cv Nabusa. When Nabusa was inoculated with the endophytes, however, gene expression was strongly upregulated. This may be because these genes are already constitutively expressed in the tolerant cultivar, resulting in similar expression between non-inoculated controls and endophyte-inoculated plants. This finding is not entirely unique. When resistance was induced systemically in susceptible and tolerant cucumber (*Cucumis sativus* L.) plants by *Pseudomonas putida* Trevisan against Fusarium wilt caused by *F. oxysporum* f. sp. *cucumerinum* J.H. Owen, plant defence responses were expressed more in the susceptible than in the tolerant cultivar (Liu *et al*., 1995).

The up-regulation of genes involved in defence signaling (*Calmodulin-*Ca^{2+}), jasmonic acid pathway (*COI1* and *LOX*), cell wall strengthening (*cellulose synthase* and *β 1,3-glucan synthase*) and transport of defence molecules (*ABC transporter*) in the current study is of great significance. Our results thereby support the finding by Athman (2006) that induced resistance is the primary means of *R. similis* control by the endophytic isolate V5w2. Cellular signaling is initiated soon after the invader has been recognized by higher plants (Gelli *et al*.,

1997; Zimmermann *et al.*, 1997). *Calmodulin-*Ca^{2+} was up-regulated from 2 to 30 dai of both EAHB cultivars by isolates Emb2.4o and V5w2, thereby indicating that defence signaling had been initiated. Calmodulin is involved in the JA-dependent and –independent wound signal transduction pathways (Leon *et al.*, 1998). *COI1* was up-regulated in the roots and rhizomes, and *LOX* in the roots of both EAHB cultivars. These genes are involved in the JA signaling pathway that regulates plant responses to abiotic stress, defences against herbivores, insects and pathogens, and wound healing (Xie *et al.*, 1998).

Genes encoding the cell wall strengthening proteins cellulose synthase and β-1,3-glucan synthase were up-regulated in roots of both cv Nabusa and cv Kayinja following colonization by endophytic isolates Emb2.4o and V5w2. Cellulose forms an important component of the expanding cell wall and is the primary determinant of the strength of the cell wall (Dhugga, 2001). Cell wall strengthening following endophyte inoculation has often been inferred from the fact that endophytic colonization is limited to the epidermal and outer cortical cells (Olivain and Alabouvette, 1999; Bao and Lazarovits 2001; Paparu *et al.*, 2006a). *Cellulose synthase* genes were also induced in *A. thaliana* following treatment with SA (Schenk *et al.*, 2000), and after infection of cotton (*Gossypium* sp.) with the causative agent of Verticillium wilt, *Verticillium dahliae* Kleb (Zwiegelaar and Dubery, 2006). Similarly, the ABC transporter gene was up-regulated in both the tolerant and susceptible cultivars 2 dai with endophytic isolates Emb2.4o and V5w2. ABC transporter proteins in eukaryotic cells help to catalyse the efflux of various compounds out of the cell. They have been implicated in herbicide detoxification, xenobiotic transport, pigment transport, alleviation of oxidative damage and the transport of antimicrobial compounds (Davies and Coleman, 2000). The up-regulation of an *ABC transporter* gene, and that of *glycolate oxidase* (another gene involved in oxidative burst) in the current study shows the probable occurrence of an oxidative burst response following root colonization by endophytic isolates Emb2.4o and V5w2.

qRT-PCR confirmed that the *ABC transporter* gene was highly up-regulated in both the susceptible and tolerant banana cultivars following root colonization by endophytic isolate V5w2, but was reduced after 33 days in *R. similis* non-challenged plants. Interestingly, when endophyte-inoculated plants of both cvs were challenged with *R. similis*, the *ABC transporter* gene was up-regulated significantly compared with nematode non-challenged plants or endophyte-free plants challenged with the nematode. This suggests the priming of the *ABC transporter* gene in banana by inoculation with isolate V5w2 against *R. similis*. In a related

study, Johnson *et al.* (2003) reported up-regulation of a gene encoding ABC transporters of the group PDR-5 in tall fescue (*Lolium arundinaceum* Schreb.) plants infected with the fungal endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams). The induction of the same gene was earlier reported in rice following infection by the rice blast fungus *Magnaporthe oryzae* (T.T. Hebert) M.E. Barr and following treatment with bensothiadiazole (Xiong *et al*., 2001).

Endophyte colonization resulted in a significant up-regulation of *β 1,3-glucan synthase* in cvs Nabusa and Kayinja 2 dai, with gene expression in the tolerant cv Kayinja twice as much as in the susceptible cv Nabusa. β-1,3-glucan (called callose in plants) constitutes the cell walls of higher plants and is reportedly deposited in cell walls in response to plant cell wall penetration by pathogens or biocontrol organisms (Benhamou *et al*., 1996; Benhamou and Garand, 2001). The fact that *β 1,3-glucan synthase* activity increased in endophyte-inoculated Kayinja plants until 33 dai indicates that cell wall strengthening occurs as a strong and lasting response to hyphal penetration of walls. In cv Nabusa, prior inoculation of plants with the endophyte resulted in the priming of wall strengthening enzymes only upon nematode challenge. This is important, as priming of defence responses indicates that the susceptible cv Nabusa can be treated with endophytes to protect them against nematode attack.

The expression profiles of *COI1* and *LOX* following endophyte inoculation and *R. similis* challenge suggests the probable involvement of JA-induced defences in fungal endophyte-induced resistance against *R. similis* in banana. *COI1* activity was up-regulated in the tolerant cv Kayinja at 33 dai in endophyte-inoculated plants, but not in cv Nabusa. However when endophyte-inoculated plants of both cultivars were then challenged with *R. similis*, *COI1* activity was significantly up-regulated compared with endophyte non-inoculated and *R. similis* challenged plants at 33 dai. This again demonstrates priming of *COI1* activity in the susceptible cultivar. *COI1* is believed to be involved in jasmonate responses (Stintizi *et al*., 2001).

LOX activity was significantly and non-significantly up-regulated in the susceptible and tolerant cvs at 33 dai, respectively, and after *R. similis* challenge in the tolerant cultivar. Similar to *COI1*, *LOX* has been shown to play a central role in the induction of JA-induced genes such as *proteinase inhibitor* (*PI*) and *polyphenol oxidase* (*PPO*) (Li *et al*., 2004). Heitz *et al*. (1997) reported that the activation of *LOX* preceded that of *PI* in the wound-signaling

pathway, confirming their regulatory role in the production of defence genes. *PI* is involved in the defence of host plants against insect herbivores (Berger *et al*., 1995), while PPO is one of the enzymes involved in the phenylpropanoid pathway (lignin synthesis) and has been reported following wounding (Constabel *et al*., 2000).

Our study confirms induced resistance as a mode of action for endophytic isolate V5w2 against *R. similis*. This supports earlier findings of Athman (2006) and Vu *et al*. (2006). However in the current study, we were able to isolate and identify molecules (genes) induced in a susceptible and tolerant banana following endophyte colonization and *R. similis* challenge of endophyte-inoculated plants.

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Table 1. Base composition of oligonucleotide primers designed for putative defence-related genes up-regulated in roots and rhizomes of East African Highland banana cultivars following inoculation with endophytic non-pathogenic *Fusarium oxysporum* isolates Emb2.4o and V5w2

TDF	Putative identity	Primer sequence (5'-3')	Tm °C	Product size	Annealing temperature °C
77	*Lipoxygenase*	AGACTGCGTACCGACAGGCT	62.3°C	201	63°C
		TGTCTGCCGAGCGAATTCA	64.1°C		
17	*Coronatine insensitive 1*	TGTAGACTGCGTACCGACTC	56.5°C	283	63°C
		CTCGCCAATGTAACCAAG	55.1°C		
68	*β 1,3-glucan synthase*	TGTAGACTGCGTACCGACA	56.3°C	163	63°C
		CCATGGGAAGGATAAGGA	55.8°C		
52	*ABC transporter*	GTAGACTGCGTACCGACAAG	56.0°C	150	63°C
		GTGGAGGAAACAAGAGGAAG	56.4°C		

Table 2. Putative identities of non-redundant transcript-derived cDNA fragments (TDFs) differentially up-regulated in roots and rhizomes of a susceptible (cv Nabusa, AAA-EA) and tolerant (cv Kayinja, ABB) banana following inoculation with endophytic non-pathogenic *Fusarium oxysporum* isolates Emb2.4o and V5w2

TDF[1]	Genbank accession	Protein similarity	Origin of similar sequence	% ID/E-value[2]	Function[3]	Time of up-regulation (dai)[4]	Tissue	Isolate	Cultivar
1	AAM 12880	GTP-binding protein	*Helianthus annuus*	92%/1e^{-30}	Protein transport	7,30	Root	Emb2.4o	Nabusa
2	AAM 61594.1	Glycolate oxidase	*Arabidopsis thailana*	72%/9e^{-28}	Oxidative burst (Defence)	2	Root	Emb2.4o	Nabusa
3	AAR 85970.1	Chlorophyll A-B binding protein	*Nicotiana tabacum*	83%/2e^{-20}	Photosynthesis	2	Root	Emb2.4o/V5w2	Nabusa
6	BAD 53949.1	Unknown protein	*Oryza sativa*	37%/4.5	Unknown	30	Root	V5w2	Nabusa
7	AAT 08714.1	Ribosomal protein	*Hyacinthus orientalis*	89%/7e^{-28}	Protein synthesis	2,7,30	Root	Emb2.4o/V5w2	Nabusa
8	AAZ 79231.1	Cellulose synthase	*N. tabacum*	47%/5e^{-12}	Primary cell wall appositions	7,30	Root	Emb2.4o	Nabusa
10	YP 556837.1	Transcriptional regulator	*Burkholderia xenovorans*	52%/1.2	Regulation	30	Root	Emb2.4o	Nabusa
14	XP 646197.1	Protein kinase	*Dictyostelium discoideum*	29%/3.5	Signal transduction	2,7,30	Rhizome	Emb2.4o/V5w2	Nabusa
15	BAB 33421.1	Senescence protein	*Pisum sativum*	60%/4e^{-15}	Cell differentiation and development	2	Rhizome	V5w2	Nabusa
17	ABK 27928.1	Coronatine-insensitive 1	*N. attenuata*	85%/3e^{-36}	Jasmonate response (Defence)	2,7,30	Root/Rhizome	Emb2.4o/V5w2	Nabusa/Kayinja

TDF[1]	Genbank accession	Protein similarity	Origin of similar sequence	% ID/E-value[2]	Function[3]	Time of up-regulation (dai)[4]	Tissue	Isolate	Cultivar
18	AAK 91819.1	Kinesin	*Zea mays*	68%/7e^{-17}	Transport	2,7,30	Root/Rhizome	Emb2.4o/V5w2	Nabusa/Kayinja
19	BAD 08712.1	Phosphatidylcholine transfer protein	*O. sativa*	62%/2e^{-18}	Transport	2,7,30	Root/Rhizome	Emb2.4o/V5w2	Nabusa/Kayinja
22,23	BAC 43353.1	Unknown	*A. thailana*	100%/0.002	Unknown	2,7,30	Root/Rhizome	Emb2.4o/V5w2	Nabusa/Kayinja
26	NP 001057011.1	Os 06g018660	*O. sativa*	79%/2e^{-11}	Unknown	2,7	Root	Emb2.4o	Nabusa
27	CAC 27142.1	Ribosomal protein	*Picea abies*	90%/8e^{-08}	Protein synthesis	7,30	Root	Emb2.4o/V5w2	Nabusa/Kayinja
28	AAC 13596.1	Endonuclease-1	*A. thailana*	84%/1e^{-29}	Cell differentiation and development	7,30	Root	Emb2.4o	Nabusa/Kayinja
29	AAP 53974.2	Cell division protein	*O. sativa*	98%/3e^{-37}	Cell differentiation and development	7,30	Root	Emb2.4o	Nabusa/Kayinja
31	NP 567643.1	Unknown	*A. thailana*	73%/0.002	Unknown	2,7,30	Root	Emb2.4o	Nabusa
32	P 00873	Ribulose biphosphate carboxylase	*Chlamydomonas reinhardtii*	60%/1e^{-08}	Photosynthesis	7	Root	Emb2.4o	Nabusa
33	ABF 70023.1	Hypothetical protein	*Musa acuminata*	92%/0.03	Unknown	2,7,30	Root	Emb2.4o/V5w2	Nabusa
34	AAQ 14245.1	Actin	*M. acuminata*	100%/5e^{-17}	Cell differentiation and development	2,7,30	Root	Emb2.4o/V5w2	Nabusa
37	NP 849822.1	CAT 2	*A. thailana*	82%/2e^{-23}	Transport	2,7,30	Root	Emb2.4o/V5w2	Kayinja
38	AAO 24249.1	Alcohol dehydrogenase 1	*Hordeum vulgaris*	55%/0.001	Metabolism	2,7,30	Root	Emb2.4o/V5w2	Kayinja
39	AAQ 14245.1	Actin	*M. acuminata*	100%/5e^{-17}	Cell differentiation	2,7,30	Root	Emb2.4o/V5w2	Kayinja

TDF[1]	Genbank accession	Protein similarity	Origin of similar sequence	% ID/E-value[2]	Function[3]	Time of up-regulation (dai)[4]	Tissue	Isolate	Cultivar
40	AAQ 14245.1	Actin	*M. acuminata*	100%/5e^{-17}	Cell differentiation and development	30	Root	Emb2.4o	Nabusa
44	AAD 000695.1	Nuclease	*Zinnia elegans*	66%/2e^{-30}	Cell differentiation and development	30	Root	Emb2.4o	Nabusa
45	CAJ 72166.1	Response regulator	*Candidatus kuenemia*	47%/7.7	Regulation	2,7,30	Root	Emb2.4o	Nabusa
47,54,58	CAA 33873.1	Actin	*O. sativa*	100%/6e^{-37}	Cell differentiation and development	2,7,30	Root	Emb2.4o	Nabusa
49	CAE 53908.1	Ring protein	*Triticum sativum*	93%/4e^{-26}	Phospholipid binding	7,30	Root	Emb2.4o	Nabusa
50,56	P 04464	Calmodulin- Ca^{2+}	*T. sativum*	98%/2e^{-23}	Defence	2,7,30	Root	Emb2.4o/V5w2	Nabusa/Kayinja
51	BAD 53978.1	Unknown protein	*O. sativa*	83%/0.32	Unknown	2	Root	Emb2.4o	Nabusa
52	AAM14842.1	ABC transporter	*A. thailana*	94%/1e^{-19}	Transport	2	Root	Emb2.4o/V5w2	Nabusa/Kayinja
53	AAN 64140.1	Ripening regulated protein	*O. sativa*	88%/5e^{-15}	Cell differentiation and development	2,7,30	Root	Emb2.4o/V5w2	Nabusa
57	ABA 18652.1	Glutamate decarboxylase	*Populus tremula*	93%/2e^{-46}	Metabolism	2,7,30	Root	Emb2.4o/V5w2	Nabusa/Kayinja
59	BAD 87632.1	Hydrolase	*O. sativa*	66%/2e^{-13}	Metabolism	30	Rhizome	V5w2	Nabusa
60	XP 001146709.1	Hypothetical protein	*Pan troglodytes*	50%/1.6	Unknown	2,7	Root	Emb2.4o	Nabusa
61	BAB 41205.1	Kinase-like protein	*O. sativa*	46%/3e^{-08}	Signal transduction	2,7,30	Root	Emb2.4o	Nabusa

TDF[1]	Genbank accession	Protein similarity	Origin of similar sequence	% ID/E-value[2]	Function[3]	Time of up-regulation (dai)[4]	Tissue	Isolate	Cultivar
62	ABF 70097.1	Hypothetical protein	*Musa balbisiana*	71%/4e^{-06}	Unknown	7,30	Root	Emb2.4o	Nabusa
63,66	BAB 93221.1	Ribosomal protein	*O. sativa*	89%/5e^{-16}	Protein synthesis	2,7,30	Root	Emb2.4o/V5w2	Nabusa
64	CAB 62588.1	Cathepsin B-like protease	*P. sativum*	84%/2e^{-31}	Defence	7,30	Root	Emb2.4o	Nabusa
65	NP 001049249.1	Unknown	*O. sativa*	53%/2e^{-04}	Unknown	7	Root	Emb2.4o/V5w2	Nabusa
67	ABE 84189.1	Hypothetical protein	*Medicago truncatula*	57%/4e^{-11}	Unknown	7,30	Root	Emb2.4o	Nabusa
68	AAQ 17229.1	β 1,3-glucan synthase	*Lolium multiflorum*	94%/1e^{-23}	Callose synthesis	2,7,30	Root	Emb2.4o/V5w2	Nabusa
69	AAP 81215.1	Acid phosphatase	*A. thailana*	74%/6e^{-15}	Stress	2	Root	Emb2.4o	Nabusa
70	AA 072389.1	Synaptobrevin-like protein	*O. sativa*	100%/5e^{-20}	Transport	2,7,30	Root	Emb2.4o/V5w2	Nabusa
72	AAR 37366.1	Beta-tubulin	*N. attenuata*	100%/7e^{-28}	Cell differentiation and development	7	Root	Emb2.4o/V5w2	Nabusa
73	YP 652374.1	Beta-N-acetyl hexosaminidase	*Yersinia pestis antiqua*	37%/7.8	Defence	2,7,30	Root	Emb2.4o/V5w2	Nabusa
74	BAD 45867.1	Protein kinase	*O. sativa*	75%/2e^{-23}	Signal transduction	2,7,30	Root	Emb2.4o	Nabusa
76	Q 95E94	Methylenetetrahydrofolate reductase 1	*Z. mays*	91%/7e^{-36}	Protein synthesis	30	Root	Emb2.4o	Nabusa
77	AAG 18376.1	Lipoxygenase	*Zantedeschia aethiopica*	51%/3e^{-14}	Defence (Jasmonate response)	2,7,30	Root	Emb2.4o/V5w2	Nabusa/Kayinja

[1]TDFs with no significant sequence similarities are excluded.

[2]E-value = sequence alignment significance (Karlin and Altschul, 1990).

[3] "Unknown" denotes significant similarity to proteins of unknown function.

[4]Days after inoculation.

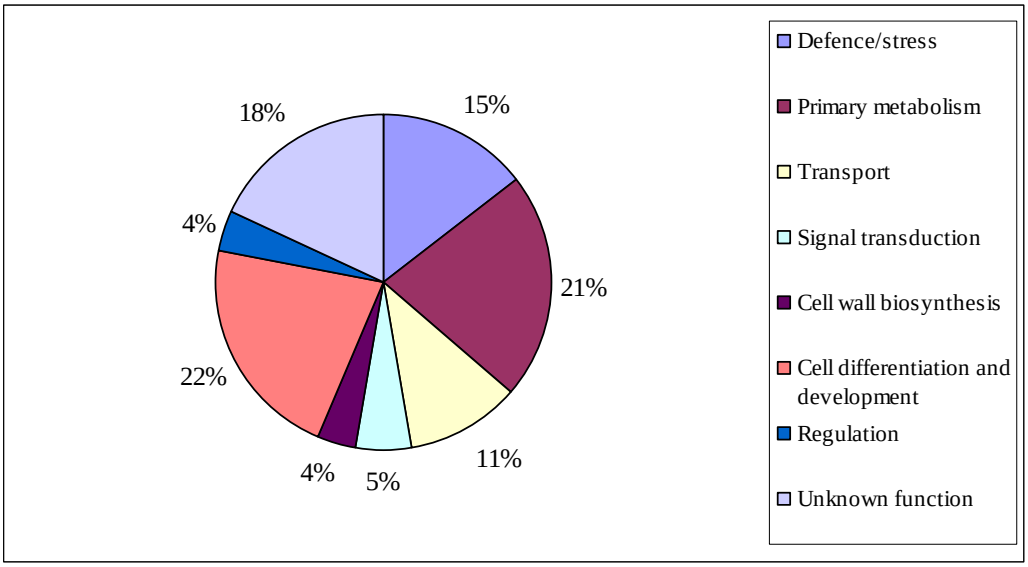

Figure 1. Pie chart showing the functions of transcript-derived cDNA fragments up-regulated in roots and rhizomes of susceptible (cv Nabusa, AAA-EA) and tolerant (cv Kayinja, ABB) bananas following inoculation with endophytic non-pathogenic *Fusarium oxysporum* isolates Emb2.4o and V5w2.

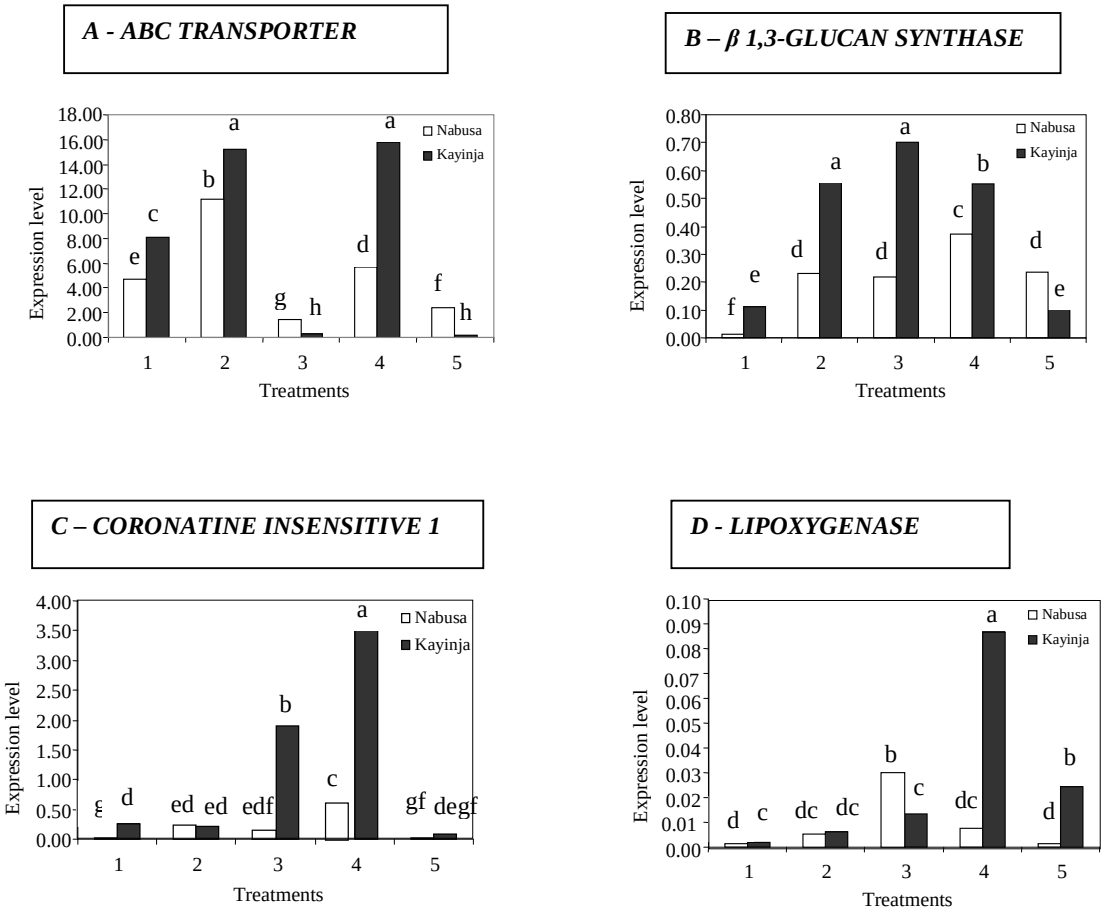

Figure 2. Confirmation of the expression patterns of four TDFs by relative quantification of transcript abundance using qRT-PCR in roots of banana cultivars susceptible (Nabusa, AAA-EA) and tolerant (Kayinja, ABB) to *Radopholus similis*. Treatment 1 = non-inoculated plants (0 h), 2 = plants inoculated with non-pathogenic *Fusarium oxysporum* strain V5w2 at 2 days after inoculation (dai), 3 = plants inoculated with strain V5w2 at 33 dai, 4 = plants inoculated with strain V5w2 and challenged with ***R. similis*** 30 dai and harvested 3 days after nematode challenge, and 5 = endophyte-free plants challenged with ***R. similis*** on day 30 and harvested 3 days later. (A) *ABC transporter*, (B) *β 1,3-glucan synthase,* (C) *coronatine insensitive 1* and (D) *lipoxygenase* genes. Bars carrying different letters are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

CHAPTER 4

Activities of phenylpropanoid pathway enzymes in susceptible and tolerant bananas (*Musa* spp.) following inoculation with a non-pathogenic *Fusarium oxysporum* endophyte and challenge with *Radopholus similis*

ABSTRACT

Phenylpropanoid pathway enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) are involved in plant defence pathways leading to lignification, synthesis of secondary metabolites including salicylic acid and phytoalexins, wound healing, and the oxidative burst. The activities of PAL, POX and PPO enzymes were analysed in roots of banana cultivars susceptible (Nabusa, AAA-AE) and tolerant (Yangambi, AAA) to the burrowing nematode ***Radopholus similis,*** following inoculation with endophytic ***Fusarium oxysporum*** isolate V5w2 and challenge with ***R. similis***. Constitutive expression of PAL and PPO were similar between the susceptible and tolerant cultivars, while constitutive POX activity was higher in the tolerant cv. PAL activity was suppressed in both cultivars 7 days after endophyte inoculation (7 dai), but was significantly up-regulated in the susceptible cv Nabusa at 30 days post nematode challenge (dpnc) in endophyte-inoculated plants challenged with ***R. similis***. In the tolerant cultivar, PAL activity was up-regulated in ***R. similis***-challenged plants at 7 and 30 dpnc irrespective of endophyte inoculation. POX and PPO were transiently up-regulated in cv Nabusa 7 dai, exceeding levels observed in non-inoculated plants of the same cv. Similar to PAL, POX activity was up-regulated at 7 dpnc in endophyte-inoculated cv Nabusa plants challenged with ***R. similis.*** In the tolerant banana cv Yangambi, POX and PPO activities were similarly up-regulated in ***R. similis***-challenged plants at 7 dpnc, irrespective of endophyte inoculation. The findings of this study implicate PAL, POX and PPO in banana defence against the root burrowing nematode ***R. similis***. Our findings further demonstrate the ability of endophytic ***F. oxysporum*** isolate V5w2 to directly induce POX and prime PAL in a susceptible banana cultivar for greater up-regulation following ***R. similis*** challenge.

INTRODUCTION

Enzymes produced in the phenylpropanoid pathway are known to be involved in plant disease resistance (Nicholson and Hemmerschmidt, 1992). Three of the more important enzymes are phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO). PAL catalyses the deamination of phenylalanine to cinamic acid in the first step of the phenylpropanoid pathway which leads to formation of lignin. PAL is also a precursor for phenylpropanoid-derived secondary plant products such as salicylic acid and isoflavonoid phytoalexins, which are involved in defence of host plants (Ward *et al.*, 1991). POX plays a crucial role in plant cell wall biosynthesis by polymerising cinnamyl alcohols into lignin and forming cross-links between the various protein components of the cell wall (Bradley *et al.*, 1992). They are also involved in wound-healing (Sherf *et al.*, 1993), lignification (Walter, 1992) and production of antimicrobial radicals (Peng and Kuć, 1992). Due to the crucial role they play in defence, POX induction is an early event in plant-microbe interactions (Cook *et al.*, 1995; Harrison *et al.*, 1995). POX is also known to be associated with metabolic processes such as ethylene biosynthesis, phenol oxidation and chlorophyll catabolism (Ross *et al.*, 1995).

PPO is an enzyme that catalyses the oxidation of o-diphenolic compounds to o-quinones which rapidly polymerise to produce black or brown pigments (polyphenols) (Vaughn and Duke, 1984). In healthy tissues, PPO only occurs in plastids, but upon insect injury or wounding, PPO reacts with phenolic substrates to form polyphenols that cause tissue darkening during lesion formation. They are, therefore, involved in limiting pathogen spread to healthy host cells. PPO was induced in hybrid poplar plants (*Populus trichocarpa* Torr. and A. Gray x *Populus deltoids* Bartram and Marsh) following mechanical damage simulating insect damage (Constabel *et al.*, 2000). The role of foliar PPO in plant defence against leaf-eating insects (Noctuidae) was reported by Felton *et al.* (1989) in tomato (*Lycopersicum esculentum* Mill.). During insect feeding, the mixing of PPO and phenolic substrates generates the highly reactive o-quinones that are able to covalently modify free amino and sulfhydryl groups in dietary proteins within the gut and mouth of the insect. The resulting phenolic adducts prevent efficient assimilation of the alkylated amino acids, reducing nutritive quality of protein (Felton *et al.*, 1992).

Banana nematodes, in particular the burrowing nematode *Radopholus similis* (Thorne) Cobb, are important pests of East African Highland bananas (EAHB) (Gold *et al.*, 1994). The best possible means to control nematodes involves an integrated pest management (IPM) strategy consisting of cultural control, host plant resistance and biological control (Speijer *et al.*, 1994). Cultural control methods, such as the use of clean planting material and crop sanitation practices might contribute greatly to nematode control, but their use by farmers is limited due to their associated costs (Gold *et al.*, 2001). The use of clean planting materials (tissue-culture plantlets, pared and hot-water treated suckers) initially reduces nematode levels (Gold *et al.*, 2001), but plants are quickly re-infestated by nematodes already present in infested fields.

Tolerance to nematodes has been identified in wild banana varieties, but most EAHB cultivars were proven to be susceptible to the pest (Ortiz *et al.*, 1995; Kiggundu *et al.*, 2003). EAHB plants, however, harbour endophytes that have the ability to induce a natural resistance response against nematodes (Schuster *et al.*, 1995). Endophytes are organisms which, at some stage in their life cycle, colonize plant tissues without causing any visible symptoms (Petrini, 1991). Non-pathogenic *Fusarium oxysporum* (Schlecht.: Fries) endophytes in EAHB plants has further shown antagonistic activity against *R. similis* (Niere, 2001; Athman, 2006). During the *in vitro* production of bananas, the tissue culture plants are separated from their endophytes (Pereira *et al.*, 1999). Beneficial endophytes, however, can be reintroduced into the sterile plant to restore natural plant health before field planting.

A correlation between phenylpropanoid pathway enzymes and nematode resistance in banana (*Musa* spp.) was established by Trudgill (1991) and more recently confirmed by Wuyts *et al.* (2006). Resistance to *R. similis* in Yangambi (*Musa acumunita*, AAA) and Gros Michel (*M. acumunita*, AAA) is reportedly associated with high levels of constitutive phenolic cells, which are products of the phenylpropanoid pathway (Fogain and Gowen, 1996; Fogain, 2000). Fogain and Gowen (1996) and Fogain (2000) also observed a positive correlation between lignification and nematode resistance in cv Pisany jari buaya. Endophytic *F. oxysporum* isolates furthermore have been shown to protect a susceptible EAHB cultivar (Nabusa, AAA-EA) against *R. similis* by increasing phenolic compounds in root cell walls (Athman, 2006). Yet, genes transcribing POX and PAL, the precursors of lignin, were not up-regulated 3

days after endophyte-inoculated EAHB plants were challenged with *R. similis* (Chapter 2). The objective of the current study, therefore, was to determine the ability of endophytic non-pathogenic *F. oxysporum* to induce PAL, POX and PPO activity in *R. similis*-susceptible and -tolerant EAHB cultivars upon challenge with *R. similis.*

MATERIALS AND METHODS

Inoculation of banana roots with non-pathogenic *Fusarium oxysporum*

Tissue culture banana plants of *R. similis*-susceptible cv Nabusa and -tolerant cv Yangambi were inoculated with a potassium chlorate resistant mutant of the non-pathogenic endophytic *F. oxysporum* isolate V5w2 (V5w2 CHR 9) (Chapter 5). The banana plants were propagated using the standard shoot-tip culture protocol described by Vuylsteke (1998). Four weeks after rooting, tissue culture plants were removed from the rooting medium and their roots and rhizomes washed with tap water to remove culture medium. They were then transferred to a hydroponic system in 250-ml plastic cups and fertilized with Poly-Feed (Haifa chemicals, Haifa Bay, Israel) (1 g Poly-Feed l^{-1} sterile tap water) to enhance root growth (Paparu *et al.*, 2006). Isolate V5w2 CHR 9, selected for its ability to reduce *R. similis* infection in banana roots (Athman, 2006), was cultured on half strength potato dextrose agar (PDA) (19 g PDA and 19 g agar l^{-1} distilled water) in order to prepare a fungal spore suspension of 1.5 x 10^6 spores/ml (Chapter 2).

Four weeks after transplantation in the hydroponic system, banana seedlings were removed from their cups for inoculation with the non-pathogenic *F. oxysporum* endophyte. Plants of each cv were washed in tap water and randomly separated into four groups, each containing 16 plants. For each cv, two groups of plants were dipped in a spore suspension of 1.5×10^6 spores ml^{-1}. The other two groups were dipped in sterile distilled water (SDW) and acted as controls. After 4 h, all plants were planted in steam-sterilized forest loamy soil in 5-L potting bags and kept in the screenhouse, where they were watered daily. After 7 days, four plants per treatment were randomly harvested and their roots pooled. Ten roots per treatment were then randomly chosen for *F. oxysporum* re-isolation, from which the endophyte was re-isolated from eight pieces per root on chlorate medium (Chapter 2). The experiment was repeated once.

Challenge of banana with *Radopholus similis*

Endophyte-inoculated and non-inoculated plants were challenged with ***R. similis*** after 8 weeks, Pure cultures of ***R. similis*** nematodes, maintained at the International Institute of Tropical Agriculture (IITA) in Namulonge, Uganda (0º32'N, 32º35'E), were cultured on carrot (*Daucus carota* L.) discs according to the method of Speijer and De Waele (1997). The nematodes were suspended in SDW in a beaker, and the number of juveniles and females were estimated in a 2-ml suspension using a light microscope (100× magnification). Three holes of ~3-5 cm deep were made in the potting soil at the base of each plant at an equal distance from one another, and 6 ml nematode suspension containing a total of 500 juveniles and females were pipetted in the holes, after which the holes were covered with soil. Plants were not watered until 24 h after challenge to ensure that nematodes were not washed away. Thereafter, plants were maintained in the screenhouse for 60 days and watered daily.

At harvest (7, 30 and 60 days post nematode challenge (dpnc)), plants were removed from their bags and their roots and rhizomes washed with tap water. Thereafter, roots of four plants per treatment were pooled, and five roots were randomly selected for ***R. similis*** damage assessment and estimation of population density. ***Radopholus similis*** damage was expressed as percentage necrotic root tissue, as defined by Speijer and Gold (1996). In brief, roots (~10 cm in length) were cut longitudinally and percentage of visible necrotic tissue estimated. Each root represented a maximum percentage root necrosis of 20%, and the five roots added up to 100% root necrosis. The five segments used for root necrosis assessment were further used to determine nematode densities after extraction with the modified Baermann funnel method (Hooper ***et al.,*** 2005).

Enzyme determination

For all treatments, enzyme activity was assayed 7 days after inoculation (dai) with isolate V5w2 CHR 9 and at 7, 30 and 60 dpnc. From the roots pooled for each treatment at harvest, approximately 15 roots were randomly selected, chopped, placed in a polythene bag and frozen in liquid nitrogen for enzyme determination. Roots were either stored at -80^{o}C until grinding, or were ground immediately in liquid nitrogen using a pre-chilled mortar and pestle. Ground samples were placed in 50-ml

Falcon tubes (Biosciences GmbH, Dresden, Germany) and stored at -80^{o}C until analysis.

PAL activity assay: PAL was extracted from banana roots according to the method described by Wuyts *et al*. (2006) with modifications. Sub-samples of 0.5 g were mixed with an equal volume of polyvinypolypyrrolidone (PVPP) and suspended in 1 ml ice-cold borate buffer (0.05 M at pH 8.8) (0.9 M Tris, 0.9 M Boric acid, 0.025 M EDTA). The samples were centrifuged at 24,000 g for 30 min at 4^{o}C. The supernatant was collected and stored at -80^{o}C until analysis. For the photometric assay, 200 µl of the enzyme extract was added to 800 µl of a 0.015-M L-phenylalanine solution in 0.025 M borate buffer at pH 8.8. The mixture was incubated at 37^{o}C for 1 h. The reaction was stopped by adding 50 µl concentrated HCl (2.0 N). All samples were prepared in triplicates. The blank consisted of a reaction prepared with D-phenylalanine. The absorbance of the samples was read at 290 nm against the blank using a BioMate 3 spectrophotometer (Thermo Electron Scientific Instrument Corporation, Madison, USA). A standard curve of cinnamic acid was prepared under the assay conditions and PAL activity expressed as the quantity (nmol) of cinnamic acid formed in the reaction in 1 h and per protein content of the sample (nmol cinnamic acid h^{-1} mg $protein^{-1}$).

POX activity assay: POX extraction from banana roots was performed according to the method described by Wuyts *et al*. (2006) with modifications. Sub-samples of 0.5 g were taken and mixed with an equal volume of polyvinypolypyrrolidone (PVPP) and suspended in 1 ml ice cold sodium phosphate buffer (0.05 M at pH 6.0). The samples were centrifuged at 16,000 g for 20 min at 4^{o}C. The supernatant was collected and stored at -80^{o}C until analysis. The reaction mixture for the photometric assay consisted of 0.25% (v/v) guaiacol and 0.3% (v/v) H_2O_2 in 1,000 µl sodium phosphate buffer (0.05 M at pH 6.0) at 27^{o}C. To start the reaction, 20 µl of the extract was added to 980 µl of the reaction mix. All samples were prepared in triplicates. The blank consisted of a reaction mix without the enzyme extract. The absorbance of the samples was read at 470 nm for 5 min at 30 s intervals, using a BioMate 3 spectrophotometer. POX activity was determined from the linear part of the reaction curve over time and expressed as the change in optical density (OD) per second and per protein content of the sample ($\Delta OD470_{nm}\ s^{-1}$mg $protein^{-1}$).

PPO activity assay: PPO was extracted from banana roots according to Wuyts *et al*. (2006) with modifications. Sub-samples of 0.5 g were taken and mixed with an equal volume of polyvinypolypyrrolidone (PVPP) and suspended in 1 ml ice cold sodium phosphate buffer (0.2 M at pH 7.0) containing 0.25% (v/v) Triton-X. The samples were centrifuged at 24,000 g for 30 min at 4^{o}C. The supernatant was collected and stored at -80^{o}C until analysis. For the photometric assay, 10 µl of the extract was mixed with 1,000 µl dopamine solution (0.005 M) in sodium phosphate buffer (0.05 M, pH 7.0 at 27^{o}C). All samples were prepared in triplicates. The blank consisted of a reaction mix without the enzyme extract. The absorbance of the samples was read at 480 nm for 2 min at 15 s intervals, using a BioMate 3 spectrophotometer. PPO activity was determined from the linear part of the reaction curve over time and expressed as the change in OD per second and per protein content of the sample ($\Delta OD480_{nm}$ s^{-1} mg $protein^{-1}$).

Protein content of enzyme extracts: Total protein content of PAL, POX and PPO extracts was determined according to the dye-binding method of Bradford (1976), using bovine serum albumin (BSA) as standard. For all assays, 10 µl of the extract was mixed with 200 µl Bradford reagent (Sigma-Aldrich) and 790 µl of SDW. The mixture was mixed gently and incubated for 15 min at 25^{o}C. Optical density for each sample was measured at 595 nm. Samples were prepared in triplicates. Protein content was estimated using a standard curve prepared using BSA.

Data analysis

Percentage colonization was analyzed using logistic regression and α-levels for pairwise mean comparisons calculated using Dunn-Sidak-corrected 95% confidence intervals. Nematode densities and percentage necrosis were analyzed using Kruskal-Wallis analysis of variance and a Statterthwaite's approximation t-value used to compare means (Sokal and Rohlf, 1995; SAS Institute, 1989). Where an ANOVA was done, PAL, POX and PPO activities were log_{10}-transformed and multiple mean comparisons between treatments were performed using Tukey's studentized range test. Means for treatment combinations were compared using contrasts (SAS Institute, 1989).

RESULTS

Root colonization by *Fusarium oxysporum*

Non-pathogenic *F. oxysporum* colonized the roots of both endophyte-inoculated and endophyte non-inoculated Nabusa and Yangambi plants in the screenhouse in Namulonge. Root colonization by *F. oxysporum* was, however, significantly higher in the endophyte-inoculated compared with endophyte non-inoculated plants 7 dai (χ^2 = 77.49, $P < 0.0001$; χ^2 = 28.05, $P < 0.0001$ for replicates 1 and 2, respectively). For endophyte-inoculated plants, root colonization was between 93.8 and 100.0% in replicate 1, and between 0.0 and 6.8% for endophyte non-inoculated plants 7 dai (data not presented). In replicate 2, root colonization varied between 64.3 and 81.3% in endophyte-inoculated, and between 8.8 and 12.0% in endophyte non-inoculated plants 7 dai. No significant differences were observed in root colonization by *F. oxysporum* between endophyte-inoculated and non-inoculated plants at 7 dpnc. At 7 dpnc, 4.4-17.5% root colonization was recorded for cv Nabusa plants inoculated with isolate V5w2, compared with 1.9-2.5% *F. oxysporum* colonization in non-inoculated control plants (data not presented). For Yangambi plants, 6.3-7.5% *F. oxysporum* colonization was recorded for endophyte-inoculated plants 7 dpnc, while 0% *F. oxysporum* colonization was recorded for the non-inoculated control plants. *Fusarium oxysporum* could no longer be re-isolated from roots at 30 dpnc.

Radopholus similis population densities and root necrosis

Nematode densities in banana roots differed significantly between replicates 1 and 2 (t = 2.22, $P = 0.032$). In replicate 1, no nematodes were extracted from banana roots 7 dpnc, and no significant differences were observed in nematode numbers among treatments 30 dpnc. Endophyte non-inoculated plants, however, contained higher nematode numbers 30 dpnc (Table 1). At 60 dpnc, endophyte-inoculated plants of cv Nabusa had significantly fewer nematodes in roots compared with endophyte non-inoculated plants (χ^2 = 9.97, $P = 0.019$). In Yangambi, nematode numbers were substantially lower in endophyte-inoculated plants, but this reduction was not significantly different from that in endophyte non-inoculated control plants. In replicate 2, few nematodes had entered the roots by 7 and 30 dpnc, and no significant differences were observed in nematode numbers among treatments. After 60 days, however, endophyte-inoculated plants of cv Nabusa had significantly lower nematode

numbers compared with endophyte non-inoculated plants (χ^2 = 9.42, *P* = 0.020), but there was no significant difference in nematode numbers between endophyte-inoculated and non-inoculated Yangambi plants. No significant differences were observed in percentage root necrosis between replicates 1 and 2 (t = 1.79, *P* = 0.77) (data not presented) and between treatments 7, 30 and 60 dpnc (Table 2).

Enzyme determination

No significant differences were observed in PAL, POX and PPO activity between replicates 1 and 2 (t = 0.01, *P* = 0.98 for PAL; t = 1.93, *P* = 0.091 for POX and t = 0.46, *P* = 0.64 for PPO).

PAL activity assay: PAL activity in the EAHB cv Nabusa and Yangambi remained significantly similar over a period of 4 months, with a few exceptions (Fig. 1). Three months after the first assay was performed (30 dpnc), PAL activity was significantly higher in roots of the cv Nabusa compared with the Yangambi (t = 7.75, *P* < 0.0001). Also, PAL activity in Nabusa was significantly higher at 3 months than at any other time point measured (Fig. 1). PAL activity 4 months after the first measurement (60 dpnc) remained significantly higher than that initially taken (7 dai) (t = 3.10, *P* = 0.0033) and taken after 2 months (7 dpnc) (t = 2.73, *P* = 0.0088). In Yangambi, PAL activity was gradually reduced (non-significantly) in the first 3 months, and increased significantly 4 months after the first assay.

When inoculated with the endophyte V5w2 CHR 9, PAL activity in Nabusa (susceptible cv) was significantly reduced compared with endophyte non-inoculated control plants after 7 days (F = 4.55, *P* = 0.045) (Fig. 1). PAL activity in endophyte-inoculated plants only increased significantly after 4 months (60 dpnc) (t = 8.43, *P* < 0.0001). In Yangambi, PAL activity was not significantly different between endophyte-inoculated and non-inoculated control plants 7 dai. Thereafter PAL activity increased significantly at 60 dpnc (t = 6.63, *P* < 0.0001). Nabusa and Yangambi responded differently pertaining to PAL production when challenged with ***R. similis*** than when inoculated with V5w2 CHR 9. In Nabusa, PAL activity was similar in plants challenged and non-challenged with ***R. similis***, except at 30 dpnc (Fig. 1). This, however, is the same point in time that PAL activity was expressed significantly higher in non-endophyte inoculated Nabusa plants than at other time intervals. In

Yangambi, PAL activity was significantly higher in *R. similis*-challenged plants 7 dpnc ($t = 8.66$, $P < 0.0001$), but not at any of the other time intervals.

PAL activity was significantly up-regulated in endophyte-inoculated and nematode-challenged Nabusa plants 30 dpnc compared with endopyte non-inoculated plants (2.3 fold) ($t = 2.56$, $P = 0.014$), endophyte-inoculated but nematode non-challenged plants (8.1 fold) ($t = 11.23$, $P < 0.0001$) and endophyte non-inoculated but nematode challenged plants (5.3 fold) ($t = 10.19$, $P < 0.0001$) (Fig. 1). The enzyme, however, was not significantly up-regulated at any other time interval. In Yangambi, PAL activity was significantly higher 7 dpnc in endophyte-inoculated plants challenged with *R. similis,* compared with endophyte-inoculated and *R. similis* non-challenged plants ($t = 2.47$, $P = 0.017$), and endophyte non-inoculated and *R. similis* non-challenged plants ($t = 3.61$, $P = 0.0007$). However, when compared with endophyte non-inoculated control plants challenged with the nematode, no significant differences were observed in PAL activity.

POX activity assay: The expression of POX in endophyte non-inoculated and nematode non-challenged plants did not differ significantly over a period of 4 months in the *R. similis*-susceptible banana cv Nabusa (Fig. 2). In the *R. similis*-tolerant Yangambi, however, POX activity increased steadily (non-significantly) until 30 dpnc, and then reduced significantly after 4 months (60 dpnc). POX activity in Yangambi was also significantly higher at two time intervals; 7 ($t = 2.52$, $P = 0.012$) and 30 ($t = 3.05$, $P = 0.0027$) dpnc, compared with that in Nabusa plants (Fig. 2).

Endophyte inoculation significantly induced POX activity in cv Nabusa ($F = 15.13$, $P < 0.0001$) plants 7 dai compared with endophyte non-inoculated plants (Fig. 2). Enzyme activity remained high 7 and 30 dpnc in Nabusa, but was significantly reduced after 4 months (60 dpnc). Similarly, POX activity was significantly up-regulated in Yangambi 7 dai ($F = 4.52$, $P < 0.0001$). However, POX activity was significantly reduced 2 months after endophyte inoculation (7 dpnc), but increased significantly thereafter, though much less than levels at 7 dai.

In Nabusa, POX activity increased by 2- fold 7 dpnc in endophyte-inoculated plants following *R. similis* challenge, compared with endophyte non-inoculated plants

challenged with *R. similis* (t = 3.39, *P* = 0.0009) (Fig. 2). The increased POX activity observed following *R. similis* challenge was not significantly different from that for endophyte-inoculated only plants. For the tolerant banana Yangambi, POX activity was significantly higher in endophyte-inoculated and *R. similis*-challenged plants at 7 dpnc, compared with endophyte-inoculated only plants (t = 2.27, *P* = 0.025). Interestingly, for Yangambi, POX activity was significantly reduced in endophyte-inoculated plants challenged with *R. similis* compared to endophyte non-inoculated plants challenged with the nematode 7 dpnc (t = 2.76, *P* = 0.0064) and non-significantly at 60 dpnc.

PPO activity assay: PPO activity in Nabusa and Yangambi control plants was reduced over time. Significantly more PPO was produced at the first enzyme assay (7 dai) than at 7 dpnc (t = 3.37, *P* = 0.0010), 30 dpnc (t = 3.79, *P* = 0.0002) and 60 dpnc (t = 6.32, *P* < 0.0001) (Fig. 3). In Yangambi, PPO activity was also significantly reduced from 7 dai to 7 dpnc (t = 6.31, *P* < 0.0001), 30 dpnc (t = 8.23, *P* < 0.0001) and 60 dpnc (t = 8.41, *P* <0.0001) (Fig. 3). PPO activity did not differ between Nabusa and Yangambi other than at 30 dpnc where PPO activity was significantly higher in Yangambi than in Nabusa (t = 3.38, *P* = 0.0009).

Nabusa plants, when inoculated with V5w2 CHR 9, had a significantly increased PPO activity compared to endophyte non-inoculated plants 7 dai (F = 15.37, *P* < 0.0001) (Fig. 3). The opposite was true for Yangambi in which endophyte-inoculated plants produced less PPO than endophyte non-inoculated plants (F = 4.27, *P* < 0.0001). No significant differences were observed in PPO activity between the two treatments at 7, 30 and 60 dpnc (Fig. 3). When control plants were challenged with *R. similis*, PPO activity was reduced significantly 30 dpnc in Nabusa. In the tolerant cv, however, activity increased significantly by 86% 30 dpnc (t = 2.77, *P* = 0.0063) and 81% 60 dpnc (t = 2.42, *P* = 0.017), compared with non-challenged control plants (Fig. 3). Endophyte inoculation did not prime PPO activity for up-regulation upon *R. similis* challenge in either Nabusa or Yangambi.

DISCUSSION

The role of PAL, POX and PPO in protecting susceptible (cv Nabusa) and tolerant (cv Yangambi (Km-5) banana plants against ***R. similis*** was demonstrated in the current study. In addition, the role of the non-pathogenic ***F. oxysporum*** endophyte V5w2 CHR 9 in inducing and priming defence-related enzymes in banana was validated. Protection of cv Nabusa against ***R. similis*** proliferation seems to be dependant on inoculation of banana roots with strain V5w2 CHR 9, while enzyme up-regulation in cv Yangambi was attained in absence or presence of the endophyte.

PAL production was reduced significantly in the susceptible cv Nabusa 7 dai, while there was no significant difference in PAL activity between endophyte inoculated and non-inoculated Yangambi plants. However, PAL activity increased significantly 60 dpnc in both. This slow up-regulation of PAL in nematode non-challenged plants is indeed an interesting, but inexplicable, phenomenon. Firstly, endophytic ***F. oxysporum*** could not be isolated from banana roots at this late stage, and no nematodes were inoculated to challenge the plants' defence responses. There was, thus, no apparent reason why PAL should be so strongly up-regulated in banana plants, at such a stage after endophyte inoculation. In the instance where PAL was highly up-regulated in endophyte-inoculated and ***R. similis*** challenged cv Nabusa plants 30 dpnc, plant defence responses could have been primed in an effort to significantly suppress nematode reproduction. Secondly, PAL activity generally appears to be expressed relatively rapidly following plant colonization by microorganisms (He ***et al.***, 2002; Shoresh ***et al.***, 2005), indicating their role in plant defence responses. For example PAL activity increased in barley (***Hordeum vulgare*** L.) roots within 24 h of root colonization by the pathogen ***Erysiphe graminis*** f. sp. ***hordei*** (EM. Marchal) and non-pathogen ***Erysiphe pisi*** DC. Similarly, endophytic ***Trichoderma asperellum*** Samuels, Hieckfeldt and Nirenberg colonization of cucumber (***Cucumis sativus*** L.) roots resulted in increased PAL activity in roots 1 and 24 h after inoculation (Shoresh ***et al.***, 2005). This was not the case in the current study.

The priming of PAL by isolate V5w2 CHR 9 following ***R. similis*** challenge in cv Nabusa potentially indicates the involvement of PAL in nematode resistance in

banana. However, in cv Yangambi PAL activity increment seemed to be in response to *R. similis* challenge, as increased PAL production was observed in both endophyte-inoculated and endophyte non-inoculated plants challenged with the nematode. In an earlier study (Chapter 2), *PAL* gene activity was not altered 2 dpnc. This may have been because the nematodes had not entered the roots, as this study demonstrated the presence of only a few nematodes in roots 7 dpnc. PAL is involved in the synthesis of cell wall bound phenolic compounds, and Athman (2006) reported increased phenolic deposition in roots of cv Nabusa following *R. similis* challenge of endophyte-inoculated plants. Wuyts *et al.* (2006) also reported increased PAL activity 7 dpnc in cv Yangambi. Up-regulation of *PAL* transcript levels following pathogen challenge has been reported in other plants as well. In beans (*Phaseolus vulgaris* L.), for instance, root colonization by *G. intraradices* did not affect *PAL* transcript levels, but subsequent challenge with *Rhizoctonia solani* Kühn resulted in significant up-regulation of *PAL* transcripts in mycorrhizal plants (Guillon *et al.*, 2002).

Transient up-regulation of POX activity was observed in both the susceptible cv Nabusa and tolerant cv Yangambi following endophyte inoculation of roots. The up-regulated activity reduced to the levels in non-inoculated plants 2 months after endophyte inoculation (7 dpnc). In an earlier study, transient up-regulation of *POX* gene activity was observed 2 dai of cv Kayinja plants (tolerant to *R. similis*) with isolate V5w2, with POX activity reduced to levels in non-inoculated plants a month later (Chapter 2). This indicates that the initial plant reaction towards colonization by fungal endophytes is a defence response, as POX is known to be a key enzyme in the early oxidative response of plants to pathogens (Blilou *et al.*, 2000). Evidence of the occurrence of an oxidative burst following fungal endophyte root colonization has been provided. Development of a hypersensitive response (Douds *et al.*, 1998) and production of reactive oxygen species (H_2O_2) around hyphal tips (Salzer *et al.*, 1999) were reported for endomycorrhizal root colonization in alfalfa (*Medicago sativa* L.) and *M. truncatula* Gaerth, respectively. In a related study involving the endo-mycorrhizal fungus (AMF) *Glomus mosseae* (Nicol. and Gerd), POX activity was up-regulated in tobacco roots (*Nicotiana tabacum* L.) during appresoria formation (Blilou *et al.*, 2000). The up-regulated activity, however, reduced to the level in the control 11 dai.

Radopholus similis challenge of endophyte non-inoculated cv Nabusa plants only significantly increased POX production 60 dpnc, but resulted in a non-significant up-regulation of activity in cv Yangambi 7 dpnc. In contrast, when the banana plants were inoculated with the endophyte, *R. similis* challenge increased POX activity significantly 7 dpnc compared to non endophyte-inoculated and *R. similis*-challenged plants in cv Nabusa, but not in Yangambi. These findings support the existence of endophyte-mediated defences in the nematode-susceptible cv Nabusa against *R. similis*. Nematode challenge of endophyte-inoculated cv Yangambi plants resulted in increased POX activity 7 dpnc when compared with plants inoculated with the endophyte but not challenged with the nematode. However, this was significantly lower when compared with POX activity for the control plants. Nematode challenge of control plants resulted in a non-significant increment of POX, compared with non-challenged controls. In agreement with our finding, Wuyts *et al.* (2006) observed increased POX activity in roots of *R. similis* challenged (endophyte-free) cv Yangambi 7 dpnc, compared to the constitutive expression. Aguilar *et al*. (2000) further reported increased POX activity in a tolerant banana cv 24 hours following challenge with *F. oxysporum* f.sp. *cubense*.

The constitutive expression of PPO was similar between cv Nabusa (susceptible) and cv Yangambi (tolerant) 7 dai, but was then significantly reduced over time. Endophyte inoculation, however, had contrasting effects on PPO activity in cv Nabusa and Yangambi. In cv Nabusa, endophyte inoculation resulted in transient up-regulation of PPO activity, but in a significant down-regulation in cv Yangambi 7 dai. After that, PPO activity declined over time in both banana cultivars. Nematode challenge significantly up-regulated PPO activity only 30 dpnc in endophyte non-inoculated cv Yangambi plants compared with non-challenged control plants. This increment, however, was still lower than the constitutive expression of PPO 7 dai. Wuyts *et al.* (2006) reported increased PPO activity 7 dpnc in cv Yangambi, the only time in the course the experiment when activity was significantly higher than the constitutive expression. In another study, Matielle (1994) reported significantly higher PPO activity in the nematode-susceptible cv Poyo (*M. acuminata* AAA), compared with the tolerant cv Gros Michel (*M. acuminata* AAA) 2 months after nematode challenge. In the event of wounding, PPO rapidly polymerizes to produce black/brown pigments which are involved in wound sealing to limit secondary

infection and pathogen spread. In our study and that of Wuyts *et al*. (2006) and Matielle (1994), constitutive PPO expression is observed to be high. Based on the function of PPO, we speculate that any increments following pest/pathogen challenge are associated with the extent of wounding. Thus, the generally low PPO levels in our study following nematode challenge may be attributed to the low percentage root necrosis observed.

The reduction of ***R. similis*** population densities in endophyte-inoculated banana roots can be attributed to the increased activities of PAL and POX in the susceptible cv Nabusa. Their reduction in Yangambi, also, can be associated with an increase in PAL, POX and PPO activity. It, therefore, appears that PAL, POX and PPO play an important role in banana defence responses against ***R. similis***. Secondly, in the susceptible cultivar the enzymes are primed by non-pathogenic ***F. oxysporum*** endophyte V5w2 CHR 9 for up-regulation following ***R. similis*** challenge. Isolate V5w2 CHR 9 was re-isolated at high frequencies from roots of endophyte-inoculated plants, and has previously been shown to induce systemic resistance in banana against ***R. similis*** (Athman, 2006). It, therefore, is a good candidate for the bio-enhancement of banana tissue culture plants against ***R. similis***.

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Table 1. Nematode densities in roots of banana cv. Nabusa (AAA-EA) and Yangambi (AAA) following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with 500 *Radopholus similis* females and juveniles

Cultivar	Treatment[1]	7 DPNC[2]		30 DPNC[2]		60 DPNC[2]	
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
Nabusa	Control + RS	0	0	8,746.5 ± 2800 a	83.3 ± 83.3 a	11,7367.7 ± 6361.4 a	6,913.9 ± 881.5 a
Nabusa	E + RS	0	83.3 ± 83.3 a	4,248.3 ± 594.7 a	0	999.6 ± 140.2 b	3,915.1 ± 546.1 b
Yangambi	Control + RS	0	249.9 ± 125.1 a	8,080.1 ± 546.2 a	0	6,080.9 ± 304.6 ba	499.8 ± 381.8 c
Yangambi	E + RS	0	83.3 ± 83.3 a	7,247.1 ± 746.5 a	499.8 ± 381.8 a	3,415.3 ± 182.1 b	249.9 ± 125.1 c

Values represent means ± SE.

[1] Control = endophyte non-inoculated plants, RS = *Radopholus similis*-challenged plants and E = plants inoculated with endophytic isolate V5w2 CHR 9.

[2] DPNC = days post nematode challenge.

Means within a column followed by different letters are significantly different ($P \leq 0.05$, Tukey's studentized range test).

Table 2. Percentage necrosis for roots of banana cv Nabusa (AAA-EA) and Yangambi (AAA) following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with 500 *Radopholus similis* females and juveniles

Cultivar	Treatment[1]	7 DPNC[2]	30 DPNC[2]	60 DPNC[2]
Nabusa	Control + RS	5.5 ± 3.1 a	23.5 ± 2.4 a	34.5 ± 10.1 a
Nabusa	E + RS	4.5 ± 1.6 a	22.0 ± 5.0 a	19.0 ± 5.1 a
Yangambi	Control + RS	8.0 ± 3.6 a	27.0 ± 5.0 a	38.0 ± 5.8 a
Yangambi	E + RS	3.0 ± 2.5 a	27.0 ± 4.7 a	22.5 ± 2.3 a

Values represent means ± SE.

[1] Control = endophyte non-inoculated plants, RS = *Radopholus similis*-challenged plants and E = plants inoculated with endophytic isolate V5w2 CHR 9.

[2] DPNC = days post nematode challenge.

Means within a column followed by different letters are significantly different ($P \leq 0.05$, Tukey's studentized range test).

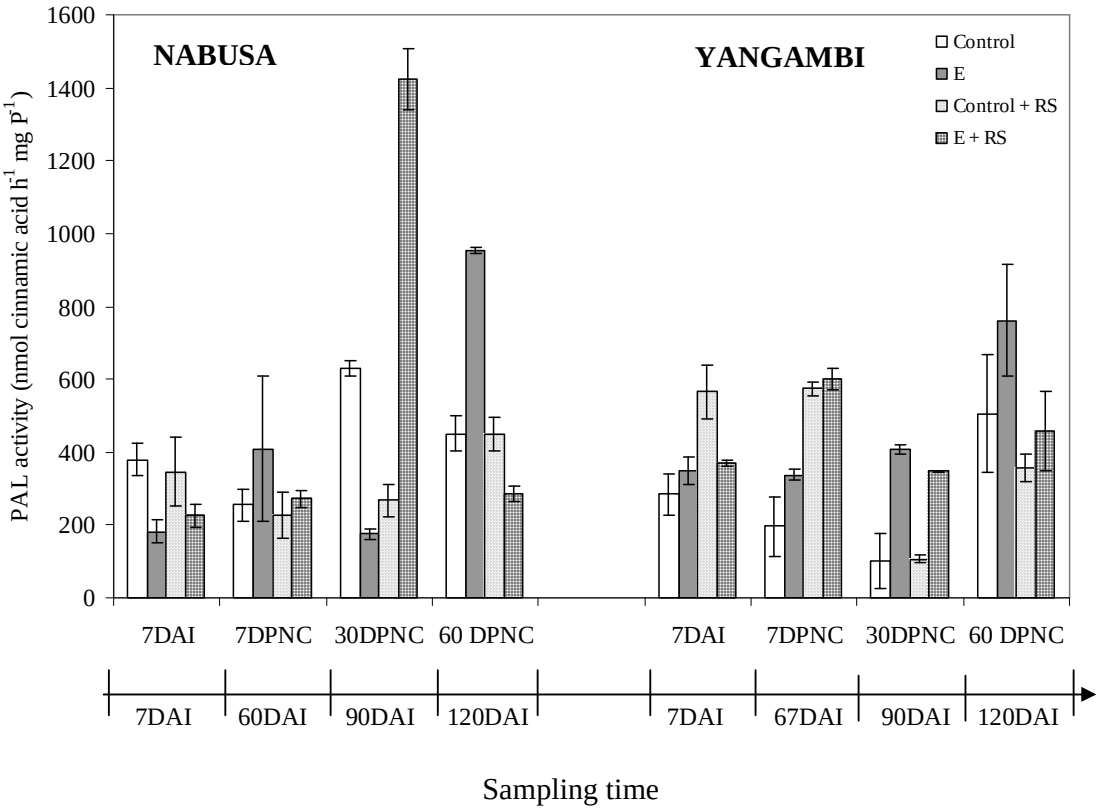

Figure 1. Phenylalanine ammonia-lyase activity in roots of banana cv Nabusa (AAA-EA) and Yangambi (AAA) at different time intervals following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with 500 *Radopholus similis* females and juveniles. Sampling time DAI = days after endophyte inoculation, dpnc = days post nematode challenge, Control = plants dipped in sterile water, E = plants inoculated with endophytic isolate V5w2 CHR 9 and RS = plants challenged with *R. similis.*

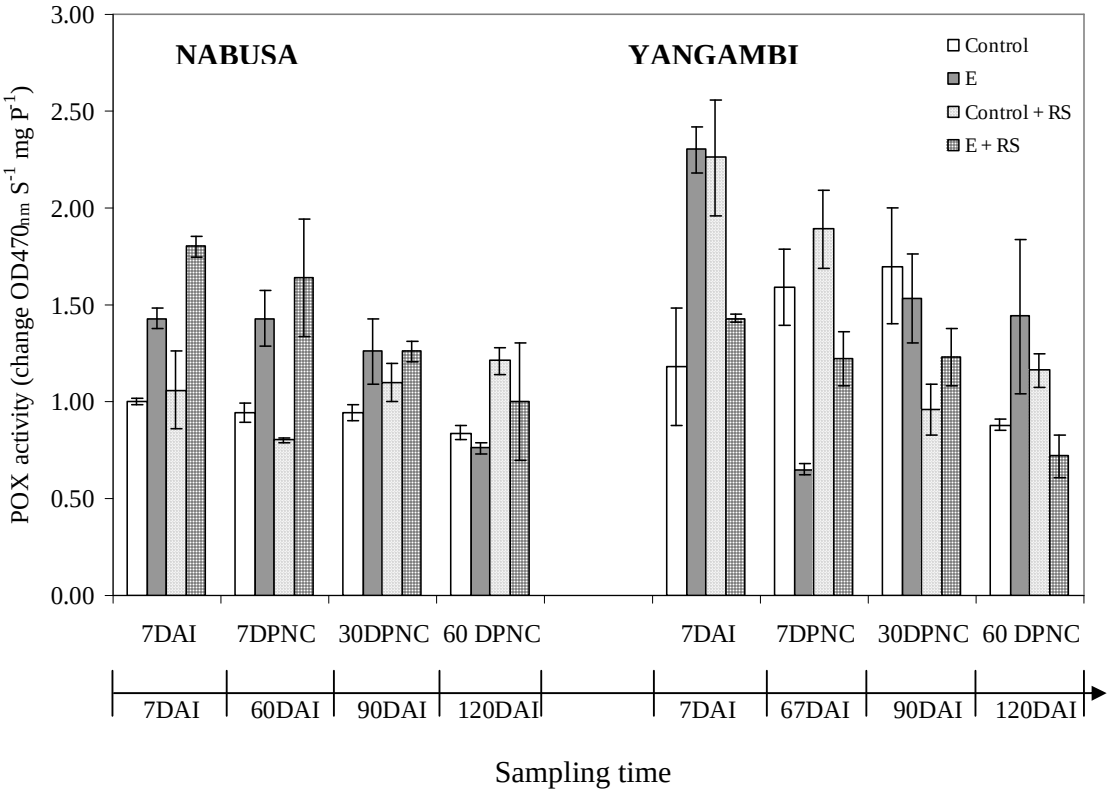

Figure 2. Peroxidase activity in roots of banana cv Nabusa (AAA-EA) and Yangambi (AAA) at different time intervals following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with 500 *Radopholus similis* females and juveniles. Sampling time DAI = days after endophyte inoculation, dpnc = days post nematode challenge, Control = plants dipped in sterile water, E = plants inoculated with endophytic isolate V5w2 CHR 9 and RS = plants challenged with *R. similis.*

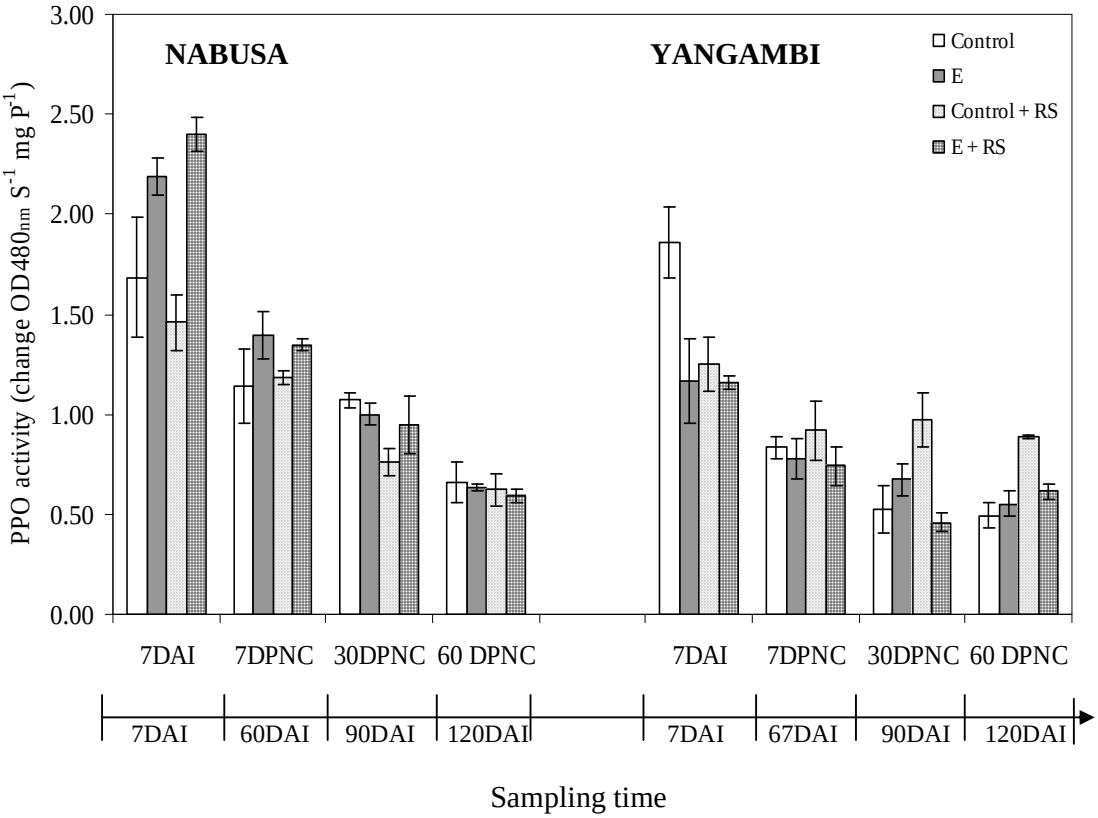

Figure 3. Polyphenol oxidase activity in roots of banana cv Nabusa (AAA-EA) and Yangambi (AAA) at different time intervals following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with 500 *Radopolus similis* females and juveniles. Sampling time DAI = days after endophyte inoculation, dpnc = days post nematode challenge, Control = plants dipped in sterile water, E = plants inoculated with endophytic isolate V5w2 CHR 9 and RS = plants challenged with *R. similis.*

CHAPTER 5

Marking endophytic non-pathogenic *Fusarium oxysporum* isolates for chemical resistance and with fluorescent proteins for use in plant colonization studies

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Chapter submitted to Biocontrol Journal

ABSTRACT

Non-pathogenic isolates of *Fusarium oxysporum* are known to colonize plant roots endophytically. To study the colonization patterns and distribution of artificially inoculated *F. oxysporum* endophytes in plants, it is necessary to be able to distinguish inoculated isolates from naturally occurring *F. oxysporum*. In the current study, *F. oxysporum* isolates were transformed with the green (GFP) and red fluorescent protein (DsRed) genes, and benomyl- and chlorate-resistant isolates were also developed. The benomyl- and chlorate-resistant mutants, and the fluorescently-labelled transformants, were able to grow on potato dextrose agar amended with 20 mg Benlate® l^{-1}, 30 g chlorate l^{-1} and 150 µg hygromycin ml^{-1}, respectively. Single spores of all mutants remained stable after several transfers on non-selective media. Most mutants and transformants produced colony diameters that did not differ significantly from that produced by their wild-type progenitors after 7 days of growth on non-selective media. A few, however, had growth rates that differed significantly from the wild-types. Plant colonization studies showed that root and rhizome tissue colonization by most benomyl- and chlorate-resistant mutants was similar to that of their wild-type isolates. Unlike GFP transformants, DsRed transformants were difficult to visualize *in planta*. Both the mutants and transformants can be used for future studies to investigate colonization, distribution and survival of *F. oxysporum* endophytes in banana plants.

INTRODUCTION

Fusarium oxysporum Schlecht.: Fries is responsible for Fusarium wilt diseases of many important agricultural crops. Some strains of *F. oxysporum*, however, occur as saprophytes in the environment and colonize healthy plant roots as non-pathogenic endophytes (Olivain and Alabouvette, 1999). Several non-pathogenic *F. oxysporum* endophytes have been isolated from East African Highland banana (*Musa* spp., genomic group AAA-EA) in farmers' fields in Uganda (Schuster *et al*., 1995; Griesbach, 2000). These isolates have been successfully re-introduced into tissue-cultured banana plants (Griesbach, 2000; Niere, 2001; Paparu *et al*., 2006a), and some have demonstrated potential to control pests such as *Radopholus similis* (Cobb) Thorne (Athman, 2006).

Colonization of plant roots and rhizomes by artificially inoculated non-pathogenic *F. oxysporum* endophytes has proved difficult to study. In banana, roots and rhizomes often become infected with additional saprophytic strains of the same species, making it difficult to distinguish introduced isolates from naturally occurring strains (Griesbach, 2000; Niere, 2001; Paparu *et al*., 2006a). The endophytes of interest, however, can be marked before they are inoculated into roots. Benomyl-resistant mutants, for instance, have been widely used for ecological studies of many fungal species. Coates *et al*. (1993) used benomyl-resistant mutants of *Colletotrichum gloeosporioides* Penz. and Sacc. to demonstrate latency in avocado fruit (*Persea americana* Mill.), and Postma and Luttikholt (1993) used *Fusarium* isolates resistant to benomyl to study control of fusarium wilt of carnation (*Dianthus caryophyllus* L.). Chlorate-resistant mutants of *F. oxysporum* can also be used for *in vivo* colonization studies (Puhalla, 1985; Zamani *et al*., 2004). According to Puhalla (1985), most chlorate-resistant mutants are stable, with only 2-4% reversion to the wild-type. However, there is no report on the use of chlorate mutants in plant colonization studies.

The green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* (Murbach and Shearer) and the red fluorescent protein (DsRed) gene from the coral *Discosoma* sp. have been widely used to study plant-microbe interactions. The GFP gene has been successfully used to study *in planta* biology of phytopathogenic fungi, including *Magnaporthe grisea* (T.T. Hebert) M.E. Barr in barley (*Hordeum vulgare* L.) (Bourret *et al*., 2002; Czymmek *et al*., 2002), *F. oxysporum* f.sp. *radicis-lycopersici* in tomato (*Lycopersicon esculentum* L.) (Lagopodi *et al*., 2002) and *F. oxysporum* f.sp. *cubense* W.C. Snyder and H.N. Hansen in banana (Visser *et al*., 2004). Transformation of filamentous fungi with the DsRed genes

(DsRed-Express and DsRed2) is a more recent development (Mikkelsen *et al*., 2003; Nahalkova and Fatehi, 2003). Nahalkova and Fatehi (2003) marked *F. oxysporum* f.sp. *lycopersici* W.C. Snyder and H.N. Hansen with the DsRed2 gene to study the infection process of tomato roots, whereas Olivain *et al.* (2006) studied the colonization of tomato roots by pathogenic and non-pathogenic *F. oxysporum* isolates labelled with the DsRed2 and GFP genes, respectively. Both the GFP and DsRed proteins show stable and species-independent fluorescence and their visualization is fast, effectively permitting real-time analysis of plant-fungal interactions. The spectral properties of these proteins are also distinct enough so that they can be viewed simultaneously within the same sample (Mikkelsen *et al*., 2003).

The first objective of the current study was to mark *F. oxysporum* endophytic isolates by generating *F. oxysporum* isolates that (1) are benomyl and chlorate resistant and (2) express the fluorescent protein genes GFP and DsRed. The second objective was to characterize the mutants and transformants with regard to *in vitro* mycelial growth rate, colony colour and colonization of banana root and rhizome tissue.

MATERIALS AND METHODS

Fungal isolates

Non-pathogenic *F. oxysporum* endophytes isolated from East African Highland banana plants in Uganda (Schuster *et al*., 1995) were used to generate chemical-resistant mutants and fluorescent protein transformants. The five wild-type isolates used included Emb2.4o, Eny1.31i, Eny7.11o, V4w5 and V5w2. These isolates have shown potential as biocontrol agents against the banana weevil (*Cosmopolites sordidus* (Germar)) and the banana root nematode (*R. similis*) (Griesbach, 2000; Niere, 2001; Athman, 2006), and are currently being further assessed at the International Institute of Tropical Agriculture (IITA) in Uganda. All isolates are maintained in soil and on filter paper.

Generation of benomyl-resistant mutants

Benomyl-resistant mutants of non-pathogenic *F. oxysporum* isolates were generated using a modified method of Coates *et al.* (1993). The isolates were each grown on half strength potato dextrose agar (PDA) (19 g PDA (Sigma-Aldrich, Steinheim, Germany) and 19 g agar (Sigma-Aldrich, Steinheim, Germany) l^{-1} distilled water) in 90-mm-diameter Petri dishes, and incubated in the laboratory at approximately 25^{o}C and a photoperiod of 12/12 h light/dark routine for 7 days. To generate benomyl-resistant mutants, 20 ml sterile distilled water (SDW)

was added to each culture and their surfaces scraped to harvest spores and mycelia. The suspensions were filtered through sterilized cheesecloth into 1000-ml sterile beakers, and then adjusted to final concentrations of 3-8 x 10^6 spores ml^{-1}. Ten ml of each spore suspension was then dispensed into empty Petri dishes and exposed to UV radiation (Philips 15W, 253 nm) at a distance of 70 mm for 3 min. After irradiation, the spores were spread on PDA containing 20 mg 50% Benlate® (Du Pont De Nemours, Paris, France) l^{-1} PDA. Petri dishes were inverted and incubated at 25°C in the dark, checking for emergence of resistant colonies between 7-10 days. Benomyl-resistant mutants were sub-cultured on PDA containing 20 mg 50% Benlate®, single-spored and stored on filter paper and in Eppendorf tubes in 15% glycerol at -60°C (Leslie and Summerell, 2006).

Generation of chlorate-resistant mutants

The method described by Puhalla (1985) was used to generate chlorate-resistant mutants. Emb2.4o and V5w2 were cultured on PDA containing 30 g potassium chlorate l^{-1} PDA in the laboratory for 7-14 days. Fast growing sectors of each isolate were putatively selected as chlorate resistant and sub-cultured on minimal media (MM) (Correll *et al*., 1987). Single spore isolates were cultured from mutants that grew thinly on MM, and stored on filter paper and in Eppendorf tubes in 15% glycerol at -60°C (Leslie and Summerell, 2006).

Transformation with GFP and DsRed genes

The method used for transformation of *F. oxysporum* isolates was based on the method of Lu *et al*. (1994), using a few modifications.

Preparation of protoplasts: Spore suspensions were prepared for *F. oxysporum* isolates Emb2.4o and V5w2 as described above. A 100-ml spore suspension of 2 x 10^6 spores ml^{-1} was mixed with 100 ml of 2 × YEG (0.8 g yeast extract and 4 g glucose in 200 ml distilled water). This suspension was dispensed in 100 ml aliquots into 200-ml Erlenmeyer flasks that were rotated at 50 rpm at 30ºC for 5-6 h. At approximately 1% spore germination, the spores were collected by centrifugation at 805 g for 10 min. The supernatant was discarded and the pellet washed with 30 ml 0.7 M NaCl, using the same centrifugation settings. The pellet of germinating spores was subsequently mixed with cell wall-degrading enzymes prepared in 20 ml NaCl (0.7 M) and shaken at 50 rpm at 30ºC for 2 h. The enzyme solution consisted of 1.42 mg chitinase (Sigma-Aldrich, St. Louis, USA), 0.67 g driselase (Sigma-Aldrich), 1 g lysing enzyme (Sigma-Aldrich), 0.8 g ß-1,3-glucanase (InterSpex Products, San Mateo, USA) and 0.15 g cellulase (Yakult Pharmaceuticals, Tokyo, Japan). Prior to adding all the enzymes to

the 20 ml NaCl, the starch carrier present in the driselase was removed by first incubating the driselase suspended in 0.7 M NaCl on ice for 15 min, followed by centrifugation at 652 g for 5 min. The supernatant was decanted, and the remainder of the enzymes added to the supernatant.

Protoplast formation was checked under the microscope (20× magnifications) every hour. When protoplasts were released from at least 75% of the germinating spores, usually after 1-2 h, the solution was spun at 652 g for 10 min at 5ºC. The supernatant was discarded and the pellet washed with 30 ml 0.7 M NaCl, by spinning the solution at 652 g for 10 min at 5ºC. Subsequently, the pellet was washed twice in 30 ml ice cold STC buffer [54.65 g sorbitol, 1.84 g $CaCl_2$, 5 ml 0.5M Tris-HCl (pH 7.5) and 250 ml distilled water] using the same centrifugation settings. The protoplast pellet was re-suspended in 700 µl STC and placed on ice.

Fungal transformation: Emb2.40 and V5w2 were both transformed with GFP and DsRed-Express genes. GFP transformants were obtained by transforming protoplasts with vector pCT74 (kindly provided by J.M. Lorang, Oregon State University, Corvallis, USA), which confers versatile, high-level constitutive gene expression in Ascomycota fungi (Lorang *et al.*, 2001). Vector pCT74 expresses GFP under control of the *Tox*A promoter of *Pyrenophora tritici-repentis* (Died.) Drechsler, and also contains a selectable marker gene, hygromycin B phototransferase (*hyg*B), under control of the *trpC* promoter of *Aspergillus nidulans* G. Winters. DsRed transformants were obtained through co-transformation with vector pPgpd-DsRed (kindly provided by L. Mikkelson, Royal Veterinary and Agricultural University, Frederiksberg, Denmark) and vector pHyg8. In vector pPgpd-DsRed the DsRed-Express gene is under control of the constitutive promoter *PgpdA* and the terminator *trpC*, which both originate from *A. nidulans* (Mikkelsen *et al.*, 2003). Co-transformation of protoplasts with vector pPgpd-DsRed and vector pHyg8, containing the *hyg*B resistance gene, was required since pPgpd-DsRed does not contain the *hyg*B resistance gene. Vector pHyg8 (A. McLeod., Stellenbosch University, Matieland, South Africa) was constructed by cloning the blunt-ended *Sal*I fragment from pCT74, containing the *hyg*B gene driven by the *A. nidulans trp*C promoter, into pBluescript (Stratagene, La Jolla, USA) digested with *Eco*RV.

Protoplasts were transformed using a polyethylene glycol/calcium chloride method (Lu *et al.*, 1994). One hundred µl of the protoplasts was placed in 12-ml test tubes containing 20 µg pCT74 (for obtaining GFP transformants) or 20 µg pPgpd-DsRed and 10 µg pHyg8 (for

obtaining DsRed-Express transformants). The mixtures were incubated for 10 min on ice, after which three aliquots (200, 200 and 800 µl) of PEG/Tris (12 g PEG, 400 µl 500 mM Tris-HCl [pH 7.5], 1 ml 1 M $CaCl_2$ and 250 ml SDW) were added to each transformation tube. The transformation reactions were incubated at 25ºC for 10 min, before adding 2 ml STC.

Selection of transformants: Seven hundred µl of the transformed protoplasts were gently mixed into 20 ml regeneration media (50^{o}C) in a 90-mm-diameter Petri dish, using a pipette tip. Regeneration media consisted of three separate solutions (A, B and C) that were combined after autoclaving. Solution A consisted of 250 mg yeast extract, 250 mg casein hydrolysate and 12.5 ml distilled water, solution B contained 85.5 g sucrose and 12 ml distilled water, and solution C contained 4 g agar and 112.5 ml distilled water. Petri dishes were incubated at 25ºC overnight, and overlaid the following morning with 1% water agar containing 150 µg hygromycin ml^{-1} (Calbiochem, La Jolla, CA) and incubated for 7-10 days at 25ºC. Emerging transformant colonies were transferred to freshly prepared PDA containing 150 µg hygromycin ml^{-1}.

The fluorescence of transformants was assessed using an epifluorescence microscope (Carl Zeiss, Mannheim, Germany). The microscope is equipped with filter set 10 (488010-0000) and filter set 15 (488015-0000) with spectral properties matching those of GFP (450-490 nm excitation, 515-565 nm emission) and DsRed-Express (512/46 nm excitation, 590 nm emission). Images were captured using an AxioCam HR camera (Carl Zeiss).

Several transformant colonies that showed GFP and DsRed fluorescence were single-spored on water agar amended with 150 µg hygromycin ml^{-1}. The single-spored transformants were sub-cultured five times on PDA to determine the stability of fluorescence. Cultures that showed high and stable fluorescence were stored in 15% glycerol in Eppendorf tubes at -60^{o}C (Leslie and Summerell, 2006).

Growth of mutant isolates and fluorescent transformants on PDA

Colony morphology and growth rates of all endophytic *F. oxysporum* mutants (benomyl- and chlorate-resistant) and fluorescent transformants that remained stable on selective media were compared to their respective wild-type isolates on PDA. Each mutant, transformant and wild-type isolate was inoculated from frozen glycerol stocks onto a 90-mm-diameter Petri dish containing PDA. Petri dishes were incubated at 25°C with 12/12 h light/dark routine for 7 days. An agar block (0.5 cm^{3}) from fully grown colonies was then transferred to the centre of

a 90-mm-diameter PDA Petri dish and incubated for 7 days under the above conditions, using five replications per isolate. Colony diameters were measured with a digital calliper (Mitutoyo Ltd, Hampshire, UK) on day 7 after inoculation.

Colonization of tissue culture plants by benomyl- and chlorate-resistant mutants

Root and rhizome colonization by benomyl-resistant mutants was studied using the banana Yangambi (Km-5) (AAA) plants, while that by chlorate-resistant mutants was studied using cv Nabusa (AAA-EA) plants. Tissue culture plantlets were propagated using a standard shoot-tip culture protocol for banana (Vuylsteke, 1998). Four weeks after rooting, tissue culture plants were removed from the rooting medium and their roots and rhizomes washed with tap water. For all the treatments, plants were grown in a hydroponic system in 250-ml plastic containers to enhance root growth (Paparu *et al.*, 2006a). The plants were removed from the hydroponic system after 4 weeks and their roots washed in sterile distilled water.

Spore suspensions (1.5×10^6 spores ml^{-1}) for plant inoculation were prepared as described above. Only mutants that had colony and growth characteristics similar to the wild-type isolates were considered for root and rhizome colonization studies. Benomyl-resistant mutants of *F. oxysporum* isolates Emb2.4o (BR 1, BR 2, BR 3 and BR 8), Eny1.31i (BR 1, BR 3, BR 4 and BR 5), Eny7.11o (BR 1, BR 2, BR 4 and BR 7), V4w5 (BR 1, BR 2, BR and BR 4) and V5w2 (BR 1, BR 4, BR 6 and BR 8), and chlorate-resistant mutants of Emb2.4o (CHR 2, CHR 3, CHR 4 and CHR 6) and V5w2 (CHR 2, CHR 4 CHR 9 and CHR 12) were each inoculated into four plants. Banana roots and rhizomes were dipped in spore suspensions for 4 h and returned to the hydroponic system. For a positive control, each wild-type isolate was inoculated into four plants, and for the negative control four plants were dipped in SDW. All experiments were conducted twice.

The plants were removed from their hydroponic cups 4 weeks after inoculation, their roots and rhizomes rinsed with tap water and stored overnight at 4^oC until re-isolation. Re-isolation of *F. oxysporum* was carried out by surface sterilizing the roots and rhizomes in 5% NaOCl for 1 min followed by 1 min in 75% EtOH. Roots were rinsed three times in SDW and placed on sterile filter paper. From each root (four per plant) and rhizome, six pieces per plant (approximately 0.5 cm long for roots and 0.5 cm^3 for rhizomes) were cut and inserted halfway in PDA supplemented with filter-sterilized novobiocin sodium salt (0.02 g l^{-1}) (Sigma-Aldrich), in 90-mm-diameter Petri dishes. These re-isolations were also done from the control plants (water and wild-type inoculated), as well as mutant inoculated plants. All *F. oxysporum*

isolated from mutant inoculated plants were sub-cultured on PDA containing the selective agent (50% Benlate® or chlorate). Petri dishes were incubated at 25°C with 12/12 h light/dark routine for 7 days. Colonies growing from plated root and rhizome pieces were viewed under a compound microscope (100× and 400× magnification). *Fusarium oxysporum* was identified based on its characteristic morphological features, such as the production of sickle-shaped macroconidia with attenuated apical cells, microconidia borne in false heads on short monophialides, and the formation of single or pairs of chlamydospores (Nelson *et al.*, 1983). To identify mutant isolates, all *F. oxysporum* growing from roots and rhizomes inoculated with the mutant isolates were sub-cultured on PDA containing 20 mg 50% Benlate® or 30 g potassium chlorate l^{-1} and isolates that still grew on these selective media were identified as benomyl- and chlorate-resistant, respectively.

Colonization of tissue culture plants by fluorescent transformants

Plants of cv Nabusa were used to study plant colonization by both GFP- and DsRed-transformed isolates. Eight plants were each inoculated with GFP transformants G 2F (Emb2.4o) and G 31 (V5w2), and DsRed transformants R 1D (Emb2.4o) and R 21 (V5w2). Transformants were inoculated as described above, and root samples harvested for microscopy at 1, 2, 3 and 7 days after inoculation. For each analysis two roots were sampled per plant. The entire experiment was conducted twice. For microscopic studies, hand-made longitudinal sections of approximately 0.5 cm long were cut from the root base, mid-root and root tip of each root. Up to 30 root sections were prepared for each root. Sections were placed on a microscope slide in a thin water film and held in position by a line of Vaseline placed on the slide. The Vaseline also helped in holding the cover slip over the root sections. Root colonization was studied using an epifluorescence microscope and a laser scanning confocal microscope (Carl Zeiss) equipped with filter sets with spectral properties matching those of GFP and DsRed. Images were captured using an AxioCam HR camera (Carl Zeiss).

Data analysis

Mean colony diameters of the mutated, transformed and wild-type *F. oxysporum* isolates were measured on day 7. Analysis of variance and multiple mean comparisons were performed using Tukey's studentized range test with SAS (SAS, 1989). Percentage root and rhizome colonization were compared between the experiments using logistic regression. Where repeat experiments yielded different results for a given variable, analysis was conducted separately. Differences in plant colonization among wild-types and their respective mutants and among mutant isolates generated from each wild-type were detected using Dunn-Sidak corrected

95% confidence interval at a significant α level of 0.0073 and 0.0085, respectively (SAS, 1989; Sokal and Rohlf, 1995).

RESULTS

Benomyl-resistant mutants

Between one and five benomyl-resistant colonies of *F. oxysporum* developed on each Petri dish following exposure of spores to UV irradiation. After the colonies were transferred to freshly-prepared Benlate®-containing PDA, stable mutants were chosen for further studies. Four benomyl-resistant mutants each were chosen for growth and plant colonization studies from Emb2.4o, Eny1.31i, Eny7.11o, V4w5 and V5w2. Colony colour and shape for all selected mutants was similar to that of the wild-type isolates (Fig. 1A and B). Colonies presented the characteristic purplish mycelium of *F. oxysporum* grown on PDA (Joffe, 1974). At 7 days, no significant difference was detected in colony diameter between most mutants and their respective wild-type isolates. However, mutants BR 8 of Emb2.4o; BR 1 and 4 of Eny1.31i; BR 7 of Eny7.11o; BR 1, 3 and 4 of V4w5 and BR 4 and 6 of V5w2 grew significantly slower than the wild-type isolates. Mutants BR 1 and 8 of V5w2, however, grew significantly faster than the wild-type (Table 1).

Total root colonization by all *F. oxysporum* endophytes (both wild-type and marked isolates) differed significantly between replicates (χ^2 = 18.40, $P < 0.0001$), with better colonization taking place in the first (78.3%, n = 96), compared with the second replicate (72.3%, n = 100). Root colonization by benomyl-resistant *F. oxysporum* also differed significantly (χ^2 = 11.06, P = 0.0009) between replicates 1 (58%, n = 96) and 2 (55.3%, n = 99). Rhizome colonization by neither the wild-type *F. oxysporum* (χ^2 = 0.39, P = 0.53), nor the benomyl-resistant *F. oxysporum* (χ^2 = 0.75, P = 0.39) differed significantly between replicates. Rhizome colonization by all *F. oxysporum* was 46.0% (n = 96) and 47.7% (n = 100) in replicates 1 and 2, respectively. Rhizome colonization by benomyl-resistant *F. oxysporum* was 28.8% (n = 96) and 31.2% (n = 99) in replicates 1 and 2, respectively. Colonization of roots of non-inoculated plants by *F. oxysporum* was 12.5% in replicate 1 and 9.7% in replicate 2, and that of rhizomes was 2.4% in both replicates.

Plants inoculated with mutant isolates were sometimes colonized by *F. oxysporum* other than those artificially inoculated. Percentage colonization by *F. oxysporum* other than the introduced ones, referred to as “naturally occurring”, was determined by sub-culturing all *F.*

oxysporum growing from roots and rhizomes of plants inoculated with the mutant isolates on PDA containing Benlate®. For isolates Emb2.4o, Eny1.31i, Eny7.11o and V5w2, the percentage of re-isolated *F. oxysporum* identified as naturally occurring from both root and rhizome tissues ranged from 0-18.8%. Mutant BR 1 and 4 of V4w5 had between 38.9-93.1% root and rhizome tissue colonization by naturally occurring *F. oxysporum* (Tables 2 and 3).

Root and rhizome colonization by most mutants of isolates Emb2.4o, Eny1.31i, Eny7.11o and V5w2 was not significantly different from that by the wild-types, but root colonization by all mutants of isolate V4w5 was significantly higher than that by the wild-type ($\chi^2 = 141.1$, $P < 0.0001$). Similarly, differences were observed in root and rhizome colonization among mutants generated from the same wild-type isolate (Tables 2 and 3). It should, however, be noted that for plants inoculated with the wild-type isolates, naturally occurring *F. oxysporum* could not be identified. As a consequence, actual colonization by wild-types could not be determined.

Chlorate-resistant mutants

Chlorate-resistant mutants were readily generated for endophytic *F. oxysporum* isolates Emb2.4o and V5w2. Fast-growing sectors of colonies inoculated on PDA containing chlorate were isolated and cultured on MM. For both isolates, mutants that grew rapidly on selective media (Fig. 1C) were chosen for growth and plant colonization studies. Colony colour and growth after 7 days for the mutant colonies were similar to that of the wild-type isolates (Table 1).

Total root colonization by *F. oxysporum* isolates differed between the replicates ($\chi^2 = 35.48$, $P < 0.0001$), with better root tissue colonization occurring in replicate 2 (62.2%, n = 39) compared with replicate 1 (46.6%, n = 35). Root colonization by chlorate-resistant *F. oxysporum* mutants also differed significantly between replicates 1 (35.3%, n = 35) and 2 (46.7%, n = 39) ($\chi^2 = 24.26$, $P < 0.0001$). Total rhizome colonization by *F. oxysporum* and that by chlorate-resistant *F. oxysporum* mutants did not differ between replicates 1 and 2. Rhizome colonization by *F. oxysporum* was 29.5% (n = 35) and 28.7% (n = 39) in replicates 1 and 2, respectively, and by chlorate-resistant *F. oxysporum* mutants was 17.6% (n = 35) in replicate 1, compared with 21.5% (n = 38) in replicate 2 (Table 4).

There was no significant difference in root colonization between *F. oxysporum* wild-type isolates and chlorate-resistant mutants in replicate 1 (Table 4). In replicate 2, root colonization

by mutant CHR 6 of wild-type isolate Emb2.4o was significantly higher than the wild-type (χ^2 = 19.39, $P < 0.0001$) and mutant CHR 2 (χ^2 = 15.97, $P < 0.0001$). No significant differences were observed in root colonization between the wild-type isolate V5w2 and its mutant isolates. Among mutants of V5w2, root tissue colonization by CHR 12 was significantly different from CHR 2 (χ^2 = 11.94, $P < 0.0006$). For both experiments, there was no significant difference in rhizome colonization between wild-types and their mutants (Table 4).

Naturally occurring *F. oxysporum* colonized both the roots and rhizomes of non-inoculated plants (Table 4). In replicates 1 and 2, *F. oxysporum* colonization of roots of non-inoculated plants was 7.4% and 4.2%, respectively, while rhizome colonization for both replicates was 11.9%. For plants inoculated with the chlorate-resistant mutant isolates, the percentage of naturally occurring *F. oxysporum* isolated from roots and rhizomes varied between 0-13.9% and 0-11.2% for both replicates 1 and 2, respectively.

GFP and DsRed transformants

GFP- and DsRed-transformed *F. oxysporum* colonies grown on hygromycin-selective media produced bright and stable fluorescence after 7 days. Single-spore cultures maintained their hygromycin resistance after six successive transfers on non-selective media. The green and red fluorescence expressed by GFP- and DsRed- transformed isolates, respectively, were easily detected in both mycelia and spores (Fig. 2A and B). The GFP-transformed isolate G 2F and the DsRed-transformed isolates R 1D and R 5D from Emb2.4o had similar growth to the wild-type on non-selective media. The GFP-transformed isolate G 1D, however, grew significantly slower than its wild-type. All transformed isolates of wild-type isolate V5w2 grew significantly faster than the wild-type after 7 days (Table 1).

Spores of the GFP-transformed isolates germinated on the banana root surface 2 and 3 days after inoculation (Fig. 2C and D). On day 4, a dense mycelial growth became visible in the epidermis (Fig. 2E). Where the tips of roots were broken prior to dipping in an endophyte spore suspension, mycelia could be seen growing parallel to the inner walls of the xylem vessels (Fig. 2F). Unlike GFP transformant visualization, the visualization of DsRed transformants within the root tissue was difficult due to autofluorescence, making it impossible to obtain photographic images.

DISCUSSION

Benomyl- and chlorate-resistant mutants of non-pathogenic *F. oxysporum* endophytes with biological control properties, as well as GFP and DsRed transformants, were generated in this study. Most mutants and transformants had growth rates and growth characteristics similar to that of the wild-type isolates. More importantly, resistant mutants were able to colonize plant roots and rhizomes similar to their wild-type progenitors, and with few exceptions, remained resistant to benomyl or chlorate upon re-isolation from the plant. For these reasons we believe that they are suitable for use in plant colonization studies.

Artificial inoculation of tissue culture banana plants with *F. oxysporum* endophytes is often hampered by the colonization in the same plants by naturally occurring strains of the same species (Griesbach, 2000; Niere, 2001; Paparu *et al.*, 2006a). Mycelia were previously also observed in non-inoculated banana roots following fungal cell wall staining of root and rhizome sections during histological studies (Paparu *et al.*, 2006b). Colonization of the roots of non-inoculated plants by saprophytic strains of *F. oxysporum* is a common event in screenhouse studies as a result of the ubiquitous nature of the fungus (Olivain and Alabouvette, 1999; Salerno *et al.*, 2000). In the current study, colonization of endophyte-inoculated plants by naturally occurring strains of *F. oxysporum* seems to have been insignificant when colonization of non-inoculated plants by such strains is considered. Colonization frequencies by naturally occurring *F. oxysporum* strains in banana roots and rhizomes ranged between 0 and 18.8%.

A great number of naturally occurring *F. oxysporum* strains were isolated from the roots and rhizomes of banana plants inoculated with two benomyl-resistant mutants of isolate V4w5 (BR 1 and BR 4). For these two mutants, root and rhizome colonization by naturally occurring *F. oxysporum* ranged between 38.9 and 93%, which is much higher than the colonization frequencies of banana roots and rhizomes inoculated with any of the other mutants. We speculate that the mutants BR 1 and BR 4 lost their resistance to benomyl after colonizing plants, and that the percentages observed thus include both wild-type and naturally occurring strains of *F. oxysporum*. Loss of resistance to benomyl had been observed in the earlier stages of mutant generation. For instance, the benomyl-resistant mutant BR 1 had a higher colony diameter on day 7 compared with the wild-type isolate, but failed to grow on PDA amended with 20 mg l^{-1} 50% Benlate® after storage at 4^{o}C. To our knowledge, the

ability of benomyl-resistant mutants to lose their resistance to the selective chemical after plant colonization has not been reported before.

When marked with the GFP gene, endophytic non-pathogenic *F. oxysporum* isolates were clearly visible on root surface and in epidermis when visualised under a epifluorescent- and laser scanning confocal microscope. The observation that the non-pathogenic *F. oxysporum* isolates did not colonize the cortex and vascular system when roots were left intact, but reached the inner walls of the xylem in roots only when their tips were broken, suggests that active growth of the endophyte is limited to the root epidermis. The restriction of non-pathogenic *F. oxysporum* isolates to the outer root cells was previously reported by Benhamou and Garand (2001) and Paparu *et al.* (2006b). This is unlike colonization of plant roots by pathogenic *F. oxysporum* in the absence of wounding, where massive invasion of the root epidermis, cortex, endodermis and the parenchyma cells in cucumber (*Cucumis sativus* L.) inoculated with *F. oxysporum* f.sp. *pisi* W.C. Snyder and H.N. Hansen has been reported (Benhamou and Garand, 2001).

Based on growth characteristics and *in planta* visibility, the GFP transformant G2F will be useful in future studies. Although several earlier studies have shown that GFP- and DsRed-modified endophytic microorganisms do not differ from their wild-type progenitors in plant colonization (Hsiang and Chastagner, 1991; Coates *et al.*, 1993; Postma and Luttikholt, 1999; Hallmann *et al.*, 2001; Nahalkova *et al.*, 2003; Visser *et al.*, 2004) and biological control (Coates *et al.*, 1993; Postma and Luttikholt, 1999; Hallmann *et al.*, 2001) properties, transformant G2F should be characterized further with regard to these aspects before being used in future studies.

Regardless of the clear visibility of the morphological structures of agar cultures of *F. oxysporum* endophytes expressing DsRed when viewed using epifluorescent microscopy, the transformed fungus was not easily discerned when inoculated into a banana root, probably due to autofluorescence around the cell walls. Autofluorescent plant bodies are often observed in the epidermis of infected plant tissues. They are presumed to be plant phenolics and an indicator of host response to infection (Czymmek *et al.*, 2002). The occurrence of phenolic substances in banana roots inoculated with *F. oxysporum* endophytes was reported by Athman (2006). It is, therefore, likely that the autofluorescence observed was due to defence responses within the cell wall, induced by endophyte colonization.

The benomyl- and chlorate-resistant non-pathogenic *F. oxysporum* endophytes that retained their chemical resistance properties following plant colonization in our study will be of great value for studying biological control and host-pathogen interactions in less advanced laboratories. It is, however, important that for future studies the pest control abilities of these mutants be compared with that of the respective wild-type isolates. For future colonization studies, we recommend that chlorate-resistant rather than benomyl-resistant mutants be used for plant colonization studies, as none of the chlorate-resistant mutants lost their resistance during storage or following plant colonization. Based on growth characteristics, and root and rhizome colonization ability, we recommend the use of mutants CHR 6 and CHR 4 for *F. oxysporum* isolate Emb2.40; and CHR 9 and CHR 12 for isolate V5w2.

Marking of endophytic fungi with chemical resistance and fluorescent genes can be of great value when comparing the ability of fungal isolates to colonize plant tissue, inoculation methods, and for histological studies related to colonization patterns and plant resistance. With marked isolates it will be possible to perform dual or multiple endophyte inoculations, where, for instance, a single plant is inoculated with both a chlorate- and benomyl-resistant isolate. Percentage colonization by each isolate can then be determined. Previously, *Aspergillus nidulans* G. Winter, *Pencillium paxilli* Bainier, *Trichoderma harzianum* Rifai, *T. virens* and pathogenic and non-pathogenic *F. oxysporum* isolates from tomato were marked with GFP and DsRed markers for use in multiple inoculation studies (Mikkelsen *et al.*, 2003; Olivain *et al.*, 2006). The use of fluorescent protein markers, however, can be limited in countries where regulations on the importation of genetically modified organisms and intellectual property rights are not fully formalised. Furthermore, the limited availability of advanced equipment in developing countries, such as fluorescent microscopes, can also be a constraint when applying technologies that require such equipment. In these cases, the use of isolates marked with benomyl or chlorate resistance can be of great value, as it is fast, reliable and does not require any sophisticated equipment.

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Table 1. Growth of five *Fusarium oxysporum* wild-type isolates and their respective chemical resistant mutants (chlorate and benomyl) and fluorescently-marked transformant isolates on potato dextrose agar

Isolate	Benomyl-resistant mutants			Chlorate-resistant mutants			Fluorescent transformants		
	Mutant[2]	colony diameter (cm)[1]		Mutant[2]	colony diameter (cm)[1]		Mutant[2]	Colony diameter (cm)[1]	
Emb2.4o	WD	7.1 ± 0.1	a	WD	7.8 ± 0.1	a	WD	7.9 ± 0.1	a
Emb2.4o	BR 1	7.3 ± 0.1	a	CHR 2	7.9 ± 0.0	a	G 1D	6.9 ± 0.1	b
Emb2.4o	BR 2	7.2 ± 0.1	a	CHR 3	8.0 ± 0.0	a	G 2F	7.7 ± 0.1	a
Emb2.4o	BR 3	7.3 ± 0.1	a	CHR 4	8.0 ± 0.0	a	R 1D	7.8 ± 0.1	a
Emb2.4o	BR 8	6.3 ± 0.1	b	CHR 6	7.9 ± 0.0	a	R 5D	7.8 ± 0.1	a
Eny1.31i	WD	7.7 ± 0.2	a						
Eny1.31i	BR 1	6.9 ± 0.2	b						
Eny1.31i	BR 3	7.8 ± 0.1	a						
Eny1.31i	BR 4	6.7 ± 0.1	b						
Eny1.31i	BR 5	7.7 ± 0.1	a						
Eny7.11o	WD	7.5 ± 0.14	ab						
Eny7.11o	BR 1	7.4 ± 0.2	b						
Eny7.11o	BR 2	7.7 ± 0.1	ab						
Eny7.11o	BR 4	7.9 ± 0.1	a						
Eny7.11o	BR 7	5.4 ± 0.1	c						
V4w5	WD	4.7 ± 0.1	a						
V4w5	BR 1	4.1 ± 0.1	bc						
V4w5	BR 2	4.4 ± 0.1	ab						
V4w5	BR 3	4.3 ± 0.1	b						
V4w5	BR 4	3.7 ± 0.1	c						
V5w2	WD	4.5 ± 0.1	c	WD	7.9 ± 0.6	a	WD	5.9 ± 0.5	b
V5w2	BR 1	7.4 ± 0.2	a	CHR 2	7.9 ± 0.1	a	R 21	8.0 ± 0.0	a
V5w2	BR 4	3.2 ± 0.1	d	CHR 4	8.0 ± 0.0	a	R 22	8.8 ± 0.1	a
V5w2	BR 6	3.4 ± 0.1	d	CHR 9	7.9 ± 0.0	a	G 31	8.0 ± 0.0	a
V5w2	BR 8	5.2 ± 0.1	b	CHR 12	8.0 ± 0.0	a	G 32	8.0 ± 0.0	a

[1]Values represent mean colony diameter 7 days after growth on PDA, at ± 25°C. For each isolate, means within a column followed by the same letter are not different (P = 0.05, Tukey's Studentized range test).
[2]WD = wild-type, BR = benomyl-resistant, CHR = chlorate-resistant, R = DsRed-Express transformant, G = GFP transformant.

Table 2. Colonization of tissue culture Yangambi (Km-5) (AAA) roots by endophytic *Fusarium oxysporum* mutants resistant to benomyl and their respective wild-type isolates

Isolate	Mutant[1]	Replicate 1						Replicate 2					
		Total colonization (%)[2,3]		Benomyl-resistant (%)[2]		Natural occurring (%)[2,4]		Total colonization (%)[2,3]		Benomyl-resistant (%)[2]		Natural occurring (%)[2,4]	
Control		12.5				12.5		9.7				9.7	
Emb2.4o	WD	90.3	a					76.4	ab				
Emb2.4o	BR 1	95.8	a	95.8	a	0.0	a	79.2	ab	77.8	ab	1.4	a
Emb2.4o	BR 2	69.4	a	69.4	a	0.0	a	75.0	b	75.0	b	0.0	a
Emb2.4o	BR 3	100.0	a	100.0	a	0.0	a	93.1	a	93.1	a	0.0	a
Emb2.4o	BR 8	68.1	b	68.1	a	0.0	a	86.1	ab	84.7	ab	1.4	a
Eny1.31i	WD	66.7	b					78.3	ab				
Eny1.31i	BR 1	83.3	ab	81.5	ab	1.9	a	70.8	ab	61.1	b	9.7	a
Eny1.31i	BR 3	94.4	a	94.4	ab	0.0	a	88.9	a	88.9	a	0.0	a
Eny1.31i	BR 4	76.4	b	76.4	b	0.0	a	50.0	b	50.0	b	0.0	a
Eny1.31i	BR 5	79.2	ab	79.2	ab	0.0	a	76.4	a	76.4	a	0.0	a
Eny7.11o	WD	88.9	a					80.6	a				
Eny7.11o	BR 1	86.9	a	86.1	a	0.0	a	79.2	a	79.2	a	0.0	a
Eny7.11o	BR 2	83.3	a	83.3	a	0.0	a	83.3	a	83.3	a	0.0	a
Eny7.11o	BR 4	84.7	a	84.7	a	0.0	a	79.2	a	79.2	a	0.0	a
Eny7.11o	BR 7	87.5	a	86.2	a	11.3	a	94.4	a	94.4	a	0.0	a
V4w5	WD	16.7	c					41.7	b				
V4w5	BR 1	93.1	a	0.0	c	93.1	a	69.4	a	7.0	c	62.5	a
V4w5	BR 2	58.3	b	56.7	a	1.7	c	51.4	ab	38.9	b	12.5	c
V4w5	BR 3	60.4	b	54.2	a	6.3	c	72.2	a	65.3	a	6.9	c
V4w5	BR 4	54.2	b	15.3	b	38.9	b	69.4	a	23.6	b	45.8	b
V5w2	WD	97.0	a					58.3	c				
V5w2	BR 1	94.4	ab	94.4	a	0.0	a	84.7	b	84.7	b	0.0	a
V5w2	BR 4	84.7	ab	83.3	a	1.4	a	66.7	bc	57.0	c	0.0	a
V5w2	BR 6	52.8	c	47.2	c	5.6	a	48.6	c	48.6	c	0.0	a
V5w2	BR 8	77.8	b	77.8	b	0.0	a	90.7	a	90.7	a	0.0	a

For each isolate, means within a column followed by the same letter are not different (P = 0.0073 for overall colonization and P = 0.0085 for colonization by benomyl-resistant mutants, Logistic regression and Dunn-Sidak method). [1]WD = wild-type isolate, BR = benomyl-resistant mutant. [2]Percentage colonization is the average root pieces colonized for four plants per mutant, three roots per plant and six pieces per root. [3]Total colonization = colonization by both naturally occurring and marked *F. oxysporum*. [4]Naturally occurring = re-isolated *F. oxysporum* from mutant inoculated plants on PDA, and which did not grow on the expected selective media when sub-cultured.

Table 3. Colonization of tissue culture Yangambi (Km-5) (AAA) rhizomes by endophytic *Fusarium oxysporum* mutants resistant to benomyl and their respective wild-type isolates

Isolate	Mutant[1]	Total colonization (%)[2,3]		Benomyl-resistant (%)[2]		Natural occurring (%)[2,4]	
Control		2.38				2.38	
Emb2.4o	WD	25.0	a				
Emb2.4o	BR 1	35.4	a	31.3	a	0.0	a
Emb2.4o	BR 2	20.8	a	18.8	a	4.2	a
Emb2.4o	BR 3	37.5	a	37.5	a	2.1	a
Emb2.4o	BR 8	25.0	a	25.0	a	0.0	a
Eny1.31i	WD	45.8	a				
Eny1.31i	BR 1	28.6	a	26.2	b	2.4	a
Eny1.31i	BR 3	56.3	a	56.3	a	0.0	a
Eny1.31i	BR 4	39.6	a	39.6	ab	0.0	a
Eny1.31i	BR 5	54.6	a	52.1	ab	2.1	a
Eny7.11o	WD	61.9	a				
Eny7.11o	BR 1	52.1	a	50.0	a	0.0	a
Eny7.11o	BR 2	39.6	ab	39.6	ab	2.1	a
Eny7.11o	BR 4	37.5	ab	37.5	ab	0.0	a
Eny7.11o	BR 7	20.8	b	20.8	b	0.0	a
V4w5	WD	64.3	a				
V4w5	BR 1	77.1	a	8.3	c	68.8	a
V4w5	BR 2	37.1	b	33.3	b	4.2	b
V4w5	BR 3	78.6	a	78.6	a	0.0	b
V4w5	BR 4	70.8	a	16.7	bc	54.2	a
V5w2	WD	66.7	a				
V5w2	BR 1	66.7	a	66.7	a	0.0	b
V5w2	BR 4	43.8	ab	25.0	b	18.8	a
V5w2	BR 6	37.5	b	29.2	b	8.3	b
V5w2	BR 8	54.2	ab	52.1	a	2.1	b

For each isolate, means within a column followed by the same letter are not different (P = 0.0073 for overall colonization and P = 0.0085 for colonization by benomyl-resistant mutants, Logistic regression and Dunn-Sidak method).

[1]WD = wild-type isolate, BR = benomyl-resistant mutant.

[2]Percentage colonization is the average colonization of rhizome pieces for eight plants per mutant and six pieces per rhizome (experiments 1 and 2 combined).

[3]Total colonization = colonization by both naturally occurring and marked *F. oxysporum*.

[4]Naturally occurring = re-isolated *F. oxysporum* from mutant inoculated plants on PDA, and which did not grow on the expected selective media when sub-cultured.

Table 4. Colonization of tissue culture banana roots and rhizomes of cv Nabusa (AAA-EA) by endophytic *Fusarium oxysporum* mutants resistant to chlorate and their respective wild-type isolates

Isolate	Mutant[1]	Root colonization-Replicate 1						Root colonization-Replicate 2					
		Total colonization (%)[2,3]		Chlorate-resistant (%)[2]		Natural occurring (%)[2,3]		Total colonization (%)[2,3]		Chlorate-resistant (%)[2]		Natural occurring (%)[2,4]	
Control		7.4				7.4		4.17				4.17	
Emb2.4o	WD	43.1	a					50.0	b				
Emb2.4o	CHR 2	41.7	a	30.6	a	11.1	a	58.3	b	54.2	b	4.2	a
Emb2.4o	CHR 3	38.9	a	36.1	a	2.8	a	66.7	ab	66.7	ab	0.0	a
Emb2.4o	CHR 4	51.9	a	51.9	a	0.0	a	69.4	ab	69.4	ab	0.0	a
Emb2.4o	CHR 6	51.4	a	41.7	a	9.7	a	86.1	a	86.1	a	0.0	a
V5w2	WD	57.4	a					68.1	ab				
V5w2	CHR 2	50.0	a	38.9	a	11.11	a	52.8	ab	38.9	b	13.9	a
V5w2	CHR 4	38.9	a	37.1	a	1.85	a	47.2	ab	47.2	ab	0.0	a
V5w2	CHR 9	59.7	a	58.3	a	1.39	a	62.5	ab	54.2	ab	8.3	a
V5w2	CHR 12	38.9	a	33.3	a	5.56	a	75.0	a	68.1	a	6.9	a

Isolate	Mutant[1]	Rhizome colonization					
		Total colonization (%)[2,3]		Chlorate-resistant (%)[2]		Natural occurring (%)[2,4]	
Control		11.9				11.9	
Emb2.4o	WD	43.8	a				
Emb2.4o	CHR 2	27.1	a	22.9	a	4.2	b
Emb2.4o	CHR 3	31.3	a	31.3	a	0.0	b
Emb2.4o	CHR 4	38.1	a	26.2	a	11.2	a
Emb2.4o	CHR 6	41.7	a	31.3	a	10.4	a
V5w2	WD	23.8	a		a		
V5w2	CHR 2	25.0	a	22.9	a	2.1	a
V5w2	CHR 4	19.0	a	16.7	a	2.4	a
V5w2	CHR 9	16.7	a	16.7	a	0.0	a
V5w2	CHR 12	26.2	a	26.2	a	0.0	a

For each isolate, means within a column followed by the same letter are not different (P = 0.0073 for overall colonization and P = 0.0085 for benomyl mutants, Logistic regression and Dunn-Sidak method). [1]WD = wild-type isolate, CHR = chlorate-resistant mutant. [2]Percentage root colonization is the average colonization of root pieces for four plants per mutant, three roots per plant and six pieces per root, and percentage rhizome colonization is the average colonization of rhizome pieces for eight plants per mutant and six pieces per rhizome (experiments 1 and 2 combined). [3]Total colonization = colonization by both naturally occurring and marked *F. oxysporum*. [4]Naturally occurring = re-isolated *F. oxysporum* from mutant inoculated plants on PDA, and which did not grow on the expected selective media when sub-cultured.

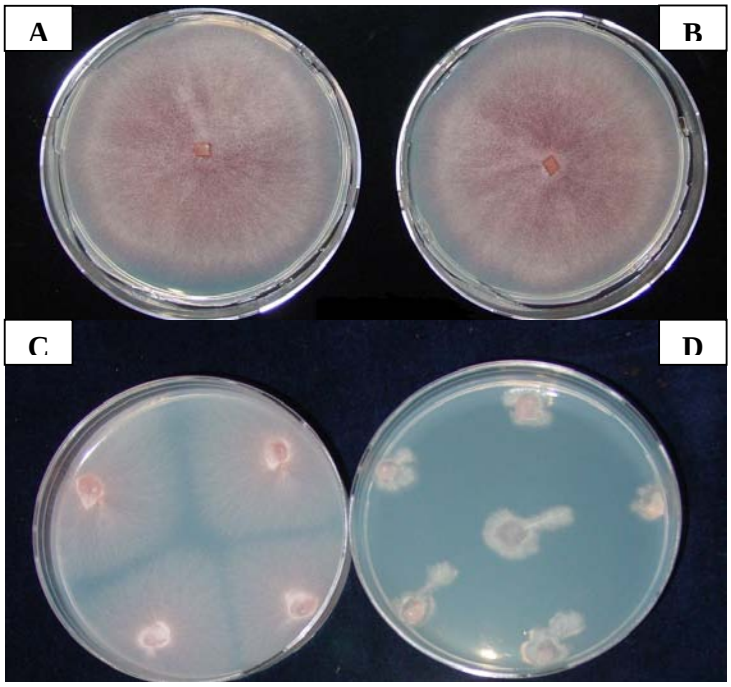

Figure 1. Growth of (A) wild-type endophytic *Fusarium oxysporum* Eny7.11o on PDA 7 days after inoculation, (B) a benomyl-resistant mutant of endophytic *Fusarium oxysporum* Eny7.11o (mutant BR 2) on PDA 7 days after inoculation (dai), (C) chlorate-resistant mutant of endophytic *Fusarium oxysporum* V5w2 (mutant CHR 9) on media amended with 30 g potassium chlorate l^{-1}, 3 dai and (D) wild-type endophytic *Fusarium oxysporum* V5w2 on media amended with 30 g potassium chlorate l^{-1}, 3 dai.

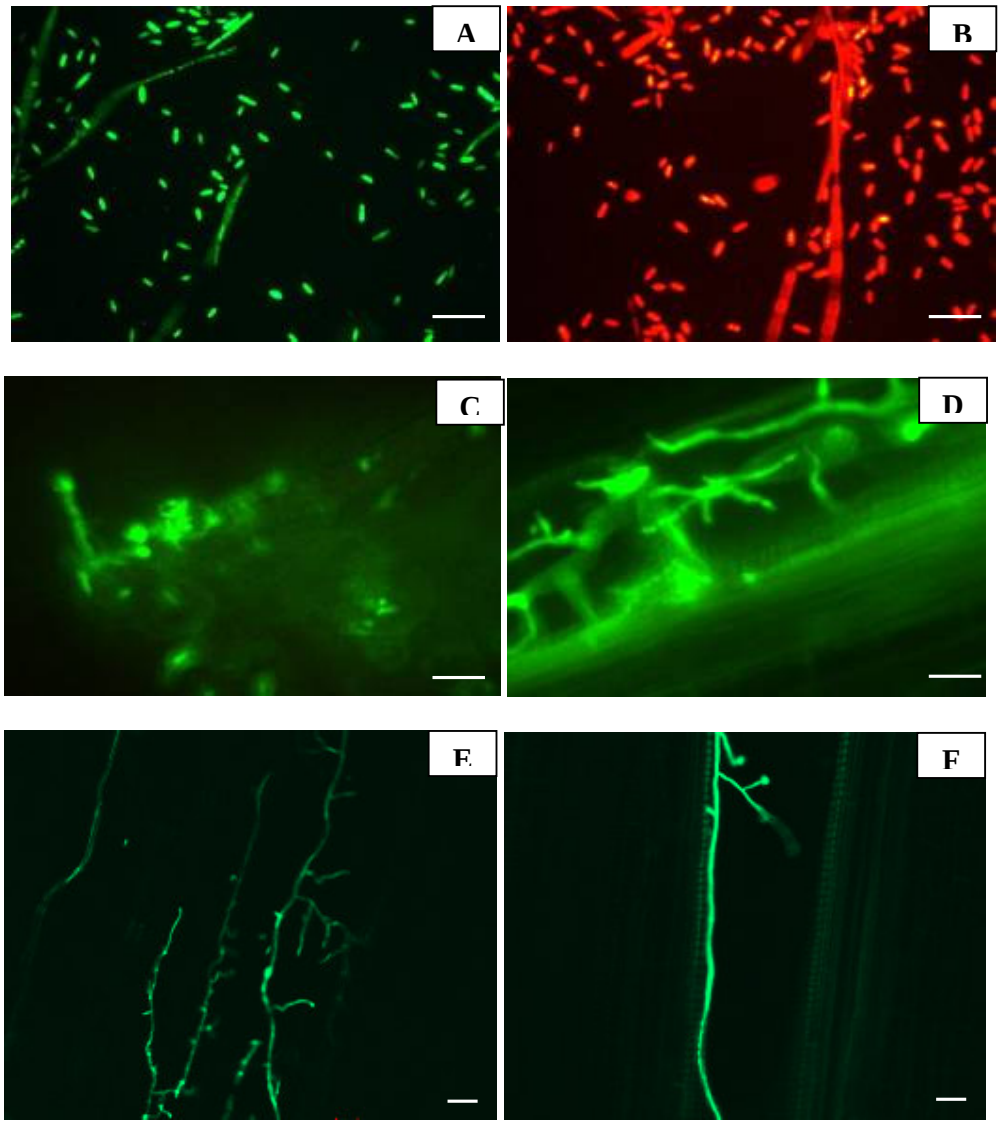

Figure 2. Fluorescent microscope images of (A) spores and mycelia of endophytic *Fusarium oxysporum* GFP transformant G 31 and (B) spores and mycelia of endophytic *Fusarium oxysporum* DsRed transformant R 1D (scale bars represent 50 µm), spores of transformant G 31 expressing GFP germinate on banana root surface 2 (C) and 3 (D) days after inoculation (dai) (scale bars represent 5 µm), longitudinal root section showing fungal mycelia 4 dai in the hypodermis (E) along the inner walls of the root xylem (F) (scale bar represents 20 µm).

CHAPTER 6

Dual inoculation of *Fusarium oxysporum* endophytes: effect on plant colonization, plant growth and control of *Radopholus similis* and *Cosmopolites sordidus*

Chapter submitted to Biocontrol Science and Technology Journal

ABSTRACT

The burrowing nematode (*Radopholus similis*) and the banana weevil (*Cosmopolites sordidus*) are major pests of banana (*Musa* spp.) in the Lake Victoria basin region of Uganda. Biological options to control the two pests include the use of non-pathogenic *Fusarium oxysporum* endophytes of banana. In the current study, the ability of endophytic *F. oxysporum* isolates Emb2.4o and V5w2 to control the banana weevil and *R. similis*, alone and in combination, was investigated. Plant colonization by the endophytes was determined by inoculating their chemical-resistant mutants (Emb2.40 BR8 and V5w2 CHR 9), separately and in combination, onto banana roots. Plant growth promotion was determined by measuring plant height, girth, number of live roots and fresh root weight at harvest, and control of banana pests was determined by challenging endophyte-inoculated plants with the weevil and the nematode. Root colonization was highest in plants inoculated with both endophytes, compared with those inoculated with only one of the endophytes. Root colonization was better for isolate V5w2 CHR 9 than Emb2.4o BR 8. Dually inoculated plants showed a significant increase in height, girth, fresh root weight and number of functional roots following nematode challenge. Nematode numbers in roots were reduced 12 weeks after challenge of 8-weeks-old endophyte-inoculated plants. Similarly, weevil damage was non-significantly reduced in the rhizome periphery, the inner and outer rhizome, and pseudostem base when dually-inoculated plants were compared to single endophyte-inoculated plants and controls. We conclude that dual inoculation of bananas with endophytic isolates Emb2.4o BR 8 and V5w2 CHR 9 increases root colonization by the endophytes, reduces *R. similis* numbers and banana weevil damage, and enhances plant growth in the presence of *R. similis* infestation.

INTRODUCTION

The most important pests of banana in Uganda are the banana weevil (*Cosmopolites sordidus* Germar) and the burrowing nematode *Radopholus similis* (Cobb) Thorne (Gold *et al.*, 1993; 1994). The banana weevil is distributed in all banana growing areas of the tropics and sub-tropics at elevations of 1,000-1,400 m above sea level (Gowen, 1995), and affects wild and cultivated bananas and plantains. The weevil causes severe damage in economically important plantains (AAB genome), East African Highland cooking bananas (EAHB) and Ensete (Gold and Bagabe, 1997). Yield losses may reach 100% (Koppenhofer *et al.*, 1994) and result from sucker mortality, reduced bunch weight, snapping of heavily infested plants and shorter plantation longevity (Ogenga-Latigo and Bakyalire, 1993; Mbwana and Rukazambuga, 1999). *Radopholus similis* occurs in most banana growing regions of the world (Gowen, 2005). It attacks Cavendish cultivars in commercial plantations, and plantains and cooking bananas cultivated in lowlands in East Africa and Central America (Sarah *et al.*, 1996). Root damage due to *R. similis* infestation results in reduced bunch weights and complete loss of plants where toppling occurs as a result of severe root damage (Sarah *et al.*, 1996).

An integrated pest management approach is being used to control populations of the two pests (Speijer *et al.*, 1994; Gold *et al.*, 2001). Control options for the banana weevil include the use of clean planting material such as tissue culture plantlets and pared hot water-treated field suckers (Seshu Reddy *et al.*, 1998; Gold *et al.*, 2001), good crop husbandry (weeding, sucker removal, pruning, mulching, the use of manure and destruction of residues) (Masanza *et al.*, 2004), trapping of adult weevils (Gold *et al.*, 2002) and chemical control (Collins *et al.*, 1991; Gold *et al.*, 1999; 2001). For nematodes, current control strategies include habitat management (field sanitation), use of clean planting material and chemical control (Furadan) (Speijer *et al.*, 1994). Biological control strategies include the use non-pathogenic *Fusarium oxysporum* Schlecht.: Fries endophytes (Schuster *et al.*, 1995; Niere, 2001; Athman, 2006).

The use of microbial agents for biological control of banana pests has been investigated in *in vitro* and screenhouse trials before. Isolates of the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin have been demonstrated to kill banana weevils when applied in a solid substrate at the base of the plant (Nankinga, 1995). Endophytic non-pathogenic *F. oxysporum* have similarly been demonstrated to control both banana weevils and *R. similis* (Schuster *et al.*, 1995; Griesbach, 2000; Niere, 2001). The endophytes offer a unique opportunity for pest control, as they can be established inside tissue culture banana plants,

which is both environmentally friendly and affordable. Two endophytes, Emb2.4o and V5w2, have been identified as effective in controlling weevils and nematodes, respectively. Endophytic *F. oxysporum* reduces *R. similis* populations primarily by means of induced resistance (Athman, 2006), and the banana weevil through antibiosis to eggs and larvae (Griesbach, 2000; Kapindu, personal communication). They also have the ability to stimulate plant growth and vitality (Niere, 2001).

In banana fields where nematodes and weevils occur simultaneously, more than one endophyte, specific to each pest, needs to be established in the same plant. The combined use of endophytes for biological control of a pathogen or pest can sometimes be more effective than using a single isolate, because they contribute different beneficial properties to plant defence (Ramamoorthy *et al.*, 2001). Zum Felde *et al.* (2006) reported reduced *R. similis* populations in pot trials where banana plants were inoculated dually with endophytic *F. oxysporum* isolates S9 and P12 and *Trichoderma atroviride* (formerly *T. harzianum* Rifai) isolates S2 and MT-20. In another report, Dubois *et al.* (2004) demonstrated the potential of dually-inoculated non-pathogenic *F. oxysporum* isolates to increase root weight and reduce *R. similis* necrosis. However, the success of dual inoculation was not assessed in the study by Dubois *et al.* (2004), because there was no way of differentiating inoculated isolates upon re-isolation from plants. In some cases, however, combined inoculation of biological control agents in a single plant can be less effective than inoculating them separately. This may often result from antagonism between biocontrol organisms leading to reduced control (Chen *et al.*, 2000) or loss of control (Viaene and Abanoi, 2000), when compared with controls. In plant colonization studies, Wille *et al.* (2002) showed that *Bromus erectus* Huds. singly inoculated with *Epichloë bromicola* Leuchtmann and Schardl isolates resulted in a higher percentage of infected tillers than dual inoculations.

In order to determine the colonization, survival and competition between endophytes in banana plants, endophytic *F. oxysporum* isolates need to contain markers with which they can be detected in plant material. In this study, chemical-resistant mutants have been used to determine the effect of dual endophyte inoculation on plant colonization, plant growth and control of *R. similis* and *C. sordidus*.

MATERIALS AND METHODS

Plant colonization

Fungal endophyte isolates: Two non-pathogenic endophytic *F. oxysporum* isolates were mutated to investigate the ability of the endophytes to colonize and survive in banana roots. Emb2.4o BR 8 and V5w2 CHR 9 are benomyl- and chlorate-resistant mutants, respectively, with growth and plant colonization characteristics similar to their wild-type isolates Emb2.4o and V5w2 (Chapter 5). The wild-type isolates are known to reduce attack of banana plants by the banana weevil and *R. similis* (Griesbach, 2000; Niere, 2001; Athman, 2006). Emb2.4o BR 8 and V5w2 CHR 9 were grown on half strength potato dextrose agar (PDA) (19 g PDA and 19 g agar l^{-1} distilled water) in the laboratory (natural light at ± 25^{o}C) for 7 days. Sterile distilled water (SDW) was then added to each culture, the fungal spores dislodged, filtered through sterile cheese cloth, and spore concentrations adjusted to 0.75 and 1.5 x 10^{6} spores ml^{-1}.

Endophyte inoculation: A screenhouse experiment was conducted at the research farm of the International Institute of Tropical Agriculture (IITA) in Uganda (0º32'N, 32º35'E)**.** Tissue cultured plants of the EAHB cv Kibuzi (*Musa* spp., AAA-EA) were propagated using a standard shoot-tip culture protocol for banana (Vuylsteke, 1998). Four weeks after rooting, plants were removed from the rooting medium and their roots and rhizomes washed using tap water. Plants were then replanted in 250-ml plastic cups to enhance root growth (Paparu *et al*., 2006). After 4 weeks, plants were inoculated with *F. oxysporum* isolates Emb2.4o BR 8 and V5w2 CHR 9, separately and in combination, by dipping their roots and rhizomes for 4 h in a 400 ml spore suspension held in 1000-ml glass beakers. Plants inoculated with only one of the isolates were inoculated with a spore concentration of 1.5 x 10^{6} spores ml^{-1}. For dual inoculated plants, the spore suspensions for the two isolates were mixed together prior to inoculation. After inoculation, plants were kept in the screenhouse for 4 weeks and watered daily. Ten plants were used per treatment and the experiment was repeated once.

Endophyte re-isolation: Four weeks after inoculation (4 wai), all plants were harvested for endophyte re-isolation. For each plant, four roots and the entire rhizome were used in re-isolations. The roots and rhizomes of inoculated banana plants were first disinfected in 5% NaOCl for 1 min and in 75% EtOH for 1 min. They were then rinsed thrice in sterile distilled water and placed on sterile tissue paper. For each root, two pieces of approximately 0.5 cm

long were cut from the tip, middle and base (total of six root pieces per root). For the inner and outer rhizome, six pieces each were re-isolated.

Surface-disinfected root and rhizome pieces were inserted halfway in PDA supplemented with antibiotics (0.1 g penicillin G, 0.2 g streptomycin sulfate and 0.05 g chlortetracycline l^{-1}) in 90-mm-diameter Petri dishes and incubated in the laboratory for 7 days. Fungi growing from plated root and rhizome pieces were viewed under a compound microscope (100× and 400× magnification) and identified as *F. oxysporum* (Nelson *et al*., 1983). All *F. oxysporum* isolates from endophyte-inoculated plants were sub-cultured on PDA containing either 20 mg 50% Benlate® (Du Pont De Nemours, Paris, France) or 30 g potassium chlorate l^{-1} PDA to determine colonization of banana roots and rhizomes by the mutants. Where plants were dually inoculated with Emb2.4o BR 8 and V5w2 CHR 9, all re-isolated *F. oxysporum* cultures were sub-cultured separately on PDA containing each selective chemical.

Plant growth and pest control

Banana plants of cv Nabusa were inoculated with Emb2.4o BR 8 and V5w2 CHR 9, either separately or in combination, at a spore concentration of 1.5 x 10^6 spores ml^{-1} as described above. The plants were then challenged with *R. similis* and the banana weevil, individually and in combination. Four wai, four plants were randomly sampled from each inoculation treatment and *F. oxysporum* re-isolated from roots and rhizomes as described above to assess inoculation success. Re-isolations were done again from all plants at harvest. The experiment was repeated once.

Radopholus similis *culture and inoculation*: Plants were challenged with nematodes 8 weeks after endophyte inoculation. Pure nematode cultures obtained from carrot discs (Speijer and De Waele, 1997) were suspended in SDW in a beaker, and the number of juveniles and females in a 2 ml suspension estimated using a light microscope (100× magnification). To inoculate plants with nematodes, three holes (3-5 cm deep) were made in potting soil at the base of the plant at equal distance from one another. A total of 1,000 juveniles and females per plant were then pipetted into these holes and the holes covered with soil. Plants were not watered for 24 h to ensure that the nematodes were not washed away. The plants were maintained in the screen house for 12 weeks and watered daily.

Cosmopolites sordidus *culture and inoculation*: Banana weevils were trapped from banana fields at Namulonge using split pseudostems (Griesbach, 2000). Female and male weevils were differentiated on the basis of rostrum characteristics as described by Longoria (1968). Only females were used for plant infestation. Eight weeks after plant inoculation with the endophytes, each plant was infested with 10 female banana weevils. The weevils were placed on the surface of the potting soil in buckets containing single plants. To prevent escape of weevils, a mosquito net was put at the bottom of the buckets before adding soil, and after infestation plants were covered with mosquito nets (250 × 250 cm) (Fig. 1). The plants were maintained in the screen house for 12 weeks and watered daily.

Plant growth assessment: Plant height was recorded every 4 weeks until harvest, while girth, number of roots and total root weight were recorded at harvest. Height was measured as the distance from the point where the youngest leave emerges from the pseudostem to the base of the plant, while girth was measured at the base of the pseudostem. After the roots were washed with tap water they were cut, counted and fresh root weight measured.

Pest damage assessment: At harvest plants were removed from buckets and their roots and rhizomes examined for nematode damage. *Radopholus similis* damage was expressed as percentage necrotic root tissue (Speijer and Gold, 1996). Five randomly selected roots were cut to approximately 10-cm long pieces and used to assess percentage root necrosis. The roots were cut longitudinally and the percentage of visible necrotic tissue was estimated. Each root represented a maximum percentage root necrosis of 20% and the five roots were added up to 100% root necrosis. The five segments used for root necrosis assessment were further used to determine nematode numbers. The modified Baermann funnel method (Hooper *et al.*, 2005) was used to extract nematodes from roots. Nematodes were extracted overnight, rinsed into 100-ml glass bottles and kept at 4^{o}C until they were counted. Prior to counting, nematode suspensions were reduced to 25 ml and the average nematode population density determined from three 2-ml aliquots. For banana weevil damage, the method of Nankinga (1995) was used to assess damage in the peripheral rhizome, inner and outer rhizome, and inner and outer pseudostem base.

Data analysis

Colonization of banana roots by non-pathogenic *F. oxysporum* was analysed using logistic regression and alpha-levels for pair wise mean comparisons conducted using Dunn-Sidak corrected 95% confidence intervals. In making comparisons between single and dual

inoculations, colonization by wild-type *F. oxysporum* was excluded. For all parameters in which a t-test statistic was used to compare differences between sample means, a Statterthwaite's approximation t-value was used when the variances were unequal (Sokal and Rohlf, 1995; SAS Institute, 1989).

Data with uneven variance was subjected to a normality test to ensure homogeneity of variance prior to statistical analysis. Growth data was analysed separately for each pest challenge, because nematode- and banana weevil-challenged plants received different treatments after challenge. Plant height increase (height at harvest minus height at inoculation) was sqrt-transformed, subjected to ANOVA, and together with girth and root weight analysed separately for each replicate. Multiple mean comparisons between treatments were performed using Tukey's studentized range test (SAS Institute, 1989). Root number was analyzed using Kruskal-Wallis analysis of variance.

Nematode counts were calculated per 100 g of root sample and together with percentage root necrosis analysed using Kruskal-Wallis analysis of variance. Data from each replicate was analysed. Treatment means were compared using a t-test. Peripheral, inner and outer rhizome damage caused by the banana weevil was arcsine-sqrt-transformed, while outer and inner pseudostem damage was sqrt-transformed before ANOVA. Differences in mean percentage damage to each plant tissue among the different treatments were separated using Tukey studentized range test (SAS Institute, 1989).

RESULTS

Plant colonization

Banana roots were better colonized by the endophytic *F. oxysporum* isolates than rhizomes (Fig. 2). In replicate 1, percentage root- and rhizome colonization were 45.6 and 13.4%, respectively, and 22.8 and 3.1%, respectively, in replicate 2. The percentage root colonization by naturally occurring strains of *F. oxysporum* varied between 0 and 9.2%.

Root colonization was significantly higher in plants inoculated with both endophytes at a spore concentration of 0.75×10^6 spores ml^{-1} than in plants inoculated with only one of the endophytes (Fig. 2). Dual inoculation at a concentration of 0.75×10^6 spores ml^{-1} also resulted in significantly higher root colonization than that at a concentration of 1.5×10^6 spores ml^{-1} in replicate 2, but not in replicate 1. In both replicates, higher percentage root colonization was

observed for isolate V5w2 CHR 9 when inoculated together with Emb2.4o BR 8, but the isolate colonized rhizomes better when inoculated singly (Fig. 2). On the contrary, isolate Emb2.4o BR 8 colonized both roots and rhizomes better when inoculated singly (Fig. 2). In both replicates, for all isolates, whether inoculated singly or dually, there was no preference for root base, middle or tip (χ^2 = 0.42, *P* = 0.81; χ^2 = 3.57, *P* = 0.17 for replicates 1 and 2, respectively).

Rhizome colonization was significantly higher in all endophyte-inoculated plants, compared with non-inoculated controls (Fig. 2). Colonization by isolate V5w2 CHR 9 did not differ significantly among endophyte-inoculated plants, but that by isolate Emb2.4o BR 8 differed significantly among endophyte-inoculated plants in replicate 1. Both isolates colonized the inner and outer rhizome equally well.

For the pest challenge experiment, successful root and rhizome colonization was observed for mutant isolates Emb2.4o BR 8 and V5w2 CHR 9. Roots were significantly better colonized by the endophytes than rhizomes, with root colonization amounting to 34.7 and 44.9% for replicates 1 and 2, respectively, 4 wai. Rhizome colonization for replicates 1 and 2 were 6.9 and 16.7%, respectively (Fig. 3). In replicate 1, percentage root colonization was not significantly different among endophyte treatments, but in replicate 2 percentage root colonization was significantly lower in plants inoculated with Emb2.4o BR 8, compared with those inoculated with V5w2 CHR 9 or with both isolates. From 4 wai to harvest (20 wai), endophyte colonization was significantly reduced in both banana roots and rhizomes, and no significant differences were found in colonization among treatments. Colonization of roots in endophyte non-inoculated plants by naturally occurring isolates of *F. oxysporum* was significantly lower than root colonization by the endophytes 4 wai in both replicates 1 and 2 (Fig. 3).

Plant growth

In banana plants not challenged with nematodes and/or the banana weevil, endophyte non-inoculated (control) plants produced faster-growing plants with significantly more roots (replicate 2 only) than endophyte-inoculated plants (Table 1). When endophyte-inoculated plants were challenged with the weevil, plant growth was reduced compared to endophyte non-inoculated plants (significant in replicate 2 only). However, endophytes dually inoculated into plants enhanced plant growth significantly compared to endophyte non-inoculated plants and plants inoculated with only one endophyte when they were challenged with nematodes,

separately or in combination with weevils, in replicate 1 (Table 1). No great differences in growth response were observed between banana plants inoculated with V5w2 CHR 9 and Emb2.4o BR 8.

For control plants (endophyte non-inoculated), pest challenge had a significantly negative effect on plant growth in some instances. For example, in replicate 2 height was significantly reduced for control plants challenged with both the nematode and weevil ($F = 9.41$, $P \leq 0.0001$) (Fig. 4). Similarly, weevil challenge significantly reduced the number of roots for endophyte non-inoculated plants in both replicates (Fig. 4). In replicate 2, fresh root weight was significantly reduced for control plants challenged with both the nematode and weevil.

Pest control

Radopholus similis numbers were significantly lower in plants inoculated with isolate V5w2 CHR 9 and those inoculated dually with V5w2 CHR 9 and Emb 2.4o BR 8 compared with endophyte non-inoculated plants and plants inoculated with Emb2.4o BR 8 in replicate 1 (Table 2). Nematode numbers in replicate 2, however, did not differ significantly among nematode-challenged treatments. Root necrosis in plants inoculated with V5w2 CHR 9 was non-significantly reduced compared to the other treatments, but not significantly. When endophyte-inoculated plants were challenged with both nematodes and weevils, the number of nematodes was not significantly lower, nor was the damage caused by nematodes (Table 2). The exception was the amount of necrosis in banana roots inoculated with V5w2 CHR 9 compared to those subjected to other treatments.

No significant reduction in weevil damage to the pseudostem periphery and the outer rhizome was obtained when banana plants were inoculated with endophytic isolates V5w2 CHR 9 and Emb2.4o BR 8 (Fig. 5). However, dual inoculation with the endophytes resulted in a significant damage reduction in the inner rhizome compared with other inoculation treatments in replicate 1, but not in replicate 2. Pseudostem damage was substantially lower than that recorded for the rhizome. Significant reductions were, nevertheless, observed in both outer and inner pseudostem for plants inoculated with both endophytes compared to plants inoculated with one endophyte only (Fig. 5).

DISCUSSION

Endophytic non-pathogenic isolates of *F. oxysporum* used to reduce damage caused by *R. similis* (V5w2 CHR 9) and weevils (Emb2.4o BR 8) were able to stimulate growth and protect banana plants better when inoculated together than when inoculated singly. Significant increments were observed in plant height, girth, fresh root weight and number of live roots for dually-inoculated plants. Multiple spp. inoculation of *Pseudomonas* endophytes in banana reportedly resulted in increased plant height, girth, and leaf area (Harish *et al.*, 2008). The effects of endophyte colonization on plant growth range from positive (increased growth), neutral (no effect) to negative (reduced growth). The latter is often observed where growth conditions such as water (Cheplick *et al*., 2000; Ahlholm *et al*., 2002) and light (Pinto *et al*., 2000) are limited. Increased growth of endophyte-inoculated plants was observed by Redman *et al*. (2001) for tomato plants inoculated with *Colletotrichum magma*; Debattista *et al*. (1990) for *Acremonium* sp. colonization of tall fescue; Niere (2001) for *F. oxysporum* endophyte colonization of EAHB; and Waller *et al*. (2005) for *Piriformospora indica* Sav. Verma colonization of barley (*Hordeum vulgare* L.)

Isolate V5w2 CHR 9 was a better root colonizer than Emb2.4o BR 8, whether inoculated alone or in combination with Emb2.4o BR 8. When inoculated together, the two isolates co-existed well in both roots and rhizomes, but behaved differently. Root colonization by V5w2 CHR 9 was increased by dual inoculation, while that by Emb2.4o BR 8 was reduced in the presence of V5w2 CHR 9. According to Read and Taylor (2000), within host densities of a parasite can be affected by a co-infecting strain, indicating possible competition. In *Bromus erectus* Huds., studies on dual inoculation of four isolates of *Epichloë bromicola* Leuchtmann and Schardl revealed that dual inoculations altered infection rates. In the presence of a fast colonizing isolate, colonization by a slow colonizer was observed to be less likely or very limited. The above phenomenon is similar to that observed in the current study. Despite the above, root colonization was significantly higher in plants inoculated with both isolates compared with those inoculated singly. The increased endophytic colonization for dually-inoculated plants was associated with increased plant growth (height and girth) and *R. similis* population reduction in replicate 1.

An interesting trend emerged pertaining to nematode numbers in endophyte-inoculated plants. When inoculated with V5w2 CHR 9 and with a combination of V5w2 CHR 9 and Emb2.4o BR 8, nematode numbers were reduced when compared to those inoculated with Emb2.4o BR

8 and the controls. One can argue that the nematode control is a result of better root colonization by V5w2 CHR 9 than by Emb2.4o BR 8. However, this may not be the case, as dually-inoculated plants in replicate 2 attained less control of ***R. similis*** despite the higher root colonization recorded, in comparison with replicate 1. Thus, factors other than increased plant colonization may be responsible for the reduced ***R. similis*** numbers following dual inoculation of ***F. oxysporum*** endophytes. One explanation is that the synergistic effect in pest control by dually-inoculated isolates might be due to the additive effect of their combined modes of action (Meyer and Roberts, 2002). In a related study, dual inoculations of ***F. oxysporum*** isolates displaying different modes of action resulted in a 63% reduction in ***R. similis*** numbers in pot trials 8 weeks after challenge (Zum Felde ***et al.***, 2006). In the same experiment by Zum Felde ***et al***. (2006), dual inoculation of ***T. atroviride*** isolates similarly reduced ***R. similis*** numbers by 61%. A greater possibility, however, is that the mode of action of isolate V5w2 CHR 9 against ***R. similis*** involves induced resistance (Athman, 2006; Vu ***et al.***, 2006), and might have caused a reduction in nematode damage irrespective of the amount of root colonization. The mode of action whereby Emb2.4o BR 8 controls ***R. similis*** and the banana weevil has not yet been determined. We further speculate that the increased plant height and girth for dually-inoculated plants challenged with the nematode observed in replicate 1 may have been the consequence of reduced nematode numbers in roots.

When plants were challenged with both the nematode and the weevil, nematode numbers were significantly reduced compared with treatments where the nematode was applied alone. In this case, damage caused by the banana weevil to the rhizome might have negatively influenced the nematodes that feed in the roots. According to Masters ***et al.*** (1993), the interaction between above- and below ground consumers is affected by quantitative and qualitative changes in the shared host plant. Qualitative changes result in systemic changes in the root that negatively affect development and reproduction of root-feeding organisms (Soler ***et al.***, 2007). In the current study, quantitative changes in the plant due to weevil feeding might have reduced root biomass and caused root death, as significant reductions in root number and weight were found in weevil-damaged plants. Root reduction or removal furthermore limits nutrient availability for root inhabiting organisms, hence affecting the latter negatively (Masters ***et al.***, 1993). A reduction in root quality also might have affected nematode population growth negatively.

Dual inoculations resulted in weevil damage reductions in both replicates, though non-significantly in replicate 2. Isolate Emb2.4o BR 8 inoculated singly did not show much

effectiveness in reducing banana weevil damage. This is contrary to the findings of Kapindu (personal communication) who used it in *in vitro* experiments. The lack of banana weevil control displayed by isolate Emb2.4o BR 8 in the current study may be due to its failure to colonize the rhizome effectively, or the isolate may not be effective *in vivo* as it proved to be *in vitro*.

Banana plants free of endophytes had a significantly higher plant height compared with endophyte-inoculated plants when not challenged with *R. similis* and the banana weevil. The growth advantage of endophyte-free plants under pest-free conditions may be attributed to costs incurred by endophyte-inoculated plants upon endophyte colonization. Endophyte colonization of plants is known to induce morphological and physiological defence mechanisms in the host (induced resistance), which is a well documented mode of action of bacterial and fungal endophytes (Duijff *et al.*, 1998; He *et al.*, 2002; Johnson *et al.*, 2003; Wang *et al*., 2005; Athman, 2006). Induced resistance has some associated costs to the host plant (Baldwin and Hamilton, 2000). Better defended plants are reported to have a lower fitness (for instance in terms of seed production) compared to less defended ones when both are grown under pest-free conditions (Simms and Fritz, 1990; Agrawal, 2000; Heil *et al*., 2000).

In banana plants not inoculated with endophytes, a significantly lower plant height (replicate 2 only), number of live roots, and fresh root weights (replicate 2 only) occurred following pest challenge. Extensive rhizome tunnelling resulting from the feeding activity of the banana weevil larvae might have resulted in root damage and subsequent root death. We, therefore, speculate that the reduced plant height may have been a result of the interference in water and nutrient transport up-wards from the roots due to extensive rhizome tunnelling and root death.

From the current study, we conclude that dual inoculation of *F. oxysporum* mutant isolates Emb2.4o BR 8 and V5w2 CHR 9 resulted in increased root colonization and tended to promote growth in presence of *R. similis* challenge. More studies, however, are necessary to know whether effects on growth are positive or neutral. Dual endophyte inoculations further reduced *R. similis* numbers and banana weevil damage in screenhouse, and now need to be tested in the field. If proven valuable, banana bio-enhancement with endophytic isolates Emb2.4o BR 8 and V5w2 CHR 9 can be used for tissue culture EAHB planted in fields severely affected by nematodes and weevils in Uganda.

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Table 1. Growth of tissue culture banana plants (cv Nabusa, AAA-EA) 20 weeks after plant inoculation and 12 weeks after pest challenge

Inoculation treatment[1]	Nematode challenge		Weevil challenge		Nematode and weevil challenge		Non-challenged	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
Height (cm)								
Emb2.4o BR 8	38.9 ± 1.3 b	37.6 ± 1.6 b	42.2 ± 1.9 a	42.2 ± 1.2 ab	36.1 ± 1.8 c	39.0 ± 1.1 a	34.7± 1.9 b	43.8 ± 1.4 ab
V5w2 CHR 9	34.2 ± 1.9 b	41.5 ± 1.5 ab	44.9 ± 1.9 a	35.5 ± 1.6 c	40.4 ± 2.0 bc	37.1 ± 1.1 a	41.3 ± 1.1 ab	40.7 ± 0.8 b
Dual	55.1 ± 1.5 a	42.0 ± 1.1 ab	41.1 ± 1.9 a	38.2 ± 1.0 bc	55.7 ± 1.5 a	38.7 ± 1.0 a	36.1 ± 2.3 ab	43.3 ± 1.3 ab
Control	36.8 ± 2.1 b	46.5 ± 1.6 a	44.6 ± 2.1 a	44.5 ± 1.4 a	43.8 ± 2.3 b	38.2 ± 0.6 a	42.4 ± 2.2 a	47.2 ± 1.5 a
Girth (cm)								
Emb2.4o BR 8	12.4 ± 0.4 b	15.7 ± 0.5 a	12.7 ± 0.7 a	15.4 ± 0.2 a	11.3 ± 0.4 c	14.9 ± 0.5 a	11.8 ± 0.3 a	15.4 ± 0.3 a
V5w2 CHR 9	12.9 ± 0.5 b	15.8 ± 0.4 a	12.5 ± 0.5 a	14.4 ± 0.6 a	12.7 ± 0.5 c	14.0 ± 0.4 a	11.5 ± 0.3 a	15.8 ± 0.4 a
Dual	16.6 ± 0.3 a	15.9 ± 0.2 a	12.1 ± 0.4 a	13.8 ± 0.5 a	16.5 ± 0.3 a	14.3 ± 0.7 a	11.3 ± 0.4 a	14.8 ± 0.2 a
Control	10.9 ± 0.2 c	16.4 ± 0.2 a	13.2 ± 0.5 a	14.6 ± 0.4 a	14.7 ± 0.5 b	14.4 ± 0.6 a	11.7 ± 0.3 a	15.6 ± 0.3 a
Root number								
Emb2.4o BR 8	33.2 ± 1.9 b	32.0 ± 2.0 a	15.2 ± 1.7 a	32.0 ± 2.5 a	17.8 ± 2.0 b	22.6 ± 1.3 a	34.4 ± 1.8 a	32.8 ± 1.8 b
V5w2 CHR 9	32.8 ± 2.2 b	27.6 ± 2.0 a	14.8 ± 1.6 a	25.8 ± 2.9 ab	15.6 ± 2.1 b	17.0 ± 2.5 a	36.0 ± 2.7 a	35.4 ± 2.2 b
Dual	48.8 ± 2.5 a	29.4 ± 1.9 a	16.5 ± 1.6 a	19.9 ± 3.0 a	28.8 ± 3.3 a	20.7 ± 2.5 a	31.1 ± 3.1 a	34.3 ± 1.0 b
Control	28.6 ± 2.8 b	31.2 ± 2.1 a	13.8 ± 1.7 a	35.1 ± 2.7 a	24.1 ± 2.6 ab	25.6 ± 4.1 a	32.4 ± 1.6 a	42.3 ± 1.5 a
Fresh root weight (g)								
Emb2.4o BR 8	111 ± 10 b	168 ± 11 ab	70 ± 8 a	170 ± 7 ab	103 ± 10 b	123 ± 8 a	132 ± 18 a	171 ± 6 b
V5w2 CHR 9	143 ± 9 b	148 ± 13 b	80 ± 12 a	136 ± 13 b	97 ± 13 b	94 ± 9 a	143 ± 10 a	173 ± 10 b
Dual	207 ± 9 a	147 ± 5 b	88 ± 12 a	130 ± 15 b	178 ± 19 a	131 ± 17 a	128 ± 12 a	176 ± 6 b
Control	106 ± 6 b	186 ± 7 a	112 ± 14 a	196 ± 17 a	132 ± 17 b	135 ± 15 a	117 ± 11 a	218 ± 8 a

Height represents change in height between plant inoculation and harvest, girth represents the circumference of the pseudostem base at harvest, and fresh root weight represents weight of all live roots. For each growth parameter, means in a column followed by different letters are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

[1]Dual = Emb2.4o BR 8 + V5w2 CHR 9

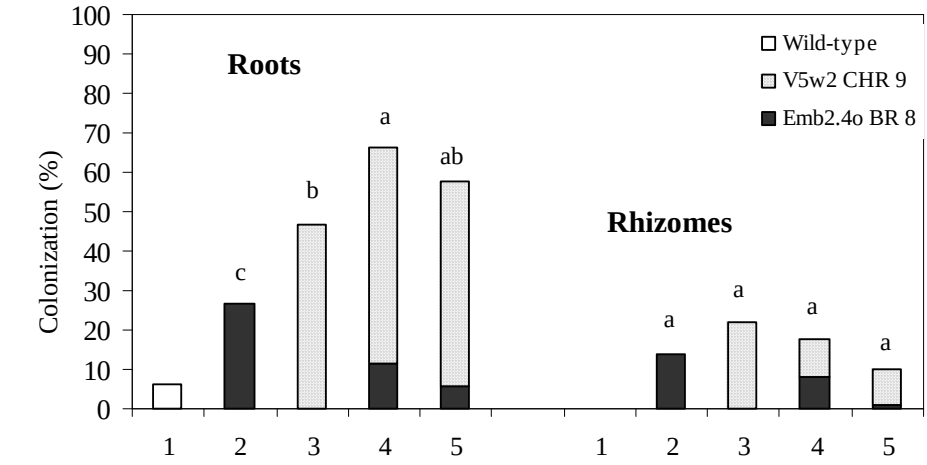
Table 2. Nematode numbers and root necrosis in tissue culture banana plants (cv Nabusa, AAA-EA) 20 weeks after endophyte inoculation and 12 weeks after *Radopholus similis* challenge of 8-week-old plants

Inoculation treatment[1]	Pest	Total nematodes (x100)/100 g root			Necrosis (%)	
		Replicate 1	Replicate 2		Replicate 1	Replicate 2
Control	Nematode	217.7 ± 94.3 a	217.3 ± 75.7 a		14.4 ± 5.3 bc	22.8 ± 4.9 a
Emb2.4o BR 8	Nematode	249.4 ± 72.7 a	223.5 ± 53.4 a		14.3 ± 3.5 bc	27.3 ± 4.6 a
V5w2 CHR 9	Nematode	55.9 ± 23.7 b	218.1 ± 69.4 a		6.9 ± 2.1 c	16.3 ± 2.7 a
Dual	Nematode	79.8 ± 32.3 b	122.2 ± 2.3 ab		15.4 ± 3.8 bc	24.7 ± 5.3 a
Control	Nematode and weevil	25.49 ± 8.2 b	19.5 ± 8.7 b		15.4 ± 2.4 bc	23.0 ± 4.9 a
Emb2.4o BR 8	Nematode and weevil	24.8 ± 12.1 b	9.2 ± 3.2 b		20.2 ± 3.0 b	28.0 ± 6.0 a
V5w2 CHR 9	Nematode and weevil	38.7 ± 16.2 b	12.2 ± 6.5 b		32.6 ± 4.2 a	24.2 ± 7.9 a
Dual	Nematode and weevil	15.5 ± 4.4 b	22.8 ± 9.1 b		11.3 ± 2.9 bc	15.8 ± 3.4 a

Values represent means ± SE, n = 10.
[1]Dual = Emb2.4o BR 8 + V5w2 CHR 9.
Means in a column followed by different letters are significantly different $P \leq 0.0019$ (Dunn-sidak correction).

Figure 1. Two-month-old tissue-culture banana plants (cv Nabusa, AAA-EA) enclosed in mosquito nets after infestation with 10 female banana weevils.

Replicate 1



Replicate 2

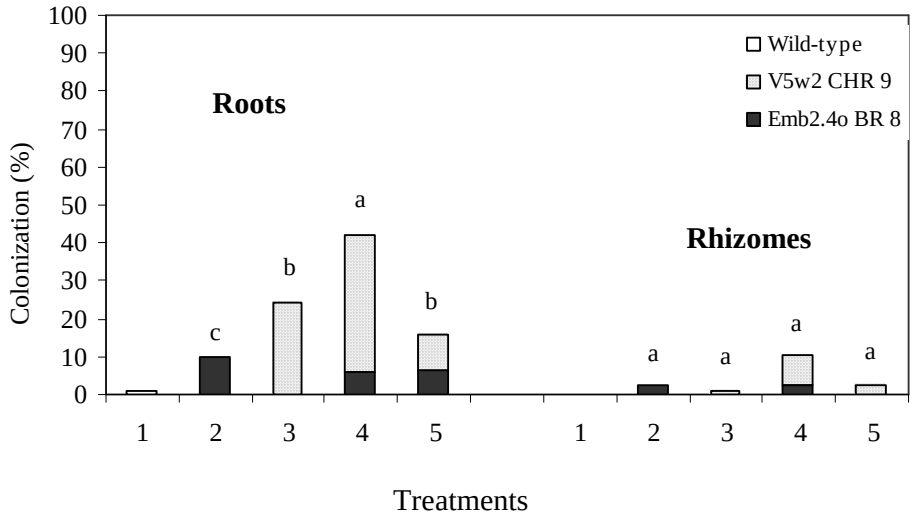
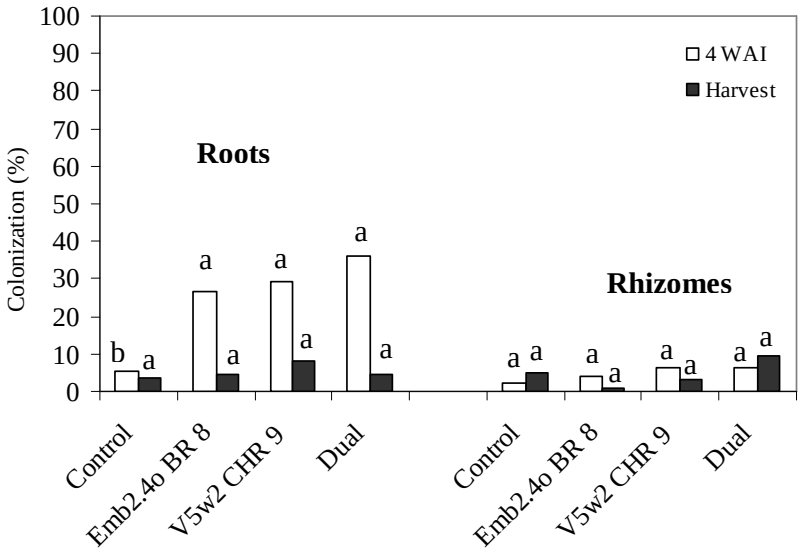


Figure 2. Colonization of roots and rhizomes of tissue-culture banana plants (cv Kibuzi, AAA-EA), 4 weeks after inoculation with *Fusarium oxysporum* isolates Emb2.4o BR 8 and V5w2 CHR 9. Treatment 1 = non-inoculated control plants 2 = plants inoculated with Emb2.4o BR 8 with a spore suspension of concentration 1.5×10^6 spores ml^{-1}, 3 = plants inoculated with isolate V5w2 CHR 8 with a spore suspension of concentration 1.5×10^6 spores ml^{-1}, 4 = plants inoculated with isolates Emb2.4o BR 8 and V5w2 CHR 9 with a spore suspension of concentration 0.75×10^6 spores ml^{-1} and 5 = plants inoculated with isolates Emb2.4o BR 8 and V5w2 CHR 9 with a spore suspension of concentration 1.5×10^6 spores ml^{-1}. For each plant organ, bars followed by different letters within an experiment are significantly different at $P \leq 0.005$ (Dunn-sidak correction).

Replicate 1



Replicate 2

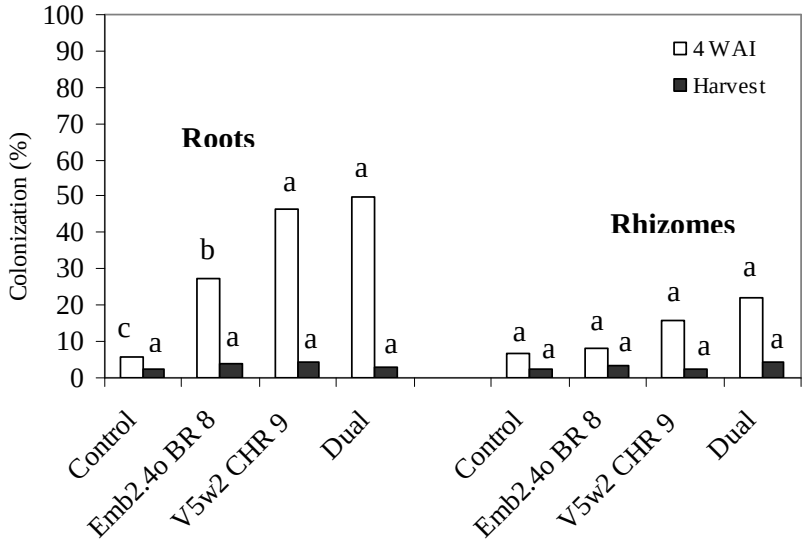
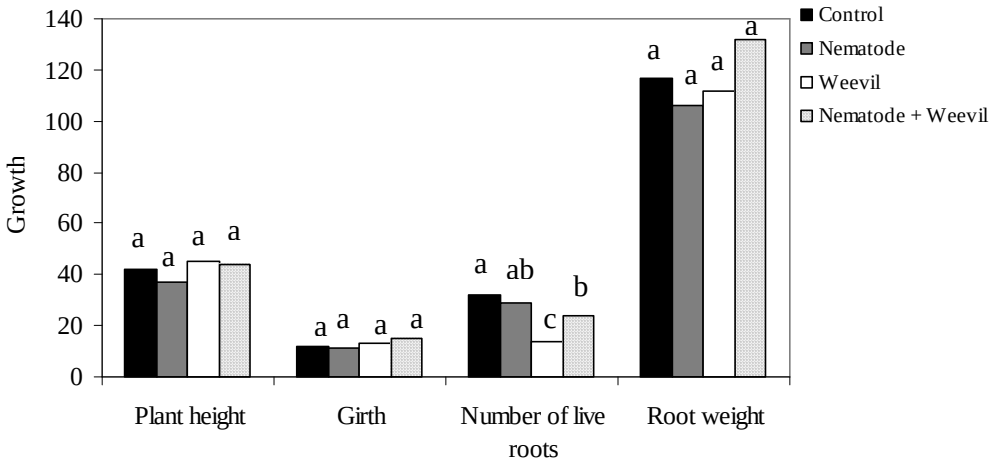


Figure 3. Colonization of tissue cultured banana plants (cv. Nabusa *Musa* spp., AAA-EA) by *Fusarium oxysporum* 4 weeks after inoculation and at harvest. Dual = plants inoculated with isolates Emb2.4o BR 8 and V5w2 CHR 9. Bars followed by different letters for each plant organ and isolation time are significantly different at $P \leq 0.009$ (Dunn-sidak correction).

Replicate 1



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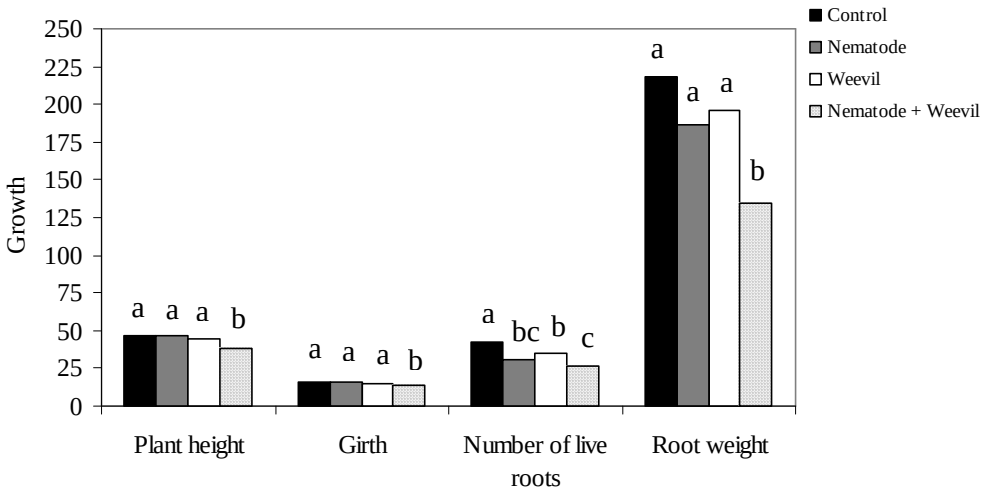
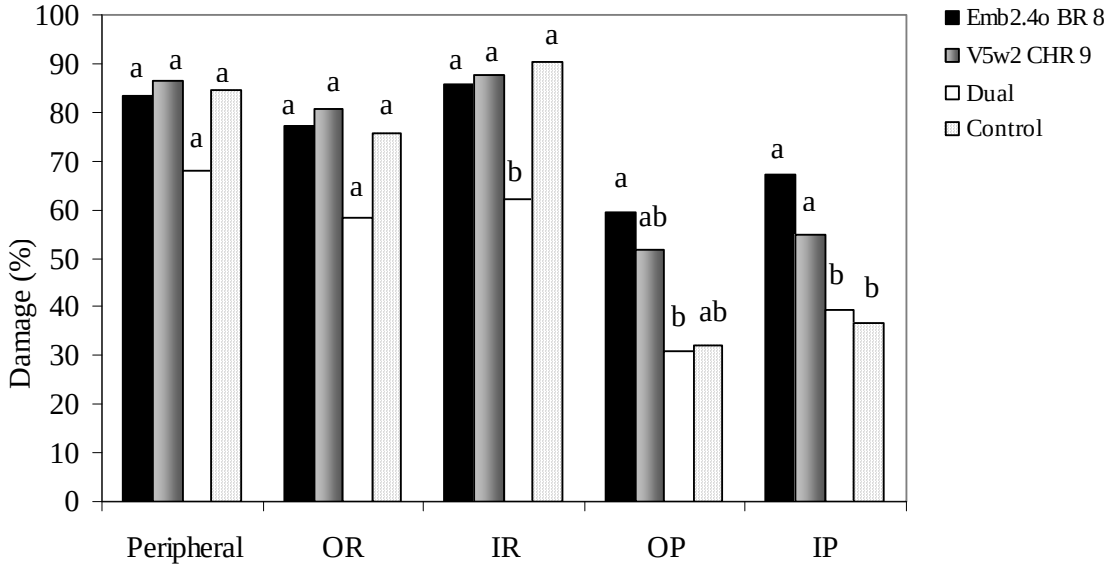


Figure 4. The effect of *Radopholus similis* and *Cosmopolites sordidus* challenge on the growth of tissue cultured banana plants (cv Nabusa, AAA-EA). Height (cm) represents change in height between planting and harvest, girth (cm) represents the circumference of the pseudostem base at harvest, and fresh root weight (g) represents weight of all live roots. For each growth parameter, bars followed by different letters are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

Replicate 1



Replicate 2

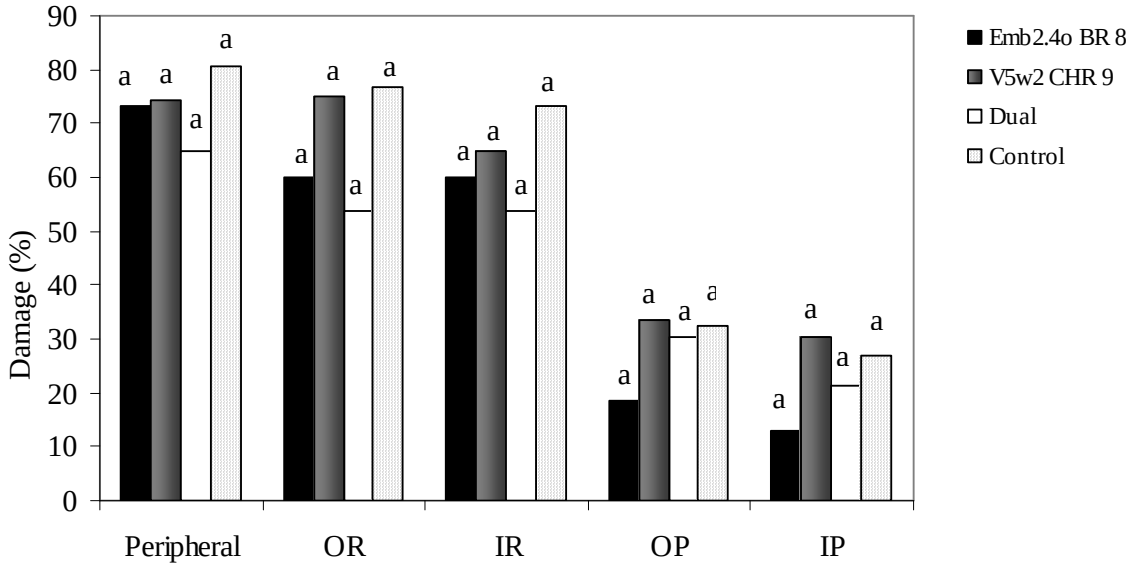


Figure 5. Weevil damage in tissue cultured banana plants (cv Nabusa, AAA-EA) 20 weeks after inoculation with non-pathogenic *Fusarium oxysporum* endophytes and 12 weeks after infestation of 8-week-old plants with 10 female banana weevils. OR = outer rhizome base, IR = inner rhizome base, OP = outer pseudostem and IP= inner pseudostem. For each plant organ, bars followed by different letters are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

SUMMARY

In the interactions between fungal endophytes and their hosts, the host may benefit through protection against pathogens and pests, growth promotion and tolerance to abiotic stresses. Non-pathogenic *Fusarium oxysporum* endophytes of banana have been shown to reduce the damage caused by the *Cosmopolitus sordidus* and the burrowing nematode *Radopholus similis.* The mode of protection against the burrowing nematode involves induced resistance, but the molecular basis of this resistance yet to be demonstrated. It has further been reported that protection of the host by multiple endophytes can lead to better control of target pests, probably because of the multiple modes of action involved. This phenomenon, however, has not been fully demonstrated for *F. oxysporum* endophytes of banana. This study aimed to investigate the molecular and biochemical basis of endophyte protection of East African Highland bananas (EAHB) against *C. sordidus* and *R. similis*.

Expression of banana defence-related genes following endophyte inoculation and *R. similis* challenge varied greatly between the nematode-susceptible cv Nabusa and the nematode-tolerant cv Kayinja. In cv Nabusa, only the *peroxidase* (*POX*) and *lectin* genes were responsive to endophyte colonization of roots, or *R. similis* challenge. *POX* and lectin activities were significantly down-regulated 2 and 33 days after endophyte inoculation (dai), respectively. In cv Kayinja, endophyte colonization resulted in transient up-regulation of *POX* and a down-regulation of *endochitinase* (*PR-3*), *lectin*, *pectin acetylesterase* (*PAE*), *phenylalanine ammonia-lyase* (*PAL*) and *PIR7A* (*peroxidase*). Similar to systemic acquired resistance, *PR-1* and *catalase* activities were up-regulated in the cv Kayinja 33 dai.

Genes involved in signal transduction, cell wall strengthening, jasmonic acid pathway and defence molecule transport were differentially expressed in endophyte-inoculated plants. The expression profiles of four defence-related genes following endophyte inoculation and *R. similis* challenge were studied using quantitative real-time PCR. *ABC transporter*, *β-1,3-glucan synthase, coronatine insensitive 1* (*COI1*) and *lipoxygenase* (*LOX*) were up-regulated following endophyte inoculation. *β-1,3-glucan synthase* and *COI1* were highly up-regulated following *R. similis* challenge of endophyte-inoculated plants of the susceptible cv Nabusa, while *COI1* and *LOX* were highly up-regulated following nematode challenge of endophyte-inoculated plants of the tolerant cv Kayinja. However *ABC transporter* gene activity was not up-regulated following nematode challenge of plants of both cultivars.

UP-regulation of phenylpropanoid pathway enzymes PAL, POX and PPO has been observed in roots following colonization by both pathogenic and non-pathogenic fungi. In the current study, endophyte inoculation resulted in down-regulation of PAL activity in both a susceptible (cv Nabusa) and tolerant (cv Yangambi) banana. In cv Nabusa, endophyte inoculation primed PAL activity for up-regulation 30 days post nematode challenge (dpnc). However, in cv Yangambi PAL activity was up-regulated 7 dpnc irrespective of endophyte inoculation. Endophyte inoculation transiently up-regulated POX in cv Nabusa, but activity reduced to the levels in the controls 30 dai. Similar to PAL, ***R. similis*** challenge of endophyte-inoculated plants of Nabusa caused significant up-regulation of POX 7 dpnc. Nematode challenge of control plants of cv Yangambi resulted in a non-significant up-regulation of POX compared with non-challenged controls, but a significant up-regulation compared to all endophyte-inoculated plants. PPO activity was transiently up-regulated in cv Nabusa and down-regulated in cv Yangambi 7 dai. For all treatments, PPO activity was significantly reduced between 7 dai and 120 dai (60 dpnc).

Fusarium oxysporum endophyte isolates Emb2.4o and V5w2 were successfully marked with benomyl- and chlorate resistance and transformed with fluorescent protein genes, while Eny1.31i, Eny7.11o and V4w5 were marked with benomyl resistance only. Most mutants and fluorescent protein transformants maintained resistance to the selective chemical on PDA and after plant colonization. Benomyl- and chlorate-resistant mutants were successfully used to determine actual plant colonization percentages by inoculated endophytes. Similarly, GFP transformants were successfully used to ascertain the pattern of endophytic root colonization *in vivo*.

In plants dually inoculated with isolates Emb2.4o BR 8 and V5w2 CHR 9, both isolates were recovered from roots and rhizomes 4 weeks after inoculation, but isolate V5w2 CHR 9 proved a better colonizer of the two tissue types. Root colonization by isolate V5w2 CHR 9 was boosted when inoculated dually with Emb2.4o BR 8, while that by Emb2.4o BR 8 was reduced in the presence of V5w2 CHR 9. Where growth advantages were observed for dually inoculated plants, it occurred where plants were challenged with ***R. similis***. In the absence of pests, control plants showed better growth than endophyte-inoculated plants. On the other hand, weevil challenge of control plants resulted in significant reductions in plant height, number of live roots and root fresh weight. Dual endophyte inoculation resulted in a significant reduction in ***R. similis*** populations in nematode only challenged plants, compared

with plants inoculated with Emb2.4o BR 8 singly and control plants challenged with the nematode. In one replicate banana weevil damage to the outer and inner pseudostem base, and the inner rhizome were significantly reduced for dually-inoculated plants.

APPENDIX

Minimal media (MM)

Basal media (Correll *et al*., 1987)

KH_2PO_4	1	g
KCL	0.5	g
$MgSO_4.7H_2O$	0.5	g
$FeSO_4.7H_2O$	10	mg
Sucrose	30	g
Agar	20	g
Trace element solution	0.2	ml
Distilled water	1,000	ml

Mixing the following ingredients in 95 ml of distilled water makes trace element solution. Citric acid, 5g; $ZnSO_4.7H_2O$, 5g; $Fe(NH_4)_2(SO4)2$, 1g; $CuSO_4.5H_2O$, 0.25g; $MnSo_4.H_2O$,50mg; H_3BO_3, 50mg; $NaMoO_4.2H_2O$, 50mg.

Minimal media (Correll *et al*., 1987)

Basal medium	1,000	ml
$NaNO_3$	15	g