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Endophytic *Fusarium oxysporum*: Phylogeny and induced defense responses in banana plants against *Radopholus similis*

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Endophytic *Fusarium oxysporum*: Phylogeny and induced defense responses in banana plants against *Radopholus similis*

The burrowing nematode *Radopholus similis* is a destructive pest of banana causing severe yield losses in plantations worldwide. *R. similis* infestation of the banana root is significantly reduced, when the root system is colonized by specific non-pathogenic *Fusarium oxysporum* endophytes. This enhanced resistance towards the nematode acts in a systemic manner. Until now, it is not clear how and why only specific isolates of this species of *Fusarium* induce what is called systemic induced resistance (SIR). In order to obtain a better understanding of this unique interrelationship, mutualistic *F. oxysporum* endophytes were phylogenetically compared both to each other and to the pathogenic forms of *F. oxysporum* f.sp. *cubense*. Molecular and biochemical aspects of SIR responses in the banana cultivar 'Valery' (AAA) were assessed.

RAPD, RFLP and sequence analysis of different DNA sequences were evaluated for their potential to infer the phylogeny of *F. oxysporum* isolates, which were characterized as being mutualistic by conferring enhanced resistance against *R. similis*. Most isolates originated from Uganda, Africa. Sequence analysis of the partial IGS region was identified as the best approach for inferring the phylogenetic relations of the Fusarium isolates to each other. It could be shown that non-pathogenic were clearly distinct from the pathogenic isolates and that isolates generally clustered according to their geographical origin by forming an independent African clade. However, in contrast to the scattered and genetic diverse mutualists and commensalists from Uganda, pathogens were arranged in an exclusive cluster showing only little genetic diversity. Therefore, pathogenicity can be considered a monophyletic trait, whereas mutualism must be considered polyphyletic.

NPR1 and *PR-1* homologs of *Musa* AAA were chosen as marker genes for ISR and SAR respectively. Real-time PCR was used to monitor their expression in the leaves and roots of the banana cultivar 'Valery' (AAA) in response to treatments with chemicals, *R. similis* and mutualistic, commensalistic or pathogenic *F. oxysporum* isolates. Expression studies showed that, SAR and not ISR, was elicited by certain chemical inducers. The presence of *R. similis* alone did not lead to SAR or ISR. Nematode penetration was significantly reduced by the mutualistic Fo162 and the pathogenic Fo001, by 39% and 45%, respectively. *PR-1* transcripts accumulated in response to the pathogen Fo001, suggesting the onset of SAR as part of the incompatible interaction between Fo001 and the Fusarium wilt resistant cultivar Valery. The accumulation of *NPR1* and *PR-1* transcripts did not increase in the presence of the mutualist Fo162, thus indicating that both ISR and SAR were not involved during SIR.

Root exudates extracts of Valery, colonized by Fo162, were collected on a XAD-4 matrix, extracted and both analyzed by RP-HPLC and tested *in vitro* biotest systems for their chemotactical activity towards *R. similis*. The RP-HPLC analysis of the root exudate extracts identified the identical set of 26 compounds for both treatments. Significant differences in compound accumulation were observed between the treatments. Nevertheless, the individual root exudate extracts did not show any repellent or attractant activity towards *R. similis* in an *in vitro* biotest system.

ISR and SAR seem to be irrelevant in the enhanced resistance against *R. similis* that is induced by mutualistic *F. oxysporum* isolates. Neither gene expression studies nor root exudate profiling indicated the actual mechanism that accounts for the enhanced resistance of banana against *R. similis*. However, the findings of the phylogenetic as well as the gene expression studies, along with routine VCG tests, support the safe use of mutualistic Fo162 as a *R. similis* management tool in banana production in future.

Fusarium oxysporum Endophyten: Abstammungsverhältnisse und induzierte Abwehrreaktionen gegen *Radopholus similis* in der Bananenpflanze

Der Wurzelnematode *Radopholus similis* ist ein aggressiver Schädling der Banane und verursacht massive Ertragsverluste in Anbaugebieten weltweit. Besondere apathogene endophytische Pilze der Spezies *F. oxysporum* können den Nematodenbefall durch Kolonisierung des Bananenwurzelsystems reduzieren, wobei die erhöhte Resistenz gegen *R. similis* systemisch induziert wird. Jedoch ist unklar warum nur einige Endophyten diese sogenannte Systemisch Induzierte Resistenz (SIR) auslösen und worauf Sie basiert. Um diese Fragen zu beantworten, wurden zum einen die Abstammungsverhältnisse von apathogenen Isolaten untereinander und im Vergleich zu pathogenen Isolaten analysiert, zum anderen wurden molekulare und biochemische Aspekte der induzierten Abwehrreaktion am Beispiel der Bananensorte Valery untersucht.

RAPD, RFLP, DNA-Sequenzanalyse sowie die Analyse verschiedener DNA-Regionen sollten die Abstammungsverhältnisse mutualistischer, commensalistischer oder pathogenen *Fusarium* Spezies beschreiben. Den besten Ansatz zur Beschreibung der verschiedenen *Fusarium* spp. lieferte die Sequenzanalyse der IGS-Region. Durch den starken Einfluss des geografischen Ursprungs, kam es zur Bildung eines separaten afrikanischen Astes. Dabei unterschieden sich die nicht pathogenen Isolate deutlich von den Pathogenen. Im Gegensatz zu den genetisch diversen apathogenen Endophyten bildeten die Pathogene ein eigenes Cluster mit nur geringer genetischer Diversität. Daher wird in diesem Ast des Stammbaumes die Eigenschaft der Pathogenität als monophyletisches und Mutualismus als polyphyletisches Merkmal betrachtet.

In Studien zur Genexpression wurde homologe *NPR1* und *PR-1* Sequenzen von *Musa* AAA als Marker für jeweils Induzierte Systemische Resistenz (ISR) oder 'Systemic Acquired Resistance' (SAR) identifiziert und der Einfluss von Chemikalien, *R. similis* und mutualistischer, commensalistischer oder pathogener *F. oxysporum* Isolate auf deren Expression in Blättern und Wurzeln von Bananen der Sorte Valery ermittelt. Hierbei wurde nur SAR, aber nicht ISR durch die chemischen Induktoren ausgelöst. Die Penetrationraten von *R. similis* wurden durch das mutualistische Isolat Fo162 und das Pathogen Fo001 signifikant um 39% bzw. 45% reduziert. Während die Penetration von *R. similis* alleine weder ISR noch SAR auslöste, wurde die Akkumulation von *PR-1* Transkripten durch das Pathogen Fo001 erhöht, was auf eine Beteiligung von SAR bei der inkompatiblen Reaktion der resistenten Bananensorte Valery gegen Fo001 schließen lässt. Die Akkumulation von *NPR1* oder *PR-1* Transkripten wurde durch den Mutualisten Fo162 nicht signifikant erhöht. Folglich spielen SAR und ISR keine Rolle bei SIR in Banane.

In einem weiteren Versuch wurden Wurzeln von Valery gezielt mit dem Mutualisten Fo162 besiedelt. Die Wurzelexudate wurden durch eine Matrix gebunden, anschließend extrahiert, mittels RP-HPLC analysiert und in einem *in vitro* Testsystem gegen *R. similis* getestet. Die Analyse der Extrakte zeigte je Kontrolle und Behandlungen eine identische Zusammensetzung von 26 Verbindungen, welche sich lediglich in der jeweils nachgewiesenen Menge unterschieden. In den Biotests zeigte keines der Extrakte eine abschreckende oder anlockende Wirkung auf *R. similis*.

Die Studien zur Genexpression zeigen, dass weder ISR noch SAR eine Rolle bei der erhöhten Resistenz durch SIR spielten. Auch die Analyse der Wurzelexudate gab keinen Aufschluss über die Wirkmechanismen der erhöhten Resistenz der Banane gegen *R. similis*. Dagegen unterstützen die Ergebnisse der phylogenetischen als auch der Studien zur Genexpressionsstudie, im Einklang mit den Daten aus vorausgegangenen VCG Tests, die risikofreie Anwendung des nicht pathogenen Mutualisten Fo162 bei der Bekämpfung von *R. similis* im Bananenanbau.

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Abbreviations and Symbols

A	Ampere
Å	Angstrom (10^{-10} m)
ACC	1-Aminocyclopropane-1-carboxylic acid
AIC	Akaike Information Criterion
ANOVA	Analysis of variance between groups
A	Ampere
Avr	Avirulent
BI	Bayesian Inference
BTH	Benzol (l,2,3) thiadiazole-7carbothioic acid S-methyl ester
℃	degree Celsius
CI	Consistency index
CT	Cycling threshold
DA	Dalton (molecular weight unit)
DAD	Diode Array Detector
DAI	Days after Inoculation
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Desoxyribonucleotide
DPI	Days post Infection
EDS	Enhanced disease susceptibility
EDTA	Ethylene-diamin-tetra-acetat
Foc	*Fusarium oxysporum* f.sp. *cubense*
g	Gravity (9.81 m/s^2)
GS	Genetic similarity
hLRTs	Hierarchal Likelihood Ratio Tests
HPLC	High Performance Liquid Chromatography
HR	Hypersensitive response
INA	2,6-dichloroisonicotinic acid
Indel	Insertion and/or deletion
IPM	Integrated pest management
ISR	Induced systemic resistance
IFo	Inducing *Fusarium oxysporum*

IGS	Intergenic spacer
ITS	Internal transcribed spacer
JA	Jasmonic acid
l	Litre
lnL	Log naturalis of Likelihood
LSD	least significant difference
M	Molar
mAU	Milli Ampere Units
MeJA	Methyl Jasmonate
Milli-Q	Water purification systems manufactured by Millipore Corporation.
mL/g	Pore volume
mRNA	Messenger RNA
mtS/LrDNA	mitochondrial Smal/Large ribosomal DNA
NDR	Non-race specific disease resistance
NPR1	No expresser of PR-genes
P	Nominal level of significance
PAD	Phytoalexin-deficient
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pf	Final population
PGPB	Plant growth promoting bacteria
pi	Initial population
PP	Posterior probabilities
PR	Pathogenesis-related
qPCR	quantitative PCR or real-time PCR
R	Resistance
RAPD	Random amplified polymorphic DNA
RB	Reaction buffer
RCF	Relative centrifugal force (Synonym g)
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction length polymorphism
RI	Retention index
Rn	Normalized Reporter Signal during real-time PCR

RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpm	Rounds per minute
RQ	Relative Quantification
rSSU	ribosomal Small Sub Unit
RT	Reverse transcription
s	Second
SA	Salicylicc acid
SAR	Systemic Acquired Resistance
SAHN	Sequential Agglomerative Hierarchical Nested
SNA	Synthetic nutrient-poor agar
SIR	Systemic induced resistance or ‘Biological Enhancement’
SNP	Single nucleotide polymorphism
t	Time
TAE	Tris-acetate-EDTA
TMV	Tobacco Mosaic Virus
UPGMA	Unweighted Pair group Method with Arithmetic Average
VCG	Vegetative compatibility group
V	Voltage
Ø	Diameter

1 General Introduction

Despite worldwide intensification of agricultural production and tremendous progress towards increasing yields of major food crops, the goal of reducing the problems associated with malnutrition is far from being solved (FAO, 2004). To date still 776 million people from developing countries remain undernourished. In contrast, the general conclusion of the FAO report 'World agriculture: towards 2015/2030', was that future growth of global agriculture will be able to cope with the predicted demand for food in the future and that global shortages are considered unlikely. However, serious problems at national and regional level have been identified and are expected to worsen, if not resolved by the development of practical solutions. Nevertheless, for some countries, growth rates of food production are not expected to keep pace with food demand. In general three major factors contribute to growth potential: Water, fertilizer and pesticide, the latter two, however have detrimental side effects on the environment (Chapin et al., 2000; Parmesan and Yohe, 2003). Crop losses are also caused by both abiotic and biotic factors. It is estimated that annual crop losses due to plant diseases alone are currently 220 billion dollars, thus reducing the actual attainable crop production by 14.1% (Oerke, 2005). At the same time, consumers are becoming increasingly aware of the need for safe and nutritious food. To increase food production while reducing pesticide input, alternative technologies for integrated pest management (IPM) are required.

1.1 Banana: The important but sensitive food source

According to the FAO report for crop prospects and food situation, 32 countries suffer from food insecurity and require external support. 20 of those countries originate from Africa, illustrating the unequal distribution of global food resources. These African countries include the countries around the Great Lakes (Lake Victoria), Tanzania, Gabon and Uganda. In 2008 food shortages for Uganda were characterized as 'Localized crop failure, insecurity' (FAO, 2008). Cooking bananas are especially important in this region and people consume close to one kg of bananas every day (Bioversity-International). For example, most of the Ugandan population relies on cooking bananas as the major staple-food (Gold et al., 1999).

Banana (*Musa* spp.) is an herb that belongs to the family of *Musacea*. Morphologically it consists of a branched belowground stem, the rhizome or corm and roots. The banana stem is in fact a pseudostem composed of tightly packed

leaves and can reach up to 15 m (Jones, 1999). The large and complex inflorescence consists of rows of hermaphroditic flowers, of which only the female will produce fruits also known as 'fingers'. Depending on genetic, abiotic and biotic factors, the banana bunch weight (i.e. the inflorescence now bearing fruit) can vary from 15-45 kg. Although the mother pseudostem dies after fruiting, banana is considered a perennial crop, so called suckers emerge from buds at the corm and form a new pseudostem during the following cropping cycle (Simmonds, 1966).

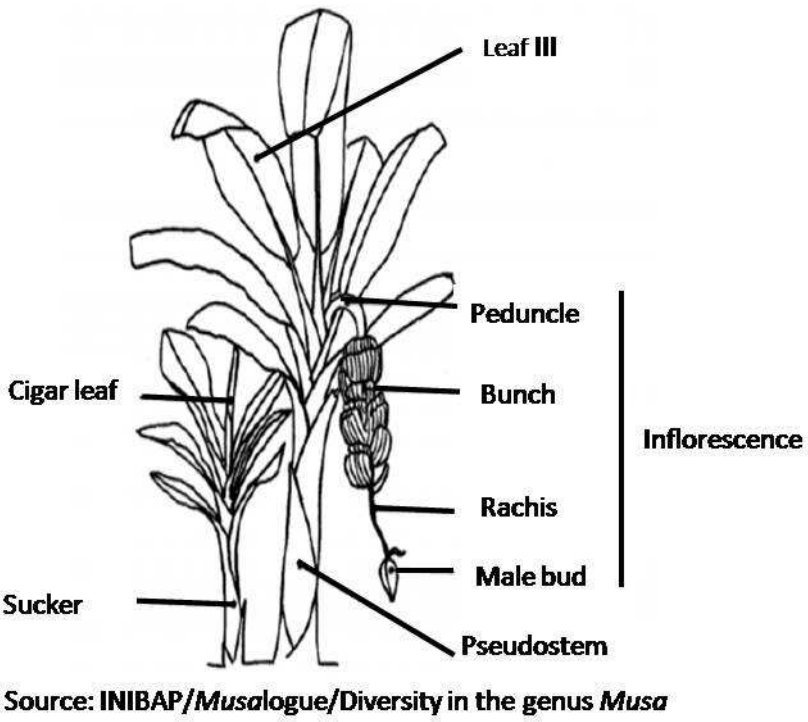


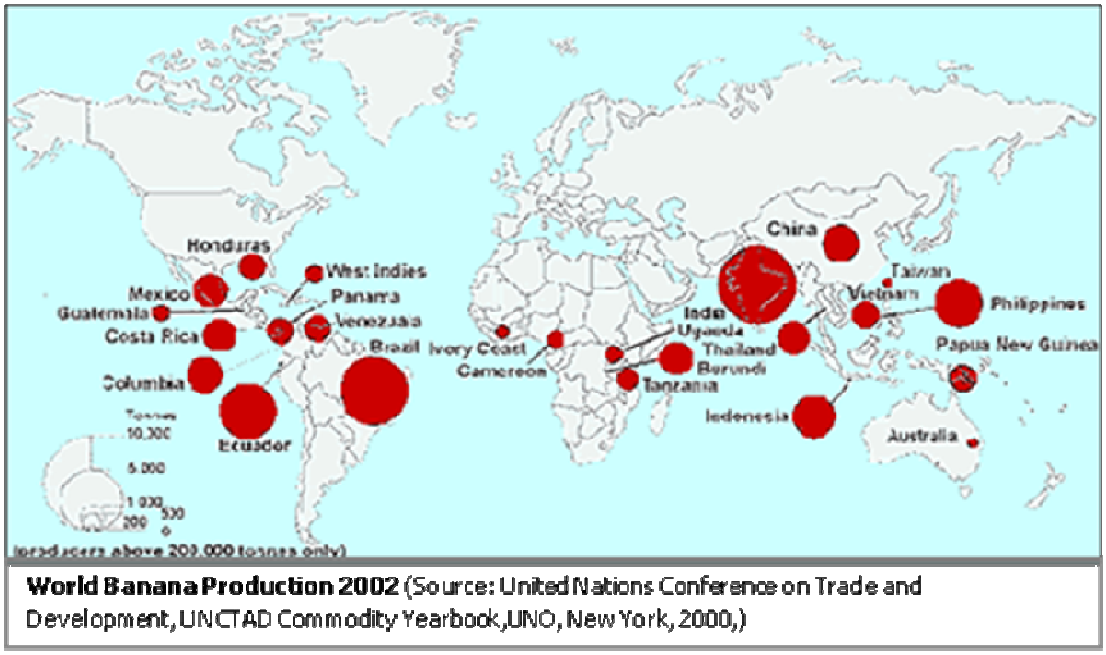
Figure 1: Banana plant with sucker, inflorescence and the banana bunch

Archaeological and palaeoenvironmental studies demonstrate a very long history of banana cultivation as early as 7000 b.c. and possibly even 10 000 b.c. (Denham et al., 2003). Papua New Guinea is most likely the centre of origin of banana. From here the banana has spread over the Indian peninsula into eastern and western Africa, then to central and South America and finally to the pacific islands (INIBAP, 2000). Banana now thrives in over 120 tropical and subtropical countries, characterized by a minimum rainfall of 1250 mm and average minimal temperatures of 15℃ (Gowen, 2005). Although wild bananas are in edible; many hybrids of edible bananas are grown and consumed. Edible bananas are parthenogenic, therefore sterile and are propagated vegetatively (Simmonds, 1962; Simmonds, 1966). Bananas of the genus of *Musa* can be divided into four sections that include both seeded and seedless types: *Eumusa* (2n=22), *Rhodoclamys* (2n=22), *Callimusa*

(2n=20) and *Australimusa* (2n=20). Most of the cultivated bananas arose from *Eumusa* and the genus *Musa* now contains 11 species (Horry et al., 1997). Most of *Eumusa* bananas are derived from *Musa acuminata, Musa balbisiana* or a hybrid of the two. The currently applied nomenclature indicates the genomic origin by designating 'A' for the *M. acuminata* genome and 'B' for the *M. balbisiana* genome. The level of ploidy of each genotype is therefore indicated by assigning A or B for each genome present (Gowen, 2005; Simmonds, 1966). *M. acuminata* is the most widespread of the *Eumusa* species and its centre of diversity is thought to be Malaysia (Simmonds, 1962) or Indonesia (Horry et al., 1997; Nasution, 1991).

Bananas are produced for different purposes, like dessert bananas (AAA and AAB), East African cooking and beer bananas (AAA-EA), cooking banana (ABB) and plantains (AAB) (Ortiz, 1997). Cultivars of the Cavendish subgroup, belonging to the triploid AAA of *M. acuminata*, are the economically most important varieties of dessert bananas. The best known cultivars of the Cavendish subgroup are Grand Naine, Valery, Williams, Apple and Dwarf Cavendish. These cultivars have replaced the formerly produced AAA subgroup Gros Michel, which was highly susceptible to the Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* race 1. Fusarium wilt is also known as Panama disease (Daniells et al., 2001). A very distinct type of cooking bananas (Plantain AAB) is cultivated in the wet tropical zones of West and Central Africa. Another distinct group of bananas (AAA-EA) is found in the East African Highlands, which is considered a secondary centre of banana diversity (De Langhe, 1996; Simmonds, 1966). The East African Highlands mainly comprise Uganda, Rwanda and the Kagera Region in Tanzania. East African Highland bananas are mainly produced by small scale and resource-limited farmers, that use no or only limited amounts of pesticides and fertilizers. In this specific region of Africa has the worldwide highest per capita consumption of banana. Essentially, all of the bananas produced in this area are traded and consumed locally (Davies, 1995; Karamura, 1999; Mbwana and Rukazambuga, 1999). The global banana production (Figure 2), including dessert, cooking, brewing banana and plantains, is estimated at 116 million metric tons, therefore the banana ranks place 8 amongst the world most important staple foods (FAOSTAT, 2008).

However, yields in global banana production and in particular those of the East African Cooking Bananas are being reduced by bacterial and fungal diseases, insect and nematode pests (Buddenhagen, 1960; Hooks et al., 2008; Sarah et al., 1996; Sikora et al., 1989).



World Banana Production 2002 (Source: United Nations Conference on Trade and Development, UNCTAD Commodity Yearbook, UNO, New York, 2000,)

Figure 2: Major banana producing countries worldwide, production volumes are illustrated by the size of circles.

The most notorious diseases are the fungal Panama disease caused by *Fusarium oxysporum* f.sp. *cubense* race 1, the Black Sigatoka or Black Leaf Streak caused by the fungus *Mycosphaerella fijiensis* (Smith et al., 1997), the Bacterial wilt caused by *Pseudomonas solanacearum* Smith (Race 1) (Frey et al., 1996), the Banana Bunchy Top Virus (BBTV) (Hooks et al., 2008), the banana weevil *Cosmopolites sordidus* and several root lesion nematodes like *Radopholus similis* and *Pratylenchus* spp. (Sikora et al., 1989).

1.2 Nematode pests of banana and *Radopholus similis* (Cobb) Thorne

A total of 150 nematode species, belonging to 43 different genera, are reported to be associated with *Musa spp*. (Gowen and Quénéhervé, 1990). Nematode parasitism in banana roots is generally characterized by the simultaneous infestation of several species and annual average yield losses due to nematodes are estimated as high as 20%. The burrowing nematode *Radopholus similis* is one of the most destructive pests, causing the so called Toppling disease (Gowen, 2000; Sasser and Freckman,

1986). Local loss can be much higher and is dependent on factors like soil texture, drainage system, fertilization, climatic conditions as well as nematode species and pest density (O'Bannon and Esser, 1985). The most widespread and damaging nematode species are *Radopholus similis* and species of *Pratylenchus, Meloidogyne* and *Helicotylenchus* (Gowen, 2005; Speijer and De Waele, 1997; Speijer et al., 1999a).

Within the *Nematoda*, the migratory burrowing nematode *Radopholus similis* (Cobb) Thorne is placed in the order of *Tylenchida* and the family of *Pratylenchidae*. It is also well known under the synonyms *Tylenchus similis* (synonym of *R. similis sensu lato*) and *R. similis* banana race (synonym of *R. similis sensu stricto*). The common names are: Banana burrowing nematode, Toppling disease nematode (english), Anguillule mineuse du bananier (french) and nematodo coco or nematodo barrendador (spanish). *R. similis* is the most important root health damaging organism of banana worldwide (Sarah et al., 1996) and is listed as No. 126 in the EPPO A2 list of quarantine pests (EPPOa). The transport of infected plant material is the major cause for nematode transmission together with the movement of bulk soil on plant roots (EPPOb).

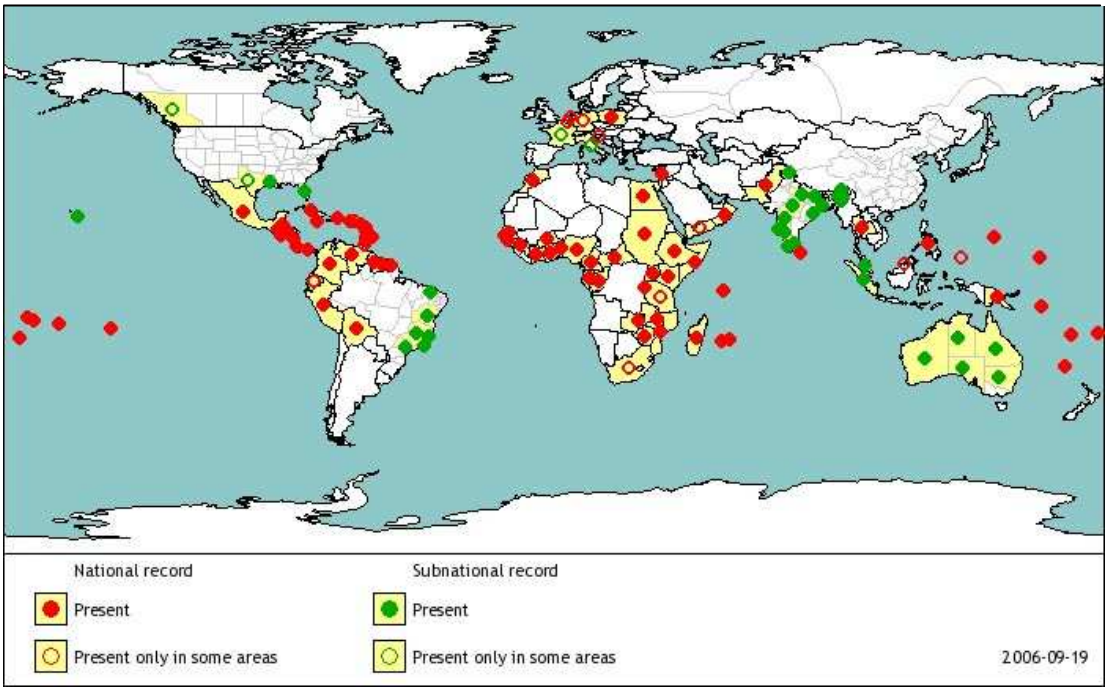


Figure 3: EPPO map showing the global distribution of the quarantine pest *Radopholus similis* based on national and sub-national records (source: http://www.eppo.org).

R. similis (Cobb) Thorne was first reported from banana fields in the Fiji Islands in 1893. Today it is found in most tropical and sub-tropical banana producing regions worldwide and can even be found in glasshouses in temperate areas (O'Bannon, 1977). Figure 3 shows a recent update of the global distribution of *R. similis* based on national and sub-national records.

R. similis completes its entire life cycle within the root cortex. Under favourable conditions and at 24℃- 30℃ one generation cycle takes 20-25 days. Females can lay 4-5 eggs per day. Juveniles hatch 8-10 days after egg deposition. The development from the juvenile to adult is completed within 13 days (Gowen and Quénéhervé, 1990). Only females and juveniles are infective and can, if conditions in roots become adverse, migrate into the soil in search for new roots. Once the root is invaded, *R. similis* moves intercellularly and feeds on the surrounding cells (Figure 4A and 4B), but does not enter the stele (Sarah et al., 1996).

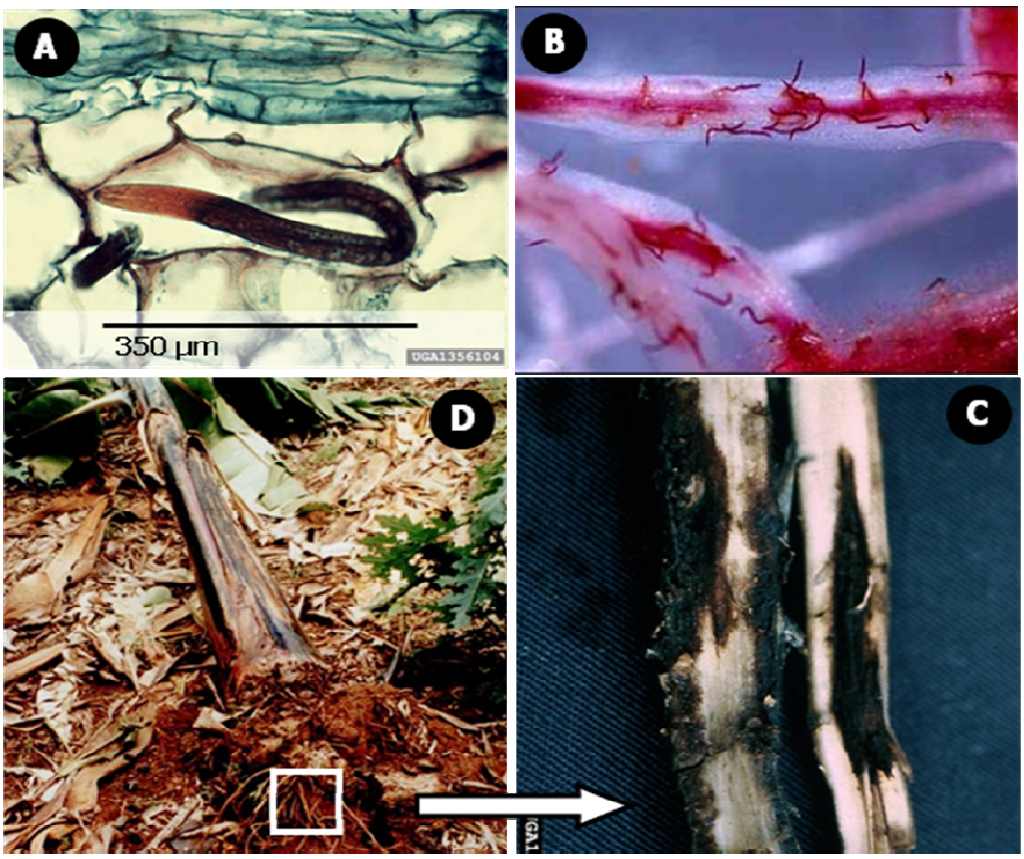


Figure 4: *Radopholus similis* feeding at a banana root cell (A) and stained sample of banana roots colonized by *R. similis* (B). Necrosis of banana root cause by *R. similis* infestation (C) and toppling (D) of a banana plant as result of root system degradation.

The most obvious symptom of *R. similis* infection is the up-rooting and toppling of banana plants (Figure 4D), which is also considered the major reason for yield losses in banana and plantains (Price, 1995). Disturbed water and nutrient uptake and prolonged vegetative cycles due to poorly developed roots cause severe yield decline over the cropping cycle (Gowen and Quénéhervé, 1990). Secondary infections of the plant by microorganisms occur during root feeding and lead to necrosis of the infected rhizomes and roots (Figure 4C) and in some cases the entire corm, hence the name Blackhead disease (Sarah, 2000).

Losses in global banana production due to *R. similis* were estimated to reach 20% (Sasser and Freckman, 1986), representing 23.2 metric tons 2006 (FAOSTAT). In Ivory Coast losses can reach 75% in poor and eroded soils (Sarah et al., 1996). *R. similis* accounted for yield losses of up to 30-35% per cycle in on-station trials and in Uganda losses of 50% on East African Highland bananas have been reported (Speijer et al., 1999a; Speijer et al., 1999b). These losses are of particular concern in Africa, since banana provides staple food for 100 million people in Sub-Saharan Africa (INIBAP, 1998).

To some extent, the control of nematodes can be achieved by nematicides, which either have a paralysing or lethal effect (Whitehead, 1998). 20-75% increases in banana yield were realized due to nematicide application in nematode infested production areas, thus documenting the dramatic yield losses caused by *R. similis* and other nematode species (Broadley, 1979; Gowen, 1994; McSarlay and Parrado, 1986; Sarah, 1989). Nevertheless, significant yield losses are still experienced, because *R. similis* completes its entire life cycle in the protective environment of the root tissue (Gowen and Quénéhervé, 1990). In addition, the reduced efficacy of nematicides due to enhanced bio-degradation of the active compounds by soil microbes is becoming an increasing problem in many growing areas with frequent application history (Karpouzas et al., 2004a; Karpouzas et al., 2004b; Ou et al., 1994; Suett et al., 1999). Finally nematicides are highly toxic and they impact the environment negatively. Therefore, organophosphates and carbamates have been separated from the market in Australia (Stirling and Pattison, 2008) and similar restrictions for their use were launched by the ‘Rotterdam Convention’ of the

European Union (24.02.2004), which are documented in the EU directive 91/424/EEC.

Due to the previously discussed deficiencies in nematode control by means of chemical treatments, the need to develop new integrated nematode pest management techniques has become evident. Monitoring the presence of nematodes is one part of such an integrated management approach. The initial spread of nematodes can be prevented by the use of clean planting material. Nematode free tissue culture banana plants, hot water treatments or the application of fenamifos to banana suckers from the field can help to reduce nematode pressure. The prevention of nematode spread is also strongly dependent on the farmer's awareness of the nematode as a pest. Extension programs help farmers to develop measures to prevent nematode introduction and their subsequent spreading. Breeding for nematode resistance was being used in the past (Cavendish cv. Gros Michel) (De Waele and Speijer, 1999) and is presently available for some cultivars (i.e. Yagambi km5, Pisang jari buaya and Calcutta 4) (Wuyts et al., 2007)), but not for those of commercial importance (i.e. Grand Naine, Valery, Williams). However, resistance breeding and the use of nematode resistant varieties often is limited by qualitative properties of the banana fruit, which does not match the requirements of the export market (zum Felde 2008). Soil organic amendments and nutrient management aim at both improving plant health and increasing biological diversity in soils and both will finally lead to plant growth promotion and activation of the antagonistic potential that exists in the soil (Sikora, 1992a; Stirling and Pattison, 2008).

The most abundant organisms dwelling soils are fungi and bacteria. Plant-health prompting rhizobacteria and endomycorrhizal fungi promote plant health, leading to increased pest tolerance. Antagonists comprise competitors, predators, parasites, pathogens or basically any organism that would repel, inhibit or kill the nematode pest. Bacterial and fungal parasites, fungal egg pathogens and predacious trapping fungi are potential antagonists of nematodes in the soil (Sikora, 1992b). However, all these antagonists share similar limitations in the control of *R. similis*. They do not reach the nematode, which is protected by its habitat, the root tissue (Gowen and Quénéhervé, 1990).

The use of antagonistic endophyte treated planting material is a fairly recent option for nematode control in banana. The introduction of mutualistic endophytic fungi into the banana root system showed plant growth promoting effects, and more important, effective reduction of *R. similis* penetration rates, thus limiting the pest pressure by restricting the nematode population (Vu et al., 2006). Although the endophyte approach has shown promising results against nematodes in banana and tomato (Dababat, 2008; Dababat and Sikora, 2007; Hallmann and Sikora, 1994; Niere, 2001; Niere et al., 1999; Vu et al., 2006; zum Felde 2008), this approach needs further research for practical exploration.

1.3 Plant associated *Fusarium* species: Pathogens and endophytes

Fusarium oxysporum Schlechtendahl emend. Snyder and Hansen is a cosmopolitan anamorphic fungus (Correll, 1991), comprising both pathogenic and non-pathogenic isolates (Gordon and Martyn, 1997). Pathogenic forms of *F. oxysporum* cause wilt and root rot diseases of more than 100 economically important plant hosts (Booth, 1971). Fusarium wilt is initiated by an abundant and systemic colonization of the xylem, thus reducing the general water transport that finally leads to the typical wilting of the plant (Beckman, 1990).

Pathogenic isolates of *F. oxysporum* are classified in various ways, with the classification forma specialis (f. sp.) indicating host specialization. *F. oxysporum,* f. sp. *cubense* causes Fusarium wilt of banana and is considered the most damaging *Fusarium* f.sp. from an economical point of view (Booth, 1971). Based on their virulence to specific banana cultivars, the various *F. oxysporum* f. sp. *cubense* isolates are further sub-divided into the races 1, 2, 3 and 4. *F. oxysporum* f. sp. *cubense* race 1 is the causal agent of the Panama disease which almost destroyed the entire banana export trade in South America up until the 1960s (Stover, 1962). This disease still ranks among the most destructive plant diseases of all ages (Simmonds, 1966). Before 1960, the banana production was mainly based on Gros Michel cultivars (*Musa* AAA) that were highly susceptible to the Panama disease. Dessert banana production was finally saved by the replacement of the susceptible Gros Michel cultivars by race 1 resistant Cavendish cultivars like Grand Naine, Williams and Valery (Ploetz and Pegg, 1997). Today, banana production is threatened by the recently emerged tropical race 4 (TR4). TR4 is able to seriously

infect the current economically important cultivars that are resistant to race 1 (Ploetz, 1994).

In addition to the *F. oxysporum* f.sp. *cubense* classification by races, pathogenic strains of *Foc* were also characterized into groups of vegetative compatibility (VCG). This classification method was proposed by Puhalla (Puhalla, 1985) and is based on asexual exchange of genetic information or heterokaryosis, which can occur during the event of vegetative hyphae fusion (anastomosis) of two strains with very similar genotypes (Buxton, 1968; Kuhn et al., 1995). According to their compatibility with known tester strains, new pathogenic isolates are then divided into different VCGs. In general each VCG is described by a five digit number: The first three digits correspond to host specialization or forma specialis (i.e. 012 for *cubense*), and the last one or two digits correspond to an individual VCG within the specific forma specialis. *F. oxysporum* f.sp. *cubense* is separated into 24 different VCGs (Katan and Katan, 1988; Kistler et al., 1998; Ploetz and Correll, 1981) and the currently known races are grouped into specific VCGs, thus making VCG typing a valid alternative for the classical pathogenicity tests on host plants (Katan and Katan, 1988).

Apart from such pathogenic endophytes, non-pathogenic fungal endophytes can be isolated from many host plants (Schulz et al., 1999) and few, if any plants in natural ecosystems are growing without the association with an or more endophytic fungi (Petrini, 1986). The term endophyte (greek: ‘endo’-inside and ‘phyton’-plant) is generally defined as an organism that can, at one point within its lifecycle, be isolated from asymptomatic plant tissue (Petrini, 1991) and endophytic fungi are classified as parasites or pathogens (detrimental), commensalists (neither harmful nor beneficial) or mutualists (beneficial) (Lewis, 1985). Non-pathogenic endophytes have received less attention than pathogens; nevertheless endophytes have been described for many fungal species. Among mutualistic endophytic fungi the family of *Clavicipitaceae* and especially the anamorphic *Neotyphodium* of *Epichloë* is the best studied (White et al., 2000). This family has attracted special attention due to their mutualistic symbiosis with grasses (Clay and Leuchtmann, 1989; Clement et al., 1994) and was suspected to confer insect resistance because they produce a number of entomotoxic substances (Clay, 1991). Therefore the use of these endophytes in biocontrol of insects was studied intensively. Mutualistic endophytic

fungi were recognized also in other fungal genera and were able to induce a wide range of beneficial effects on their plant hosts, such as drought and heavy metal tolerance, enhanced nutrient acquisition (Read, 1999; Read and Camp, 1986), growth enhancement (Belesky et al., 1987), disease resistance (Carrol, 1986; Freeman and Rodriguez, 1993; Redman et al., 1999) and resistance to herbivorous insects (Latch, 1993). The root-colonizing endophytic fungus *Piriformospora indica* combines several beneficial effects including improved salt-stress tolerance, disease resistance and yield promotion in barley (Waller et al., 2005).

It is known that the majority of *F. oxysporum* isolates in nature are non-pathogenic inhabitants of the soil (Kistler, 1997) or they can be isolated from the endorhiza of asymptomatic roots (Gordon et al., 1989). When searching for mutualistic endophytes for *R. similis* control, non-pathogenic *F. oxysporum* were isolated from healthy tissue of tomato and banana plants (Amin, 1994; Hallmann and Sikora, 1994). In contrast to compatible interactions of banana with pathogenic forms of *Fusarium oxysporum* f.sp. *cubense* (leading to the occlusion of the vascular tissue and finally the wilting of the plant) the non-pathogenic *Fusarium oxysporum* isolates do not penetrate the vascular tissue and their growth is restricted to the hypodermis and cortex (Paparu et al., 2006). This may largely explain their non-pathogenic character. To really confirm the safe use of such isolates for biocontrol, pathogenicity tests or alternatively VCG testing must be performed (Katan and Katan, 1988). Strains of non-pathogenic *Fusarium oxysporum* that were incompatible to the pathogenic tester strains were selected for further screenings in order to identify beneficial effects and to assess their potential as biocontrol agents against plant parasitic nematodes (Hallmann and Sikora, 1994; Niere, 2001; Pocasangre, 2000). Colonization of tomato roots by some of the screened isolates significantly reduced infestation rates of the sedentary root-knot nematode *Meloidogyne incognita* by up to 50% (Hallmann and Sikora, 1994) as well as the infestation by the endoparasitic burrowing nematode *Radopholus similis* in banana in greenhouse pot trials by up to 80% (Niere et al., 1999; Pocasangre, 2000). Split root experiments with both tomato and banana plants showed that nematode infection of the root was reduced at the responder side (half of the banana root that did not receive the fungal treatment, nevertheless the defense response is induced by the fungus in a systemic manner) (Dababat and Sikora, 2007; Vu et al., 2006). It was therefore concluded that the

fungal colonization of the plant root at the inducer side induced a systemic defense response of the plant that resulted in the enhanced resistance against either *M. incognita* or *R. similis*. To better understand the mode of action, choice experiments were conducted, which showed that root exudates were repellent or less attractive for the nematodes when the root system was colonized by the mutualistic *F. oxysporum* strain Fo162 (Dababat, 2008). First field experiments with banana plants inoculated with beneficial endophytes, also including non-pathogenic isolates of *Fusarium oxysporum*, led to the conclusions that inoculation of seedlings with endophytes prior to field planting provides sufficient nematode control to eliminate one nematicide application per year (Menjivar Barahona, 2005). Another economically important trait of the endophyte is the transfer of 'resistance' from mother to daughter plants under field conditions (zum Felde 2008).

Ever since Sikora proposed the use of mutualistic non-pathogenic *F. oxysporum* to improve plant resistance against nematodes (Sikora and Schuster, 1999; Sikora et al., 1999), numerous effective isolates have been identified and the protective activity of mutualistic *F. oxysporum* endophytes is well documented now (Dababat, 2008; Hallmann and Sikora, 1994; Niere et al., 1999; Pocasangre, 2000; Sikora, 1992b; Sikora et al., 1989; Vu et al., 2006; zum Felde, 2008). Today the term 'Biological Enhancement' is used to describe the targeted introduction of antagonists into the pathozone of plants. Biological enhancement has become an accepted biological management tool and contributes to a more ecological and sustainable IPM of nematode diseases in banana (Pocasangre et al., 2006; Sikora et al., 2008).

1.4 Plant defense responses to pathogen attack

In their natural environment plants have to cope with many potential pathogens. Due to the efficient structural, chemical and inducible defense mechanisms, disease development is nevertheless a sporadic event (Van Loon, 2000). Structural barriers include the cuticle, which is a complex matrix coated by waxes in which esterified fatty acids and rigid lignin depositions are present. Additionally, antimicrobial compounds, known as phytoanticipins, form a chemical barrier against the pathogen (Osbourn, 1996; VanEtten et al., 1994). In addition to this first line of defense, plants possess a second line of defense, which is activated upon pathogen attack (Durrant and Dong, 2004; Truman et al., 2007). Cross-linking of cell wall components, the 'hypersensitive response' (HR) in association with the generation of 'reactive oxygen

species' (ROS), the accumulation of secondary metabolites like tannins, an array of other secondary metabolites (phytoalexins) and also the production of the pathogenesis-related (PR) proteins can occur upon pathogen attack (Van Loon et al., 1994; Van Loon et al., 2006; Van Loon and Van Strien, 1999). Resistance against specific races of pathogens depends on the recognition of pathogen avirulence (AVR) gene products by resistance (R) gene products of the plant (Nurnberger et al., 2004). Successful recognition of the pathogen leads to a incompatible interaction, which is based on the rapid initiation of an array of defense responses by the plant, which usually peak in the generation of ROS and localized cell death, also known as hypersensitive response (Dangl and Jones, 2001). If such a gene-for-gene recognition system is not established, a less specific defense response is elicited, which is referred to as basal or innate induced resistance (Nurnberger et al., 2004).

1.4.1 Pathways of the basal or innate induced resistance

Van Loon and van Strien defined induced resistance as a 'physiological state of enhanced defensive capacity, which is triggered by specific environmental stimuli and results in the activation of the plant´s innate defense system against biotic challenges' (Van Loon and Van Strien, 1999). Such an enhanced state of resistance can be effective against many fungal, bacterial and viral diseases, against herbivorous insects and also plant parasitic nematodes (Vallad and Goodman, 2004).

The best described and defined forms of induced resistance are Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR). Both responses can be distinguished from each other based on the elicitor, the involved regulatory pathways and signaling molecules (Pieterse and Van Loon, 2004). SAR is induced by virulent, avirulent and non-pathogenic microbes or by chemicals like salicylic acid, 2,6-dichloro-isonicotinic acid (Métraux et al., 1991) or Benzo-(1,2,3)- thiadiazol-t-carbothionic acid S-methyl ester (BTH) acid S-methyl ester (BTH) (Kunz et al., 1997). Signal transduction within SAR involves the signaling molecule salicylic acid (SA). Any disruption of SA accumulation prevents the induction of SAR. However, INA and BTH were identified as functional analogs of SA. Both were able to replace SA in SA-deficiency mutants of *Arabidopsis* and tobacco, since they induced the onset of SAR despite the SA deficiency (Sticher et al., 1997).

In contrast to SAR, ISR is induced by plant growth promoting bacteria (PGPB). In *Arabidopsis*, ISR is triggered by root colonizing bacteria, i.e. *Pseudomonas fluorecens* WCS417r, and confers resistance against the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani* and the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pieterse et al., 1996). This pathway clearly differs from the SAR pathway, is not mediated by SA and requires the responsiveness to jasmonic acid (JA) or ethylene (ET) (Pieterse et al., 1998; Pieterse et al., 1996). However, strains of several *Pseudomonas* species also colonize the plant root system and induce ISR without provoking visible damage (Pieterse et al., 1996; Van Loon et al., 1998). In contrast to SA dependent SAR, the expression of ISR mediated by WCS417r is not associated with the transcriptional activation of PR-genes or other known defense related genes (Pieterse et al., 1996; VanWees et al., 1997).

However, ISR is only one possible outcome of induced resistance regulated by the JA/ET-pathway. Challenge of *Arabidopsis* leaves with the fungus *Alternaria brassicicola* induced the local and systemic synthesis of the plant 'defensin' gene PDF1.2 (belongs to the PR-12 family). PDF1.2 synthesis was also induced by exogenous application of methyl-jasmonate (MeJA) and ethylene (ET) and not by application of SA or INA. JA/ET activation of the defensin PDF1.2 is independent of the regulation by NPR1 (Penninckx et al., 1996; Penninckx et al., 1998).

SAR, ISR and the induction of plant 'defensin' PDF1.2, differ from each other by the involved signal molecules or proteins and can, to a certain extent, inhibit each other. For example, induction of PDF1.2 was found to be limited by SA accumulation, resulting in reduced JA synthesis and activity. However, some cross-communication has been observed and induction of SAR and JA/ET-dependent defense responses in the same plant can lead to additive and increased protection (Penninckx et al., 1996; Pieterse and Van Loon, 2004; Van Wees et al., 2000).

A key element of this cross-communication is the non-expressor of pathogenesis-related genes (NPR1), also known as non-inducible immunity (NIM1) (Figure 5). NPR1 was identified to be involved in regulation of ISR and SAR (Pieterse and Van Loon, 2004; Pieterse et al., 2002). In contrast, JA/ET regulated induction of PDF1.2 is not dependent on regulation by NPR1 (Penninckx et al., 1996).

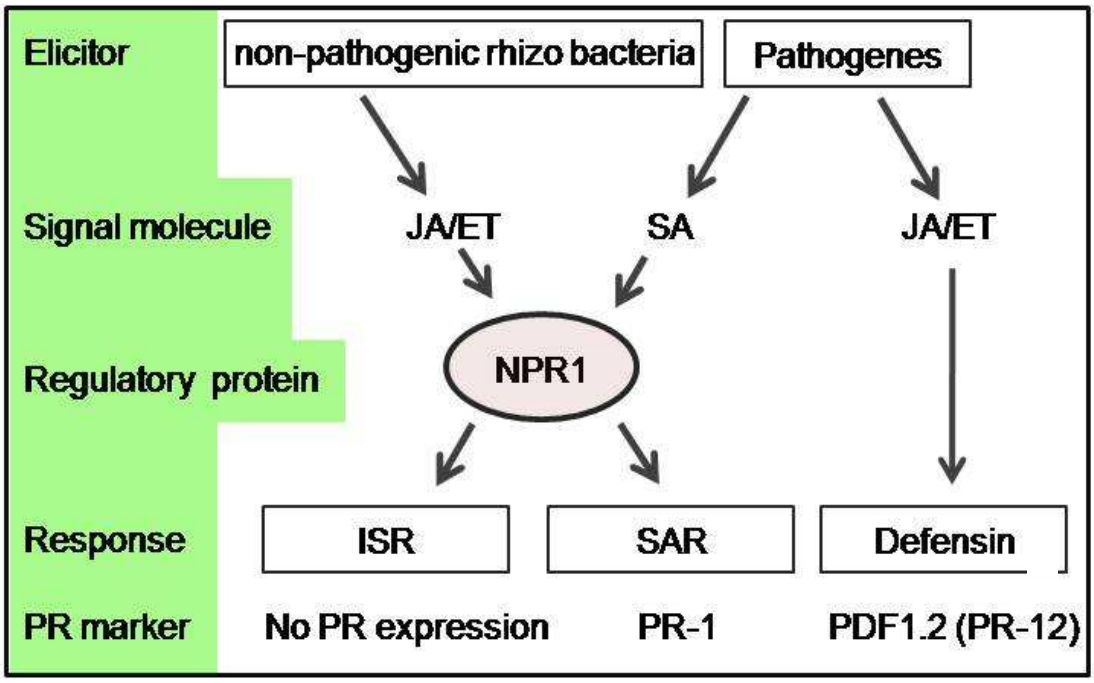


Figure 5: Model of systemic induced resistance in plants.

SA and, in some cases, JA/ET regulated pathways of induced resistance are associated with the accumulation of certain plant proteins, also known as 'pathogenesis related' (PR) proteins, while WCS417r mediated ISR is not associated with the transcriptional activation of PR-genes or other known defense related genes (Pieterse et al., 1996; VanWees et al., 1997).

PR-proteins can be involved in plant defense against pathogens and adaptation to environmental stresses (Van Loon et al., 2006). Although the regulatory NPR1 protein and the PR proteins and their association with events of induced systemic resistance in plants have received considerable attention in the past, there are still many inconsistencies and questions remaining in the overall model, especially with respect to the responses in different plant species.

1.4.2 PR proteins

In 1970, PR proteins were discovered for the first time in association with a hypersensitive response (HR) of tobacco leaves after infection with tobacco mosaic virus (TMV) (Gianinazzi et al., 1970; Van Loon and van Kammen, 1970). PR proteins (formerly known as 'b' proteins) have attracted intense interest in view of their possible involvement in plant resistance to pathogens. Initially, it was assumed that these proteins were induced in resistant plants, which also showed a HR in response to viral, fungal or bacterial infection. Later, PR proteins were also found in susceptible plants in response to abiotic stress factors (Van Loon, 1985). After their first detection in dicots, PRs also have been identified in monocots and are now considered to have a ubiquitous distribution in the plant kingdom (Ahl et al., 1982). PRs show distinct biochemical properties, like being stable at low pH (< 3) and at high temperatures or being highly resistant to protease activity. They are present in all plant organs, including roots (Van Loon and Van Strien, 1999; Van Wees et al., 1999). Within cells, PRs can be found in vacuoles, although the apoplast is the main accumulation site. PRs can also be found in primary and secondary cell walls of infected plants, as cell wall depositions in response to fungal attack (papillae) and even in the cell walls of invading fungal pathogens or the spaces formed between cell wall and the invaginated plasma membrane of the fungus (Edrevea, 2005).

In total 17 families of PR proteins are currently recognized. Families PR-1 to PR-11 and PR-17 were initially recognized and classified in tobacco and tomato, with the families PR-8 and PR-10 also described for cucumber and parsley. PR-12 to PR-16 were identified and characterized in radish, *Arabidopsis* and the monocot barley. Table 1 summarizes the information on the PR members regarding their properties and their target site. To be included as a new family of PRs, a protein currently has to comply to the following criteria: 1) Upon induction by a pathogen, the protein must accumulate in a tissue of the host where it is absent under non-pathogenic conditions and 2) induced expression must be confirmed either in two different plant-pathogen combinations or in a single plant-pathogen combination, but by two independent laboratories (Van Loon and Van Strien, 1999). A common feature of most PRs is their anti-fungal activity. However, some PRs also show antiviral, antibacterial, insecticidal and, most interestingly nematicidal properties. In general, the hydrolytic, proteinase inhibitory and membrane permeabilizing ability of PRs is often considered

Table 1: Overview of the currently known PR-families.

Family	Type member	Properties	Target
PR-1	Tobacco PR-1a	Anti fungal	Unknown
PR-2	Tobacco PR-2	ß-1,3-Glucanase	ß-1,3-Glucanase
PR-3	Tobacco PR PQ	Chitinase class I,II,IV-VII	Chitin
PR-4	Tobacco PR 'R'	Chitinase class I,II	Chitin
PR-5	Tobacco PR S	Thaumatin-like	Membrane
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	**
PR-7	Tomato P69	Endoproteinase	**
PR-8	Cucumber chitinase	Chitinase class III	Chitin
PR-9	Tobacco l-f-p*	Peroxidase	**
PR-10	Parsley 'PR 1'	'Ribonuclease-like'	**
PR-11	Tobacco PR-1a	Chitinase class I	Chitin
PR-12	Radish Rs-AFP3	Defensin	Membrane
PR-13	Arabidopsis THI2.1	Thionin	Membrane
PR-14	Barley LTP4	Lipid-transfer protein	Membrane
PR-15	Barley OxOa (germin)	Oxalat oxidase	**
PR-16	Barley OxOLP	'Oxalat oxidase-like'	**
PR-17	Tobacco PRp27	Unknown	**

Source http://www.bio.uu.nl/~fytopath/PR-families.htm, slight modifications
* l-f-p- lignin-forming-peroxidase
** No *in vitro* antimicrobial activity reported

as important feature in the defense against pathogens. Fungal cell walls for example, contain glucans, chitin and proteins, which can be negatively affected by hydrolytic enzymes such as ß-1, 3-glucanases, chitinases and proteinases. The activity of PR-8 is involved in disruption of gram positive bacteria. The proteinase inhibitory effects of PR-6 could play a role in insect or nematode defense, since PR-6 inhibits proteins that are secreted by the parasites (Abad et al., 1996; Selitrennikoff, 2001; Van Loon, 2001; Van Loon and Van Strien, 1999; Vigers et al., 1992).

Components released by fungal, bacterial, viral pathogens, as well as insects and nematodes are potent inducers of PR protein synthesis. Especially glucan and chitin fragments of fungal cell walls, glycoproteins, peptides and proteins secreted by fungi are well characterized elictors. PR synthesis can be induced by proteins, encoded for avirulence genes of fungi and bacteria, cell wall degrading enzymes and by chemicals (salicylic acid, polyacrilic and fatty acids, inorganic salts), physical stimuli (wounding, UV-B radiation, osmotic shock, low temperature, water stress), hormones (ethylene, jasmonates, abscisic acid, kinetin, auxins) and reactive-oxygen species

(ROS). In addition, the accumulation of PR, due to flowering and senescing, seed germination and somatic embryogenesis has been reported, but its occurrence seemed to be limited to development stages and was only expressed in a tissue specific manner (reviewed in Edrevea, 2005).

1.4.3 PRs as marker genes

Expression of PR proteins can be induced by at least two pathways, one regulated by SA the other regulated by JA/ET (Van Loon et al., 2006). While the accumulation of PR-1, PR-2 and PR-5 is specifically regulated by SA. PR-3, PR-4 and PR-12 are typically co-regulated by JA and ET and their regulation is, at least in *Arabidopsis*, clearly distinct from that of the SA-dependent *PR* genes (Thomma et al., 2001). However, for tobacco it has been demonstrated that different members within the same protein family respond differently to SA or JA/ET (Niki et al., 1998; Seo et al., 2001). Assuming a clear association of PRs with specific defense responses or signaling pathways, mRNA transcripts coding for marker proteins can serve as markers and will help to identify the observed defense response during SIR. Since all *PR-1* genes in plants seem to be solely inducible by SA, the expression of *PR-1* genes or proteins was being used as marker for the induction of SAR (Vernooij et al., 1994). *NPR1* transcript accumulation can serve as a general marker, indicating the induction of both SAR and ISR types of induced resistance. Finally *PR-3, PR-4* and the ‘defensin’ PDF1.2 (PR-12 family) could indicate the involvement of JA/ET induced defense responses (Lay and Anderson, 2005).

2 Research objectives

Phylogenetic analysis, expression studies and biochemical approaches were initiated to acquire a better in-depth knowledge on the diversity and characteristics of *F. oxysporum* endophytes and to identify the mechanism of the induced systemic resistance in banana plants that acts against *R. similis.* Therefore the overall research objectives were:

1) Identification of the most suitable molecular method to construct a phylogenetic overview over the mutualistic, commensalistic and pathogenic *F. oxysporum* isolates.

2) Clarify the phylogeny of the mutualistic *F. oxysporum* endophytes that have been isolated and screened for nematode control, with special focus on the phylogenetic relation between mutualistic isolates to each other and to pathogenic *Foc* isolates.

3) Characterize the expression of banana gene transcripts, associated with known induced defense responses, after exposure to specific chemical inducers.

4) Characterize the expression of these banana gene transcripts, in response to root colonization by the mutualistic *F. oxysporum* endophyte Fo162, the commensalistic *F. oxysporum* C39 and the pathogenic *F. oxysporum* f.sp. *cubense* Fo001 and *R. similis* in context with the results obtained from the expression studies of the chemical references.

5) Collection of banana root exudates and identification of possible alterations in their composition after root colonization by the mutualistic *F. oxysporum* Fo162 and assessing these root exudate extracts for their repellent or attractant activity towards *R. similis in vitro*.

3 Evaluation of different molecular techniques for phylogenetic analysis of fungal endophytes

3.1 Introduction

Ever since the emergence of biological systematics in the 18th century, morphological and physiological features have been important and very effective parameters. However, it has become apparent that these characteristics have their limitations in taxonomical studies. For example, in fungal taxonomy the difference in morphological characteristic between two species can be difficult to determine, thus requiring skilled and experienced staff. Genomic analysis and the development of molecular nucleic acid techniques have revolutionized fungal systematics in many different aspects. Now systematics can go easily beyond the species level and classify specific individuals, isolates or populations. Furthermore, the speed and relative simplicity by which the current taxonomical analysis can be performed is unprecedented (Edel, 1998).

Direct restriction fragment length polymorphism (RFLP) analysis was one of the first techniques initiating the development of molecular systematics. With the emergence of polymerase chain reaction (PCR), RFLP was further developed together with novel techniques, like AFLP, DDGE and RAPD analysis (Fisher and Lerman, 1983; Klich et al., 1991; Kohn, 1992; Mullis et al., 1986; Mullis and Faloona, 1987; Orita et al., 1989; Saiki et al., 1988; Southern, 1975; van Belkum, 1994; White et al., 1990; Williams et al., 1990). With the current rate, complete DNA sequence analysis of certain informative regions has become interesting. Taxonomic and phylogenetic studies most commonly concentrate on genomic ribosomal DNA (rDNA) regions (White et al., 1990), since these contain both conserved and variable domains that allow discrimination at the genus, species or sub-species level (Edel, 1998). In addition, the rapidly growing number of relevant sequences of many different organisms and specific primers for rDNA have become available at open-source data bases (i.e. NCBI or BROAD), thus making extensive and thorough phylogenetic studies possible.

Since fungal species or isolates within a species are sometimes difficult or impossible to differentiate by morphological characteristics (i.e. the complex morphospecies of *Fusarium oxysporum*), the taxonomy based on molecular characters is an important

tool in mycology and is becoming more and more powerful with growing numbers of sequences available at online resources.

3.1.1 RAPD analysis

Random amplified polymorphic DNA (RAPD) is a method that generates characteristic fingerprints by amplification of genomic DNA, using short (5-10 bp) arbitrary primers. The amplification pattern observed during electrophoresis reflects the number and distribution of genomic sites, which are matching the primer sequence, thus leading to primer annealing and amplification of the target DNA (Williams et al., 1990). These band patterns resemble phenotypic characters that can be converted into distance matrices and enable phylogenetic comparison among strains of fungi according to their genetic similarity (GS), when statistically analyzed. Depending on the choice of primer, RAPD fingerprints can identify organisms at different taxonomic levels, including the species level (Hadrys et al., 1992; Lehmann et al., 1992). With respect to fungi, RAPDs have also been used at intraspecific levels and successfully differentiated isolates within different fungal genera (Bayman and Cotty, 1993; Duncan et al., 1993; Guthrie et al., 1992; Wyss and Bonfante, 1993), including *F. oxysporum* and *F. solani* species (Assigbetse et al., 1994; Crowhurst et al., 1991). While a correlation of RAPDs with physiological properties, like host specificity (race) could be established for *F. oxysporum* f. sp *vasinfectum,* the identical set of primers was not informative for race-specific patterns in *F. oxysporum* f.sp. *pisi* or *dianthi* (Assigbetse et al., 1994; Grajalmartin et al., 1993; Manulis et al., 1994), thus preventing the envisaged use of pre-designed or ‘ready to go’ primer sets for trait characterization of related species.

In addition to RAPD, related PCR-fingerprinting based techniques, like amplified fragment length polymorphism (AFLP), arbitrarily primed PCR (AP PCR), DNA amplification fingerprinting (DAF) and micro- and mini-satellites are currently available, but all of them share similar limitations. Patterns of bands can be rather complex and variations can be difficult to analyze. Especially minor weak bands, resulting from varying amplification specificity or DNA confirmation, can be responsible for low reproducibility. For reproducible results, the PCR fingerprinting procedure needs a high level of standardization, since it is more sensitive to reaction conditions compared to conventional PCR amplifications. *Taq* DNA polymerase, the thermal cycler, Mg^{2+} concentration, number and stringency of temperature cycles as

well as primer concentration and the concentration and quality of the template are factors that can affect the reproducibility of the RAPD analysis (Macpherson et al., 1993; Meunier and Grimont, 1993). Co-migration of two distinct fragments of identical size is another problem. Even considering these difficulties, RAPD analysis has the advantage that multiple loci are analyzed, thus representing the whole genome as a specific banding pattern, without any need for target sequence information. This makes it a rapid tool for phylogenetic inference among genetic closely related organisms (reviewed in Edel, 1998).

3.1.2 RFLP analysis

Restriction fragment length polymorphism (RFLP) is a method for sequence polymorphism identification. Currently, mostly PCR products of different fungal strains are digested by restriction endonucleases, cleaving DNA at specific sites recognized by a sequence of 4-6 bp length. Identical to the RAPD analysis, the observed restriction patterns resemble phenotypic characters, which can be visualized and scored by gel electrophoresis, converted into distance matrices, which finally enable comparison among strains of fungi according to their genetic distance or similarity when statistically analyzed. The estimation of genetic distances can however be error-prone due to co-migrating and small sized fragments which often remain undetected by electrophoresis (reviewed in Edel, 1998). Techniques like the denaturing gradient gel electrophoresis (DGGE) (Fisher and Lerman, 1983) or single strand confirmation polymorphism (Orita et al., 1989) can be used to detect variations in sequences such as single base substitutions.

3.1.3 Sequencing analysis

Direct sequencing from PCR products and comparison of their nucleotide sequences is the most precise method for polymorphism detection (Bevan et al., 1992; Rao, 1994). Single nucleotide polymorphisms (SNPs) are a marker class that is based on substitutions, insertions or deletions of bases (Indels). SNPs are of great relevance for phylogeny and taxonomy and represent the most widespread type of sequence variation in genomes, i.e. accounting for approximately 90% of the genetic variation in the human genome (Brookes, 1999; Brumfield et al., 2003; Collins et al., 1998) Evaluation of SNP frequency in five primarily coding DNA regions of the pathogenic fungus *Coccidioides immitis* resulted in average in one SNP found in every 159 base pair (Koufopanou et al., 1997), thus exceeding by far the SNP frequency of one per

500-1000 bp in coding or one per 200-500 bp in non-coding DNA of a diverse collection of various organisms (Brumfield et al., 2003). However, the number of detected SNPs in a sequence alignment strongly depends on number and relative taxonomic positions of the involved organisms.

3.1.4 Ribosomal RNA coding region

Several properties are making the region of the ribosomal DNA the current choice for taxonomic and phylogenetic studies. rDNA is present in all living cells and codes for essential structural RNA elements within ribosomes that are essential for proper cell functioning. This area of the rDNA is submitted to a form of coevolution that is potentially representative for the whole organism. The rDNA region holds both conserved and variable domains. Additionally, the individual variable areas differ again in their sequence variability, making the whole rDNA region ideal for the identification of organisms at different taxonomic levels. The nuclear fungal rRNA coding region is generally organized in tandem repeat units. Each unit is formed by three rRNA coding genes, the small nuclear 18S rRNA, 5.8S rRNA and large nuclear 28S rRNA coding genes (Figure 6). Within one unit, the internally transcribed spacers (ITS1 and ITS2) separate these three genes, whereas the two repeats are separated from each other by the non-coding and non-transcribed intergenic spacer region.

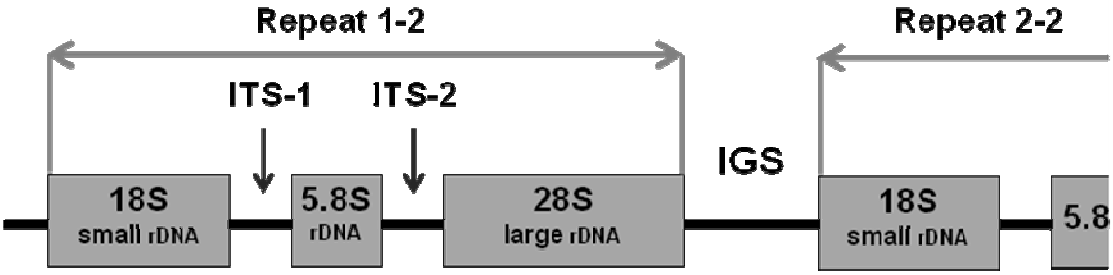


Figure 6: Organization of the ribosomal DNAs (rDNAs).

All three rRNA coding regions are highly conserved along organisms and show only little sequence variability. Therefore they only allow comparison of distantly related organisms. Different from the coding regions, the non-coding regions like IGS and ITS are able to evolve more rapidly (reviewed in Edel, 1998). Amplification of the ITS region, including ITS1, 5.8S rDNA and ITS2, yields in 600-1000 bp fragments depending on the type of primers used (White et al., 1990). Sequences of ITS have been used to describe taxonomic and phylogenetic relationships among different genera of fungi and oomycetes, i.e. rust diseases (Zambino and Szabo, 1993),

Phytophthora (Lee and Taylor, 1992), *Sclerotinia* (Carbone and Kohn, 1993), *Penicillium* (Lobuglio et al., 1993) and *Fusarium* species (Dissanayake et al., 2009; Edel et al., 1997; Enya et al., 2008; Mbofung et al., 2007; Waalwijk et al., 1996). RFLP analysis of the ITS was shown to be suitable for identification of fungi at the species level and is considered a powerful taxonomic indicator at the inter species level. However, sequence variation at the intra species level is generally low or even undetectable for the ITS (Rehner and Samuels, 1994; Zambino and Szabo, 1993).

The IGS shows lower degrees of conservation compared to the ITS and is informative for characterization of *F. oxysporum* at species level (Kurtz et al., 2007). RFLP data and sequences of PCR-amplified IGS DNA were used for discrimination and phylogenetical inference from closely related fungi, including *F. oxysporum* species (Anderson and Stasovski, 1992; Edel et al., 1995; Henrion et al., 1992), *F. oxysporum* f. sp *cubense* (O'Donnell et al., 1998b) and human pathogenic members of the *F. oxysporum* complex (O'Donnell et al., 2004). Insertions and deletions are common mutations in the IGS (Edel et al., 1995; Henrion et al., 1992) and the resulting length differences of PCR fragments can be considered an additional molecular criterion for taxonomic studies. However, the presence of Indels can bias PCR and RFLP studies and fragment length variations must be identified for the analyzed PCR product prior to restriction enzyme analysis to avoid misinterpretations of size polymorphisms.

Most taxonomic studies concentrated on the analysis of rRNA coding genes and their spacer regions. However, other target genes also have shown to be informative for phylogenetic studies in fungi. The analysis of mitochondrial large ribosomal DNA (mtLrDNA) for example resulted in similar phylogenetic tree topologies of *Penicillium* as information inferred from ITS regions. To differentiate *Fusarium* species, more conserved genes such as histones and ß-tubulin were analyzed together with the ITS. The amplification of specific genes also was used for discrimination of pathotypes within a pathogenic species (reviewed in Edel, 1998). Another potential informative target gene is the translation elongation factor (EF-1α), which has been shown to be useful in inferring the phylogeny of *F. oxysporum* f. sp *cubense* (O'Donnell et al., 1998a) and human pathogenic members of the *F. oxysporum* complex (O'Donnell et al., 2004), alone and in combination with IGS sequence data.

In conclusion, the choice of target genes for phylogenetic studies depends on the taxonomic levels of the analyzed taxa.

3.2 Scope

The scope of this chapter was to test different molecular techniques and genomic regions for their use to infer the phylogeny of a collection of mutualistic, commensalistic or pathogenic endophytic *Fusarium* isolates. For each technique, the reproducibility of the obtained data was assessed, together with the highest information content (number of polymorphic sites), tree resolution, data processing and data accessibility.

3.3 Material and Methods

3.3.1 Fungal strains an isolates

A selection of *Fusarium* strains, originating from different countries and showing different properties in terms of biocontrol or pathogenicity were used for this analysis (Table 2).

Table 2: Fungal isolates used for the evaluation of the different molecular techniques and the phylogenetic analysis.

Isolate	Species	Origin	Host	Character
Fo001[a]	*F. oxysporum* f. sp. *cubense*	South Africa	Banana	Pathogen[1]
JC1[a]	*F. oxysporum* f. sp. *cubense*	USA Florida	Cv.Apple	Pathogen[1]
II5[a]	*F. oxysporum* f. sp. *cubense*	Indonesia	Banana	Pathogen[1]
A1[b]	*F. oxysporum*	Indonesia	Banana	Biocontrol[2]
Fo162[c]	*F. oxysporum*	Kenya	Tomato	Biocontrol[2]
S9[d]	*F. oxysporum*	Costa Rica	Banana	Biocontrol[2]
P12[d]	*F. oxysporum*	Costa Rica	Banana	Biocontrol[2]
V4w5[e]	*F. oxysporum*	Uganda	Banana	Biocontrol[2]
V5w2[e]	*F. oxysporum*	Uganda	Banana	Biocontrol[2]
C 39[d]	*G. fujikuroi* ▲	Cuba	Banana	No or low control[3]
III3w3[e]	*F. oxysporum*	Uganda	Banana	No or low control[3]
IV3w2[e]	*F. oxysporum*	Uganda	Banana	No or low control[3]
IV4w4[e]	*F. oxysporum*	Uganda	Banana	No or low control[3]
V1w7[e]	*F. oxysporum*	Uganda	Banana	No or low control[3]
V2w2[e]	*F. oxysporum*	Uganda	Banana	No or low control[3]
V4w4-2[e]	*F. oxysporum*	Uganda	Banana	No or low control[3]
Eny 1.31i[f]	*F. oxysporum*	Uganda	Banana	No or low control[3]
Emb 2.4 o[f]	*F. oxysporum*	Uganda	Banana	No or low control[3]
Eny 7.11o[f]	*F. oxysporum*	Uganda	Banana	No or low control[3]

[a] Strains and VCG information provided by the Tropical Research and Education Center of the University of Florida (Homestead, USA); [b] Strains isolated by N. Amin in Sulavesi district, Indonesia (1994); [c] Strains isolated by J. Hallmann in Kenya (1999); [d] Strains isolated by L. Pocasangre in Cuba, (2002); [e] Strains isolated by R.-P. Schuster in the Mpigi district, Uganda (1993); [f] Strains isolated by M. Griesbach in the Ntungamo district, Western Uganda (2001); [1] Known pathogens to banana with unknown biocontrol activity towards *R. similis*; [2] Isolates with significant biocontrol activity (*R. similis* penetration reduced by~60%) in greenhouse trials. [3] Isolates with low or no biocontrol activity towards *R. similis*; ▲ Initially unidentified *Fusarium* spp. now identified as *Gibberella fujikuroi* (anamorph: *F. fujikuroi*) by BLAST search of the partial IGS sequence.

All fungal isolates used in this study originated from single spore colony stocks and were stored in the INRES, Department of Soilecosystem Phytopathology and Nematology, University of Bonn. In general three different strategies for fungal conservation were applied. For short term storage fungi were maintained on potato dextrose agar (PDA, see table 3) or synthetic nutrient poor agar (SNA, see table 4). For maintenance a 5 mm PDA plug with mycelium (growth zone) from an older plate was inoculated onto the centre of the respective medium. For medium and long term storage the isolates were stored at the -80℃ deep freezer using the Microbank preservation system (Cryobank TM, Master Group, Merseyside, UK).

Table 3: Potato dextrose agar (PDA)

24	g	Potato dextrose broth (Difco)
17	g	Agar
1000	ml	Aqua dest. pH 6.0

Table 4: Synthetic nutrient-poor agar (SNA)*

1.0	g	KH_2PO_4
1.0	g	KNO_3
0.5	g	$MgSO_4*7H_20$
0.5	g	KCl
0.2	g	Glucose
0.2	g	Sucrose
0.6	ml	NaOH (1M)
17.0	g	Agar
1000	ml	Aqua dest. pH 6.0

* modified by Nirenberg (1976)

For long term storage the isolates were prepared as follows: 1 ml of sterile water was added to a 5 day old fungal culture grown on PDA and spores were detached from the mycelium using a sterile metal rod. This spore suspension was transferred to the preservation vial. After inverting ten times, the liquid phase was separated and the tube was stored in a -80℃ freezer. For preservation up to 20 years, the fungal isolates were stored in soil tubes at 4 ℃. Whereby, 20 ml glass tubes were filled 2/3

with a sand and field soil (1:1; v/v) mixture and 1 ml of tap water. After sterilization at 121℃ for 60 min, the mycelium from the growth zone of a PDA culture was added. Tubes were incubated in the dark at 25℃ for 3 days to allow mycelial growth. After the mycelium had penetrated the soil, tubes were stored at 4℃.

3.3.2 DNA extraction

Fungal strains isolated from single spores (Table 2) were separated from the -80℃ stock and grown on PDA (Sigma) for seven days at 25℃ in the dark. Both mycelia and conidia were collected from the PDA plates by adding 1 ml sterile distilled water and scratching the fungus from the substrate surface using the straight edge of a sterile 30 mm spatula. Fungal material was transferred into a 15 ml round bottomed falcon tube and immediately frozen in liquid nitrogen. Mycelium and spores were freeze dried and ground into a fine powder using a vortex and a 10 mm spatula. 10-20 mg of fungal powder was used to extract the genomic DNA using the Wizard Magnetic DNA Purification System for Food (Promega) according to the manufacturer`s instructions. Successful extraction of genomic DNA was validated by agarose gel electrophoresis and comparison to a 1 kb Ladder (Promega). Electrophoresis was performed at 100V and 200 mA for 60 min using a 1% (w/v) agarose gel prepared in TAE, supplemented with 0.25 µg/ml ethidium bromide (AppliChem) (Sambrook and Russell, 2001). The isolated genomic DNA was stored in sterile distilled water at 4℃ and -20℃.

3.3.3 RAPD analysis

For RAPD analysis, the decamer standard primers A4, A5, A12, A20, C5, C10 and C15 (Roth) were used (Table 5).

Table 5: Decamer primers used for RAPD-PCR analysis of the *Fusarium* species.

Primer	Sequence 5`- 3`	Melting point [C°]
A4	AAT CGG GCT G	32
A5	AGG GGT CTT G	32
A12	TCG GCG ATA G	32
A20	GTT GCG ATC C	32
C5	GAT GAC CGC C	34
C10	TGT CTG GGT G	32
C15	GAC GGA TCA G	32

For the PCR reaction 1 µl of purified fungal genomic DNA was used as template in a 25 µL PCR reaction volume containing 5× Reaction Buffer (Promega), 1.5 mM $MgCl_2$ (included in 5× RB), 200 µM of each dNTP (Peqlab), 0.2 µM of the decamer primer (Roth) and 1.0 unit of the GoTaq polymerase (Promega) (Table 6).

Table 6: Master Mix for RAPD-PCR

Component	Volume [µl]
Sterile ddH_2O	16.3
5× Reaction Buffer	5
dNTP mix 2.5 mM	2
Random primer 10 µM	0.5
GoTaq polymerase 5 U/µl	0.2
DNA template	1
Final volume	25

PCR amplifications were performed in a T-Gradient thermocycler (Biometra) with an initial denaturation time of 240 s at 94 ℃ and 30 cycles of 94℃ for 20 s, 36℃ for 20 s and 72℃ for 90 s and a final extension step of 5 min at 72℃ (Table 7). The amplification was verified by gel electrophoresis using a 1% (w/v) agarose gel in TAE, supplemented with 0.25 µg/ml ethidium bromide (AppliChem) and the fragments were separated by electrophoresis at 60 V and 200 mA for 60 min (Sambrook and Russell, 2000). DNA fragments were compared to the 1 kb ladder (Promega), visualized by UV light and recorded using a digital camera. The presence or absence of bands was scored and compiled in excel files.

Table 7: Thermocycling program for RAPD-PCR using Roth decamer random primers

Step	**Temperature [℃]**	**Time [s]**	
Initial denaturation	94	240	
Denaturation	94	20	**30 cycles**
Annealing	36	20	
Elongation	72	90	
Final elongation	72	300	
Hold	4	Hold	

3.3.4 RFLP-analysis of IGS and ITS

F. oxysporum specific primers were used to amplify the ribosomal internal transcribed spacer and the partial intergenic spacer region by PCR (Table 8). Both primer pairs were synthesized by Sigma Genosys and stocks were adjusted to a concentration of 100 µM.

Table 8: Primers used for DNA amplification of ITS or IGS

Target region	Primer	Primer Sequence (5`-to-3`)	Amplicon [bp]
IGS	PNFo	CCCGCCTGGCTGCGTCCGACTC	1700
	PN22	CAAGCATATGACTACTGGC	
ITS	ITS 1	ATGGGTAAGGAAGACAAGAC	1000
	ITS 4	GGAAGTACCAGTCATCATGTT	

Amplification of the fragments was performed in a 50 µl PCR reaction, containing 1 unit of GoTaq polymerase (Promega), 5x Reaction Buffer (Promega), 1.5 mM $MgCl_2$ (included in the 5x RB), 0.2 mM of each dNTP (Peqlab), 0.2 µM of each forward and reverse primer (Sigma Genosys). These components were added in the order given in table 9, except for the DNA which was added first of all. PCR amplifications were performed using a T-Gradient thermocycler (Biometra) and cycling conditions are given in table 10.

Table 9: PCR reaction for IGS and ITS amplification

Component	Volume [µl]
Sterile ddH_2O	32.8
5xReaction Buffer	10
dNTP mix 0.2 mM	4
Forward primer 10 µM	1
Reverse primer 10 µM	1
GoTaq polymerase 5 U/µl	0.2
DNA template	1
Final volume	50

Table 10: Thermocycler program for the amplification of IGS and ITS fragments

	IGS		ITS		
Step	**Temp. [℃]**	**Time [s]**	**Temp. [℃]**	**Time [s]**	
Initial denaturation	95	240	95	240	
Denaturation	95	90	95	60	
Annealing	52	52	60	60	**35 cycles**
Elongation	72	90	72	60	
Final elongation	72	300	72	300	
Hold	4	Hold	4	Hold	

For RFLP analysis, 25 µl of IGS or ITS PCR fragments were digested in a final reaction volume of 30 µl, by adding 0.7 µl of restriction enzyme, which corresponded to 7 units, 3 µl 10× Reaction Buffer, 0.3 µl of BSA (all Promega) and 1 µl of sterile ddH_2O and incubating at 37℃ for two hours (Table 11). Six restriction enzymes were chosen for restriction analysis: *Hae*III, *Hin*fI, *Hin*dIII, *Hin*6I, *Hpa*II and *Dra*I.

Table 11: Master Mix for restriction enzyme digest used for RFLP analysis

Component	Volume [µl]
Sterile ddH_2O	1
10x Reaction Buffer	3
Acetylated BSA, 10 U/µl	0.3
DNA template (PCR product)	25
Restriction enzyme 10 U/µl	0.7
Final volume	30

After digestion, 25 µl of the PCR product was loaded onto a 2% (w/v) agarose gel in 1×TAE, supplemented with 0.25 µg/ml of ethidium bromide (AppliChem) and the fragments were separated by electrophoresis at 60 V and 200 mA for 90 min (Sambrook and Russell, 2000). As a reference for fragment size the Peqlab 50 bp ladder was used. The DNA fragments were visualized by UV light and recorded using a digital camera.

3.3.5 Sequencing of the IGS

Sequencing was performed for the amplified IGS fragments only. Amplification of the IGS was performed as described in chapter 3.3.4. Amplified IGS fragments were purified using the illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) from the PCR product directly. 500 µl of capture buffer type 2 was mixed with 45 µl PCR product and loaded onto a GFX MicroSpin column that was placed in a collection tube. After centrifugation at 16000×g and 30 seconds (s) the flow through was discarded and the GFX MicroSpin column was returned to the collection tube. To eliminate impurities from the DNA present on the capturing matrix, 500 µl of wash buffer type 1 was loaded onto the GFX MicroSpin column. After another centrifugation step at 16000×g and 30 s the flow through and the collection tube were discarded and the MicroSpin column was placed into a sterile DNase-free 1.5 ml microcentrifuge tube (Eppendorf). To recover the purified DNA, the capturing matrix was eluted by adding 50 µl of DNase-free water followed by an incubation time of 60 s prior to centrifugation at 16000×g and 60 s. The concentration and quality of purified DNA was determined using the Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, USA). DNase-free water was used as reference. According to the requirements of the sequencing company, the DNA concentrations were adjusted to 20-40 ng/µl using DNase-free water. Sequencing was performed by the GATC sequencing service (GATC-Biotech, Konstanz). The chromatographic sequencing data was manually inspected for sequence quality using BioEdit (Hall, 1999). For species determination, sequences were individually analyzed by BLAST search on the NCBI server. For the assembly of sequences and transformation into fasta format Vector NTI Advance (Invitrogen) was used.

3.3.6 Processing of RAPD and RFLP data matrices

The IGS and ITS RFLP and the RAPD dataset were processed using NTEdit and NTSYSpc Numerical Taxonomy System, Version 2.2 (Exeter software). The presence or absence of markers was scored for each isolate and the results were compiled in a excel file. Prior to calculation of the genetic similarity (GS), the excel files were converted into an NTSYSpc compatible data matrix. For each marker system the pair-wise genetic distances between taxa was calculated using the DICE coefficient of similarity (Dice, 1945). GS matrices were subjected to the unweighted pair group method with arithmetic average (UPGMA) cluster analysis (Sneath and

Sokal, 1973) followed by the sequential agglomerative hierarchical nested (SAHN) cluster analysis (Sneath and Sokal, 1973) implemented in NTSYSpc 2.2.

3.3.7 Sequence analysis of the IGS

IGS sequences were aligned, edited and finally converted into a nexus format using ClustalX (Thompson et al., 1997). MrModeltest was used to identify the best fitting model for nucleotide substitution of the IGS dataset (Nylander, 2004). MrModeltest is partially implemented in Paup 4.10 beta (Swofford, 2003). Bayesian Inference (Huelsenbeck and Ronquist, 2001) started from a random tree and isolate C39 from Cuba, which was typed as *Fusarium fujikuroi* by BLAST analysis, served as the outgroup. The analysis was performed with 5.000.000 generations at a sample frequency of 1000, using 5 independent heated chains, melting temperature of 0.2 and a swap frequency of 1. Burn-in was set to 25% of total sample size, discarding all trees generated prior to the burn-in period. The phylogenetic tree was composed using Treeview (Page, 1996), TreeEdit (Rambaut and Charleston, 2001), Photoshop 7.0 (Adobe) and Powerpoint (Microsoft) for additional annotation of information.

3.4 Results

3.4.1 RAPD analysis

The RAPD analysis of the 19 fungal isolates identified a total of 154 binary characters, which were coded as 1 for a present fragment and 0 for an absent fragment. Due to the high density and varying intensity of bands (Figure 7), the scoring of band patterns generated by RAPD-PCR was rather complex.

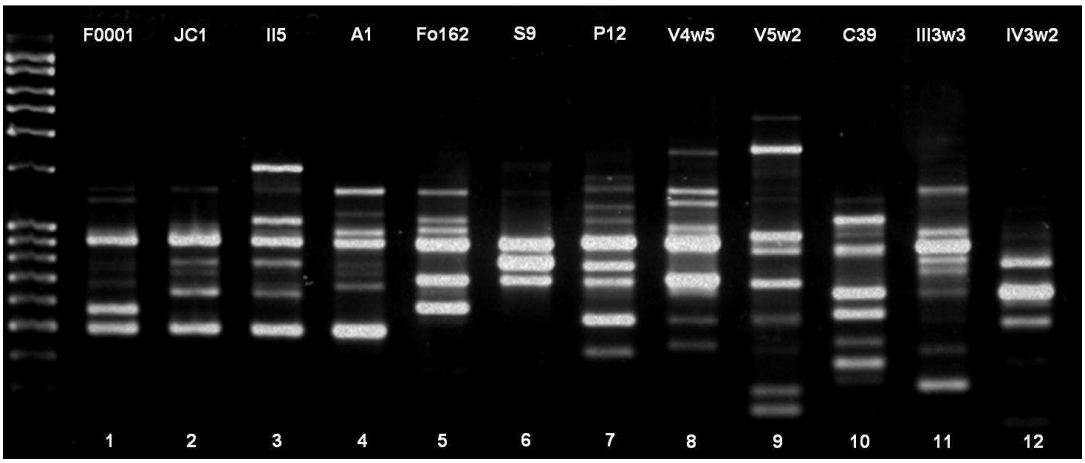


Figure 7: Example for the complexity of band patterns generated by amplification of the genomic DNA of 12 fungal endophytes by using the RAPD primer A5.

UPGMA (Sneath and Sokal, 1973) cluster analysis followed by SAHN cluster analysis resulted in the midpoint rooted dendrogram displayed in Figure 8.

The taxa were fully resolved and the resulting tree was divided into two groups, designated as clade A and B. Clade A contained the pathogens Fo001, JC1, II5, the mutualistic endophytes A1 and S9 and the commensalistic endophyte V1w7, showing a genetic similarity ranging from 69 to 84%. Clade B contained the mutualists Fo162, P12, V5w2 and V4w5 and the commensalists IV3w3, V2w2, IV4w4, III3w3, V4w4-2, Eny1.31i, Emb2.4o and Eny7.11o. All these isolates originated from Africa, except for the mutualist P12 originating from Costa Rica. Isolates from clade B showed a GS ranging from 72 to 91% and shared a common ancestor with clade A (68% GS). The endophyte C39 was the most distant taxon and was therefore used as outgroup in the sequence analysis.

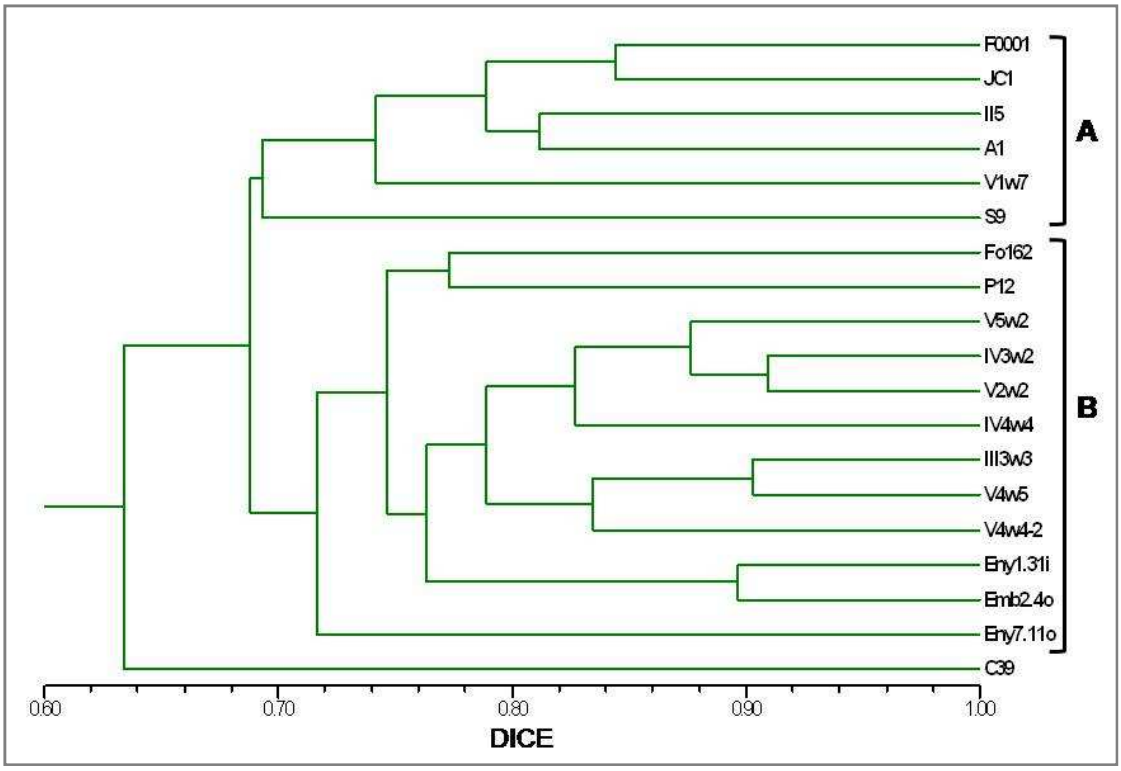


Figure 8: Midpoint rooted dendrogram resulting from the RAPD analysis of 19 pathogenic or non pathogenic *F. oxysporum* isolates. Bars indicate the similarity [%] among taxa based on the Dice coefficient of genetic similarity.

3.4.2 RFLP analysis of the ITS

The PCR reaction using the *F. oxysporum* specific ITS primers ITS1 and ITS4 produced a single fragment with the predicted size of approximately 1.0 kb. The RFLP of the ITS with the restriction enzymes *Hae*III, *Hin*fI, *Hin*dIII, *Hin*6I, *Hpa*II and *Dra*I resulted in a total of 22 markers for the 19 isolates. Thus, the number of informative polymorphisms was low for all of the restriction enzymes used in this analysis (Figure 9).

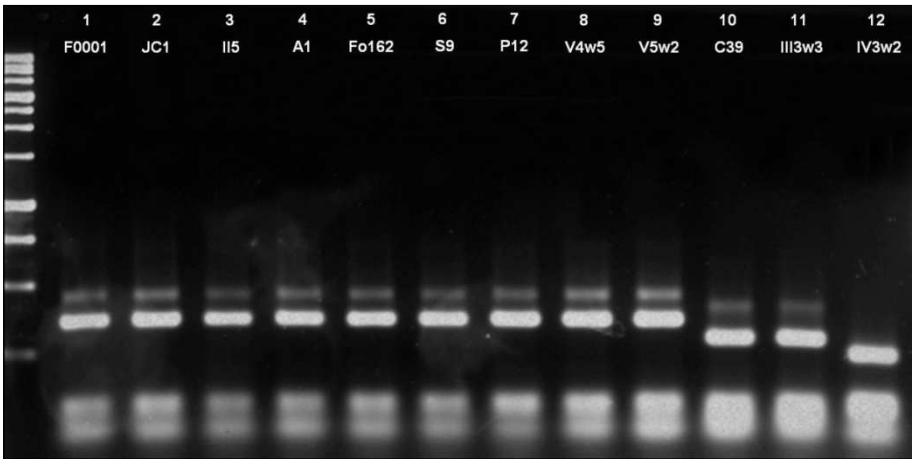


Figure 9: Example for the low number of informative markers identified after RFLP analysis of the amplified ITS fragments of 12 fungal endophytes using *Hpa*II.

After cluster analysis of the molecular markers according to SAHN a midpoint rooted dendrogram was generated (Figure 10).

The cluster analysis revealed a phylogenetic tree with four different taxonomic groups, clades A, B, C and D. Clade A consisted of the two pathogens Fo001 and JC1 (GS 93%) and is consequently monophyletic for pathogenicity. Clade A showed 89% GS to clade B, which contained a total of 14 isolates, II5, Fo162, P12, Emb2.4o, Eny7.11o, Eny1.31i, V4w5, V4w4-2, V2w2, V1w7, IV4w4, IV3w2, S9 and V5w2. Although, clade B showed a high degree of diversity in terms of biocontrol traits, pathogenicity and sampling origin (Table 2), it remained unresolved. Thus, not providing information about the phylogenetic relatedness among the taxa. Clade C is formed by the mutualist A1 alone, sharing a common ancestor with the isolates of clade B (GS~93%). Both clade C and B shared a common ancestor with the 'Pathoclade' A (GS~89%) and the three share a common ancestor (GS~81%) with clade D, which was solely formed by the endophyte III3w3. The endophyte C39 was

the most distant taxon to all of the previously mentioned taxa found in clades A-D. In general, the tree resulting from ITS-RFLP data showed low resolution of phylogenetic relationships among the individual isolates that were analyzed.

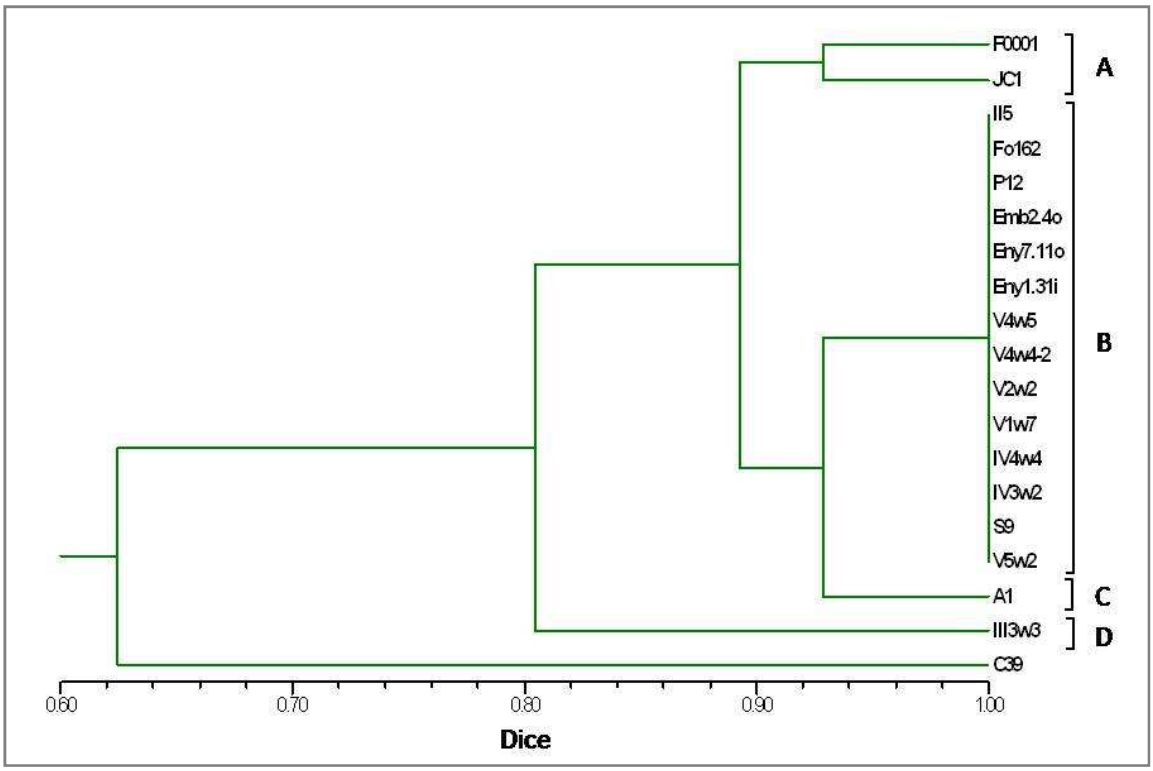


Figure 10: Midpoint rooted dendrogram of ITS fragments of 19 pathogenic or non-pathogenic *F. oxysporum* endophytes. Bars indicate genetic the similarity [%] amongst taxa based on the Dice coefficient of similarity.

3.4.3 RFLP-analysis of the IGS

The PCR reaction using the *F. oxysporum* specific IGS primers PNFo and PN22 produced a single fragment with the predicted size of approximately 1.7 kb. Using the restriction enzymes *Hae*III, *Hin*fI, *Hind*III, *Hin*6I, *Hpa*II and *Dra*I a total of 36 markers could be identified within the IGS sequences (Figure 11) for the 23 isolates analyzed.

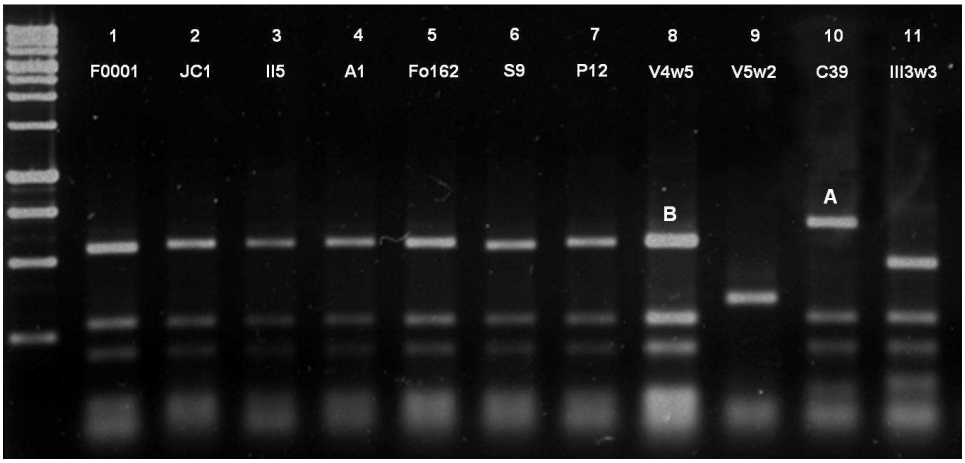


Figure 11: Typical example for the low number of informative markers identified by RFLP analysis of 11 *Fusarium* endophytes using the restriction enzyme *Hpa*II. A and B: markers with similar positions within the gel.

After cluster analysis of molecular markers according to SAHN, a midpoint rooted dendrogram was generated (Figure 12).

Isolates were divided in two different clades; referred to as clades A and B. Clade A showed a maximum similarity among the taxa of 92% and consisted of two unresolved and five resolved taxa. Subgroup 1, which contained isolates Fo001, JC1, II5 and A1, is closely related (GS 98%) to the subgroup, which contained isolates IV3w2, V1w7 and V2w2. Taxa P12 and V5w2 (both~95%), as well as V4w5, V4w4-2 and S9 (~92%), are following in decreasing order of genetic similarity. Clade B showed a max. similarity among taxa of ~92% and consisted of 6 taxa, including a group of the three unresolved taxa Fo162, Eny1.31i and Emb2.4o, having ~95% genetic similarity to the resolved taxa Eny7.11o and IV4w4 and ~92% GS with taxon III3w3. The last common ancestor of the clades A and B showed ~87% GS and taxa of both clades showed ~72% of GS with C39.

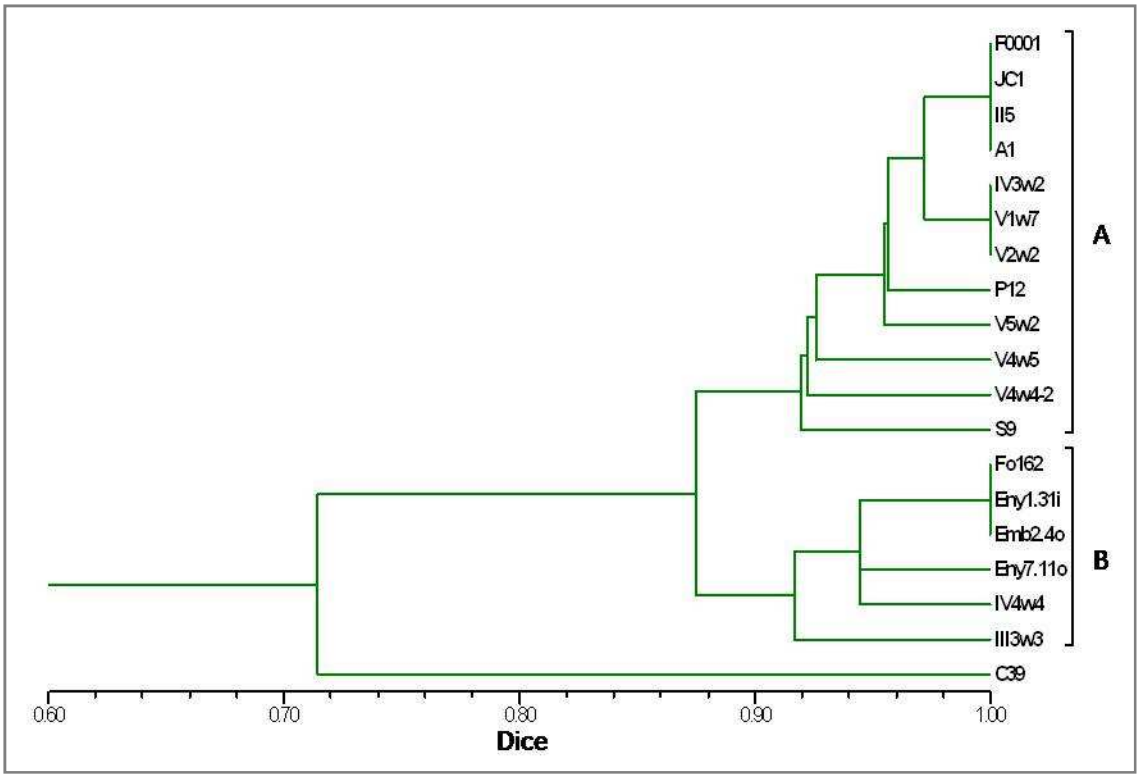


Figure 12: Midpoint rooted dendrogram resulting from IGS restriction analysis of 19 pathogenic or non-pathogenic *F. oxysporum* endophytes. Bar indicates the similarity amongst taxa based on the Dice coefficient of similarity.

3.4.4 Sequence analysis of the IGS

The PCR reaction using the *F. oxysporum* specific IGS primers PNFo and PN22 produced a single fragment with the predicted size of approximately 1.7 kb. DNA sequence analysis, followed by BLAST analysis (NCBI nucleotide search) of the IGS fragments confirmed the previously identified genera and species of all isolates, except for the isolate C39, which showed strongest homology with *Gibberella fujikuroi* (anamorph: *F. oxysporum*), accession number AY249382 (Query coverage 99%; max. ident. 91%). This isolate was therefore used as outgroup in the phylogenetic analysis of the IGS sequences. The alignment consisted of 1348 nucleotides with a total of 126 (9.34%) polymorphic sites for the 19 fungal strains. By using the Akaike Information Criterion (AIC) in MrModeltest, the Hasegawa, Kishino Yaro with gamma rates (HKY+G; -lnL=3270.4, K=5, AIC=6550.8 and base frequencies of fergA= 0.2163; fregC= 0.2193; fregG= 0.3209; fregT= 0.2435) was identified as best-fit model for nucleotide substitution (Nylander, 2004). The average of the final standard deviation of split frequencies of the Bayesian Inference (BI) was 0.00345. Both runs of the BI resulted in identical 50% majority-rule consensus trees with only marginal differences among the harmonic means of the log-likelihood

values (run 1= -5112.11; run 2= -5228.61). The phylogenetic tree was generated as 50% majority-rule dendrogram with midpoint rooting (Figure 13).

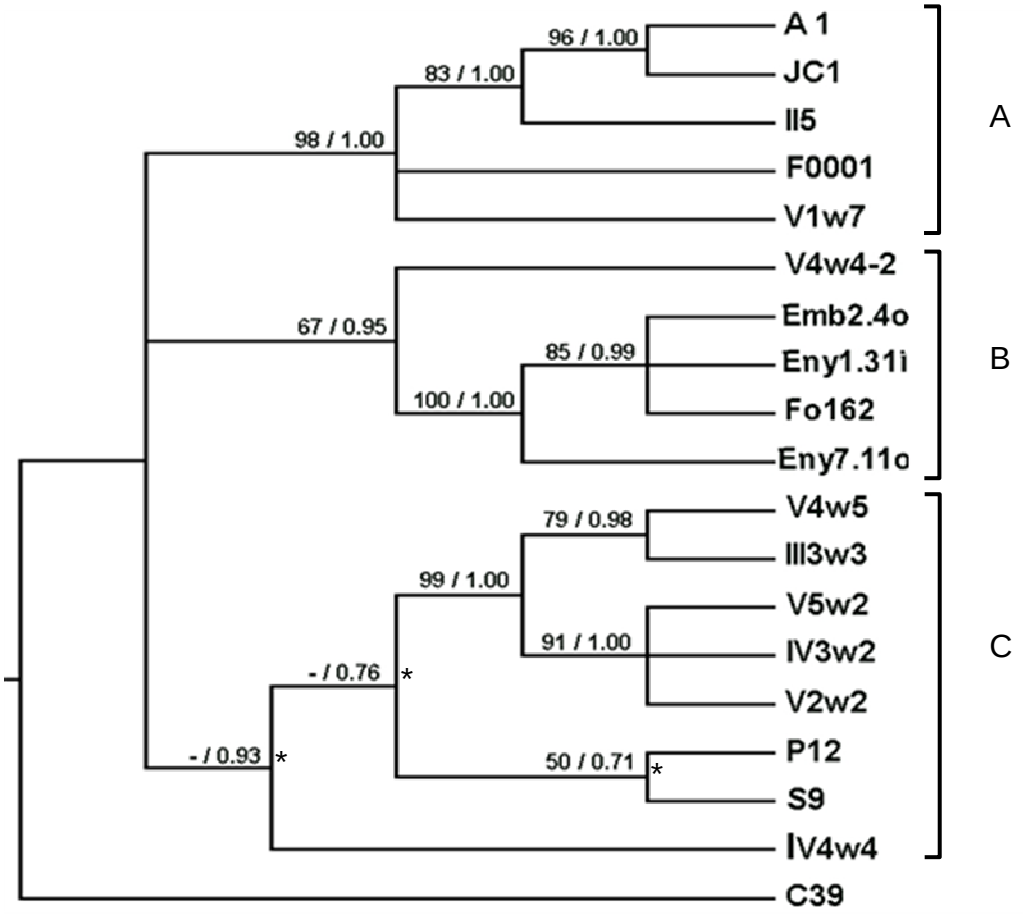


Figure 13: 50% majority-rule consensus tree, displayed as a dendrogram, inferred from a 1.35 kb fragment based on the intergenic spacer of 19 mutualistic, commensalistic or pathogenic endophytes of *F. oxysporum.* Model of nucleotide substitution was HKY+G. Values indicated at the nodes are Jackknife-support (values<50% not displayed) and Posterior Probabilities.

The dendrogram resulting from the BI showed full resolution for all taxa. The clade credibility values are represented by Posterior Probabilities (PP) and ranged from 0.71 to 1.00, thus giving strong support for all nodes. The dendrogram was divided into three clades, referred to as clade A, B and C. Except for the nodes and their branches, which were marked by asterisks*, the tree topology was supported by strong Jackknife values higher than 67%.

The description of the phylogeny between the taxa starts from the terminal nodes of the tree (high similarity) towards the internal nodes (decreasing similarity). Jackknife and PP values are annotated in brackets representing the support for each node

(=last common ancestor of the before mentioned isolates). Clade A was strongly supported by Jackknife and PP values (≤83/1.00%) and contained the three pathogens JC1 (96/100 with A1), II5 (83/1.00 with A1 and JC1) and Fo001 (98/1.00 with A1, JC1, II5 and V1w7), as well as the mutualist A1 (96/1.00) and the commensalistic endophyte V1w7 (98/1.00 to all taxa of clade A). Clade B consisted of five taxa and contained the mutualistic strain Fo162, the commensalistic endophytes Emb2.4o, Eny1.31i (85/0.99), Eny7.11o (100/1.00) and V4w4-2 (67/0.95) and its structure is strongly supported by both Jackknife and PP values. Clade C was formed by eight taxa. Nodes for the subgroup of the mutualistic V4w5 and the commensalistic III3w3 (79/0.98), the mutualist V5w2 and the commensalists IV3w2 and V2w2 (91/1.00) were well supported (99/1.00). However, there was only weak support for the node that links the mutualists S9 and P12 (50/0.71) and the support for the node connecting both to the before mentioned taxa of clade C is weak as well (-/0.76). Finally clade C is complemented by IV4w4 and its position within the tree is strongly supported by PP (0.93), but the Jackknife support is however weak. C39 was defined as outgroup and evidently formed the most distant taxon of the entire selection.

3.5 Discussion

In this chapter, three different molecular techniques, RAPD, RFLP and sequence analysis as well as 3 different targets (whole genome, ITS and IGS) were evaluated to infer the phylogeny of 19 *F. oxysporum* or related isolates. One of the most important goals with respect to the phylogenetic characterization of the fungal strains was to achieve the best possible resolution among all isolates.

IGS sequence analysis and RAPD analysis both resulted in sufficient resolution. The IGS-RFLP analysis showed at most an acceptable resolution. The inferred phylogeny resulted in similar tree topologies in all three cases. For RAPD, IGS-RFLP and IGS sequence analysis all pathogenic strains (Fo001, II5 and JC1) and the mutualistic A1 were found in one clade. Mutualists and commensalistic endophytes were mostly present in one or two clades and separated from the pathogens and A1. As strongly indicated by the phylogeny inferred from the RAPD, ITS and IGS-RFLP analysis, sequence analysis of the IGS confirmed that the endophyte C39 belongs to a closely related but different genus, which most likely is *Gibberella fujikuroi* (anamorph: *F. fujikuroi*).

By using only 6 restriction enzymes for the RFLP analysis, neither the ITS (one group with 14 unresolved taxa) nor the IGS analysis (three groups with four and two times three unresolved taxa, respectively) provided enough information for a dendrogram showing full resolution of isolates. The higher resolution achieved by IGS-RFLP analysis was a result of higher numbers of markers produced and scored (36 binary characters), when compared to the ITS-RFLP (22 binary characters). The difference in numbers of binary characters has two causes. Firstly, although both ITS and IGS are non-coding regions, IGS is in addition non-transcribed and therefore has the tendency to show lower degrees of conservation compared to the ITS (Edel, 1998). Therefore, the IGS shows higher degrees of variation and is more informative for characterization of fungi at intraspecies level. The high information content of the IGS for taxonomical studies with fungi was previously described (Anderson and Stasovski, 1992; Edel et al., 1995; Henrion et al., 1992). Secondly, assuming equal levels and distribution of sequence variation within IGS and ITS, the numbers of markers expected from the analysis of the IGS should increase 1.7-fold due to its physical fragment length of approximately 1.7 kb compared to the approximately 1.0 kb fragment for the ITS. By using more restriction enzymes for the RFLP analysis for both target fragments, the number of scored binary characters may increase and thus improve the resolution of the inferred trees. Nevertheless, the relatively low number of polymorphisms observed during ITS-RFLP can also be explained by the sequence variation of the ITS, which is generally low or even undetected at the intra-specific level (Rehner and Samuels, 1994; Zambino and Szabo, 1993). Finally, with some rare exceptions (O'Donnell, 1992), the IGS will always provide more information when compared to ITS and therefore it is the preferable target.

Considering the number of binary markers and variable sites indentified using RAPD (154 binary characters when using seven random primers) and sequence analysis of the IGS (126 informative sites), both techniques produced sufficient information to fully resolve the phylogenetic trees and to describe the relatedness among the isolates. Both techniques gave a better resolution when compared to the RFLP analysis. Especially the performance of sequence analysis of the IGS was impressive, which can be explained by the complete identification of mutations, insertions and deletions during sequence analysis. In particular length mutations are common mutations in the IGS (Henrion et al., 1992) and can be missed by using the

RAPD or RFLP techniques. The ITS region was not sequenced in this study, but the increased number of markers with sequence analysis over RFLP must be expected.

The potential of sequences analysis of PCR-amplified IGS for the discrimination and phylogenetic inference of closely related *F. oxysporum* species was not unexpected, since this technique already successfully discriminated *F. oxysporum* f. sp *cubense* (O'Donnell et al., 1998b) and human pathogenic *F. oxysporum* isolates (O'Donnell et al., 2004) in previous studies. In addition, sequence analysis can be used for the identification of single nucleotide polymorphisms (SNPs), thus enabling the discrimination of isolate groups, as was previously shown for *Erysiphe necator* (causal agent of grapevine powdery mildew) where a SNP in the β-tubulin gene is used to distinguish between two specific genotypes in european vineyards (Amrani and Corio-Costet, 2006).

Although RAPD was successful in characterizing the phylogenetic relationships and achieved full resolution of trees, the complex band patterns (Figure 7) combined with the occurrence of minor and weak bands were found to be challenging for the reproducibility and scoring of results as was already described (Macpherson et al., 1993; Meunier and Grimont, 1993). Consequently, this technique tends to be error prone. This could have contributed to the observed differences in tree topology, when compared to the tree resulting from the sequence analysis of the IGS. Although, the use of RAPD analysis for intra species characterization was proposed elsewhere (Hadrys et al., 1992; Lehmann et al., 1992); RAPD or related techniques should be used in addition to sequence analysis if some isolates show very little or no sequence differences amongst their pair-wise alignments, since RAPDs can analyze multiple genomic loci at the same time and can serve to infer the phylogeny amongst closest related organisms (Williams et al., 1990).

Similar to the problems of reproducibility encountered during RAPD analysis, RFLP also must be considered error prone, since co-migrating and small sized fragments often remain undetected by electrophoresis (Edel, 1998). There are ways to avoid the resulting misinterpretations (Fisher and Lerman, 1983, Orita et al., 1989 check), but these are quite laborious compared to identification by sequence analysis. Limitations and complexity in reproducibility of RAPD and RFLP techniques is

especially disadvantageous if datasets need to be complemented by additional taxa, isolates or data of independent laboratories or research groups. In this case, sequence analysis offers a strong advantage over any of the other techniques. Sequences can be published in data bases such as NCBI or BROAD and can be accessed via internet, enabling easy complementation and accumulation of sequence information for the species of interest. Sequences of unidentified organisms can be easily characterized and morphological identification can be confirmed by using identity matches resulting from a BLAST search. Complementation of alignments with organism sequences from data bases can significantly increase the information content of the phylogenetic analysis. An obvious disadvantage of this tool is the data base pollution with morphologically or physiological misidentified organisms leading to invalid conclusions.

Considering the overall performance of the individual techniques and target genes, the most informative, reliable and reproducible method for the phylogenetic analysis of related *F. oxysporum* and related species is by DNA sequence analysis of the IGS region. In addition, reference sequences from data bases can be integrated into the phylogenetic analysis. In the future the method also offers the option to perform SNP analysis for the rapid identification of groups of isolates.

4 Phylogenetic analysis of mutualistic, commensalistic and pathogenic *Fusarium oxysporum* endophytes

4.1 Introduction

Fusarium oxysporum Schlechtendahl emend. Snyder and Hansen is a cosmopolitan anamorphic fungus (Correll, 1991), comprising pathogenic and non-pathogenic isolates (Gordon and Martyn, 1997). Pathogenic isolates of *F. oxysporum* f. sp. *cubense* (*Foc*) cause Fusarium wilt in banana (*Musa* spp.) and are found in almost all banana producing areas worldwide (Ploetz, 1994; Stover and Simmonds, 1987). *Foc* isolates can be either divided into the races 1, 2,3 and 4 based on their virulence to specific banana cultivars or into vegetative compatibility groups, dividing *F. oxysporum* f. sp. *cubense* into 24 different VCGs (Chitwood, 2002; Fourie et al., 2009; Katan and Katan, 1988; Kistler et al., 1998; Paparu et al., 2009; Puhalla, 1985; Stinson et al., 2006; Wuyts et al., 2006). Since specific races fall within specific VCGs, the VCG typing is also indicative for the virulence to the various banana cultivars (see also chapter 1.3).

Next to pathogenic *Foc*, many strains of non-pathogenic *Fusarium oxysporum* have been identified, some of which have shown to be beneficial by reducing *Radopholus similis* penetration into banana roots (Niere, 2001; Vu, 2005). Pathogenicity and VCG tests of these endophytes are key elements to determine the safe use of selected non-pathogenic mutualistic *F. oxysporum* strains as control agents against *R. similis* in banana production. However, compared to *Foc*s the knowledge about the diversity and the phylogeny of non-pathogenic mutualistic *F. oxysporum* strains is rather limited.

To infer the phylogenic relations among non-pathogenic *F. oxysporum,* being mutualistic, commensalistic or pathogenic *Foc*s a DNA based phylogenetic analysis will be essential. Non-pathogenic *F. oxysporum* may segregate into clonal lineages, as was observed for *Foc* isolates in several DNA based phylogenetic studies (Bentley et al., 1998; Fourie et al., 2009; Groenewald et al., 2006; Koenig et al., 1997; O'Donnell et al., 1998b). Assuming a clonal population structure, the character of non-pathogenicity might be monophyletic within *F. oxysporum*, thus forming an independent clonal lineage that can be distinguished from the clonal lineages typically formed by *Foc*s in previous studies (Fourie et al., 2009; Groenewald et al.,

2006; Koenig et al., 1997; O'Donnell et al., 1998b). In addition, the trait that leads to enhanced resistance against *R. similis* could be monophyletic within the non-pathogenic isolates of *F. oxysporum*. In that case these isolates would be descendants of a common ancestor that carried the mutualistic trait and phylogenetic analysis could be used to perform a 'phylogenetic assisted pre-selection' of mutualistic isolates. Such a preselection could effectively help to significantly reduce time consuming greenhouse trials in future screenings for mutualistic antagonists.

4.2 Scope

The main objective of this study was to resolve the phylogeny of a set of well characterized non-pathogenic *Fusarium oxysporum* strains using the sequence data of their IGS regions, with emphasis on their mutualistic or commensalistic traits, geographical distribution and in particular their phylogenetic relations to pathogenic *Foc*s isolated from Uganda and other banana growing areas. This will show whether or not mutualism is a monophyletic trait within the presented set of pathogenic and non-pathogenic isolates. Since, the safe use of mutualistic endophytes as a biological tool within banana IPM is of special interest, this work is also performed to determine whether non-pathogenic *F. oxysporum* strains are phylogenetically distinct from pathogenic *Foc*s that can cause the devastating Panama wilt disease.

4.3 Material and Methods

4.3.1 Fungal endophytes and sequencing of the IGS

Non-pathogenic strains from Uganda were sampled at two different sites. Isolates sampled by R.-P. Schuster originate from the Mipigi district (0.252204, 32.317543 or alternative +0° 15' 7.93", +32° 19' 3.15") 30 km fr om the Ugandan capital Kampala and those sampled by Matthias Griesbach originate from the Ntungamo district (-0.884266, 29.650161 or alternative -0° 53' 3.36", + 29° 39' 0.58") situated at the national border to the Democratic Republic of Congo and 70 km north of the national boarder to Ruanda. For the *Foc* isolates from Uganda more detailed information concerning sampling site was not available. DNA extraction, amplification, purification and quantification for sequencing purpose, sequencing and assembling of fasta files for all isolates was performed as described previously (Chapter 3.3).

4.3.2 Data processing and phylogenetic analysis

Sequences were aligned, edited and finally converted into nexus format using ClustalX (Thompson et al., 1997). MrModeltest was used to identify the best fitting model for nucleotide substitution of the IGS dataset (Nylander, 2004). MrModeltest is partially implemented in Paup 4.10 beta (Swofford, 2003). Bayesian inference started from a random tree. The isolate C39 was defined as outgroup. Two independent runs with 5.000.000 generations, sample frequency of 1.000, 5 independent heated chains, melting temperature of 0.2 and swap frequency of one were performed with Mr Bayes. Burn-in was set to 25% of total sample size, discarding all trees generated prior to the bur-in period (Huelsenbeck and Ronquist, 2001).

4.3.3 Tracer: Inspecting the Bayesian Inference run parameters

Tracer 3.0 (Rambaut and Drummond, 2003-2005) was used to follow convergence of the two BI runs reported by Mr Bayes.

4.3.4 Jackknife-analysis

Jackknife analysis was performed with Paup 4.10 beta (Swofford, 2003). Search type for Jackknife analysis was ‘Fast stepwise addition’ considering gaps a new 5^{th} state of nucleotide, with 37% deletion of data, emulated ‘Jac’ resampling and replicate number of 10.000 (Farris et al., 1994).

Table 12: Fungal endophytes included in the phylogenetic analysis.

Isolate	Species	Origin	Host	Character	VCG*
Fo001[a]	*Foc*	South Africa	unknown	Pathogen[1]	0120
JC1[a]	*Foc*	USA Florida	Apple	Pathogen[1]	01210
II5[a]	*Foc*	Indonesia	unknown	Pathogen[1]	01213
A1[b]	*F.o.*	Indonesia	unknown	Biocontrol[2]	n.c.
Fo 162[c]	*F.o.*	Kenya	Tomato	Biocontrol[2]	n.c.
S9[d]	*F.o.*	Costa Rica	Banana	Biocontrol[2]	n.c.
P12[d]	*F.o.*	Costa Rica	Banana	Biocontrol[2]	n.c.
V4w5[e]	*F.o.*	Uganda	Banana	Biocontrol[2]	n.c.
V5w2[e]	*F.o.*	Uganda	Banana	Biocontrol[2]	n.c.
C 39[d]	G. *fu.* ▲	Cuba	Banana	No control[3]	n.c.
III3w3[e]	*F.o.*	Uganda	Banana	No control[3]	n.c.
IV3w2e	*F.o.*	Uganda	Banana	No control[3]	n.c.
IV4w4[e]	*F.o.*	Uganda	Banana	No control[3]	n.c.
V1w7[e]	*F.o.*	Costa Rica	Banana	No control[3]	n.c.
V2w2[e]	*F.o.*	Uganda	Banana	No control[3]	n.c.
V4w4-2[e]	*F.o.*	Uganda	Banana	No control[3]	n.c.
Eny 1.31if	*F.o.*	Uganda	Banana	No control[3]	n.c.
Emb 2.4 o[f]	*F.o.*	Uganda	Banana	No control[3]	n.c.
Eny 7.11o[f]	*F.o.*	Uganda	Banana	No control[3]	n.c.
CAV 2459[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,1220,01222
CAV 2469[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,01220
CAV 2477[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,0126,01222
CAV 2483[g]	*Foc*	Uganda	Banana	Pathogen[1]	0128
CAV 2490[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,0126,01220,01222
CAV 2500[g]	*Foc*	Uganda	Banana	Pathogen[1]	0128
CAV 2505[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124
CAV 2515[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124
CAV 2519[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,0125,0128
CAV 2542[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,0129,01220,01222
CAV 2543[g]	*Foc*▲▲	Uganda	Banana	Pathogen[1]	-
CAV 2545[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,0128,0129,01220
CAV 2546[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,0129,01222
CAV 2549[g]	*Foc*	Uganda	Banana	Pathogen[1]	0124,0129,01222
CAV 2556[g]	*Foc*	Uganda	Banana	Pathogen[1]	0125
CAV 2560[g]	*Foc*▲▲▲	Uganda	Banana	Pathogen[1]	01220

[a] Strains and VCG information provided by the Tropical Research and Education Centre of the University of Florida (Homestead, USA); [b] Strains isolated by N. Amin in Sulawesi district, Indonesia (1994); [c] Strains isolated by J. Hallmann, Kenya (1999); [d] Strains isolated by Pocasangre in Cuba (2002); [e] Strains isolated by R.-P. Schuster Mpigi district, Uganda (1993); [f] Strains isolated by M. Griesbach Ntungamo district, Western Uganda (1996); [g] Strains provided by Plant Pathology Institute, University Stellenbosch (South Africa), isolated from symptomatic banana plants in Uganda. *Foc-F. oxysporum* f.sp.*cubense, F.o.-F. oxysporum, G. fu.-Gibberella fujikoroi* (anamorph: *F. fujikuroi*).
[1] Pathogen to banana with unknown biocontrol activity towards *R. similis.* [2] Significant biocontrol activity (~60% reduced penetration of *R. similis* per root system) in greenhouse trials. [3] Low or no biocontrol activity towards *R. similis.****** Tested against known VCGs of *F. oxysporum* f. sp. *cubense, lycopersici* and *radicis-lycopersici* (Zum Felde, 2008). n.c.-not compatible to any known VCG of *F. oxysporum* f. sp. *cubense* or against any non-pathogenic isolate within this study (Niere, 2001).▲ Initially unidentified *Fusarium* spp. now identified as *G.fujikuroi* by BLAST search of the partial IGS sequence of C39.▲▲ Morphologically identified as *F. oxysporum* f.sp. *cubense,* identified as *F. oxysporum* f.sp. *loti* by BLAST search of the partial IGS sequence (max. ident. 92%, respectively).▲▲▲ Morphologically identified as *Fusarium oxysporum* f.sp. *cubense,* identified as *F. proliferatum, F. golbosum or G. fujikuroi* by BLAST search of the partial IGS sequence (max. ident. 92%, respectively).

4.3.5 Tree building program

Phylogenetic trees were compiled using Treeview (Page, 1996), TreeEdit (Rambaut and Charleston, 2001), Photoshop 7.0 (Adobe) and Powerpoint (Microsoft) for additional annotation of data.

4.4 Results

The alignment of the 35 *Fusarium* spp. consisted of 1513 characters (Figure 15). As best-fit model for nucleotide substitution, the general time reversible with gamma rates and equal base frequencies (GTR+G; -lnL= 4652.5088, K=9, AIC=9323.0) was identified by the Akaike Information Criterion (AIC) in MrModeltest (Nylander, 2004). A total 186 (12.3%) informative sites were identified. The final average standard deviation of split frequencies of the Bayesian Inference (BI) was 0.003053. Both runs of the BI resulted in identical 50% majority-rule consensus trees with only marginal differences amongst the harmonic means of the log-likelihood values (run1= -4325.71; run 2= -4325.51). Branch support, expressed as posterior probabilities (PP), was generally high and ranged from 0.83 to 1.00, except for the nodes 1) and 2) (Figure 14). Jackknife analysis confirmed the general tree topology according to BI, but did not achieve identical resolution (Jackknife support ≤ 60 not displayed). Isolates C39 (Outgroup, *G. fujikuroi,* anamorph: *F. fujikuroi*) and CAV 2560 (BLAST ident.: *F. proliferatum, F. golbosum* or *G. fujikuroi* max. ident. 92%, respectively) are the most distant isolates differing from the remaining isolates in 0.15-0.17 changes per site.

The Phylogram shows full resolution for all taxa. Major clades (A and B) and basic distribution of clonal lineages (I-IV) are generally congruent for both analysis methods. Clade A contains the pathogenic isolates Fo001 (South Africa; VCG 01210), JC1 (Florida/USA; VCG 0120) and II5 (Indonesia; VCG 01213), whereas clade B contains the isolates belonging to the other VCGs and the non-pathogenic isolates, except for the commensalist V1w7 and the mutualist A1. Clade B can be further divided into three clonal lineages or subclades (II-IV) that mainly comprise isolates from the countries located at the Great Lakes Region (Lake Victoria) in East Africa, but it also contains two isolates from Costa Rica, Central America. Pathogenic *Foc*s from Uganda exclusively from the subclade II and show only little genetic variation (0.001 changes per site) even when they belong to different VCGs or VCG complexes (VCGs 0124, 0125, 0126, 0128, 0129, 01220, 01222).

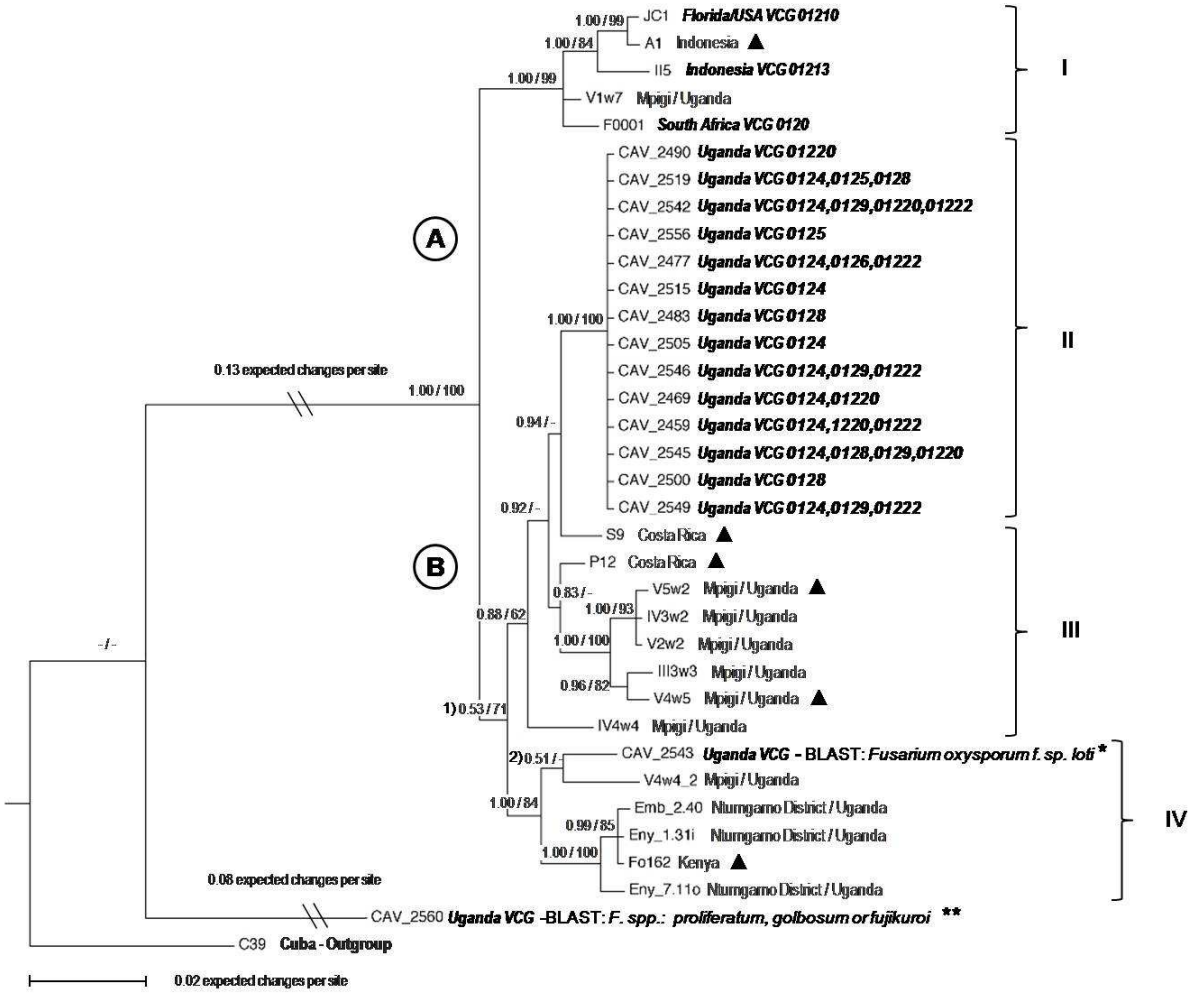


Figure 14: 50% majority rule phylogram of 35 *Fusarium* isolates inferred from a 1.51 kb fragment of the IGS sequence using Bayesian Inference under the GTR+G model of nucleotide substitution. Two major clades are indicated with A and B, sub-clades (I-IV) are indicated to the right. For each taxon VCG (only for f.sp.), geographical sampling origin and character (***pathogen***, ▲mutualist and commensalist) are indicated. Bayesian Posterior Probabilities and Jackknife-values are indicated in respective order at the internodes (values < 50% are indicated as dashes). BLAST search of the partial IGS of both, strain CAV_2543* or CAV_2560** showed higher max. identity to *Fusarium* other than *oxysporum* f.sp. *cubense.*

Subclade III shows the highest genetic variability (0.019 expected changes per site) and is formed by eight non-pathogenic *F. oxysporum*. All isolates originating from Uganda were sampled in the Mpigi district and two of these are mutualists. The two mutualistic isolates from Costa Rica are genetically more similar to the Ugandan *Focs* than the other endophytic isolates sampled in the Mpigi district. Within the non-pathogenic endophytes from Mpigi district there is a clear (1.00/100) split separating the two mutualists V5w2 and V4w5 into different sections, with closer relation (0.002-0.004 expected changes per site) to their commensalistic neighbours. However, the commensalist IV4w4 is the most distant taxon of subclade III.

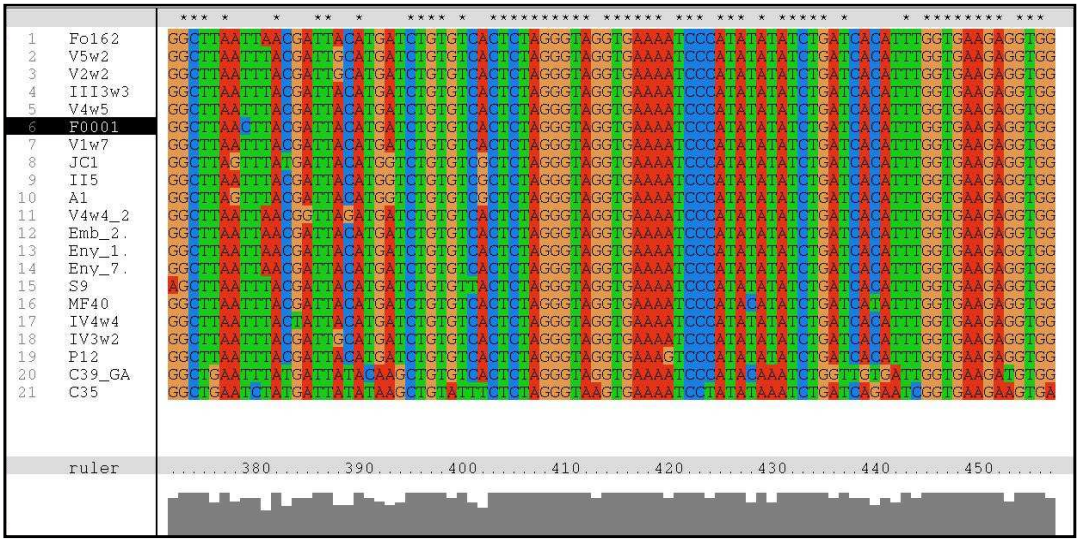


Figure 15: Section of a sequence alignment and SNPs of IGS dataset used for phylogenetic analysis

Subclade IV is split into two sections, one section harbours the commensalist V4w4_2 and the pathogen CAV 2543, which may have been misidentified morphologically, since BLAST search (max. ident. 98%) nested the isolate within strains of *Fusarium oxysporum* f.sp. *loti*, an organism causing wilt to birds foot trefoil (*Lotus coniculatus*). This pathogen however was recently proposed to be included as new forma specialis in the *Fusarium oxysporum* complex (Bergstrom et al., 1995). The second section of subclade IV is distinct and shows low genetic diversity (0.005 changes per site) among the commensalistic isolates originating from the Ntungamo district in Uganda and the mutualistic *F. oxysporum* strain Fo162 from Kenya.

4.5 Discussion

For several reasons a DNA based phylogenetic analysis of *Foc* and the so far non-described non-pathogenic isolates of *F. oxysporum* was necessary to gain insight into the genetic relations among the isolates. Generally, the phylogenetic species concepts of *F. oxysporum* f. sp. *cubense* and non-pathogenic isolates are hard to establish. Differentiation based on morphological characters or physiological features is insufficient or even impossible (Edel, 1998) and teleomorph stage of *F. oxysporum* has never been observed (Fourie et al., 2009; Leslie and Summerell, 2006). *Foc* isolates can be characterized according to their vegetative compatibility (see also chapter 1.3), but the VCG concept only applies for the forma specialis of *F. oxysporum* (Edel, 1998). Thus, the relations among both non-pathogenic isolates

alone, and more importantly, *Foc*s to non-pathogenic isolates can not be resolved. To infer the phylogenetic relations among *Foc*s and non-pathogenic isolates of the *F. oxysporum* complex a DNA based molecular approach was needed. This study is the first phylogenetic analysis that resolves the relationships among pathogenic *F. oxysporum* f.sp. *cubense* and mutualistic or commensalistic non-pathogenic isolates of *F. oxysporum*.

The measurements of genetic distances and the choice of an appropriate outgroup can be difficult for phylogenetic studies concerning *F. oxysporum* (Koenig et al., 1997). Hence, the results of chapter three, when C39 was the most distant taxon in all phylogenetic trees, was of significant importance. In the present analysis, C39 (identified as *G. fujikuroi;* anamorph: *F. fujikuroi* based on a BLAST search of the partial IGS sequence) showed to be an appropriate outgroup. This is in accordance with other phylogenetic studies in which isolates of the *Gibberella fujikuroi* complex demonstrated to be convenient outgroups within a global set of *F. oxysporum* f.sp. *cubense* isolates (Gordon and Martyn, 1997). However, the use of IGS sequence information was shown to be successful in inferring the phylogenetic relations among *F. oxysporum* and *Fusarium* species in several earlier studies (Abo et al., 2005; Edel et al., 1995; Henrion et al., 1992; Kawabe et al., 2005; O'Donnell, 1992) and showed to be the right choice to infer the phylogenetic relations between non-pathogenic *F. oxysporum* and *Foc*s from Uganda in the present study. The relatively high resolution that was obtained resulted from the specific nature of IGS, which is a relatively quickly evolving non-transcribed region (Edel et al., 1995; Pikaard, 2002). Nevertheless, the genetic similarity within the Ugandan *Foc*s was relatively high. The resolution may be further resolved by the use of AFLP analysis (see also chapter 3), as was demonstrated in other phylogenetic studies describing the relations among human pathogenic *F. oxysporum* isolates (O'Donnell et al., 2004).

Clade A contains the TR4 isolate from Indonesia (typically belonging to the VCG 01213/16 complex) and the pathogenic isolates with VCGs 01210 (Florida/USA) and VCG 0120 (South Africa). The sequence analysis showed that *Foc* isolates from Uganda (clade B) are phylogenetically distinct from the *Focs* in clade A. This clear distinction is also present in the VCG distribution. Furthermore *Foc*s from Uganda are phylogenetically distinct from non-pathogenic *F. oxysporum* from East Africa and

Costa Rica, forming a clear sub-clade or clonal lineage with only low genetic diversity. The diversity of VCG within the Ugandan *Foc*s is however still high, including seven distinct VCGs (VCGs 0124, 0125, 0126, 0128, 0129, 01220, 01222) or VCG complexes of two or more VCGs. This division between the various VCGs in the two clades, A and B, seems typical, since this has also been recently demonstrated in a similar study, comprising an extensive number of various pathogenic *F. oxysporum* isolates from all over the world (Fourie et al. 2009).

Pathogenicity and mutualism seem to be polyphyletic traits from a global point of view. Both can be found in clade A as well as in clade B. Thus both pathogenicity in *F. oxysporum* f. sp. *cubense* and mutualism of the non-pathogenic *F. oxysporum* isolates seem to have evolved in different parts of the world independently. The disribution of the mutualists in clade B shows that mutualism occurs multiple times in a scattered manner and the tree topology indicates that the trait of mutualism maybe was lost at some points. In contrast to that the Ugandan *Foc*s are arranged in a compact cluster. Thus, pathogenicity may be polyphyletic from a global point of view, whereas it seems to be monophyletic within the confined area of Uganda. Distinct clonal lineages would explain such clear separation.

The first impression might be that, except for the Ugandan *Foc*s, no indication for any clonal lineage can be recognized clearly. Nevertheless, various phylogenetic studies on global selections of *Focs* showed that several (about eight) distinct clonal lineages are recognized, which cluster according to VCGs but more or less irrespectively of their geographical sampling origin (Bentley and Bassam, 1996; Bentley et al., 1995; Bentley et al., 1998; Fourie et al., 2009; Koenig et al., 1997; O'Donnell et al., 1998b). These findings are not in conflict with the present results, since larger sets of *Focs* with global distribution were analyzed, when compared to the limited number of *Focs* and their relatively low diversity in geographical distribution in this study. For the presented set of isolates, the geographical sampling site clearly influences the clustering of isolates and was a more determining factor for the phylogeny as was observed in other studies (Bentley et al., 1998; Fourie et al., 2009). For example, even though Mpigi and Ntungamo districts are only about 250 km apart, isolates could generally be distinguished.

However, these results were not unexpected and can be explained by the relative low diversity in the geographical sampling sites which was mainly the East African Highlands. The East African Highlands are considered a secondary centre of diversity for banana and co-evolution of these *F. oxysporum* isolates with its host plant seems likely (De Langhe, 1996; INIBAP, 1998; Simmonds, 1966). The exceptional and distinct phylogenetic position of African isolates was also observed in earlier studies, when isolates from Misuku Hills, Karonfa in Malawi (VCG 01214), sampled at the cultivars Harare and Mbufu (both AAA-EH) formed a distinct clonal lineage within a worldwide collection of *Foc*s representing a diverse selection of different VCGs and sampling sites (Koenig et al., 1997).

In corroboration to these findings, it is not surprising that *Foc*s originating from Uganda are clearly distinct from the *Foc*s found in clade A. Hence, the individual *Foc*s of clade A may represent different clonal lineages. The number of isolates is however far too limited to support the idea of the clonal lineages concept which was shown for *Foc*s in other studies (Bentley et al., 1998; Fourie et al., 2009; Koenig et al., 1997). Nevertheless, pathogenic isolates were found in two distinct clades, providing evidence for multiple and independent events leading to pathogenicity as was suggested previously (Bentley et al., 1998; Fourie et al., 2009; Gordon and Martyn, 1997; O'Donnell et al., 1998b). Thus, the *Foc* found in Uganda are less related to the *Foc*s worldwide and are more close related to the non-pathogenic isolates sampled in the same region. Since, the East African Highland bananas are being considered as a secondary centre of diversity for this special *Musa* variety (AAA-EH) (De Langhe, 1996), coevolution of the Ugandan *Foc*s with EAHB does support this hypothesis.

However, the analysis also would suggest that the pathogenic *F. oxysporum* from Uganda have evolved from non-pathogenic endophytes. Such an event has only been observed for the ascomycete *Colletotrichum magna* and under artificial conditions only (Freeman and Rodriguez, 1993) and never for *F. oxysporum* (Gordon and Martyn, 1997). However, the phylogenetic analysis supports the event of co-evolution of the Uganda *Foc* with its banana host, but also the derivation of pathogens from previously non-pathogenic endophytes.

Even though the geographical origin seemed to have a strong influence, not all isolates clustered according to their sampling site. Curious is the presence of the Ugandan commensalist V1w7 in the international clade A and the nesting of the Cuban isolates S9 and P12 with the Ugandan isolates. A BLAST search of the V1w7 IGS sequence showed highest maximum similarity (99%) to *F. oxysporum* f.sp. *cubense* strain CAV632, which is a race 1 pathogen (VCG 01210) originating from Honduras. The close phylogenetic relationship of CAV632 to *Foc*s from USA, the Philippines, Malaysia and Indonesia, representing VCGs 0122, 01210, 01213 and 01216 (Fourie et al., 2009) suggest the introduction of V1w7 into Uganda. Conversely, isolates S9 and P12 were most likely exported to Cuba. The association or nesting of isolates from the Caribbean or Central America with isolates from Africa rather than other Central American isolates has been observed in earlier studies, when *Foc*s from Honduras (cv. Highgate), Brazil, Jamaica and Nicaragua (cv. Blugge AAB) formed one clonal lineage with *Foc*s from Malawi (cv. Harare) and Tanzania (cv. Pisang Awak) (Bentley et al., 1998). In this particular case, all pathogenic isolates shared the same VCG 0124, which is also present within the Ugandan sub-clade of *Foc*s in the present analysis. In addition the East African Highland cooking banana is also cultivated in Tanzania (Davies, 1995; Karamura, 1999; Mbwana and Rukazambuga, 1999), which is situated in the south of the Great Lakes Region of Lake Victoria in relative close geographical vicinity to the sampling sites in Uganda and Kenya. Thus, transmission of fungal isolates seems likely. Overall, isolates V1w7, S9 and P12 suggest the long distance transmission of organisms. The clustering of the mutualist Fo162 from Kenya with commensalists Eny 1.31, Eny 7.11 and Emb 2.4o from the Ntungamo district, western Uganda might be the result of short distance transmission.

Another interesting issue is the lack of host specificity of non-pathogenic isolates. Fo162 was isolated from tomato plants but also effectively colonizes different tomato varieties and even different banana cultivars (El-Fattah et al., 2007; Hallmann and Sikora, 1994; Vu et al., 2006; Vu, 2005). Less host specificity would automatically lead to a wider host range of such non-pathogenic isolates and could explain the relative high degree of diversity of the non-pathogenic endophytes from Uganda. In turn the relatively low genetic diversity observed within the Ugandan *Foc*s may result from their higher host specificity. High levels of diversity among non-pathogenic

isolates were also observed during vegetative compatibility tests. When 13 non-pathogenic *F. oxysporum* isolates were tested, compatibility was observed for two isolates only (Niere, 2001), thus further indicating, that non-pathogenic *F. oxysporum* are more diverse in VCG than *Foc*s.

More important than the phylogenetic relationships between the different pathogenic *Foc*s are the relations between non-pathogenic and pathogenic isolates from East Africa in this study. In clade B the pathogenic isolates are clearly distinct from the non-pathogenic forms, whereas clade A harbours too few taxa and is much more diverse in sampling origin to draw conclusions from the discovered phylogeny. Clade B contains sufficient taxa to recognize that mutualists and commensalists are distinct from the *Foc* isolates. The non-pathogenic isolates fall within several sub-clades and do not show segregation according to their mutualistic trait. Therefore it is unlikely to assume that the mutualistic trait is bound to a special lineage within non-pathogenic isolates of *F. oxysporum.* Together with the observation that the mutualist A1 was found in the distinct clade A, it was concluded that mutualism occurs polyphyletic within the *F. oxysporum* complex. Similar to the pathogenicity in Ugandan *Foc*s, the rise or loss of mutualistic traits could be associated with a co-evolutionary event, as was proposed before. When interpreting this random nesting of mutualists within the phylogram, a clonal transmission (i.e. all mutualists are descendants of one common ancestor) of the mutualistic trait must be rejected and the early detection of mutualistic isolates by ‘phylogenetic assisted pre-selection’ in order to reduce the need for laborious screening trials was not realized in this study.

However, the phylogenetic relations amongst strains of non-pathogenic *F. oxysporum* and pathogenic *F. oxysporum* f.sp *cubense* from Uganda were inferred for the first time. The distinct separation of both groups consolidated the results of previous VCG tests and emphasized the safe use of mutualistic endophytic *F. oxysporum* as a biological nematode management tool in banana IPM.

5 Mimicking of induced defense responses in banana *

5.1 Introduction

Systemic induced resistance (SIR) is a biological management tool to control *R. similis* infestation of the banana root system by artificial introduction of selected mutualistic *F. oxysporum* strains into the banana root (Niere et al., 1999; Vu et al., 2006). Although it is well documented that such fungal strains induce a systemic reaction that leads to reduced nematode penetration rates, the underlying molecular mechanisms of this phenomenon still remain unclear. It is possible that the observed SIR follows previously described pathways of induced systemic resistance responses, nevertheless it can not be excluded that the endophyte triggers an alternative and independent pathway of induced resistance.

Systemic Acquired Resistance (SAR) is one of the best described systemic induced plant defense mechanisms. SAR is generally induced by virulent, avirulent and non-pathogenic microbes or by exogenous application of specific chemicals (Sticher et al., 1997), i.e. by exogenous application of salicylic acid (SA) (Vernooij et al., 1994) or its functional analogs 2,6-dichloro-isonicotinic acid (INA) (Métraux et al., 1991) and benzo[1,2,3]thiadiazol (BTH) derivatives (Kunz et al., 1997), with S-methyl benzo [1,2,3] thiadiazol-7-carbothioic acid available as the commercial products BION®, ACTIGARD® or BOOST® (Oostendorp et al., 2001; Sticher et al., 1997). These chemicals themselves do not show antimicrobial activity, but induce the same characteristic set of SAR genes as found under pathological situations or exogenous induction by SA (Friedrich et al., 1996). The induction of SAR in dicotyledonous plants is typically associated with the systemic accumulation of SA and certain pathogenesis-related (PR) proteins (Sticher et al., 1997).

Beside the SA-dependent SAR, Induced Systemic Resistance (ISR) is another well described form of induced resistance, which is triggered by several *Pseudomonas* species, i.e. *Pseudomonas fluorescens* strain WCS417r, and by herbivorous insects (Pieterse et al., 2000; Pieterse et al., 2002; Pieterse et al., 1998; Pieterse et al., 1996; Van Loon et al., 1998).

* This experiment was conducted in collaboration and kind assistance of Dr. Andrea Ditzer (INRES-Department of Soilecosystem and Nematology)

ISR requires responsiveness to jasmonic acid (JA) or ethylene (ET), but is not associated with the transcriptional activation of *PR*-genes (Pieterse et al., 1998; Pieterse et al., 1996; VanWees et al., 1997). However, ISR is only one possible outcome of induced resistance regulated by the JA/ET-dependent pathway. The increased resistance observed after challenge of *Arabidopsis* leaves with the fungus *Alternaria brassicicola* and the concomitant local and systemic synthesis of the plant 'defensin' protein PDF1.2 (belonging to PR-12 protein family) was associated with increased levels of JA synthesis and ET production (Penninckx et al., 1996; Penninckx et al., 1998). Thus, in both the regulation of ISR and the synthesis of PDF1.2 the phytohormones JA and ET are involved. In the JA signaling pathway, methyl jasmonate (MeJA), which is a methyl ester of JA (Mason et al., 1992), is a key compound and regulates the JA biosynthetic pathway by a positive feedback mechanism (Cheong and Choi, 2003; Sasaki et al., 2001). Its exogenous application seems to increase the defense capacity of plants against some fungal and bacterial pathogens (Penninckx et al., 1996; Van Wees et al., 1999). PDF1.2 synthesis was also induced by exogenous application of MeJA and ET, but not by exogenous application of SA or its functional analog INA. This shows that the exogenous application of MeJA and ET can activate the JA/ET-dependent pathways, which are independent from the SA-inducible SAR. 1-Aminocyclopropane-1-carboxylic acid (ACC) is a natural precursor of the phytohormone ethylene (Pieterse et al., 1998); therefore ACC may trigger ET dependent defense responses in the plant.

Although different plant species respond to infections by the activation of similar defense mechanisms, their regulation may vary in detail. For example, there is a clear distinction of SA-inducible PR-1, PR-2 and PR-5 and JA/ET-inducible PR-3, PR-4 and PR-12 proteins in *Arabidopsis* (Lay and Anderson, 2005; Thomma et al., 2001), whereas in tobacco members of the same protein family are regulated differently by SA or JA/ET (Niki et al., 1998; Seo et al., 2001). For pathogen-induced SAR, the information is mainly based on dicots and relatively little information on monocots is available (Sticher et al., 1997), in particular on banana.

Exogenous application of chemicals, that were shown to induce either the JA/ET- or SA-regulated pathways, can help to characterize the expression of certain defense-related genes in banana during ISR or SAR respectively. The information generated

under controlled conditions can be compared to the responses induced by endophytes and will contribute to a better understanding of the responsible defense mechanisms induced in banana leading to reduced nematode infestation. Proteins and their coding transcripts that are indicative for certain signaling pathways and types of induced resistance or transcripts being involved in the defense to nematodes are of major interest. For example, *PR-1* has been widely accepted as SA-inducible marker gene for SAR and all *PR-1* genes in plants seem to be inducible by SA (Vernooij et al., 1994). PR-1 was induced by both pathogen and chemical SAR inducers in the monocot corn (Morris et al., 1998). Another protein, the 'Non-expressor of Pathogenesis Related genes' (NPR1) is a key element that regulates the cross-talk between ISR and SAR (Pieterse and Van Loon, 2004). PDF1.2 was frequently used as marker for the induction of a JA/ET-dependent defense-signaling pathway (Lay and Anderson, 2005), which is differs from those of ISR or SAR and which is also independent of the regulation by NPR1 (Penninckx et al., 1996; Penninckx et al., 1998). PR-6 proteins are also interesting because these proteins exhibit proteinase-inhibitory effects and are assumed to play a role in insect or nematode defense, by inactivating proteins that are released by the pest (Abad et al., 1996; Selitrennikoff, 2001; Van Loon, 2001; Van Loon and Van Strien, 1999; Vigers et al., 1992).

5.2 Scope

The first aim of the study was to identify marker genes that are typically involved in induced resistance in the monocot banana. Chemical inducers of SAR and ISR will be used to monitor the transcript accumulation of these marker genes under defined conditions. The chemical induced expression of the marker genes can then be used to determine whether ISR, SAR or an alternative mechanism is playing a role during the SIR in banana plants when colonized with non-pathogenic mutualistic *Fusarium oxysporum* endophytes in further studies.

5.3 Material and Methods

5.3.1 Plant material

Cavendish bananas (AAA) cultivar 'Valery' were used to assess the response of specific *PR*-genes to different chemical treatments. Two week old plants were provided by Vitropic (Saint-Mathieu-de-Trévieres, France). Plantlets were grown in a 50 ml mixture of sterilized sand and field soil (1:1; v/v) in a climatic chamber at 25℃ and a diurnal cycle with 16 hours light. Sterilized tap water was used for irrigation. After three weeks the soil was separated from the root system by carefully rinsing with tap water and the banana plants were transferred into 300 ml pots containing a sterilized mixture of sand and field soil (1:1; v/v). Each plant was irrigated with 100 ml of tap water and transferred to the greenhouse. The banana plants were grown for four more weeks prior to experimental use.

Table 13: Treatments and concentrations of the chemicals used for banana leaf dips.

Treatment	Silwet L-77	Concentration
Absolute control	-	-
Silwet (control)	100 ppm	-
MeJA	100 ppm	100 µM
ACC	100 ppm	1000 µM
SA	100 ppm	1000 µM
INA	100 ppm	5 µM
BION©	100 ppm	50 mg/l

MeJA–Methyl Jasmonate (Sigma, liquid: 1 M=224.3 g/l); ACC–Aminocyclopropane-1-Carboxylat (Sigma, powder: 1 M=101.1 g/l); SA–Salicylic acid (Sigma powder; 1 M=138.12 g/l); INA–2,6-Dichloroisonicotinic acid, (Sigma, powder: 1M=192.0 g/l); Benzothiadiazole (Syngenta, water dispersable granules as BION© 50 WG: 50.0 mg/l).

Banana plants were treated with five different chemical inducers (Table 13), which were dissolved in distilled water. Each treatment (except for the absolute control) was supplemented with 100 ppm of the surfactant Silwet L-77 (Lehle Seeds, USA) to improve absorption. Banana foliage was submerged by turning the plant upside down into the chemical solution for 10 s. To improve absorption, the foliage was moistened once more with tap water using an atomiser after the initial leaf treatment had dried. Control plants were treated with water only (absolute control) or with water containing 100 ppm Silwet L-77 (control). Seven plants per treatment were treated.

5.3.2 Sampling of plants

Plants were sampled 24 hours after chemical application. Soil was gently separated from the root system using tap water and both roots and leaves were collected in 15 ml tubes, immediately frozen using liquid nitrogen and stored at -80 ℃.

5.3.3 RNA extraction, DNA digestion and reverse transcription

Root and leaf samples for expression studies were lyophilized and subsequently pulverized into a fine powder using a spatula and vortex. During the sequential steps of sample processing, all samples were stored at -80℃. Approximately 3 mg of root or leaf powder was used for RNA extraction, which was performed with the 96 nucleospin RNA extraction kit (Macherey-Nagel) according to the manufacturer`s instructions. RNA was eluted in 100 µl of RNase free double distilled water.

Table 14: Master Mix for the DNaseI digest of RNA samples

Component	Volume [µl]
RNA template	7.0
10x DNaseI buffer	1.0
RNase free ddH_2O	1.0
EDTA 25 mM*	1.0
DNaseI	1.0
Total volume	10.0/11.0*

* EDTA was added in a final step

To eliminate remaining genomic DNA fragments, an additional DNaseI digest was performed (Table: 14). 7 µl of RNA template was incubated with 1 µl of 10x DNaseI buffer (Invitrogen), 1 µl of RNase-free double distilled water (Qiagen) and 1 µl of DNaseI for 15 min at RT. The DNaseI enzyme was then inactivated by incubating the samples at 65℃ for 10 min. To protect RNA from deg radation 1 µl of 25 mM of EDTA was added prior to the incubation.

cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. To each product of the DNaseI digest (11 µl) from the previous step, 8 µl of Master Mix (Table 15) and 1 µl of MultiScribe Reverse Transcriptase were added, respectively. Reactions were mixed gently and centrifuged briefly.

Table 15: Master Mix for the High Capacity cDNA Reverse Transcription reaction volume used for cDNA synthesis

Component	Volume [µl]
10x RT Buffer	2.0
25x dNTP Mix (100 mM)	0.8
10x RT Random Primers	2.0
Nuclease-free ddH_2O	3.2
Reverse Transcriptase	1.0
RNA template	11.0
Total volume	20.0

cDNA synthesis was performed in a total volume of 20 µl using the thermal cycler conditions given in table 16.

Table 16: Thermal cycling conditions used for the synthesis of cDNA.

	Step 1	Step 2	Step 3	Step 4
Temperature (℃)	25	37	85	4
Time	10 min	120 min	5 sec	Hold

After cDNA synthesis, samples were diluted in 8 µl of double distilled water (Qiagen) in order to achieve sufficient template volume for further analysis. Samples were stored at -20℃ to avoid degradation.

5.3.4 Primer design for expression analysis by real-time PCR

Primers pairs for real-time PCR were designed based on *Musa acuminata* sequences of *PR1* and *NPR1* genes that were chosen from the NCBI and the Global Musa Genomics Consortium (GMGC) data base (Table 17). For the amplification of the *18S* gene previously described primers were used (Hershkovitz et al., 1999).

Sequences of *Musa PR-1* or *NPR1* genes were aligned using the VectorNTI suite (Invitrogen) and polymorphic regions were identified. Primer Express software (Applied Biosystems) was used to design balanced pairs of *PR-1* and *NPR1* primers. The fragment length of amplicons ranged from 62 bp to 92 bp (primer sequences: Table 18).

Table 17: *Musa* sequences used to design specific primer pairs for the amplification of *PR-1*, *NPR1* and *18S* gene transcripts.

Accession number	Target	Data base description	Predicted Gene*
MA57L19[1]	*PR-1*	Pathogenesis-related 1	Mu57L19_05
MA69C10[1]	*PR-1*	Pathogenesis-related 1	Mu69C10_15
Mu72F16[1]	*PR-1*	Pathogenesis-related 1	Mu72F16_08
Mu72F16[1]	*PR-1*	Pathogenesis-related 1	Mu72F16_10
MA72F16[1]	*PR-1*	Pathogenesis-related 1	Mu72F16_13
MA57L19[1]	*PR-1*	Pathogenesis-related 1	Mu57L19_08
MA69C10[1]	*PR-1*	Pathogenesis-related 1	Mu69C10_12
EF055881[2]	*PR-1*	Pathogenesis-related 1	
DQ925843[2]	*NPR1*	NPR1-like protein	
EF137717[2]	*NPR1*	NPR1-like protein	
AF069226[2]	*18S*	*18S* SSU rRNA gene	

[1] Accession number corresponding to Global Musa Genomics Consortium (GMGC).
[2] Accession number corresponding to the NCBI data base.
* Referring exclusively to the accession numbers of GMGC.

5.3.5 Validation of complete DNA digestion using real-time PCR

To verify the complete digestion of DNA, DNaseI treated RNA (negative control) and cDNA samples (positive control) were compared in a study. A real-time PCR with primers pairs coding for *18S* and *PR-1e* gene transcripts was performed using the positive, negative and absolute control (water only) as template. Real-time PCR was performed with the 7500 Fast real-time PCR System (Applied Biosystems). The wells of the 96 well detection plates (Applied Biosystems) were filled with 1 µl of positive, negative or absolute control sample, 2 µl of both *18S/PR-1e* forward and *18S/PR-1e* reverse primer (10 µM each), 5 µl of sterile distilled water and 10 µl of 2x SYBR Green Master Mix for each sample respectively.

5.3.6 Dilution series of cDNA

A cDNA dilution series (undiluted, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}) was prepared in order to validate the continuous performance of the polymerase enzyme during the real-time PCR. The *18S* cDNA was amplified according to the protocol described at 5.3.7; performing 60 instead of 40 cycles.

5.3.7 Gene expression analysis using real-time PCR

Single strand cDNA, synthesized from plant RNA was used as a template for Relative Quantification (RQ). The expression profiles of six putative defense related banana genes *PR-1a, PR-1e, PR-1f, PR-1h, NPR1a* and *NPR1b* (Table 18) were analyzed and related to the constitutively expressed *18S* gene of the ribosomal small subunit. RQ was performed with the 7500 Fast real-time PCR System (Applied Biosystems).

Table 18: Primer sequences of *NPR1, PR-1* and *18S* primer pairs.

Target gene	**Forward primer sequence (5`-3`)**	**Reverse primer sequence (5`-3`)**	**Size [bp]**	**Position* [bp]**
PR-1a	CTCCTGGGTCTCAGAGAAGCA	CCACACAACCTGCGTGTAGTG	91	424-515
PR-1e	TCGGGTGCAGTGCAACAG	TGCCCGCTGGGTTGTAGT	59	540-599
PR-1f	TGCTCGGGTGCAATGCG	TGCCCGCTGGGTTGTAGT	63	536-599
PR-1h	GGAATCGACGCCGTCAAA	CGAGTTGCTGTTGTGGTCGTA	62	406-468
NPR1a	TTGGCCCTGCTGTAAAATGTATT	TTCCAGGACAAAATTTGAAACAGA	65	1919-2011
NPR1b	TGGGTTCTTCGTGCCAAAA	CATGGTTCTATAAGAGGCAACAACTC	65	1845-1909
18S **	AAGCCCACGCTCTGGATACAT	GCCAACACAATAGGACCGAAA	64	**

* Amplicon position within the respective alignment [bp].
** Primer pair designed by Hershkovitz et al., 1999.

The 96 well detection plates (Applied Biosystems) were loaded with 1 µl of cDNA, 2 µl of both forward and reverse primers (10 µM each), 5 µl of sterile distilled water and 10 µl of 2x SYBR Green Master Mix for each sample, respectively.

Cycling conditions for the selected ‘delta delta Cycling Threshold’ (ddCT) study for the Relative Quantification were as follows: SYBR Green was used as detector. ROX was chosen as internal reference and the *18S* gene was set as the endogenous control for the target genes *PR-1a, PR-1e, PR-1f, PR-1h, NPR1a* and *NPR1b.* Real-time PCR started with a preincubation time of 10 min at 95 ℃ followed by 40 cycles of 15 s incubation at 95℃ and 1 min of primer anne aling and extension at 60℃. Samples were performed in triplicate.

5.3.8 Gene expression analysis

Normalization of expression data was performed using the standard curve of the specific target gene and the endogenous *18S* control gene, in accordance with the ddCT method (Livak and Schmittgen, 2001).

5.4 Results

5.4.1 RNA extraction

Electrophoresis of the RNA extraction of all root and leaf samples showed the typical pattern (Figure 16) for RNA, assuming that no RNA degradation had occurred.

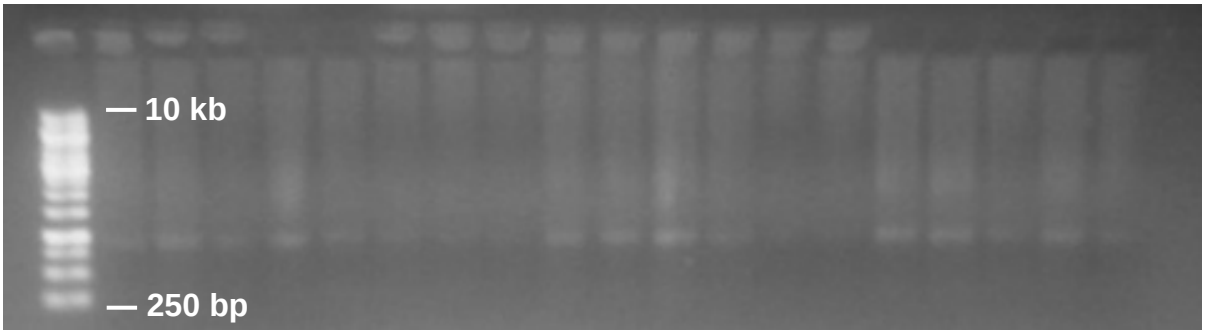


Figure 16: Example for the successful extraction of RNA from 20 root samples. First line loaded with 5 µl of the peqGOLD 1 kb DNA-ladder (Peqlab) and lines 2-21 loaded with 5 µl of root RNA samples.

5.4.2 Primer design

Gene orthologs for two *NPR1* and eight *PR-1* genes, but no orthologous sequences for *PR-6* genes, associated with insect and nematode resistance (PR-6), or *PDF1.2* (PR-12 protein family), indicative for JA/ET-depending defense pathways, could be identified from the *Musa* genome using the Global Musa Genome Consortium (GMGC) or National Centre for Biotechnology Information (NCBI) data base.

The alignment of the eight *Musa PR-1* sequences consisted of 624 bp. The highest degree of sequence variation within the alignment of the *PR-1* gene sequences was found within the position 371-624 (Figure 17).

For each gene, specific primers were chosen from polymorphic sequence regions showing significant numbers of polymorphisms. No sequence variation between Mu57L19_05 and Mu69C10_15 was observed for this region of the alignment. High

sequence homology was also observed for Mu57L19_08, Mu69C10_12 and EF055811.

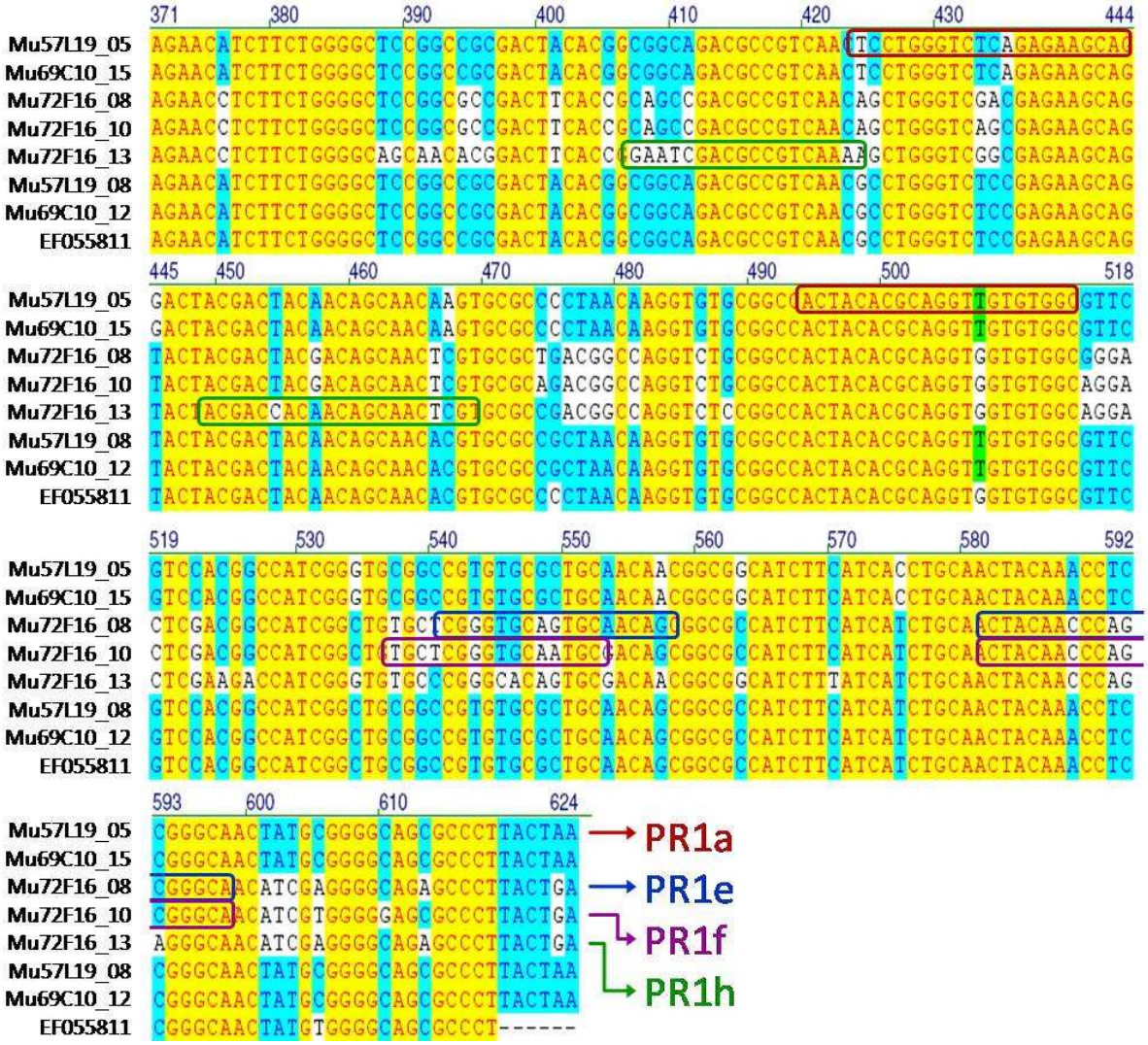


Figure 17: Alignment of eight *Musa PR-1* gene sequences showing significant sequence variation.

Four *PR-1* primer pairs were selected from the polymorphic regions of the alignment: 1) The *PR-1*a primer set was designed based on the sequence of Mu57L19_05 and amplifies a 91 bp fragment covering the alignment positions 424-515, 2) the *PR-1*e primer set was designed based on the sequence of Mu72F16_08 and amplifies a 59 bp fragment covering the alignment positions 540-599, 3) the *PR-1*f primer set was designed based on the sequence of Mu72F16_10 and amplifies a 63 bp fragment covering the alignment positions 536-599 (reverse primers for *PR-1*e and *PR-1*f are identical) and 4) the *PR-1*h primer set was designed based on the sequence of

Mu72F16_13 and amplifies a 62 bp fragment covering the alignment positions 406-468.

The alignment of the two *Musa NPR1* sequences consisted of 2073 bp. The highest degree of sequence variation within the alignment of the *NPR1* gene sequences was found within the position 1776-2073 (Figure 18).

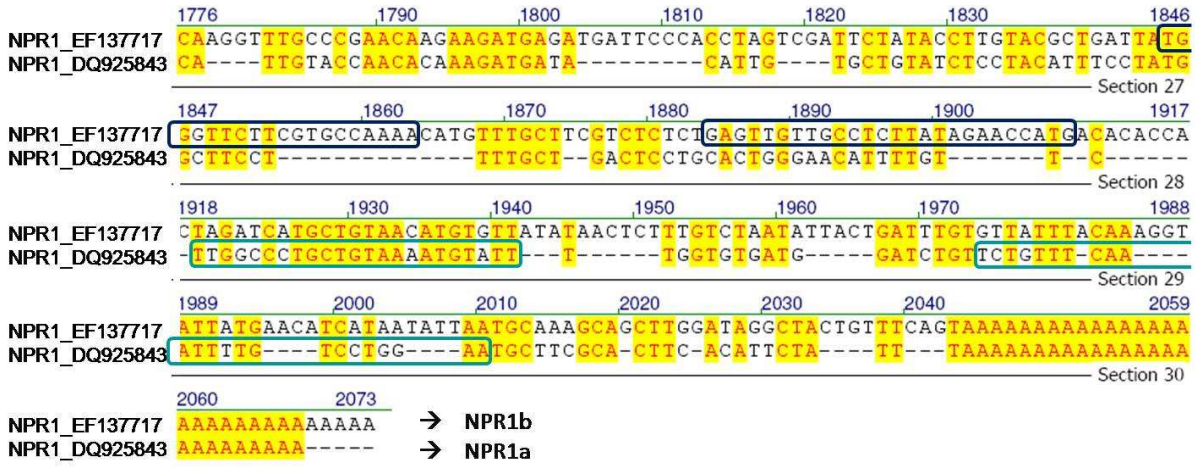


Figure 18: Alignment of two *Musa NPR1* gene sequences showing significant sequence variation.

The *NPR1* specific primers were chosen from different positions within the alignment and show significant numbers of polymorphisms for each forward and reverse primer. The primer set specific for *NPR1a* was designed based on the *NPR1* sequence DQ925843 and amplifies the alignment positions from 1919 to 2011, resulting in a 65 bp fragment. The primer set specific for *NPR1b* was designed based on the *NPR1* sequence EF137717 and its amplicon covers the alignment positions from 1845 to 1909 resulting in a 65 bp fragment.

5.4.3 Validation of DNA digest using real-time PCR

The amplifications of the *18 S* and *PR-1e* gene transcripts were used to follow the cycling threshold (CT) of templates that received different treatments. Samples receiving DNase and RT treatment reached the CT after about 8 and 23 cycles respectively, whereas samples with DNase, but without RT treatment reached CT after 34 cycles for *18S* or not at all for *PR-1e* (Figure 19). No amplification was observed for the water control (absolute control). In case water was used as template no amplification at all was observed.

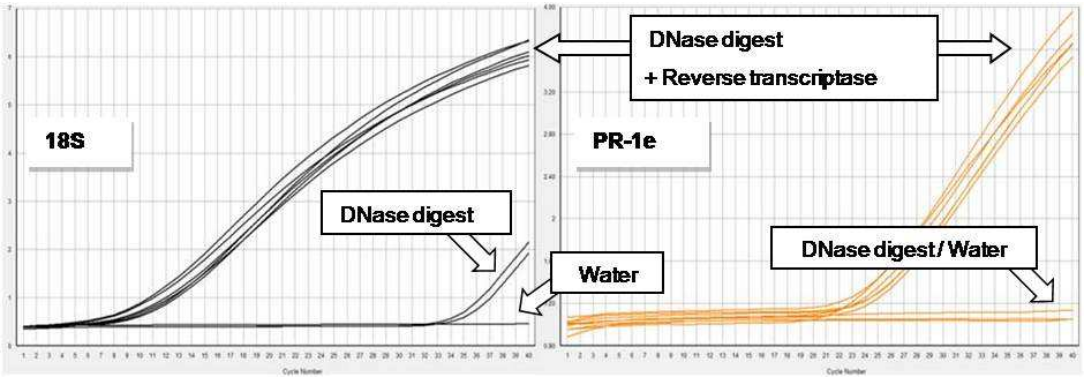


Figure 19: Amplification plot of *18S* and *PR-1e* gene transcripts when RNA receiving DNaseI and RT (CT 8 and 23); RNA receiving DNaseI without RT (CT 34 and -) or Milli-Q water alone (no amplification) was used as template.

5.4.4 Dilution series of cDNA

When the *18S* transcripts were amplified by using undiluted, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} times diluted cDNA, the Cycling Thresholds (CT) were reached after 8, 14.5, 21, 27.5 and 32.5 cycles, respectively (Figure 20). Dilution by 100 resulted in a constant CT delay, resulting in intervals of the amplification curves of 6.5 cycles, except for the 10^{-8} dilution (5 cycles), thus showing a linear relation of dilution and amplification pattern, indicating a constitutive performance of the Taq Polymerase at the relevant cDNA concentrations. Dilution by 10^{-8} resulted in sufficient amplification (Rn=3.8) of cDNA for data analysis. Enzyme performance showed stable performance over more than 40 cycles.

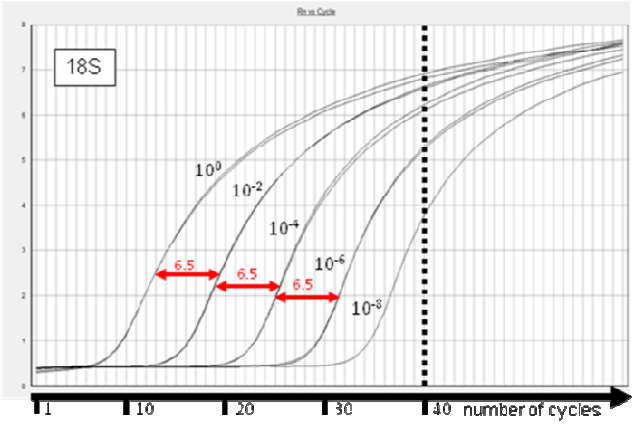


Figure 20: Amplification plot of *18S* cDNA transcripts using cDNA template dilutions of 100, which resulted in constant CT delays of 6.5 cycles.

5.4.5 Accumulation of *NPR1* and *PR1* transcripts

Banana plants (n=7) were treated (leaf dip) with the chemical treatments Silwet L77, SA and Silwet L77, INA and Silwet L77, BION and Silwet L77, ACC and Silwet L77

and MeJA and Silwet L77 (see table 13). Total RNA from leaf and root tissue was extracted and cDNA was synthesized by reverse transcription. Expression levels (unit: Relative Quantification) of mRNA transcripts coding for *PR-1* (*PR-1a, PR-1e, PR-1f, PR-1h*) and *NPR1* (*NPR1a, NPR1b*) gene sequences were analyzed in relation to the constitutive *18S* gene transcripts and compared to the mRNA transcripts of the untreated control (absolute control) for leaf and for root samples. The different chemical treatments resulted in significant differences in accumulation of transcripts, displayed by the Relative Quantification (RQ; the RQ determines the change in expression of the target sequence in the test sample relative to the same sequence in a calibrator sample) coding for pathogenesis related proteins for leaf and root samples, when compared to the control plants (Figure 21). RQ minima and RQ maxima showed only little variation for the analyzed transcripts for leaf (0.01-0.78 and 0.01-0.85) and root (0.04-0.38 and 0.04-0.31) samples across all treatments. Except for *PR-1h*, leaf samples responded stronger to the different chemical treatments when compared to root samples.

Although in most cases the responses of transcripts to the chemical treatments showed similar patterns of up- or down regulation in the root and leaf samples, the expression profiles of some proteins showed a differential accumulation in a tissue specific manner. The accumulation of *NPR1a* transcripts was reduced in leaf and root samples in response to all treatments, including Silwet (RQ=0.03-0.10 and 0.50-0.77, respectively). In leaves *NPR1a* remained almost undetected. For *NPR1b* the, BION and MeJA treatments resulted in a slight increase in accumulation (RQ=1.09 and 1.18, respectively) and a slight decrease in accumulation for INA (0.90), SA (0.82), Silwet (0.93) and ACC (0.63) in leaves. The root samples responded with collective decrease in *NPR1b* accumulation for all treatments (RQ=0.59-0.76).

PR-1a transcript accumulation was strongly increased in leaves (4.25) and roots (1.79) in response to BION. INA, SA and MeJA showed a tissue specific response, resulting in a slight increase in transcript accumulation in the leaf samples (1.49, 1.72 and 1.54, respectively) and a slight decrease in *PR-1a* transcript accumulation in roots (0.80, 0.72 and 0.88, respectively). Silwet decreased *PR-1a* accumulation in leaves (0.61), but at the same time increased its accumulation in roots (1.19).

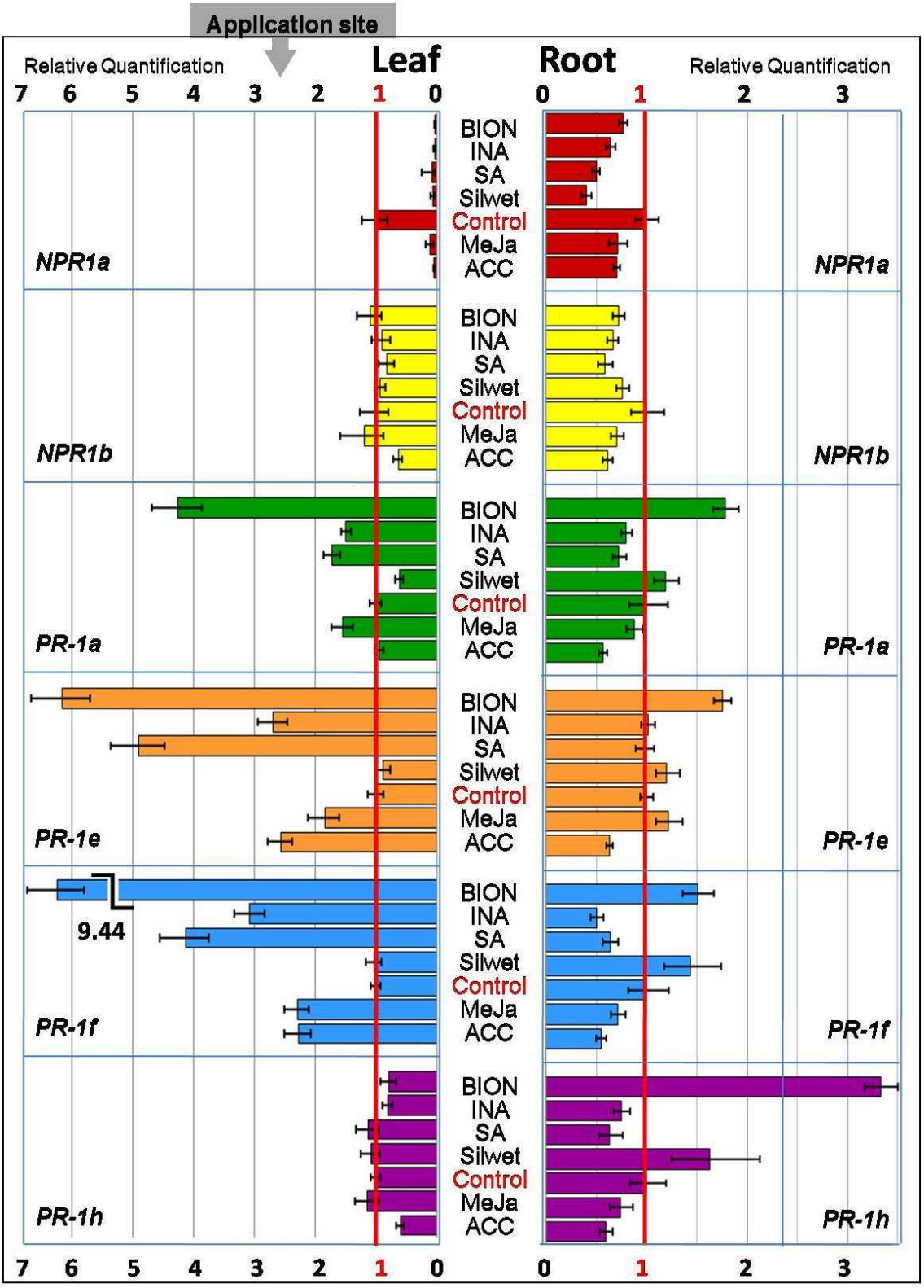


Figure 21: Relative Quantification of the cDNA transcripts of six *Musa NPR1* or *PR-1* genes, extracted from leaf and root mRNA of banana plants (n=7) in response to BION, INA, SA, Silwet, MeJA and ACC treatments, applied as a leaf dip 24 hours prior to harvest. Values are displayed as RQ, error bars represent RQ minima and maxima. Asterisk (*) indicates, that the column representing the RQ of *PR-1f* in response to the BION treatment in leaf tissue is beyond the displayed range of the graph. Therefore the column was split (indicated by the hooked line), its actual RQ-value is indicated below the column top

Leaf samples did not show any significant response to ACC (0.95), but *PR-1a* accumulation was reduced in roots (0.57).

PR-1e transcript accumulation in leaves was significantly increased in response to all chemical treatments, BION (6.17), INA (2.69), SA (4.90), MeJA (1.84) and ACC (2.57). The Silwet treatment was not significantly different (0.88) in the leaf. In roots a *PR-1e* transcript accumulation increase was observed for BION (1.76), Silwet (1.20) and MeJA (1.22), whereas accumulation decreased in response to ACC (0.63). No response was observed for INA (1.02) or SA (0.98).

A strong to moderate increase in accumulation of *PR-1f* transcripts in leaves was observed for all chemical treatments, with INA (3.07), SA (4.14) and especially BION (9.44) showing the highest levels in transcript accumulation. No significant difference was observed for the Silwet treatment (1.03) in leaves, but its transcript accumulation was increased (1.44) in roots at a similar level as the BION treatment (1.51). INA, SA, MeJA and ACC (0.50, 0.64, 0.72 and 0.55) treatments resulted in a decrease in *PR-1f* accumulation in roots.

In leaves no (SA: 1.13, Silwet: 1.08 and MeJA: 1.14) or only little changes (BION: 0.78, INA: 0.80 and ACC: 0.59) in *PR-1h* transcript accumulations were observed. In contrast, for BION (3.33) and also Silwet (1.63) the accumulation had increased in roots, whereas for INA, SA, MeJA and ACC (0.75, 0.63, 0.74 and 0.60) the *PR-1h* accumulation had decreased.

5.5 Discussion

Genome sequence data bases still show gaps for many plant species and not all PR families have been identified in each plant species examined so far. Generally the members of the various *PR* genes have been described for *Arabidopsis*, barley, bean, maize, potato, tobacco and tomato (Van Loon et al., 2006; Van Loon and Van Strien, 1999). The sequencing process of *Musa* is still ongoing, therefore severely limiting the research presented here. Finally, homologous sequences for *NPR1* and *PR-1*, but not for *PR-6* (associated with insect and nematode resistance) or *PDF1.2* (PR-12 family, indicative for JA/ET-depending defense pathways) could be extracted from the GMGC or NCBI data bases. Hence, gene expression studies were limited to the ISR and SAR or SAR indicative marker genes *NPR1* and *PR-1*, respectively.

SA-inducible SAR and JA/ET dependent ISR can be induced in parallel and NPR1 shows a regulatory function between the cross-talk of both the SAR and ISR pathway of signal transduction (Pieterse and Van Loon, 2004; Pieterse et al., 2002). Thus, the two *NPR1* homologs *NPR1a* and *NPR1b* were used as an indicator for a general event of induced resistance (Van Loon et al., 2006) and *PR-1*, widely accepted as SA-inducible marker gene for SAR (Vernooij et al., 1994), served as an exclusive indicator for SAR in *Musa*. Exogenous leaf applications of methyl jasmonate (MeJA) and ACC were used to activate JA/ET-inducible pathways. For the activation of the SA-inducible SAR, exogenous application of SA (Vernooij et al., 1994) or its functional analogs INA and BION® were used (Kunz et al., 1997; Métraux et al., 1991).

In general, the accumulation of *NRP1* and *PR-1* transcripts in leaves responded stronger to the chemical treatments than the roots (except for *PR-1e*). SAR is known to extend from the leaves into the root system (Gessler and Kuc, 1982; Tahirialaoui et al., 1993), nevertheless the lower response in roots is not unexpected since the chemicals were applied to the foliage and the accumulation of PR proteins during SAR was reported to fade with increasing distance to the inoculation site (Stintzi et al., 1993). For example, when the PR levels of tobacco leaves were analyzed after tobacco mosaic virus (TMV) infection, the levels of PR proteins in non infected leaves was only 5-10% of those detected in leaves exhibiting a hypersensitive reaction.

In contrast, the accumulation of *PR-1h* transcripts in roots showed a stronger response in a chemical specific manner, being strongly upregulated for the BION and Silwet treatment and otherwise being down-regulated. These results indicate that *PR-1h* seems to have a tissue specific function in root cells and has less importance for leaves. The dramatic decrease in *NPR1a* transcript accumulation (RQ 0.03-0.08) in leaves in response to all treatments (including Silwet) is remarkable. In signal transduction, the NPR1 protein is located downstream of SA and it is essential for the activation of *PR-1* gene transcription during both biological and chemical induction of SAR in *Arabidopsis* (Lawton et al., 1996; Pieterse and Van Loon, 2004; Pieterse et al., 1998). NPR1 proteins are directly involved in the *PR-1* gene expression during SAR by stimulating the DNA-binding activity of the TGA transcription factor to the cognate *cis* element that activates PR-1 gene expression (Pieterse and Van Loon, 2004). The accumulation of *PR-1a*, e and f transcripts was significantly increased in response to the BION, INA, SA, MeJA or ACC treatments, especially in leaves. This increase of *PR-1* transcript accumulation must theoretically be the result of an increased NPR1 activity. Nevertheless, *NPR1a* transcript accumulation was significantly decreased in response to all chemical treatments that led to an increased *PR-1* transcript accumulation. The observed decrease was stronger in leaves than in roots. This would indicate an inverse correlation of *NPR1a* expression with spatial proximity to the site of induction. The *NPR1b* expression was significantly different when compared to *NPR1a* expression, only showing a decrease after treatment with ACC in leaves, whereas transcript accumulation was significantly decreased in response to all chemical treatments in a similar manner to *NPR1a* in roots. Thus, the *NPR1a* and *NPR1b* transcript accumulation in leaves were affected differently. Slight differences in functional tasks or shifts at the moment of activation of both *NPR1s* could be the source for the observed differences. *NPR1* responses will be discussed more detailed and in the context of additional data in the following chapter.

The accumulation of *PR-1a*, *PR-1e* and *PR-1f* transcripts is similar and showed a significant increase for all chemical treatments in leaves, except for *PR-1a* in the presence of Silwet or ACC. The increased *PR-1a, PR-1e* and *PR-1f* accumulation of these transcripts indicates the induction of SAR and corroborates with observations made for SA and its functional analogs INA and BION in other studies, where SAR

and the accumulation of *PR-1, PR-2* and *PR-5* transcripts was induced by exogenous application of the very same chemicals (Friedrich et al., 1996; Oostendorp et al., 2001). Exogenous application of SA, INA and especially BTH (BION) were found to be particularly effective in inducing PR-1 protein expression in the monocot maize (Morris et al., 1998) and the activated resistance was observed to be typically very long lasting in other monocots (Oostendorp et al., 2001). Whether the significant higher accumulation levels of the *PR-1-a, -e* and *-f* homolog transcripts in response to the BION treatment were the result of a delayed or prolonged induction or due to a slower or prolonged uptake of BION cannot be answered with this study.

In root samples the transcript accumulation of *PR-1-a, -e*, *-f* and *–h* homologs in response to SA, INA and BION also followed a certain pattern, which was different from the one observed in the leaf samples. Results are characterized with the BION treatment leading to elevated *PR-1* levels in all homologs analyzed and with SA or INA showing no differences or significantly decreased *PR-1* transcript accumulation. The systemic translocation of BION within the plant and its long lasting effect (Oostendorp et al., 2001) could account for the observed significant increase in transcript accumulation of all *PR-1* homologs. Although INA is also fully systemic and independent of SA production to activate SAR responses (Métraux et al., 1991; Oostendorp et al., 2001), its effect on the *PR-1* homologs was opposite to the BION treatment and more similar to the SA responses. Decreasing concentrations or reduced mobility of INA compared to BION could explain the different responses. The exogenous application of SA was shown to be sufficient for electing SAR in leaf, except for *PR-1h*. Nevertheless, SA itself is not the translocated signal during SAR (Verberne et al., 2003; Vernooij et al., 1994), therefore it seems reasonable to expect differences in *PR-1* expression between the site of application (leaf) and sites that are located more distant (root). Exactly that was observed for the various *PR-1* transcript accumulation profiles in leaf and root tissue in this study.

General conclusions drawn from the accumulation of PR-1 transcripts in response to the SAR inducers SA, INA and BION are, that the accumulation of *PR-1a*, *e* and *f* responds to at least the BION treatment in both leaf an root tissue, thus excluding a tissue specific expression profile of these PR-1 proteins. In general, *PR-1h* seemed

to be tissue specific since, a response to the chemical treatments was only observed in root tissue.

Interestingly, the accumulation of *PR-1e* and *PR-1f* transcripts also increased after MeJA and ACC treatments, similar to the SA, INA or BION treatments in leaf and root samples. Both chemicals were intended to affect the JA and ET regulated pathway, which is clearly distinct from the SA regulated pathway of SAR. However, ACC significantly increased *PR-1e* and *PR-1f* (1.57 and 2.28) transcript accumulation in leaves. The exogenous application of MeJA also increased *PR-1a, e* and *f* transcript accumulation (1.54, 1.84 and 2.29) in leaves. Although ACC was expected to induce JA and ET-regulated defense responses rather than the SA-inducible SAR marker *PR-1*, the significant increased accumulation of *PR-1e* and *PR-1f* transcripts in leaves corroborates with findings of slight increases in *PR-1, PR-2* and *PR-5* gene expression in leaves after exogenous application of ACC to *Arabidopsis* cv. Col 0 roots (Van Wees et al., 1999). In another study with *Arabidopsis*, ethylene acted as a potentiator of SA- and pathogen-induced *PR-1* gene expression (Lawton et al., 1995; Lawton et al., 1994) and in the monocot rice the expression of four out of twelve PR-1 family members was induced in response to ACC (Mitsuhara et al., 2008). In addition, ET was required for the production or transmission of the systemic SAR signal in TMV-infected leaves of tobacco plants (Verberne et al., 2003). Therefore it seems reasonable to assume that the ACC treatment was the trigger for the increase of *PR-1e* and *PR-1f* transcript accumulation in leaves. Another study further connects ET to responses associated with SAR, when ET was proposed to be a signal for the specific expression of a basic-type *PR-1* tobacco gene (*PRB-1b*) belonging to an ethylene responsive sub-family, whose regulation differs from the acidic-type *PR-1* genes (Eyal et al., 1993). Finally, both chemicals ACC and ET seem to be associated with the expression of *PR-1* genes and the induction of SAR in some cases.

Transcript accumulation of *PR-1* homologs in leaf and root tissue responded to the exogenous treatment with MeJA analogous to the treatments with SA, INA and BION. However, the exogenous application of MeJA was not expected to result in increased *PR-1* transcript accumulation. In studies with *Arabidopsis,* exogenous applications of MeJA increased the defense capacity of plants against some fungal and bacterial pathogens by expression of jasmonate-inducible genes, like *Atvsp,*

Lox1, Lox2, Pal1 and *Pin2* or *PDF1.2*, which can be considered marker genes for one specific pathway of defense responses that is regulated by JA and ET. In contrast, the expression of the so called SAR genes *PR-1, PR-2* or *PR-5* was not observed (Penninckx et al., 1996; Van Wees et al., 1999). The findings in *Arabidopsis* are however in conflict with the characterization of JA-inducible cDNAs corresponding to one acidic and one basic PR-1 protein in rice (Agrawal et al., 2000). Other studies, investigating the response of 12 PR-1 family members in rice to SA, ACC and MeJA treatments, also showed that four PR-1 proteins were induced by treatment with ACC and seven by treatment with MeJA, compared to only five when SA was applied (Mitsuhara et al., 2008). The increase of *PR-1* transcripts or the accumulation of PR-1 proteins found in rice or banana show that the *PR-1* expression is at least for these two monocots JA-inducible. This also suggests that the expression of *PR-1* genes in monocot and dicot plants may be regulated differently.

6 Transcriptome analysis: Plant responses to *Fusarium oxysporum* and *Radopholus similis*

6.1 Introduction

Infestation of banana roots by the burrowing nematode *Radopholus similis* can be reduced by targeted introduction of selected non-pathogenic mutualistic *Fusarium oxysporum* endophytes (Menjivar Barahona, 2005; Niere, 2001; Niere et al., 1999; Vu et al., 2006; zum Felde 2008). Split-root experiments showed, that the inoculation of a mutualistic endophyte to one half and *R. similis* to the other half of spatially separated roots of the same banana plant significantly reduced *R. similis* penetration rates (Vu, 2005). These investigations demonstrated that the mutualistic endophyte elicted a systemic response within the banana plant that resulted in reduced penetration of the nematode. The observed effect of induced resistance was finally described as systemic induced resistance (SIR).

The mechanism of induced resistance in plants is defined as 'physiological state of enhanced defense capacity', which is induced upon specific environmental stimuli and results in the activation of the plant's native defense system (Van Loon and Van Strien, 1999). Induced resistance can be effective against many organisms, including viruses, bacteria, fungi, herbivorous insects and nematodes (Vallad and Goodman, 2004). The best studied mechanisms of induced resistance in plants are the systemic acquired resistance (SAR) and the induced systemic resistance (ISR). Both are differentiated based on their elicitors and the involved regulatory pathways. SAR is induced upon infection with virulent, avirulent and non-pathogenic microbes and involves the signaling molecule SA (Sticher et al., 1997; Vallad and Goodman, 2004).

ISR is induced by several *Pseudomonas* species (i.e. *Pseudomonas fluorescens* strain WCS417r) and by herbivorous insects and the signal transduction within the plant depends on jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 2000; Pieterse et al., 2002; Pieterse et al., 1998; Pieterse et al., 1996; Van Loon et al., 1998). Although ISR and SAR follow different signaling pathways, both pathways can be induced simultaneously and seem to communicate or interfere with each other to a certain extent. Cross-communication between the SA- and JA/ET-dependent pathways is realized through the regulatory function of the NPR1 protein (Pieterse and Van Loon, 2004; Pieterse et al., 2002). *NPR1* transcript accumulation is

therefore indicative for a general event of induced resistance, regardless if ISR or SAR is induced (Pieterse and Van Loon, 2004).

In contrast, the onset of SAR is clearly associated with the SA-dependent accumulation of *PR-1* gene transcripts and their proteins, which can be used as a specific marker for SA-inducible pathways leading to SAR (Thomma et al., 2001; Vernooij et al., 1994).

SIR shows parallels to both ISR and SAR. Resistance is induced locally by a non-pathogenic biotic factor (the selected mutualistic endophytes); the defense response of the plant shows a systemic effect and is directed against an organism, other than the elicitor. Even though, the effectiveness of SIR is well documented, the underlying mechanism remains unclear, since possible pathways were never analyzed at the molecular level.

6.2 Scope

Based on the results of the previous chapter, the objective of this study was to evaluate the accumulation of *PR-1* and *NPR1* gene transcripts in banana leaf and root tissue in response to *R. similis* infestation, fungal colonization by pathogenic or non-pathogenic *F. oxysporum* isolates and the combination of the two. One goal was to better understand the plant response during *R. similis* infestation and endophytic colonization and to identify the mechanisms of systemic induced resistance (SIR) elicted by the mutualistic endophyte *F. oxysporum* 162. A second goal was to characterize the transcriptional responses of banana to the root colonization by pathogenic and non-pathogenic *Fusarium* endophytes.

6.3 Material and Methods

6.3.1 Banana plants

Cavendish bananas (AAA) of the cultivar 'Valery' were used to assess the response of specific *PR* transcripts to the various biotic elicitors. Two week old plantlets were provided by Vitropic (Saint-Mathieu-de-Trévieres, France) and further propagated in a 50 ml mixture of sterilized sand and field soil (1:1; v/v) in a climatic chamber at 25℃ and a diurnal cycle with 16 hours light. Steri lized tap water was used for irrigation. After three weeks, the soil was separated from the root system by carefully rinsing with tap water and the banana plants were transferred into 300 ml pots containing a mixture of sterilized sand and field soil (1:1; v/v). Each plant was irrigated with 100 ml of tap water and transferred to the greenhouse. The banana plants were grown for four more weeks prior to experimental use.

6.3.2 Endophytic strains

Three fungal strains with different traits were selected for experimental use: The pathogenic Fo001, the mutualistic Fo162 and the commensalistic C39 (Table 19).

Table 19: Endophytic *Fusarium* isolates used in the transcriptome analysis.

Isolate	Species	Origin	Character	VCG
Fo001[a]	*F. oxysporum* f. sp. *cubense*	South Africa	Pathogen[1]	0120
Fo162[b]	*F. oxysporum*	Kenya	Biocontrol[2]	n.c.
C 39[c]	*G. fujikuroi* ▲	Cuba	Low control[3]	n.c.

[a] Isolated from banana and provided by the Tropical Research and Education Centre of the University of Florida (Homestead, USA); [b] Isolated from tomato in Kenya by J. Hallmann (1999); [c] Isolated from banana in Cuba by Pocasangre (2002); [1] Known pathogen to banana, unknown biocontrol activity towards *R. similis*; [2] Significant biocontrol activity (penetration of *R. similis* reduced by ≥60%) in greenhouse trials. [3] Low biocontrol activity towards *R. similis* (≤20).▲ Initially unidentified *Fusarium* spp. now identified as *Gibberella fujikuroi* (anamorph: *F. fujikuroi*) by BLAST search of the partial IGS sequence of C39. n.c.- not compatible to any known VCG of *F. oxysporum* f. sp. *cubense*.

Each strain originated from single spore colonies and was maintained as described in chapter 3.3.

For propagation of the individual fungal strains a 5 mm diameter PDA plug with mycelium (growth zone) was transferred to 300 ml of sterile 100% PDB medium (24 g Potato Dextrose broth (DIFCO) and 1000 ml Aqua dest. pH 6.0), which was incubated for 4 days at room temperature and 120 rounds per minute (rpm) on a

orbital shaker. Spores and mycelium were separated by pouring the liquid medium over four layers of cheese cloth. The collected spores were separated from the PDB by centrifugation at 1100×g for 15 minutes. The supernatant was then separated and spores were resuspended in 100 ml of sterile tap water. The spores were washed in three subsequent steps according to the procedure described before. Spore density within the suspension was assessed under 40 times magnification using a Thoma heamocytometer. The final spore density was adjusted to 1.0×10^6 spores per ml.

6.3.3 Propagation of *Radopholus similis*

Pure nematode cultures of *Radopholus similis* (Cobb) Thorne were obtained from Dr. De Weale, Head of the Laboratory for Tropical Crop Improvement at the Catholic University of Leuven (KUL), Belgium. The nematodes originated from samples taken in Uganda and were maintained and propagated on sterile carrot disks according to an established method (Speijer and De Waele, 1997). Fresh carrots with foliage were used for the propagation of *R. similis*. Foliage was separated and the carrots were washed with a commercial brush under tap water. Carrot tips were separated to both sides and the carrot cylinders were sterilized in 1% (v/v) of sodium hypochlorite for 60 min. All subsequent steps were performed in a vertical flow cabinet under sterile conditions. Carrots were sprayed with 70% (v/v) of ethanol, which was burned before the cortex was peeled. This procedure was repeated three times. The carrots were then cut into sterile cylinders of 5 mm size and transferred to Petri dishes with Ø 35 mm. Depending on future needs 20-1000 nematodes were inoculated to the exterior cylinder of the carrot discs. Nematode inoculated carrot disks were stored at 25℃ in the dark.

6.3.4 Experimental setup and treatment combinations

Young banana plants (*Musa* AAA cv. Valery, n=10) were inoculated with the commensalist C39, the mutualist Fo162 and the pathogen Fo001 (Chapter 6.3.5). Water was used as a control. After two weeks five plants per treatment were inoculated with 1500 *R. similis* (Chapter 6.3.6). The individual treatment combinations are given in table 20. After another two weeks, plants were sampled (Chapter 6.3.7), nematode penetration rates were determined (Chapter 6.3.8) and the accumulation of *NPR1* and *PR-1* transcripts was analyzed for leaf and root tissue (Chapter 6.3.10).

Table 20: Individual treatment combinations of the gene expression studies.

Treatment	Fungal strain	Trait	*R. similis*[3]
1	Fo001[1]	Pathogen	**+**
2	Fo162[1]	Mutualist	**+**
3	C39[1]	Commensalist	**+**
4	Control[2]	**-**	**+**
5	Fo001[1]	Pathogen	**-**
6	Fo162[1]	Mutualist	**-**
7	C39[1]	Commensalist	**-**
8	Control[2]	**-**	**-**

Number of replicates per treatment=5.
[1] Plants inoculated with 1.0×10^6 fungal spores per ml.
[2] Plants treated with sterile tap water.
[3] (**+**) 1500 nematodes 14 days post fungal inoculation, (**-**) no nematodes.

6.3.5 Inoculation of endophytic strains

Prior to inoculation of the six week old banana plants, the soil was gently separated from around the root system using tap water. To improve fungal infection, the banana roots were slightly wounded by removing approximately 5% of the root tissue using a pair of sterilized scissors. The root system was then submerged into 400 ml of a suspension containing 1.0×10^6 spores per ml of the individual isolates Fo162, C39 or Fo001. Plants were submerged for five minutes under constant agitation. Control plants were treated with sterilized water. Ten plants per treatment were inoculated.

6.3.6 Inoculation of *Radopholus similis*

R. similis was multiplied on carrot sterile disks (see chapter 6.3.3). Tap water was used to rinse free moving *R. similis* from carrot disks into 50 ml falcon tubes. The number of nematodes per ml was determined using a binocular at 40 times magnification and nematode counting chamber. Nematode densities were adjusted to the concentration needed for experimental use. For *R. similis* inoculation three holes, two cm deep and in three cm distance to the banana pseudostem, were made using a 1 ml pipette tip. A total number of 1500 nematodes were inoculated into these holes in three equal batches of 300 µl. After infiltration of the nematode suspension, the holes were carefully covered with soil.

6.3.7 Sampling of plants

Soil was gently separated from the root system using tap water. 90% of the root system was sampled to determine *R. similis* penetration rates. The rest of the roots and the entire shoots were used for gene expression studies. Samples for expression analysis were collected in 15 ml tubes. Roots were frequently subsampled and chosen at random in order to obtain unbiased and representative datasets. To prevent RNase activity and RNA degradation, samples were immediately frozen in liquid nitrogen.

6.3.8 Evaluation of *Radopholus similis* penetration rates

To determine *R. similis* penetration rates, roots were stained in 50 ml Fuchsine Acid (Merk) under slightly simmering conditions in a microwave for 5 min. Roots were then suspended in 10 ml of tap water, fragmented using the Ultra Thorax blender and the number of *R. similis* was counted at 10 times magnification using binoculars and Nordmeyer nematode counting chamber (Hooper, 1990; Sikora and Schuster, 2000).

6.3.9 Statistical analysis

Statistical analysis of the nematode penetration rates was performed based on the absolute number of penetrated *R. similis* per root system. Levene test of variance equality, ANOVA and LSD Post Hoc test were performed using SPSS 17.0 (SPSS).

6.3.10 Gene expression analysis

RNA extraction, DNA digestion, cDNA synthesis, real-time PCR cycling settings, validation of DNA digestion using real-time PCR and statistical analysis were performed as described previously in chapter 5.3. The previously designed primer pairs *PR-1a, PR-1e, PR-1f, PR-1h, NPR1a and NPR1b* were used for the amplification of transcripts by real-time PCR and except for an alternative dilution factor (undiluted, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) the dilution series of cDNA was performed according to the methods described in chapter 5.3.

6.4 Results

6.4.1 *Radopholus similis* penetration rates

Figure 22 shows the penetration rates of *R. similis* per root system 14 days after inoculation. The control roots contained 1121 *R. similis*, whereas the Fo001, Fo162 and C39 colonized roots contained 610, 684 and 890 nematodes, respectively. Levene test of variance equality identified homogeneity of variances (P=0.48) within the analyzed set of data. ANOVA and LSD were performed at a significance level of $P \leq 0.05$. ANOVA identified significant differences among the treatments (P=0.04). Two homogenous subgroups of treatments were identified by the LSD test: Group A contained the treatments with the pathogenic strain Fo001 and the mutualistic strain Fo162 with reductions of *R. similis* penetration by 45% (LSD; P=0.012) and 39% (LSD; $P \leq 0.014$) respectively. The commensalistic strain C39 reduced *R. similis* penetration rates by 20% and was not significantly different from both the control (Group B) and group A (Figure 22).

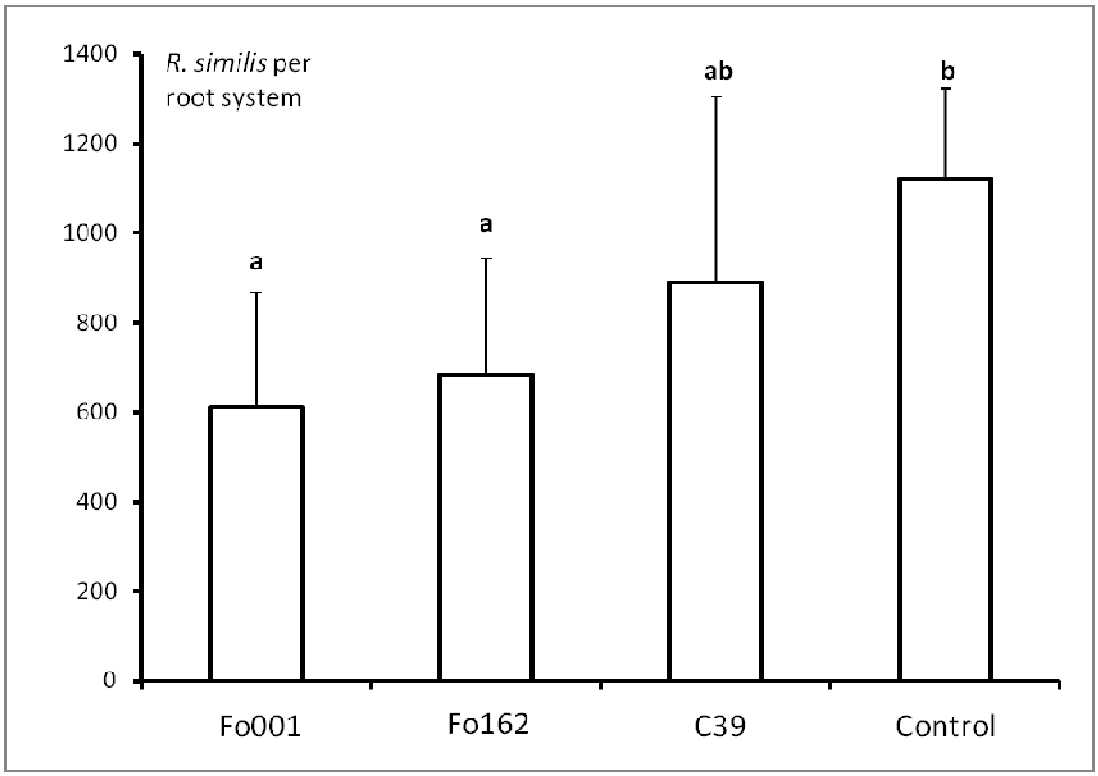


Figure 22: Effect of the root colonization by the *Fusarium* strains Fo001 (pathogen), Fo162 (mutualist) or C39 (commensalist) on the average penetration rates of *R. similis* into the root system of the banana cultivar Valery 14 days after nematode inoculation and 28 days after fungal inoculation. Error bars represent the standard deviation. Treatments with different letters are significantly different based on LSD-test ($P \leq 0.05$; n=5).

6.4.2 Validation of RNA and cDNA quality

The RNA extraction of root and leaf samples was checked by electrophoresis and resulted in typical pattern for plant RNAs, showing no indications for RNA degradation (data not shown).

In order to verify the complete DNA digestion and the successful cDNA synthesis of the RNA samples, transcripts of the *18S* banana gene were amplified by real-time PCR. The *18S* amplification plot of leaf samples (Figure 23) shows that the cycling threshold (CT) for cDNA templates ranged from 4-8 cycles. Amplification curves reached a plateau around 20 cycles for all samples, except for the negative controls (No reverse transcription), which reached the CT after 28 and 33 cycles, respectively.

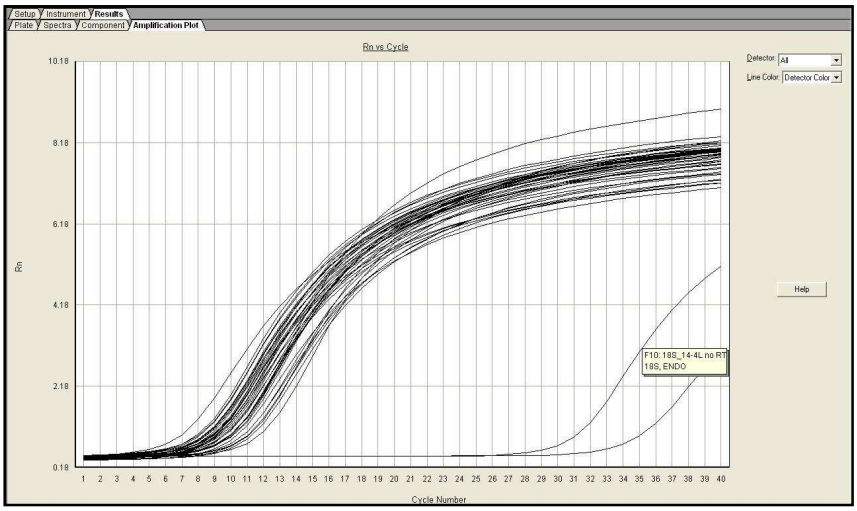


Figure 23: Amplification plot of cDNA transcripts coding for the *18S* banana gene originating from leaf tissue. CT of samples performing cDNA synthesis was reached after 4-8 cycles. The two negative controls (no cDNA synthesis) reached CT after 28 and 33 cycles.

For the cDNA of the root samples (Figure 24), the CT values for the amplification of the *18S* banana gene ranged from 5 to 10 cycles and the amplification curves reached a plateau between 15 to 18 cycles. The RT negative controls (No cDNA synthesis) reached CT after 30 and 33 cycles, respectively.

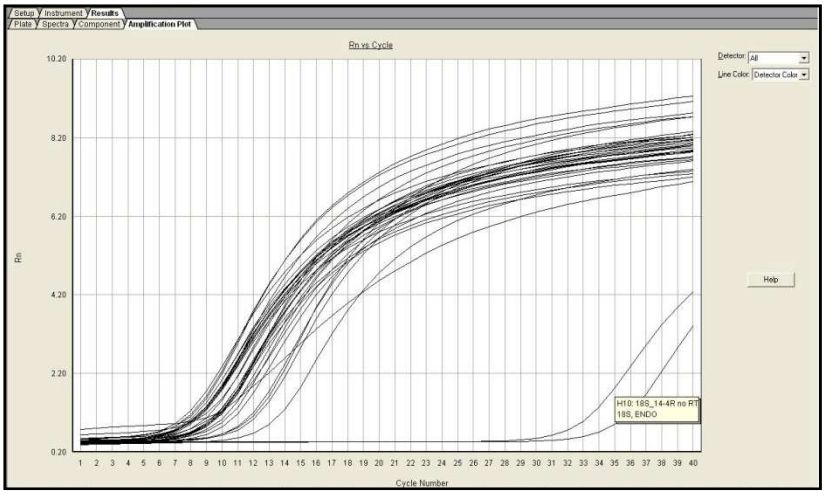


Figure 24: Amplification plot of cDNA transcripts coding for the *18S* banana gene originating from root tissue. CT of samples performing cDNA synthesis was reached after 5-10 cycles. The two negative controls (no cDNA synthesis) reached CT after 30 and 33 cycles.

6.4.3 Dilution series and polymerase performance

In order to validate the constitutive performance of the DNA polymerase during real-time PCR and to validate the quality of the cDNA templates, undiluted, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of cDNA template were compared by following the amplification of the *18S* cDNA (Figure 26). CT of the undiluted cDNA was reached after 3.24 cycles and those of the 10^{-1}, 10^{-2} and 10^{-3} times diluted cDNA were reached after 6.5, 9.6 and 12.9 cycles, respectively. The 10^{-4} dilution showed a CT of 16.5 cycles. Overall, this showed that the consecutive dilution steps of 10 resulted in the consecutive and constant CT delay of 3.3 over a broad dilution range.

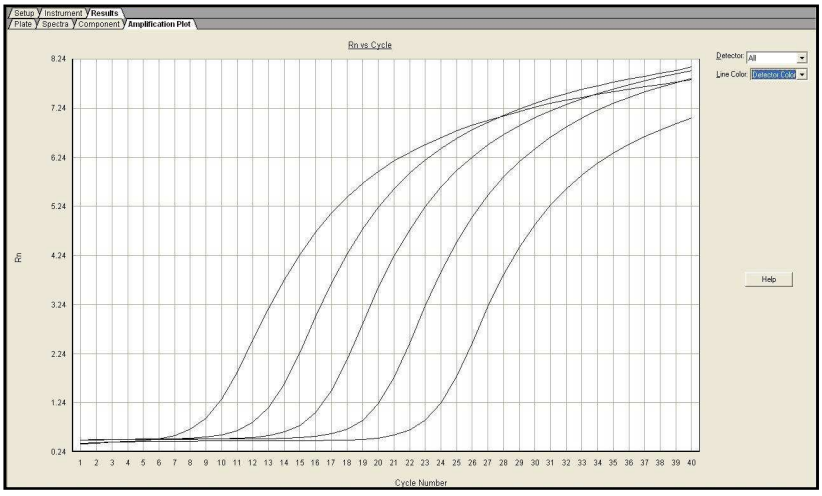


Figure 25: Amplification plot of cDNA transcripts coding for the *18S* banana gene, using undiluted, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of the cDNA templates.

6.4.4 *NPR1* and *PR-1* expression levels in response to nematode treatment

When compared to the non-infected control, the RQ of the *PR-1* or *NPR1* gene transcripts did not result in significant alterations in response to the nematode penetration in roots (Figure 26). The RQ in roots was characterized by strong differences in RQ minima and RQ maxima, whereas in leaves RQ minima and maxima showed less variation. However, only the accumulation of *PR-1a*, *PR-1f* and *PR-1h* transcripts (RQ= 0.32, 0.10 and 0.22) in leaves showed significant differences in response to the presence of *R. similis*. Remarkably, the abundance of *NPR1a* had decreased five-fold on average in response to nematode presence, whereas it had increased two-fold for *NPR1b*.

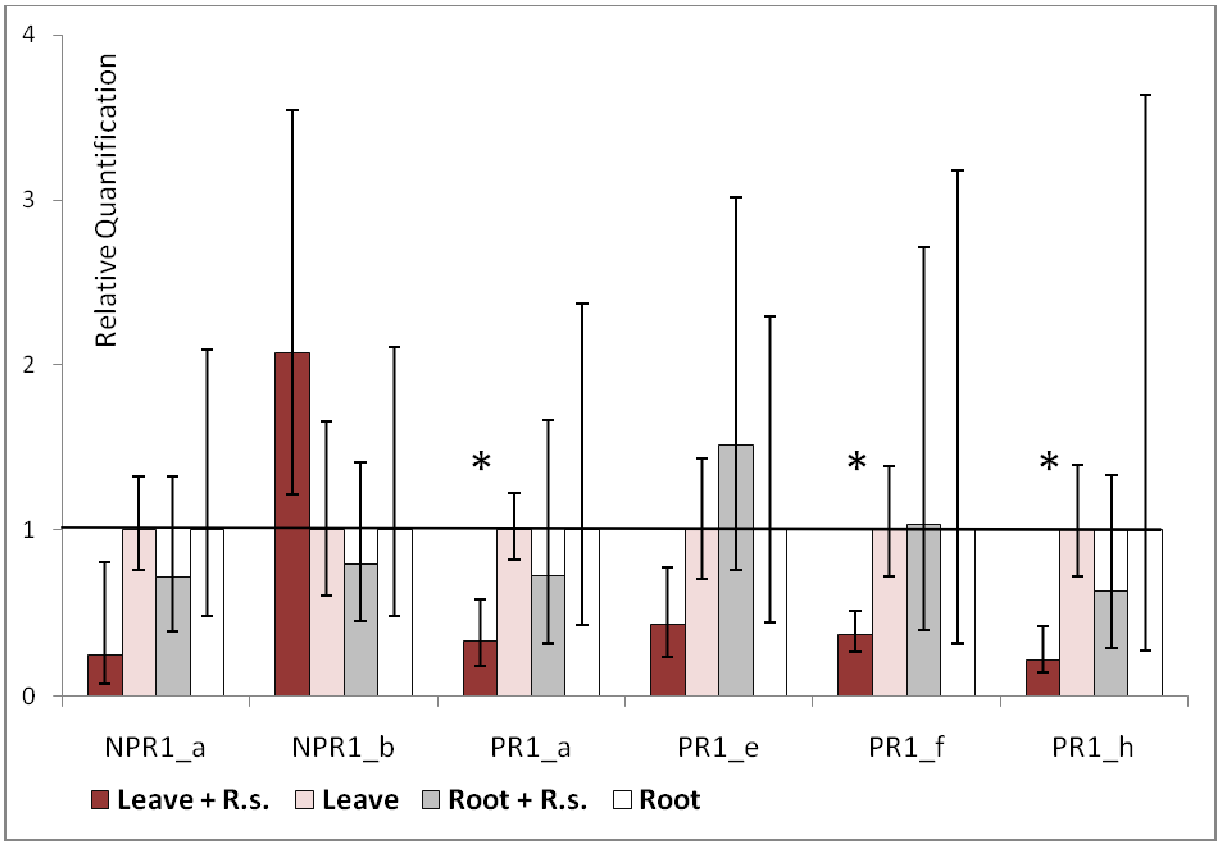


Figure 26: Relative Quantification of the cDNA transcripts coding for two *NPR1* and four *PR-1* banana genes in leaf and root tissue in the presence or absence of the nematode *R. similis* and related to the non-inoculated control (RQ=1). Error bars represent RQ minima and maxima ($P \leq 0.05$; n for leaves=5, n for roots=4).

6.4.5 Accumulation of transcripts in the presence of endophytic *Fusarium* isolates alone and in combination with *R. similis*

Results of the statistical analysis of the real-time PCR data for root and leaf samples are illustrated in figure 27. Results showed that, none of the fungal treatments had a significant influence on *NPR1*a or *NPR1b* transcript accumulation in leaves and roots. Nevertheless, the response to endophyte or *R. similis* treatments was more distinct in leaf tissue compared to the tissue of roots. In addition, the accumulation of *NPR1a* transcripts in leaf tissue increased in response to Fo001 and *R. similis* colonization of the root (RQ= 2.70), the remaining treatments led to a decrease in accumulation of *NPR1a* transcripts with RQ levels of 0.23, 0.17, 0.25, 0.25, 0.54 and 0.75 for Fo162+*R. similis,* C39+*R. similis, R. similis*, Fo001, Fo162, and C39, respectively.

The accumulation of *NPR1b* transcripts in leaves seemed to behave in a reciprocal manner, since the RQ for Fo001+*R. similis* was 0.15 and 1.01, 2.14, 2.08, 2.58, 1.71 and 1.52 for Fo162+ *R. similis*, C39+ *R. similis*, *R. similis*, Fo001, Fo162 and C39, respectively. No significant differences or recognizable pattern of *NPR1a* or *NPR1b* transcript accumulation were detected in root tissue.

Accumulation of *PR-1* transcripts in response to the individual treatments in leaves was similar and in some cases almost identical. Whenever the pathogenic isolate, Fo001, was present alone or in combination with *R. similis*, the accumulation of *PR-1* transcripts increased. In contrast, any other treatment, except for C39 in *PR-1h*, resulted in decreased transcript accumulation for all *PR-1* homologs analyzed. However, not all of the observed differences were statistically significant.

The accumulation of *PR-1a* transcripts increased in response to Fo001+*R. similis* (RQ=1.96) and also increased significantly ($P \leq 0.05$) in response to Fo001 (RQ=3.65). The colonization by the mutualistic isolate Fo162 and the commensalistic isolate C39 resulted in a significant decrease ($P \leq 0.05$) in *PR-1a* transcript accumulation in both the absence (RQ values of 0.30 and 0.19, respectively) and in the presence of *R. similis* (RQ values of 0.32 and 0.41, respectively). *PR-1a* transcript accumulation also significantly ($P \leq 0.05$) decreased in the presence of *R. similis* alone (RQ=0.36).

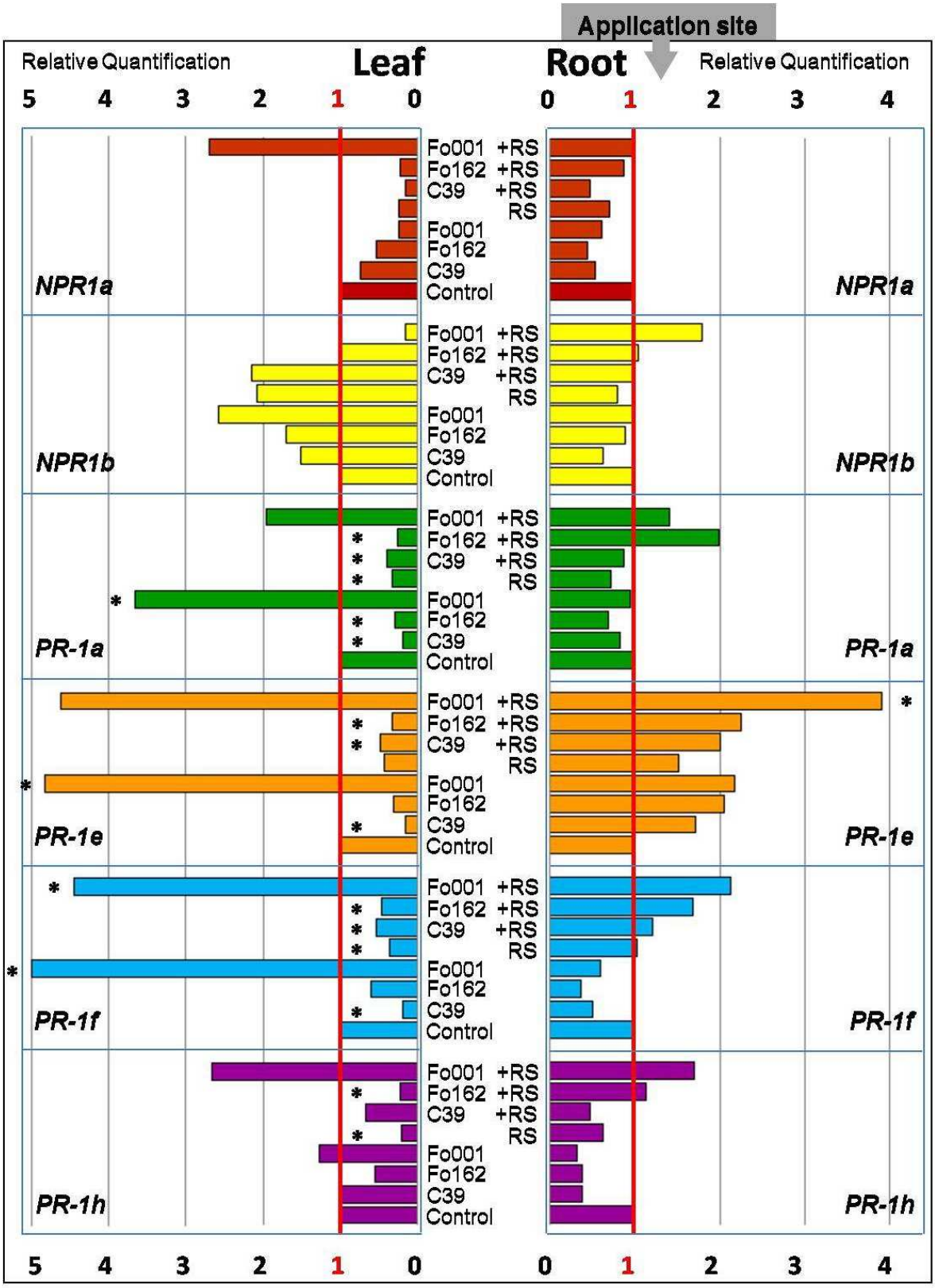


Figure 27: Relative Quantification (RQ) of the transcripts of two *NPR1* and four *PR-1* banana genes in leaf and root tissue after inoculation with the individual *Fusarium oxysporum* isolates (Fo001, Fo162 and C39) and in the absence or presence of the nematode *R. similis* (RS), related to the non-inoculated control (RQ=1). Error bars, representative for RQ min and max, are not displayed. Significant differences compared to the control are indicated by asterisk (*) ($P \leq 0.05$; n for leaves=5, n for roots=4).

The accumulation of *PR-1e*, *PR-1f* and *PR-1h* transcripts was similar to those of *PR-1a*. For *PR-1e* this resulted in an RQ increase to 4.61 with Fo001+*R. similis* and a significant increase (*P*≤0.05) to 4.82 with Fo001 alone. The presence of the isolates Fo162 and C39 resulted in significantly different (*P*≤0.05) RQ values of 0.31 and 0.16, respectively. When *R. similis* was present, RQ values were also significantly different (*P*≤0.05) for Fo162 and C39 (0.33 and 0.49), respectively. For *PR-1f*, a significant increase (*P*≤0.05) was observed upon challenge with Fo001+*R. similis* (RQ=4.44) and Fo001 alone (RQ=4.99). And *PR1-f* transcript accumulation significantly (*P*≤0.05) decreased in the presence of Fo162+*R. similis* (RQ=0.47), C39+*R. similis* (RQ=0.54) and C39 (RQ=0.20). The decrease in *PR-1f* transcript accumulation for Fo162 alone (RQ=0.61) was however not significant.

The changes in accumulation of *PR-1h* transcripts followed those of the other *PR-1* genes although the changes in RQ values were less pronounced. Both the increase upon challenge with Fo001+*R. similis* (RQ=2.67) and Fo001 alone (RQ=1.27) and the decrease upon challenge with C39+*R. similis* (RQ=0.67) and Fo162 alone (RQ=0.56) were statistically not significant. No response to C39 alone (RQ=1.00) was detected. Only, the reduced level of *PR-1h* transcripts in the presence of Fo162+*R. similis* (RQ=0.23) and *R. similis* (RQ=0.22) alone were significant different (*P*≤0.05).

In root tissue, the expression profiles of transcripts coding for *PR-1a* and *PR1-e* homologs were different from those found in leaf tissue and no significant changes were observed in *PR-1a* accumulation for all treatments. Only the presence of Fo001+*R. similis* showed a statistically significant (*P*≤0.05) alteration in the accumulation of *PR-1e* (RQ=3.91), corroborating with the findings in leaves. The accumulation had also increased in response to all other treatments with RQ values, ranging from 2.26 for Fo162+*R. similis* to 1.41 for C39 and *R. similis*. For Fo162+*R. similis*, C39+*R. similis*, Fo162 alone and C39 alone, these results were therefore reciprocal to those found for leaves. No significant differences were observed for the *PR-1f* transcript accumulation when compared to the control. Only slight to moderate increases were observed for the fungal treatments including *R. similis* with RQ values of 1.21, 1.69 and 2.13 for C39+*R. similis,* Fo162+*R. similis and* Fo001+*R. similis,*

respectively. A decrease was observed for the fungal treatments alone with RQ values of 0.61, 0.36 and 0.50 for Fo001, Fo162 and C39, respectively.

The transcript accumulation of *PR-1h* increased moderately in response to Fo001+*R. similis* (RQ=1.76) and marginally in response to Fo162+*R. similis* (RQ=1.13). For all the other treatment combinations the accumulation of *PR-1h* transcripts decreased (RQ for: C39+*R. similis,* Fo001, Fo162 and C39 with RQ values of 0.48, 0.32, 0.38 and 0.39. However, none of these differences were statistically significant.

6.5 Discussion

In this study, responses of banana gene expression after inoculation with different biotic elicitors were followed by real-time PCR analysis. As biotic elicitors the nematode *R. similis*, one pathogenic and two mutualistic *Fusarium* endophytes, of which one is effective in controlling *R. similis*, and combinations of the nematode and the individual fungal isolates were tested. The overall aim was to verify if and how the biotic elicitors induce changes in the expression of transcripts coding for the marker genes *NPR1* and *PR-1* that are commonly indicative for the systemic defense responses of ISR and SAR, respectively.

Similar to the different chemical elicitors (Chapter 5), *NPR1* and *PR-1* transcript accumulation was altered more pronouncedly in the leaf tissue; even though all biotic elicitors were located in the roots of the plants. The expression profile in leaf tissue was elicitor specific. The pathogen Fo001 induced a contrasting accumulation profile when compared to the individual mutualistic Fo162, the commensalistic C39 or the nematode elicitors. The accumulation of *NPR1* and *PR-1* transcripts in roots differed from those in leaves, being less elicitor specific and being not significantly different from the control, except for *PR-1e* in the presence of both Fo001 and *R. similis*. The limited sample size (leaf=5 and root=4) compared to the trial with the chemical elicitors in the previous chapter (n=7) may have contributed to the lack of statistical verification, which was especially weak for the analysis of the root samples.

6.5.1 Response to nematode treatment

Although the banana root system was infested by *R. similis*, no significant changes in accumulation of either the *PR-1* or the *NPR1* transcripts were observed in roots, whereas the leaf tissue responded with a significant ($P \leq 0.05$) decrease in the

accumulation of *PR-1a, PR-1f and PR-1h* transcripts. The lack of positive response in accumulation of *PR-1* and *NPR1* transcripts to the *R. similis* treatment in root tissue suggests that both, SA- and JA/ET- dependent defense pathways are not triggered by the burrowing nematode *R. similis*. This would imply that *R. similis* infestation of the roots is not inducing SAR or ISR.

In leaf tissue none of the *NPR1* transcript accumulations differed significantly, whereas *PR-1a, f* and *h* were significantly different ($P \leq 0.05$) and slightly down regulated. This decrease in the overall *PR-1* transcript accumulation only in the leaves is remarkable. The phenomenon of decreasing *PR-1* transcripts after *R. similis* infestation was also observed for roots of the banana cultivar Nabusa (genomic group AAA-EH; susceptible to *R. similis*) in earlier studies (Paparu et al., 2007) and resulted in a decrease of *PR-1* transcript accumulation in roots three days after inoculation. However, leaves were not analyzed in that study. Anyway the decrease in *PR-1* homolog expression is different from the increased *PR-1* accumulation of transcripts observed for leaf tissue in response to the BION induced SAR found in the previous chapter 5.4. The results suggest a slight repressive effect of *R. similis* infestation on the accumulation of *PR-1* transcripts that is opposite to the response observed during SAR (Chapter 5). Therefore the induction of SAR or ISR by *R. similis* must also be excluded for leaves.

6.5.2 Response to endophyte treatments alone and combined with *Radopholus similis*

NPR1 and *PR-1* transcript accumulation in leaves was highly responsive to the fungal treatments and their nematode treatment combinations and showed a clear pattern. In contrast, responses in roots were less pronounced and the accumulation pattern was different and less uniform compared to the pattern observed in leaves.

Elicitor specific accumulation of transcripts was particularly observed for the *PR-1* homologs in leaves whenever the pathogen Fo001 was included. This Fo001 specific increase in transcript accumulation of the *PR-1* homologs also indicates that the analyzed *PR-1* genes are not constitutively expressed in the banana leaf tissue. Although there seems to be a correlation between function and tissue-specific expression and the acidic or basic properties of PR-1 proteins in some cases (Eyal et

al., 1993; Tornero et al., 1997; Van Loon et al., 2006), a tissue-specific character of the analyzed *PR-1* homologs was not obvious in the present analysis.

The presence of the non-pathogenic endophytes Fo162 and C39 and *R. similis* led in most cases to a significant ($P \leq 0.05$) decrease in the accumulation of *PR-1* transcripts in leaves. SAR was characterized by increased expression of *PR-1* transcripts when using the chemical inducers (Chapter 5). Therefore it must be concluded that the non-pathogenic endophytes Fo162 and C39 did not induce SAR within the banana plants. The fact that the expression of *PR-1* homologs in response to the mutualistic Fo162 is almost identical to the expression induced by the commensalistic C39, reducing *R. similis* penetration insignificantly by only 20%, further implies that also the decrease in *PR-1* homolog expression is not related to reduced penetration rates of *R. similis* observed during the Fo162 elicted SIR. This is also supported by the inverse accumulation of all *PR-1* homologs that was observed in response to the mutualistic strain Fo162 or the pathogenic strain Fo001, both significantly reducing *R. similis* penetration rates. Therefore it is clear that there is no correlation between the expression of *PR-1* transcripts and reduced *R. similis* penetration rates.

On the other hand it is obvious that increased accumulation of all *PR-1* homologs showed a clear correlation with the presence of the pathogenic strain Fo001. Differences between the *PR-1* expression observed in response to both non-pathogenic endophytes and the pathogenic Fo001 may have their origin in the way these fungi colonize the banana plant. A study on the colonization pattern of non-pathogenic endophytes or pathogenic *Foc*s identified major differences in the way the banana root is colonized by these two types of endophytes. When the interactions of the two non-pathogenic endophytic isolates V2w2 and III4w1 with the East African Highland Banana cv. Nabusa were followed *in situ*, fungal growth was restricted to roots and rhizomes, with numerous hyphae infecting the hypodermis, fewer infecting the cortex and none at all infecting the vascular tissue (Paparu et al., 2006). In contrast to that the micro conidia of pathogenic *Foc*s are able to enter the xylem vessels of susceptible banana cultivars (such as Gros Michel), leading to reduced water transport and finally wilting of the plant.

Resistant banana cultivars initiate physical barriers that limit the pathogen’s growth

and finally prevent micro conidia formation in the xylem vessels (Beckman, 1990; Van den Berg et al., 2007; Van den Berg et al., 2009). Particularly, the expression of cell wall strengthening genes was shown to be involved in increased tolerance against Fusarium wilt and is well documented (Van den Berg et al., 2007; Van den Berg et al., 2009). Therefore, the increased accumulation of *PR-1* transcripts in response to race 1 pathogen Fo001 documents not only the onset of SAR, but it is also reasonable to assume that the increased expression of *PR-1* transcripts may be directly involved in the defense response of the race 1 resistant cultivar Valery. This hypothesis is supported by the findings of several other studies. Thus, a general antifungal activity of PR-1 proteins against several fungal pathogens is well documented for different plant species (Niderman et al., 1995) and increased levels of *PR-1* gene expression in *Musa* roots led to increased resistance against *F. oxysporum* f.sp. *cubense* (Endah et al., 2008; Van den Berg et al., 2007). In a recently published study, PR-1 proteins isolated from pumpkin rinds showed antifungal activity against *F. oxysporum* and a membrane permeabilizing activity of the protein was suggested (Park et al., 2009).

No significant differences in the accumulation of the *NPR1a* and *NPR1b* homologs in response to any treatment were observed in leaves or roots. However, the association of *NPR1* over-expression with resistance to a variety of bacterial and fungal pests is well documented (Chern et al., 2005; Chern et al., 2001; Malnoy et al., 2007; Yuan et al., 2007) and seemed to increase responsiveness to SA and SA-dependent signals leading to the induction of *PR-1* and finally SAR (Friedrich et al., 2001). The accumulation of *PR-1* transcripts in leaves was clearly increased in response to Fo001 and is most likely the result of a resistance mechanism. Nevertheless, significant differences in the expression of *NPR1a* or *NPR1b* homologs were not observed and therefore are in conflict with findings reporting a positive correlation of *NPR1* over expression and the induction of *PR-1* and finally SAR. The results of a recently published study (Spoel et al., 2009) give a more detailed and slightly different interpretation of the role of *NPR1* expression during SAR that may explain the missing correlation between *NPR1* and *PR-1* expression observed in response to Fo001. The final conclusion of this study was that it is not alone the presence of *NPR1* transcripts or NPR1 proteins, but rather NPR1 protein phosphorylation and its activity that is the determining factor for the extent of

transcription activity of *PR-1* coding mRNA. However, the results of this study presented here are not informative in terms of NPR1 phosphorylation or activity. Therefore, the low *NPR1a* or *NPR1b* transcript accumulation together with a significant increasing *PR-1* accumulation in response to the Fo001 treatments may not necessarily represent the actual correlation between NPR1 activity and *PR-1* transcripts.

In general, the present findings indicate a role of SAR in the defense response during the incompatible interaction between race 1 *Focs* and race 1 resistant banana cv. Valery. The inverse expression profiles of the mutualist Fo162 and the commensalist C39 in leaves compared to the pathogenic Fo001 give additional support for the non-pathogenic character of Fo162 and its safe use as a biological pest management tool to control *R. similis* infestation in banana production. A correlation between reduced nematode penetration rates and SAR or ISR could not be observed. Therefore it must be assumed that SIR follows an alternative and independent pathway of induced resistance in banana.

Alternatively to the initiation of systemic defense responses, mutualistic endophytes may systemically modify the banana root exudates, leading to interferences with the host finding behaviour of nematodes. This aspect will be investigated in the following chapter.

7 Profiling of banana root exudates: Shifts in root exudate composition after colonization with the mutualistic *Fusarium oxysporum* strain Fo162?

7.1 Introduction

Nematodes are among the most important pests in banana production, accounting for an annual worldwide yield loss of about 20%. The burrowing nematode *Radopholus similis* is one of the most destructive species, causing the so called Toppling disease (Gowen, 2000; Sasser and Freckman, 1986). Banana is a perennial crop and resistant varieties matching the requirements for the export market are not available. In commercial plantations nematodes are controlled by the application of nematicides, some of which are considered the most hazardous pesticides currently available. Their frequent application also made them increasingly prone to biodegradation by soil microorganisms (Karpouzas et al., 2004a; Karpouzas et al., 2004b; Patrick et al., 2001), rendering them ineffective in the long term. Alternative methods for controlling nematode infestations are therefore urgently needed. Nematode infestations can be repressed by non-pathogenic endophytic *F. oxysporum* strains, as was shown for tomato and the sedentary nematode *Meloidogyne incognita* in greenhouse studies (El-Fattah et al., 2007; Hallmann and Sikora, 1994), as well as for *R. similis* in banana under greenhouse and field conditions (Menjivar Barahona, 2005; Niere et al., 1999; Pocasangre, 2000; Vu et al., 2006; zum Felde 2008). Split root experiments with banana plants showed that these mutualistic *F. oxysporum* strains can systemically reduced nematode penetration rates (Vu, 2005). However, gene expression studies with transcripts coding for homologous sequences of the banana *PR-1* and *NPR1* genes did not show any correlation between reduced penetration rates of *R. similis* and the presence of the mutualistic endophyte Fo162 in the banana root (Chapter 6). Thus so far, the actual mode of action during SIR remains unresolved. It was hypothesized that the excretion of fungal toxins and their systemic transport may represent one possible mode of action, since *in vitro* tests with fungal culture filtrates caused paralysis and death of *R. similis* (Vu et al., 2006). Another hypothesis is that the plant responds to the presence of the fungus by producing factors that repel the nematode or at least prevent its attraction (Dababat, 2008). The latter hypothesis seems more likely than the systemic *in planta* transport of fungal toxins and their release at sufficient concentrations and suggests a disturbed host finding behaviour of the nematode.

In general the process of nematode attraction (i.e. towards a host plant) can be divided into two phases. The long distance attraction of nematodes, which is based on unspecific chemical signals (Pline and Dusenbery, 1987) and the short distance reception of the nematodes, which relies on chemo-reception (Prot, 1980) and is also influenced by plant chemicals when searching for a suitable penetration site on the root surface. Plants produce and excrete a wide array of low molecular weight signaling compounds (Rice, 1979). These allelochemicals can produce specific allelopathic toxins that have inhibitory effects towards other plant species (Hao et al., 2007; Stinson et al., 2006; Yu et al., 2000) and are also known to affect the behaviour of microorganisms, insects or vertebrates (Metlen et al., 2009). The release of specific compounds may also interfere with the host finding behaviour of nematode species like *R. similis* and *M. incognita.* Unfortunately, in most studies the allelochemicals in nematode antagonism could not be identified or isolated (Chitwood, 2002).

Nevertheless, root exudates released into the rhizosphere can either attract or repel nematodes, cause paralysis or even death and after invasion of the root the nematode is once more challenged with plant metabolites, which can affect its behaviour again (Wuyts et al., 2006). Among plant metabolites the phenylpropanoids have received much attention since these compounds are involved in plant defense responses and resistance to some pests and diseases (Nicholson and Hammerschmidt, 1992). The activity of enzymes associated with the phenylpropanoid biosynthesis pathways is increased after nematode infection (Baldridge et al., 1998; Edens et al., 1995) and an increase in accumulation of phenylpropanoids was also detected in nematode resistant cultivars, thus suggesting that these compounds are involved in plant defense response against some plant parasitic nematodes (Hung and Rohde, 1973; Plowright et al., 1996). In addition, *in vitro* test with several nematode species and various compounds of the phenylpropanoid pathway showed that dopamine was attractant for *R similis*, whereas caffeic acid was repellent and ferulic acid was even strongly motility inhibitive or toxic (Wuyts et al., 2006). Along with others, these three chemicals could be involved in affecting *R. similis* in the endophyte-banana-interaction. The identification of such compounds would strongly contribute to a better understanding

of the processes involved in the induced resistance caused by the mutualistic *F. oxysporum* endophytes

7.2 Scope

The aims of this study were to develop a system to continuously collect root exudates of Fo162 colonized and non-inoculated banana plants, to identify shifts in the banana root exudate composition caused by the mutualistic endophyte Fo162 and to evaluate the collected root exudates for their potential to alter the chemotactic response of *R. similis in vitro*.

7.3 Materials and Methods

7.3.1 Plant materials

Sterile tissue culture banana plantlets of the Cavendish (AAA) cultivar Valerie were obtained from Vitropic (Saint-Mathieu-de-Trévieres, Dijon/France). Two week old plantlets were propagated in a 50 ml mixture of sterilized sand and field soil (1:1; v/v) in a climatic chamber at 27°C and a diurnal cycle with 16 hours light. After three weeks, the soil was separated from the root system by carefully rinsing with sterilized tap water and the banana plants were transferred into 300 ml pots containing a mixture of sterilized sand and field soil (1:1; v/v). A diurnal cycle with 14 hours light was set and the temperature was maintained at approximately 27°C. Sterilized tap water was used for irrigation. After the development of the fourth or fifth leaf plants were used for the experiments.

7.3.2 Propagation of *Radopholus similis*

Pure nematode cultures of *Radopholus similis* (Cobb) Thorne were obtained from Dr. De Weale, Head of the Laboratory for Tropical Crop Improvement at the Catholic University of Leuven (KUL), Belgium. The nematodes originated from samples taken in Uganda and were maintained and propagated on sterile carrot disks (see chapter 6.3.3).

7.3.3 Inoculation of Fo162

The mutualistic *Fusarium oxysporum* strain Fo162 was used for inoculating the banana root system. The isolate originated from single spore colonies and was maintained as described in chapter 3.3. Cultivation and inoculation of Fo162 was performed as described in chapter 6.3.2 and 6.3.5 respectively.

7.3.4 Experimental setup

Two weeks after fungal inoculation, the soil was carefully separated from the banana root systems using sterile tap water. The plants were transferred into a 300 ml Schott-Duran flask supplied with 250 ml of sterile Murashige & Skoog nutrient solution (4.4 g/l; Sigma) and the pH was adjusted to 5.8. The nutrient solution was continuously circulated at a speed of 10 ml per hour using a peristaltic pump (Ecoline, Ismatec). Root exudates released into the hydroponic system were bound to XAD-4 columns over a period of seven days (Figure 28). The pH of the medium was constantly monitored and adjusted at need. The root system was aerated with

approximately 22 l of sterilized air per hour. Air was sterilized by filtering through a 0.2 µm membrane filter (NALGENE Nunc International Corporation, USA). Loss of nutrient solution due to evaporation and transpiration was compensated by adding sterile tap water. The hydroponic system was set up in a climatic chamber with 25℃ temperature and a diurnal cycle with 16 hours light.

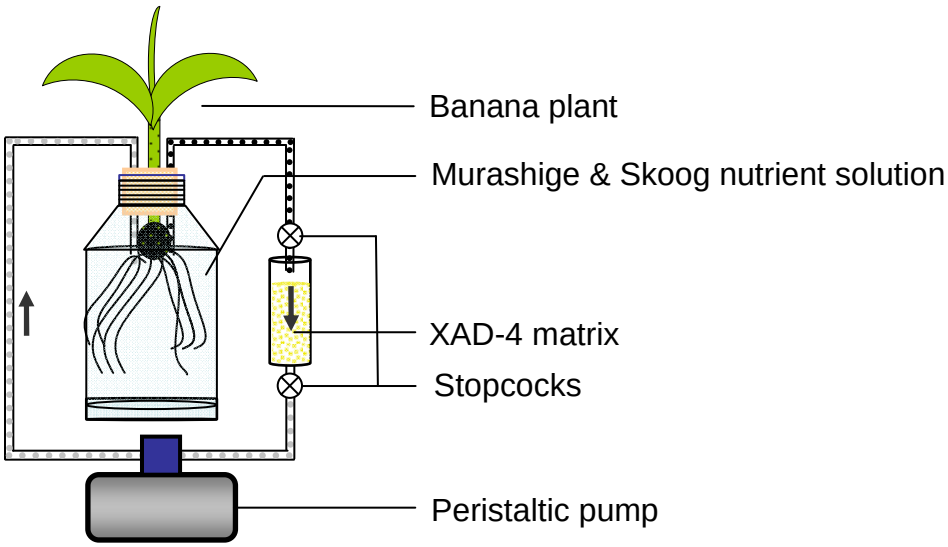


Figure 28: Hydroponic system used for cultivation of banana and the collection of root exudates.

7.3.5 XAD-4 matrix

The XAD-4 matrix (Sigma) was used to capture the released root exudates. XAD-4 is a polyaromatic adsorbent for small hydrophobic compounds, surfactants, phenols and chlorinated organics. Physical properties of XAD-4 are described in table 21. The released root exudates of each individual plant were collected on 15 g of XAD-4, which was packed onto an Econo-Column (Biorad).

Table 21: Physical specifications of XAD-4

Matrix	Styrene-divinylbenzen
Mesh size	20-60 mesh
Pore volume	~0.98 mL/g
Average pore diameter	40 Å (10^{-10} m)
Surface area	725 m²/g
Dry density	1.08 g/mL

7.3.6 Metabolite extraction from XAD-4

The XAD-4 matrix was collected and rinsed with 400 ml of sterile deionised water. The water was separated and the adsorbed components were eluted by adding 5 ml of methanol (Sigma, 99.9% spectral grade) and 12 hours of incubation time at room temperature in an orbital shaker set at 120 rpm. Root particles and other impurities were separated from the methanol phase by subsequent filtering through 45 µm and 20 µm membranes (NALGENE Nunc International Corporation, USA). Methanol was evaporated by vacuum and the pellet was resuspended in 300 µl methanol (Sigma, 99.9% spectral grade). Prior to Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) analysis, the suspension was centrifuged at 14.000×g for 5 min. 50 µl of the extract solution was subsampled for RP-HPLC analysis.

7.3.7 Caffeic acid, ferulic acid and dopamine references

In order to identify detection limits, retention times and spectra for caffeic acid, ferulic acid and dopamine (All Sigma). Pure compounds were dissolved in methanol (Sigma, 99.9% spectral grade) and 0.02, 0.2, 2, 20, 200 µg of each compound was analyzed by RP-HPLC.

7.3.8 Reverse-Phase High Performance Liquid Chromatography

RP-HPLC of the root exudate extracts was performed on a Hewlett Packard (HP) 1050 system, using a Merck LiChrospher100 C18 reversed phase column (250 by 4.0 mm, 5 µm), preceded by a LiChrospher C18 reversed phase guard column (4.0 by 4.0 mm, 5 µm). The HPLC device was controlled by ChemStation for LC 3D systems and consisted of a 1050 pump unit, a 1050 autosampler, a 1050 Diode Array Detector (DAD) and 1046A fluorescence array detector. The column was equilibrated by 90% solvent A (0.1% (v/v) trifluoroacetic acid (TFA) in water and 10% solvent B (Acetonitril). After injection, the samples were eluted at a flow rate of 1.0 ml per minute using an isocratic flow rate of 90% solvent A and 10% solvent B for two minutes, a linear gradient of 10% solvent A and 90% solvent B for 28 minutes and a final isocratic flow rate of 10% solvent A and 90% solvent B for five minutes. Before the injection of the next sample the column was re-equilibrated by a linear gradient of 90% solvent A and 10% solvent B for one minute, followed by an isocratic flow rate of 90% solvent A and 10% solvent B for four minutes. The injection volume for root exudate samples was 40 µl and 10 µl for the reference compounds. The samples were analyzed at wave lengths of 250, 270, 300, 330 and 360 nm. The UV excitation

wavelength was set to 200 nm. For compounds showing identical retention times the relative accumulation index [RAI] was calculated based on the average peak area, given as milli ampere units*time [mAU*s].

$$RAI_i = \frac{\text{Arithmetic mean (peak area Fo162)}_i}{\text{Arithmetic mean (peak area control)}_i} \quad \text{i=specific retention time}$$

7.3.9 *In vitro* bioassay

The effect of the collected root exudate extracts on *R. similis* behaviour was assessed in the following biotest (Figure 29). Petri dishes (Ø 90 mm) were filled with 10 ml of 1.0% (w/v) agar (Sigma) and 50 µl of the concentrated root exudates or solvent (methanol) were spread onto zone I or zone II in various combinations (Table 22), allowing for a 10 mm wide untreated area in the centre. Combinations were performed in triplicates.

Table 22: Experimental setup for the *in vitro* biotest system as depicted in figure 29. Fo162-Root exudate extracts of Fo162 treated plants A or B, Ree-Root exudate extracts of untreated control plants A, B or mix of A and B (1:1 v/v), Control-no application, methanol (99,9%)-solvent; n=3.

Combinations	1	2	3	4	5
Zone I	Fo162 A	Fo162 B	Control	Control	Methanol
Zone II	Ree A	Ree B	Ree A,B	Control	Methanol

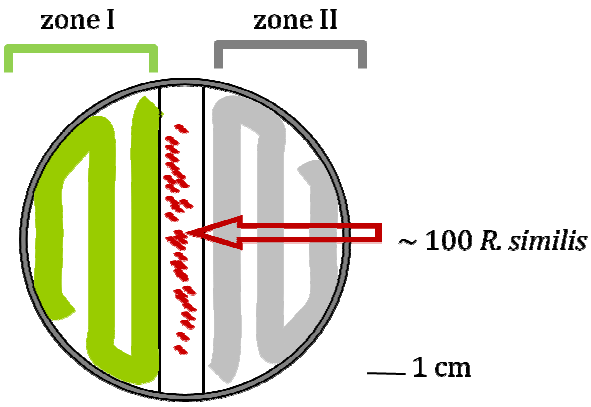


Figure 29: Biotest used to identify attracting or repellent activity of the root exudate extracts towards *R. similis*, n=3.

After evaporation of the solvents, approximately 100 individuals of *R. similis* were inoculated in a volume of 25 µl sterile tap water to the centre of the plate as illustrated in figure 29. After three hours, the number of *R. similis* per sector was recorded using a binocular.

7.3.10 Statistical analysis

Differences in the chromatographic data were assessed based on the signals at 250 nm wave length. Peaks with identical retention times were compared by analysing the peak area [mAU*s] of the two treatments using the non-parametric K-S test ($P \leq 0.05$) for independent samples (SPSS version 17.0). Differences between the individual treatments of the *in vitro* bioassay were analyzed using t-test ($P \leq 0.05$) and differences between groups were analyzed with ANOVA and the LSD test ($P \leq 0.05$ both) (SPSS version 17.0).

7.4 Results

7.4.1 pH-value of the nutrient solution

No significant shift in pH was observed during the experiment. The initial pH 5.8 shifted to approximately pH 6.0 when the XAD-4 was sampled after seven days.

7.4.2 Detection limit, retention time and spectral pattern of caffeic acid, ferulic acid and dopamine

For the detection of dopamine, caffeic acid and ferulic acid the signal strength [mAU] and peak area [mAU*s] were most intensive at 270 nm wavelength. The detection limit was 0.02 µg for both dopamine and caffeic and 0.2 µg for ferulic acid. The retention times of dopamine, caffeic acid and ferulic acid were 3.93, 9.97 and 12.58 min, respectively. The specific spectral pattern of each compound was determined (data not shown).

7.4.3 RP-HPLC-DAD analysis and profiling of extracts

When root exudate extracts were analyzed by RP-HPLC, the overall signal was most intensive at 250 nm wavelength. 26 major peaks could be identified for the chromatogram of each sample (Figure 30). According to the specific retention times of the chromatograms, the same set of peaks was identified for each individual sample.

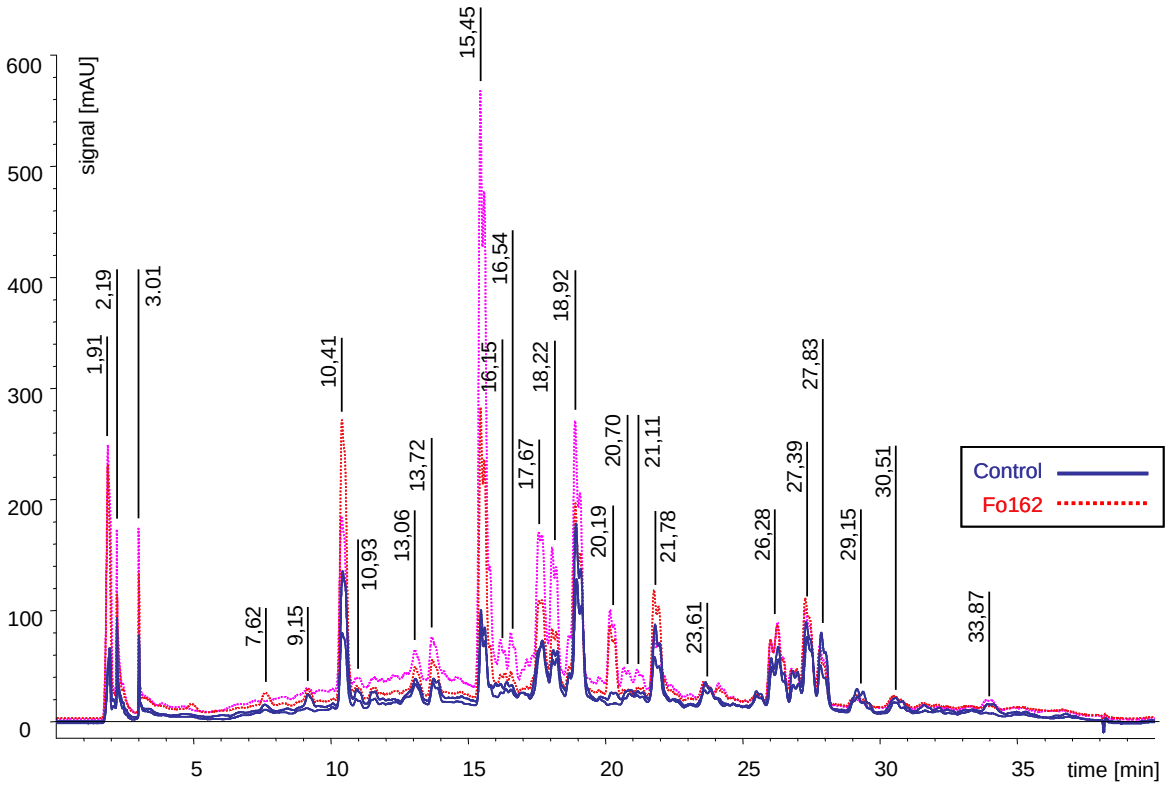


Figure 30: Chromatograms of the root exudate extracts of two control (constant lines) and two Fo162 (dotted lines) treated plants analyzed by RP-HPLC-DAD and signal wave length of 250 nm. Specific retention times [min] are annotated to the individual peaks.

The specific retention time and spectral pattern of caffeic acid, ferulic acid and dopamine was characterized initially. Nevertheless, none of the compounds was detected within the root exudate extracts of the non-inoculated control or the Fo162 treated plants.

The presence of the endophyte Fo162 in the banana root system did not result in the detection of additional peaks, but did affect the relative accumulation of several compounds that were also found in the non-inoculated control treatment.

The relative accumulation index RAI of the 26 compounds identified by RP-HPLC analysis ranged from 0.40 to 7.72 (Figure 31). Decreased RAI was observed for 12% of the detected peaks, 42% showed an increased accumulation ranging from one to two, 31% showed an increase between two and four and for 15% of the Fo162 inoculated samples RAI increased more than four fold.

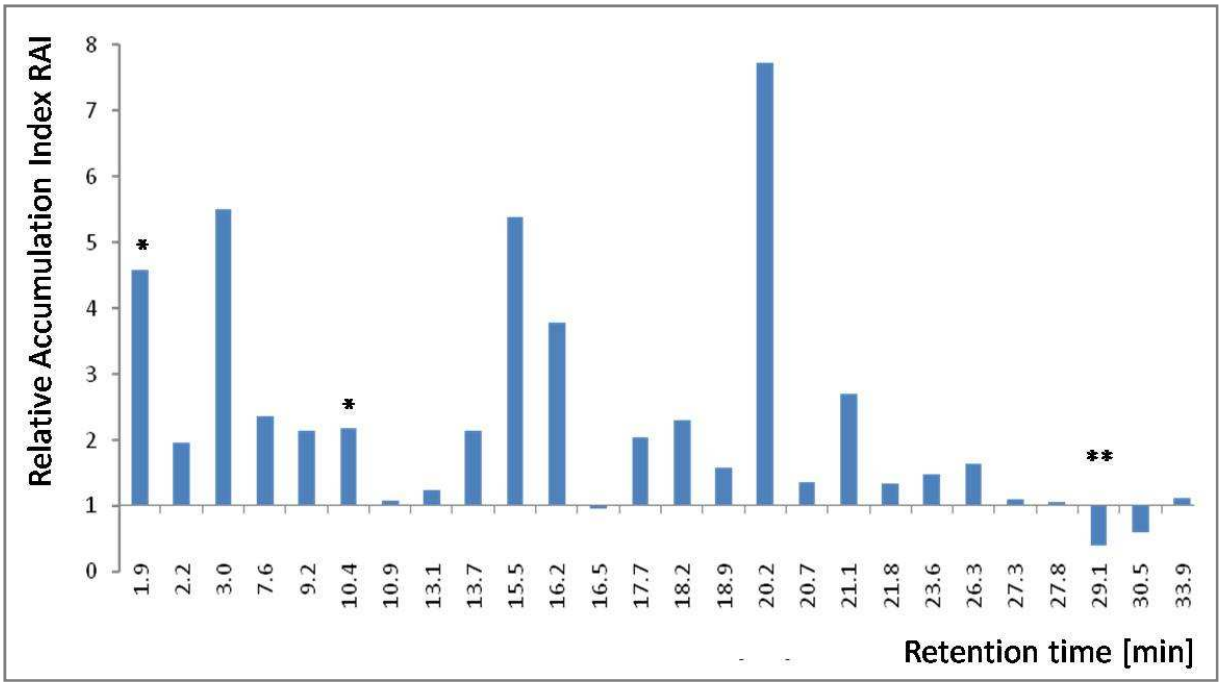


Figure 31: Relative accumulation indices of 26 peaks detected by RP-HPLC analysis (*P≤0.05; **P≤0.01; n=2).

The statistical analysis of the RAIs (Figure 31) resulted in significant differences for the peaks at 1.91 min (RAI=4.58; P=0.026), 10.41 min (RAI=2.18; P=0.046) and 29.15 min (RAI=0.40 and P=0.007) retention time.

7.4.4 *In vitro* biotest

In order to evaluate eventual attracting or repelling activity of the root exudate extracts towards *R. similis*, different combinations of root exudate extracts, solvent (methanol) and control (no application) were tested *in vitro* (Table 22 and Figure 29). For all combinations tested, on average 44% of the total applied nematodes moved to either the right or left within three hours. Maximum and minimum movement to a specific application site was observed in one of the replicates combining methanol (solvent) and Ree AB (1:1 v/v mix of root exudate extract A and B), when 36% of the applied *R. similis* were found at the Ree AB side of application compared to 7% found at the methanol side of application. This specific combination represented the combination with the strongest difference in nematode distribution in response to a specific treatment combination (Figure 32).

For the remaining treatment combinations, differences in nematode distribution as a response to the applied treatments were low (0.7%-2.2%). None of the tested

combinations gave significant differences in nematode distribution within (*t*-test) or between the groups (ANOVA).

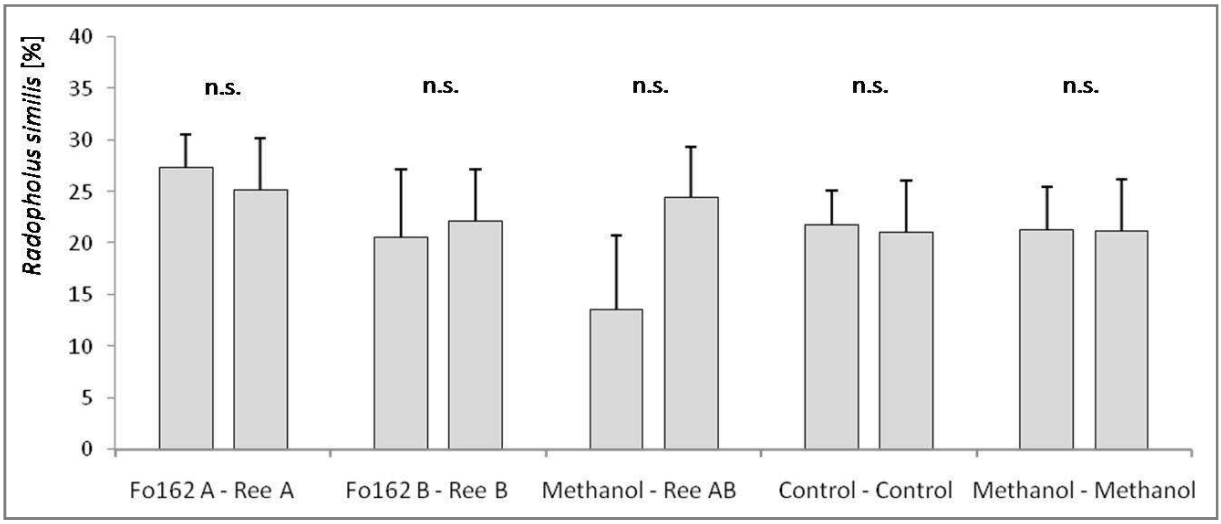


Figure 32: Final distribution of *Radopholus similis* over zone I (left) or zone II (right) in the *in vitro* biotest after three hours. Values displayed as % of the total number of inoculated nematodes. Fo162 A or B-root exudate extracts of Fo162 treated plant A or B, Ree A, B or AB-root exudate extracts of plant A, B or A and B (1:1 v/v mix), methanol-solvent, control–no application; ($P \leq 0.05$; n.s.=not significant; n=3).

7.5 Discussion

Split root experiments demonstrated that endophytic *F. oxysporum* species can cause a systemic response in the plant, resulting in reduced penetration or galling of migratory or sedentary plant parasitic nematodes in the roots of tomato and banana (El-Fattah et al., 2007; Vu et al., 2006). Choice experiments in soil showed that root exudates recovered from tomato were less attractive or more repellent towards the nematodes when roots were colonized by the mutualistic endophyte Fo162 (El-Fattah et al., 2007). This confirmed the hypothesis that the composition of root exudates is an important and determining factor with respect to the level of biological nematode control. It is currently unknown which specific root exudate factors are affected by the presence of such endophytes. Therefore the aim of the present study was to determine the chromatographic profiles of root exudates in presence and absence of the endophyte Fo162 in order to identify potential differences in their composition. A continuous trapping system similar to the one described by Hao et al. 2007 was used and the metabolites were adsorbed and concentrated on a XAD-4 matrix. This matrix is particularly suitable for the selective adsorption of hydrophobic or partially hydrophobic compounds, like plant phenolics (Hao et al., 2007). By

running this system over several days the plant metabolites were accumulated at detectable amounts. The chromatographic analysis resulted in a complex peak pattern, thus demonstrating the presence of 26 different compounds in the root exudates that were identical for all samples. The accumulation of most metabolites remained unaltered, although several metabolites did show an increased or decreased accumulation as a response to Fo162 colonization of the root system. The ambiguous accumulation of compounds indicates an active plant response to the presence of the mutualistic strain Fo162 and contradicts the accumulation of compounds as a result of general plant growth promotion that was reported elsewhere (Diedhiou et al., 2003). However, the presence of the endophyte did not result in the detection of additional compounds within the root exudates, suggesting that the fungus affects the accumulation of existing compounds, rather than the synthesis of new compounds. Fo162 may indirectly alter the synthesis of metabolites by influencing the metabolism of the plant or directly affect some of the root metabolites by mobilization due to fungal enzymes. *F. oxysporum* isolates for example, are able to degrade phenolic compounds like the antibiotic 2,4-DAPG (Schouten et al., 2004). More specifically, the endophyte Fo162 is able to degrade both ferulic acid (Schouten, 2009), caffeic acid, dopamine (Kurtz, unpublished) and salicylic acid (Schouten, unpublished). In the literature there are many reports of plant metabolites that are assumed to be involved in attracting or repelling nematodes. The activity of enzymes involved in the phenylpropanoid biosynthesis pathway increased upon nematode infection (Baldridge et al., 1998; Edens et al., 1995) or correlated with nematode resistance (Wuyts et al., 2006). Increased levels of phenylpropanoid compounds were also found in nematode resistant tomato, banana and rice cultivars (Hung and Rohde, 1973; Plowright et al., 1996; Valette et al., 1998) and in nematode resistant alfalfa plants the levels of isoflavonoids and medicarpins were increased (Baldridge et al., 1998; Edwards et al., 1995). Cell wall associated ferulic acid is assumed to be important in nematode resistance (Wuyts et al., 2007) and ferulic acid itself has strong repelling effects towards both nematodes *R. similis* and *M. incognita in vitro*. However, in other studies the major soluble compound found in resistant banana roots was dopamine, which exhibited nematode attracting activity *in vitro* (Wuyts et al., 2007). Dopamine, caffeic acid and ferulic acid were not identified by RP-HPLC analysis of the root exudate extracts. It must be assumed that the concentrations were either below the detection limit of 0.02 µg for

both dopamine and caffeic and 0.2 µg for ferulic acid or that the compounds were subsequently degraded in the *in vitro* assay or during the extraction procedure.

In the present research a biotest was used to determine the preference of *R. similis* for the extracted and concentrated root exudates. However, *R. similis* did no show significant preferences to any of the tested treatments in this biotest. In general, the nematodes were distributed evenly over the two zones infiltrated with exudates, irrespective of the applied combinations. This may indicate that the concentrated root exudates do not contain the compounds that are involved in the repellence or attraction of *R. similis* or that the concentrations of such compounds were too low. The physicochemical properties the XAD-4 matrix may not be suitable for extracting the desired allelochemicals. An alternative matrix (i.e. XAD-16) may solve this problem.

However, the negative results may also indicate that the bioassay used was not suitable to identify the chemotactic responses of *R. similis*. Assuming that the applied extracts did not contain any repellent or attractant compounds, it must be concluded that *R. similis* did not recognize any concentration gradient effective in directing its movement. In this case *R. similis* may behave similar to *Caenorhabditis elegans* when dispersing in a uniform concentration of attractant. Then the nematode density is highest at the starting point and decreases with increasing distance from the initial application site (Pierce-Shimomura et al., 1999). The dispersal behavior observed for *R. similis* in the present study is consistent with the behavior of other nematode species in similar experimental setups (Croll, 1975; Croll and Blair, 1973).

The dispersal of *C. elegans* in a spatially uniform attractant is well described as a ‘random walk’ (Pierce-Shimomura et al., 1999). When gradients are approached, periods of smooth movement are followed by periods of frequent turning, also referred to as bouts of turning or pirouettes. Changes in direction become less frequent with increasing concentration. As a result the nematode makes extended movements in the ‘right’ direction and brief movements in the ‘wrong’ direction, always reaching the peak concentration of the attractant. Chemotaxis is then the result of a series of pirouettes that always redirect the nematode towards the highest concentration of an attractant. This mechanism of chemotaxis is defined as the

'pirouette model' (Pierce-Shimomura et al., 1999). Whether or not the pirouette model works in a reciprocal way when a repelling compound is encountered, is not known.

In the present study tracks of *R. similis* movement were not recorded and the fact that *R. similis* was confronted with two zones, each lacking a gradient, does not support nematode movement according to the 'pirouette model' of chemotaxis, but rather would suggest a random walk as described for uniform concentrations of attractants. The even distribution of *R. similis* over two zones was irrespective of the tested combinations and consequently supports the theory of the 'random walk', as a result of missing gradients. Alternatively, it may also be possible that *R. similis* is either confused by two gradients of very similar concentrations or *R. similis* is generally unable to decide between such gradients.

The experiment showed that the mutualistic endophyte Fo162 significantly modified the proliferation of some compounds excreted by the banana root. Nevertheless, attractant or repellent activity of the tested root exudate extracts was not observed in the *in vitro* biotest. Although relatively simple, the experimental setup used here may be inadequate. A more complex assay in plates (Hewlett et al., 1997; Wuyts et al., 2006) or soil (El-Fattah et al., 2007) may be necessary to demonstrate repellency or lack of attraction to exudates from roots colonized by the mutualist Fo162. However, the establishment of gradients for the tested chemicals in a biotest system is an essential prerequisite, in case the 'pirouette model' was the underlying mechanism for the chemotatic response of *R. similis*.

8 General conclusions

The study on the phylogeny of endophytic *Fusarium oxysporum* strains and their role in induced defense responses in banana plants against *Radopholus similis* led to several major conclusions.

1) DNA sequence analysis of the IGS region is the most informative, reliable and reproducible method to infer the phylogeny of the pathogenic and non-pathogenic *Fusarium oxysporum* and related *Fusarium* species.

2) The phylogenetic analysis of non-pathogenic and pathogenic *F. oxysporum* endophytes showed several trends.

i) The geographic origin of isolates can have a strong influence, which accounted for the distinct separation of the isolates from the area of the East African Highlands in clade B.

ii) There is a strong possibility that this distinct separation resulted from a coevolution with the host plant, the East African Highland Banana.

iii) The mutualistic trait of those endophytes that confer the enhanced resistance against *Radopholus similis* in banana is not monophyletic.

iv) Pathogenicity was polyphyletic from a global point of view. In individual regions, such as the East African Highlands, and within a local population pathogenicity seems to evolve as a monophyletic trait.

v) Mutualistic and commensalistic *Fusarium oxysporum* strains in clade B were generally found to be phylogenetically distinct from the pathogenic *Foc*s of the same geographical area. Therefore, it is very unlikely that mutualistic *F. oxysporum* strains will convert to pathogenic forms.

3) During the study on induced systemic responses, two *NPR1* and four *PR-1* homologs were identified and characterized in response to chemical inducers.

i) Foliar applications of BION, SA and INA can induce SAR in the banana cultivar ‘Valery’.

ii) The induction of SAR by foliar application is systemic, extends into the roots and suggests that the response of some *PR-1* transcripts may be tissue specific.

iii) The accumulation of *PR-1* transcripts in banana may to some extent also be induced through the JA-regulated pathway of plant defense responses.

4) Several conclusions were drawn from the expression study of *NPR1* and *PR-1* homologs in response to *Fusarium* spp. and *Radopholus similis.*

i) By interpreting the expression of *NPR1* and *PR-1* homologs, it seems very unlikely that *R. similis* infestation of the roots induces SAR or ISR.

ii) There is no clear correlation between the *NPR1* transcript accumulation in response to the root colonization by the mutualistic Fo162 and the control of *R. similis*. ISR is most likely not involved in the control of *R. similis* during SIR.

iii) Reduced penetration rates of *R. similis* during SIR are not correlated to the accumulation of *PR-1* transcripts in response to Fo001 or Fo162. Therefore it is concluded that SAR is not causal for the reduced nematode penetration rates.

iv) It seems very likely that SAR and *PR-1* are involved in the defense response of the race 1 resistant cultivar ‘Valery’ against the race 1 pathogen Fo001.

v) The results strongly support the non-pathogenic character of the mutualistic strain Fo162.

5) The profiling of banana root exudates and their test *in vitro* led to the following conclusions.

i) Fo162 has a significant influence on some of the accumulated compounds, but did not lead to the detection of additional or lack of compounds.

ii) The detected changes in the root exudate composition did not cause repellency or lack of attraction of *R. similis* under these specific experimental conditions.

Overall, the findings of the phylogenetic as well as the gene expression studies support the safe use of the endophytic Fo162 as *R. similis* management tool in banana production. The results also documented the role of SAR in the defense response of the resistant banana cultivar Valery against the Fusarium wilt pathogen Fo001 and not against *R. similis* during SIR. Therefore, it is concluded that SIR follows an alternative pathway of induced systemic resistance that is different from SAR.

9 Outlook

The mutualistic trait that confers the enhanced nematode resistance to banana is not monophyletic. Nevertheless sequence analysis could show a clear separation of pathogenic and non-pathogenic *F. oxysporum* and will, along with VCG tests, serve as a fast and powerful tool in characterizing new *F. oxysporum* isolates in future. Even though the efficacy of several mutualistic *F. oxysporum* strains and its systemic mode of action are well documented for different plant and nematode species the expression studies with *NPR1* and *PR-1* could not identify a mechanism that is involved in the enhanced resistance of banana towards *R. similis.* Nevertheless, such mechanism may still be detectable by monitoring alternative *PRs* or defense related proteins of banana (i.e. *PR*-5). In addition, it must be considered that SAR or ISR may just be two possible outcomes of a whole array of defense responses available to the plant in order to cope with nematodes or other detrimental organisms dwelling the pathozone surrounding their roots. Finally, root exudate profiling still may reveal fungal compounds with direct antagonism against *R. similis,* when alternative matrices are used (i.e XAD-16) that adsorb different types of root exudate metabolites excreted by Fo162 treated plant roots.

‘Biological Crop Enhancement’ by mutualistic endophytes is one option for nematode IPM in banana production and future approaches for efficient nematode control my also include intensification of breeding programs, that aim at enhanced nematode tolerance like reported for the recently generated Yagambi km 5 banana cultivar that reduces the rate of *R. similis* reproduction. The clonal propagation of banana by tissue culture plantlets makes the banana an interesting candidate for genetic engineering. Once a gene conferring nematode resistance is integrated into banana plant, the specific cultivar can be easily propagated by clones without losing desired properties that are required for the export market.

However, training of farmersand extension services, nematode monitoring as well as the integrated use of all available tools for nematode control will be needed to minimize yield losses to nematode pests in the future, not alone in banana production.

10 Literature

Abad, L.R., DUrzo, M.P., Liu, D., Narasimhan, M.L., Reuveni, M., Zhu, J.K., Niu, X.M., Singh, N.K., Hasegawa, P.M. and Bressan, R.A. (1996) Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. *Plant Science*, **118**, 11-23.

Abo, K., Klein, K.K., Edel-Hermann, V., Gautheron, N., Traore, D. and Steinberg, C. (2005) High genetic diversity among strains of Fusarium oxysporum f. sp vasinfectum from cotton in Ivory Coast. *Phytopathology*, **95**, 1391-1396.

Agrawal, G.K., Rakwal, R. and Jwa, N.S. (2000) Rice (Oryza sativa L.) OsPR1b gene is phytohormonally regulated in close interaction with light signals. *Biochemical and Biophysical Research Communications*, **278**, 290-298.

Ahl, P., Cornu, A. and Gianinazzi, S. (1982) Soluble proteins as genetic markers in studies of resistance and phylogeny in Nicotiana. *Phytopathology*, **72**, 80-85.

Amin, N. (1994) Untersuchung über die Bedeutung endophytischer Pilze für die biologosche Bekämpfung des wanderenden Endoparasiten Radopholus similis (Cobb) Thorne an Bananen. Ph.D. Thesis, university of Bonn, Germany.

Amrani, L. and Corio-Costet, M.F. (2006) A single nucleotide polymorphism in the beta-tubulin gene distinguishing two genotypes of Erysiphe necator expressing different symptoms on grapevine. *Plant Pathology*, **55**, 505-512.

Anderson, J.B. and Stasovski, E. (1992) Molecular phylogeny of Northern hemisphere Armillaria species. *Mycologia*, **84**, 505-516.

Assigbetse, K.B., Fernandez, D., Dubois, M.P. and Geiger, J.P. (1994) Differentiation of Fusarium-Oxysporum F Sp Vasinfectum Races on Cotton by Random Amplified Polymorphic DNA (Rapd) Analysis. *Phytopathology*, **84**, 622-626.

Baldridge, G.D., O'Neill, N.R. and Samac, D.A. (1998) Alfalfa (Medicago sativa L.) resistance to the root-lesion nematode, Pratylenchus penetrans: defense-response gene mRNA and isoflavonoid phytoalexin levels in roots. *Plant Molecular Biology*, **38**, 999-1010

Bayman, P. and Cotty, P.J. (1993) Genetic Diversity in Aspergillus-Flavus - Association with Aflatoxin Production and Morphology. *Canadian Journal of Botany-Revue Canadienne De Botanique*, **71**, 23-31.

Beckman, C.H. (1990) Host responses to the pathogen. Pages 93-105 in: Fusarium Wilt of Banana. R. C. Ploetz, ed. APS Press, American Phytopathological Society, St. Paul.

Belesky, D.P., Devine, O.J., Pallas, J.E. and Stringer, W.C. (1987) Photosynthetic Activity of Tall Fescue as Influenced by a Fungal Endophyte. *Photosynthetica*, **21**, 82-87.

Bentley, S. and Bassam, B. (1996) A robust DNA amplification fingerprinting system applied to analysis of genetic variation within Fusarium oxysporum f.sp. cubense. . *J. Phytopathol*, **144**, 207-213.

Bentley, S., Pegg, K.G. and Dale, J.L. (1995) Genetic-Variation among a World-Wide Collection of Isolates of Fusarium oxysporum f. sp cubense Analyzed by Rapd-Pcr Fingerprinting. *Mycological Research*, **99**, 1378-1384.

Bentley, S., Pegg, K.G., Moore, N.Y., Davis, R.D. and Buddenhagen, I.W. (1998) Genetic Variation Among Vegetative Compatibility Groups of Fusarium oxysporum f. sp. cubense Analyzed by DNA Fingerprinting. *Phytopathology*, **88**, 1283-1293.

Bergstrom, G.C., Kalb, D.W., Tillapaugh, B.P. and Lucey, R.F. (1995) Fusarium wilt of birdsfood trefoil: biology and prospects for management. In Proceedings of the 11th Eastern Forage Improvement Conference. Ottawa, Ontario, Canada. pp. 43-44.

Bevan, I.S., Rapley, R. and Walker, M.R. (1992) Sequencing of PCR-amplified DNA. *PCR Methods Appl*, **1**, 222-228.

Bioversity-International. http://bananas.Bioversityinternational.org/en/why-bananas-matter-mainmenu-77.html.

Booth, C. (1971) The Genus Fusarium. Commonwealth Mycological Institute, Kew, Surry, United Kingdom.

Broadley, R. (1979) Non-volatile nematicides for control of burrowing nematode in banana plantations in north Queensland. *Austrailan Journal of Experimental Agriculture and Animal Husbandry*, **19**, 626-630.

Brookes, A.J. (1999) The essence of SNPs. *Gene*, **234**, 177-186.

Brumfield, R.T., Beerli, P., Nickerson, D.A. and Edwards, S.V. (2003) The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology & Evolution*, **18**, 249-256.

Buddenhagen, I.W. (1960) Strains of Pseudomonas Solanacearum in Indigenous Hosts in Banana Plantations of Costa Rica, and Their Relationship to Bacterial Wilt of Bananas. *Phytopathology*, **50**, 660-664.

Buxton, E.W. (1968) Parasexual recombination in the banana-wilt Fusarium *Trans. Br. Moycol. Soc.*, **45**, 133-139.

Carbone, I. and Kohn, L.M. (1993) Ribosomal DNA-Sequence Divergence within Internal Transcribed Spacer-1 of the Sclerotiniaceae. *Mycologia*, **85**, 415-427.

Carrol, G.C. (1986) The role of endophytism in plants with particular refernce to woody perennials. In: Fokema N.J., Van Den HeuvelJ., eds. Microbiology of the phyllosphere. Cambridge, UK: Cambridge University Press, 205-222.

Chapin, F.I., Zavaleta, E., Eviner, V., Naylor, R., Vitousek, P., Reynolds, H., Hooper, D., Lavorel, S., Sala, O., Hobbie, S., Mack, M. and Diaz, S. (2000) Consequences of changing biodiversity. *Nature*, **405**, 234-242.

Cheong, J.J. and Choi, Y.D. (2003) Methyl jasmonate as a vital substance in plants. *Trends in Genetics*, **19**, 409-413.

Chern, M., Fitzgerald, H., Navarre, D., Canlas, P. and Ronald, P. (2005) Overexpression of a Rice NPR1 Homolog Leads to Constitutive Activation of Defense Response and Hypersensitivity to Light. Molecular Plant-Microbe Interaction, **18**, 511-520.

Chern, M., Fitzgerald, H., Yadav, R., Canlas, P., Dong, X. and Ronald, P. (2001) Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J. **27**, 101-113.

Chitwood, D.J. (2002) Phytochemical based strategies for nematode control. *Annual Review of Phytopathology*, **40**, 221-+.

Clay, K. (1991) Endophytes as antagonists of plant pests. Pages 331-357 in: Andrews, J.H. and Hirano, S.S. (eds.). Microbial ecology of leaves. Springer-Verlag, New York, USA.

Clay, K. and Leuchtmann, A. (1989) Infection of Woodland Grasses by Fungal Endophytes. *Mycologia*, **81**, 805-811.

Clement, S.L., Kaiser, W.J. and Eichenseer, H. (1994) Acremonium endophytes in germplast of major grasses and their utilization for insect resistance. Pages 185-199 in: Bacon, C.W. and White, J.F. Jr. (eds.). Biotechnology of endophytic fungi in grasses. CRC Press, Inc.,Boca Raton, Florida, USA.

Collins, F.S., Brooks, L.D. and Chakravarti, A. (1998) A DNA polymorphism discovery resource for research on human genetic variation. *Genome Res*, **8**, 1229-1231.

Correll, J.C. (1991) The Relationship between Formae Speciales, Races, and Vegetative Compatibility Groups in Fusarium-Oxysporum. *Phytopathology*, **81**, 1061-1064.

Croll, N.A. (1975) Behavioural analysis of nematode movement. *Adv Parasitol*, **13**, 71-122.

Croll, N.A. and Blair, A. (1973) Inherent Movement Patterns of Larval Nematodes, with a Stochastic-Model to Simulate Movement of Infective Hookworm Larvae. *Parasitology*, **67**, 53-66.

Crowhurst, R.N., Hawthorne, B.T., Rikkerink, E.H.A. and Templeton, M.D. (1991) Differentiation of Fusarium-Solani F-Sp Cucurbitae Race-1 and Race-2 by Random Amplification of Polymorphic DNA. *Current Genetics*, **20**, 391-396.

Dababat, A. (2008) Importance of the mutualistic endophyte Fusarium oxysporum 162 for enhancement of tomato transplants and the biological control of the root-knot nematode Meloidogyne incognita, with particular reference to mode-of-action. PhD Thesis, INRES University of Bonn.

Dababat, A.E.F.A. and Sikora, R.A. (2007) Induced resistance by the mutualistic endophyte, Fusarium oxysporum strain 162, toward Meloidogyne incognita on tomato. *Biocontrol Science and Technology*, **17**, 969-975.

Dangl, J.L. and Jones, J.D.G. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826-833.

Daniells, J., Jenny, C., Karamura, D. and Tomekpe, K. (2001) Musalogue: Diversity in the genus Musa CIRAD: A catalogue of Musa germplasm.

Davies, G. (1995) Banana and plantain in the East African highlands. Pages 493-508 in: Gowen, S.R. (ed). Banana and plantains. Chapman and Hall, London, UK.

De Langhe, E. (1996) Focus Paper I - Banana and Plantain: the Earliest Fruit Crop? Pp. 6-8 in Networking Banana and Plantain: INIBAP Annual Report 1995. INIBAP, Montpellier, France.

De Waele, D. and Speijer, P.R. (1999) Nematode resistance in Musa. In: Frison E.A., Gold C.S.,Karamura E.B. and Sikora R.A. (Eds). Mobilizing IPM for sustainable banana production in Africa. INIBAP, Montpellier France. pp:119-126.

Denham, T.P., Haberle, S.G., Lentfer, C., Fullagar, R., Field, J., Therin, M., Porch, N. and Winsborough, B. (2003) Origins of agriculture at Kuk Swamp in the Highlands of New Guinea. *Science*, **301**, 189-193.

Dice, L.R. (1945) Measures of the amount of ecologic association between species. . *Ecology* **26**, 297-302.

Diedhiou, P.M., Hallmann, J., Oerke, E.C. and Dehne, H.W. (2003) Effects of arbuscular mycorrhizal fungi and a non-pathogenic Fusarium oxysporum on Meloidogyne incognita infestation of tomato. *Mycorrhiza*, **13**, 199-204.

Dissanayake, M.L.M.C., Kashima, R., Tanaka, S. and Ito, S. (2009) Genetic diversity and pathogenicity of Fusarium oxysporum isolated from wilted Welsh onion in Japan. *Journal of General Plant Pathology*, **75**, 125-130.

Duncan, S., Barton, J.E. and Obrien, P.A. (1993) Analysis of Variation in Isolates of Rhizoctonia-Solani by Random Amplified Polymorphic DNA Assay. *Mycological Research*, **97**, 1075-1082.

Durrant, W.E. and Dong, X. (2004) Systemic acquired resistance. *Annu Rev Phytopathol*, **42**, 185-209.

Edel, V. (1998) Use of PCR and RFLP in fungal systematics. *In Jens C. Frisvad, Paul D. Bridge and Dilip K. Arora (Eds): Chemical Fungal Taxonomy*, 52-76.

Edel, V., Steinberg, C., Avelange, I., Laguerre, G. and Alabouvette, C. (1995) Comparison of 3 Molecular Methods for the Characterization of Fusarium-Oxysporum Strains. *Phytopathology*, **85**, 579-585.

Edel, V., Steinberg, C., Gautheron, N. and Alabouvette, C. (1997) Evaluation of restriction analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA for the identification of Fusarium species. *Mycological Research*, **101**, 179-187.

Edens, R.M., Anand, S.C. and Bolla, R.I. (1995) Enzymes of the Phenylpropanoid Pathway in Soybean Infected with Meloidogyne-Incognita or Heterodera-Glycines. *Journal of Nematology*, **27**, 292-303.

Edrevea, A. (2005) pathogenesis-related proteins: research porgress in the last 15 years. *Gen. Appl. Plant Physiology*, **31**, 10-124.

Edwards, R., Mizen, T. and Cook, R. (1995) Isoflavonoid Conjugate Accumulation in the Roots of Lucerne (Medicago-Sativa) Seedlings Following Infection by the Stem Nematode (Ditylenchus-Dipsaci). *Nematologica*, **41**, 51-66.

El-Fattah, A., Dababat, A. and Sikora, R.A. (2007) Influence of the mutualistic endophyte Fusarium oxysporum 162 on Meloidogyne incognita attraction and invasion. *Nematology*, **9**, 771-776.

Endah, R., Beyene, G., Kiggundu, A., van den Berg, N., Schluter, U., Kunert, K. and Chikwamba, R. (2008) Elicitor and Fusarium-induced expression of NPR1-like genes in banana. *Plant Physiology and Biochemistry*, **46**, 1007-1014.

Enya, J., Togawa, M., Takeuchi, T., Yoshida, S., Tsushima, S., Arie, T. and Sakai, T. (2008) Biological and phylogenetic characterization of Fusarium oxysporum complex, which causes yellows on Brassica spp., and proposal of F. oxysporum f. sp rapae, a novel forma specialis pathogenic on B-rapa in Japan. *Phytopathology*, **98**, 475-483.

EPPOa. European Plant Protection Organization: http://www.eppo.org/QUARANTINE/listA2.htm.

EPPOb. Description of quarantine pests.

Eyal, Y., Meller, Y., Levyadun, S. and Fluhr, R. (1993) A Basic-Type Pr-1 Promoter Directs Ethylene Responsiveness, Vascular and Abscission Zone-Specific Expression. *Plant Journal*, **4**, 225-234.

FAO, F.a.A.O.o.t.U.N. (2004) The State of Food and Agriculture 2003-04, www.fao.org/documents/schow_cdr.asp?url_file=/docrep/006/Y5160E00.HTML.

FAO, F.a.A.O.o.t.U.N. (2008) FAO Report of Crop Prospects and Food Situation.

FAOSTAT. (2008) http://www.google.de/search?q=FAOSTAT&ie=utf-8&oe=utf-8&aq=t&rls=org.mozilla:de:official&client=firefox-a.

Farris, J.S., Kallersjo, M., Kluge, A.G. and Bult, C. (1994) Testing Significance of Incongruence. *Cladistics-the International Journal of the Willi Hennig Society*, **10**, 315-319.

Fisher, S.G. and Lerman, L.S. (1983) DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proclamation of National Academic Science USA*, **80**, 1579-1583.

Fourie, G., Steenkamp, E.T., Gordon, T.R. and Viljoen, A. (2009) Evolutionary relationships among the Fusarium oxysporum f. sp. cubense vegetative compatibility groups. *Appl Environ Microbiol*, **75**, 4770-4781.

Freeman, S. and Rodriguez, R.J. (1993) Genetic Conversion of a Fungal Plant Pathogen to a Nonpathogenic, Endophytic Mutualist. *Science*, **260**, 75-78.

Frey, P., Smith, J.J., Albar, L., Prior, P., Saddler, G.S., TrigaletDemery, D. and Trigalet, A. (1996) Bacteriocin typing of Burkholderia (Pseudomonas) solanacearum race 1 of the French West Indies and correlation with genomic variation of the pathogen. *Applied and Environmental Microbiology*, **62**, 473-479.

Friedrich, L., Lawton, K., Dietrich, R., Willits, M., Cade, R. and Ryals, J. (2001) NIM1 overexpression in arabidopsis potentiates plant disease resistance and results in enhanced effectiveness of fungicides. *Molecular Plant-Microbe Interactions*, **14**, 1114-1124.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M.G., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J.P., Kessmann, H. and Ryals, J. (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant Journal*, **10**, 61-70.

Gessler, C. and Kuc, J. (1982) Induction of Resistance to Fusarium-Wilt in Cucumber by Root and Foliar Pathogens. *Phytopathology*, **72**, 1439-1441.

Gianinazzi, S., Martin, C. and Vallee, J.C. (1970) [Hypersensitivity to viruses, temperature and soluble proteins in Nicotiana Xanthi n.c. Appearance of new macromolecules at the repression of viral synthesis]. *C R Acad Sci Hebd Seances Acad Sci D*, **270**, 2383-2386.

Gold, C.S., Karamura, E.B., Kiggundu, A., Bagamba, F. and Abera, A.M.K. (1999) Monograph on geographical shifts in highland cooking banana (*Musa*, group AAA-EH) production in Uganda. *Africa Crop Science Journal*, **7**, 223-298.

Gordon, T.R. and Martyn, R.D. (1997) The evolutionary biology of Fusarium oxysporum. *Annual Review of Phytopathology*, **35**, 111-128.

Gordon, T.R., Okamoto, D. and Jacobson, D.J. (1989) Colonization of Muskmelon and Nonsusceptible Crops by Fusarium-Oxysporum F Sp Melonis and Other Species of Fusarium. *Phytopathology*, **79**, 1095-1100.

Gowen, S., Quénéhervé, P. & Fogain, R. (2005) Nematode parasites of bananas and plantains.In: Luc, M., Sikora,R.A.&Bridge, J. (Eds). Plant parasitic nematodes in tropical and subtropical agriculture. , 611-643.

Gowen, S.R. (1994) Banana diseases caused by nematodes. Pp. 431- 460 in R. C. Ploetz, G. A. Zentmyer, W. T. Nischijima, K. R. Rohrbacher and H. D. Ohr (eds.) Compendium of Tropical fuit diseases. CAB International, Surry, England.

Gowen, S.R. (2000) Root lesion nematodes-Nematode pathogens. In: Jones D. R. (Eds). Diseases of Banana, Abacá and Ensete. CABI publishing, 303-307.

Gowen, S.R. and Quénéhervé, P. (1990) Nematode parasites of bananas, plantains and abacá. Pp. 431-460. In: Luc M., Sikora R.A. & Bridge J. (eds). Plant parasitic Nematodes in Tropical and Subtropical Agriculture. Wallingford, UK: CAB International.

Grajalmartin, M.J., Simon, C.J. and Muehlbauer, F.J. (1993) Use of Random Amplified Polymorphic DNA (Rapd) to Characterize Race 2 of Fusarium-Oxysporum F Sp Pisi. *Phytopathology*, **83**, 612-614.

Groenewald, S., Van Den Berg, N., Marasas, W.F. and Viljoen, A. (2006) The application of high-throughput AFLP's in assessing genetic diversity in Fusarium oxysporum f. sp. cubense. *Mycol Res*, **110**, 297-305.

Guthrie, P.A.I., Magill, C.W., Frederiksen, R.A. and Odvody, G.N. (1992) Random Amplified Polymorphic DNA Markers - a System for Identifying and

Differentiating Isolates of Colletotrichum-Graminicola. *Phytopathology*, **82**, 832-835.

Hadrys, H., Balick, M. and Schierwater, B. (1992) Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol Ecol*, **1**, 55-63.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95-98.

Hallmann, J. and Sikora, R.A. (1994) Influence of Fusarium-Oxysporum, a Mutualistic Fungal Endophyte, on Meloidogyne-Incognita Infection of Tomato. *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection*, **101**, 475-481.

Hao, Z.P., Wang, Q., Christie, P. and Li, X.L. (2007) Allelopathic potential of watermelon tissues and root exudates. *Scientia Horticulturae*, **112**, 315-320.

Henrion, B., Letacon, F. and Martin, F. (1992) Rapid Identification of Genetic-Variation of Ectomycorrhizal Fungi by Amplification of Ribosomal-Rna Genes. *New Phytologist*, **122**, 289-298.

Hershkovitz, M.A., Zimmer, E.A. and Hahn, W.J. (1999) Ribosormal DNA sequences and angiosperm systematics. In Molecular systematics and plant evolution, eds. P. M. Hollingsworth, R. M. Bateman, and R. J. Gornall. London: Taylor and Francis. .

Hewlett, T.E., Chen, Z.X., Dickson, D.W., Mitchell, D.J. and McSorley, R. (1997) Suppression mechanisms of Meloidogyne arenaria race 1 by Pasteuria penetrans. *Journal of Nematology*, **29**, 1-8.

Hooks, C.R.R., Wright, M.G., Kabasawa, D.S., Manandhar, R. and Almeida, R.P.P. (2008) Effect of banana bunchy top virus infection on morphology and growth characteristics of banana. *Annals of Applied Biology*, **153**, 1-9.

Hooper, D. (1990) Extraction and processing of plant and soil nematodes. In: Luc, M, Sikora, RA and Bridge, J (eds.) Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. CAB International, Wallingford, UK, pp. 45-68. .

Horry, J.-P., Ortiz, R., Arnaud, E.C., JH , Ferris, R., Jones, D.R., Mateo, N., Picq, C. and Vuylsteke, D. (1997) Banana and Plantain. Pp 67-81.

Huelsenbeck, J.P. and Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754-755.

Hung, C.L. and Rohde, R.A. (1973) Phenol Accumulation Related to Resistance in Tomato to Infection by Root-Knot and Lesion Nematodes. *Journal of Nematology*, **5**, 253-258.

INIBAP. (1998) Networking banana and plantain: Annual report 1997: International network for improvement of banana and plantain (INIBAP) Montpellier, France. pp. 72.

INIBAP. (2000) Bananas. International network for improvement of banana and plantain (INIBAP) / International plant genetic resources institute (IPRGI), Montpellier, France.

Jones, D.R. (1999) Introduction to banana, abacá and ensete. *In: Disease of banana, abacá and ensete. Jones, D. R. (Ed.) CABI Publishing, Oxon, UK, pp 1-36*.

Karamura, E.B. (1999) Workshop objectives. Pages 5-8. In: Frison, E.A., Gold, C.S., Karamura, E.B. and Sikora, R.A. (eds). Mobilizing IPM for sustainable bnanana production in Africa. INIBAP, Montpellier, France.

Karpouzas, D.G., Hatziapostolou, P., Papadopoulou-Mourkidou, E., Giannakou, I.O. and Georgiadou, A. (2004a) The enhanced biodegradation of fenamiphos in soils from previously treated sites and the effect of soil fumigants. *Environmental Toxicology and Chemistry*, **23**, 2099-2107.

Karpouzas, D.G., Karanasios, E. and Menkissoglu-Spiroudi, U. (2004b) Enhanced microbial degradation of cadusafos in soils from potato monoculture: demonstration and characterization. *Chemosphere*, **56**, 549-559.

Katan, T. and Katan, J. (1988) Vegetative-Compatibility Grouping of Fusarium-Oxysporum F-Sp Vasinfectum from Tissue and the Rhizosphere of Cotton Plants. *Phytopathology*, **78**, 852-855.

Kawabe, M., Kobayashi, Y., Okada, G., Yamaguchi, I., Teraoka, T. and T., A. (2005) Three evolutionary lineages of tomato wilt pathogen, Fusarium oxysporum f. sp. lycopersici, based on sequences of IGS, MAT1, and pg1, are each composed of isolates of a single mating type and a single or closely related vegetative compatibility group *J. Gen. Plant Pathol.* , **71**, 263-272.

Kistler, H.C. (1997) Genetic diversity in the plant-pathogenic fungus Fusarium oxysporum. *Phytopathology*, **87**, 474-479.

Kistler, H.C., Alabouvette, C., Baayen, R.P., Bentley, S., Brayford, D., Coddington, A., Correll, J., Daboussi, M.J., Elias, K., Fernandez, D., Gordon, T.R., Katan, T., Kim, H.G., Leslie, J.F., Martyn, R.D., Migheli, Q., Moore, N.Y., O'Donnell, K., Ploetz, R.C., Rutherford, M.A., Summerell, B., Waalwijk, C. and Woo, S. (1998) Systematic numbering of vegetative compatibility groups in the plant pathogenic fungus Fusarium oxysporum. *Phytopathology*, **88**, 30-32.

Klich, M.A., Lax, A.R. and Bland, J.M. (1991) Inhibition of some mycotoxigenic fungi by iturin A, a peptidolipid produced by Bacillus subtilis. *Mycopathologia*, **116**, 77-80.

Koenig, R.L., Ploetz, R.C. and Kistler, H.C. (1997) Fusarium oxysporum f. sp. cubense consists of a small number of divergent and globally distributed clonal lineages. *Phytopathology*, **87**, 915-923.

Kohn, L. (1992) Developing new characters for fungal systematics: an experimental approach for determining the rank of resolution. *Mycologia*, **84**, 139-153.

Koufopanou, V., Burt, A. and Taylor, J.W. (1997) Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus Coccidioides immitis. *Proc Natl Acad Sci U S A*, **94**, 5478-5482.

Kuhn, D.N., Cortes, B., Pinto, T. and Weaver, J. (1995) Parasexuality and heterocrayosis in Fusarium oxysporum f. sp. cubense. *Phytopathology*, **85**, 1119.

Kunz, W., Schurter, R. and Maetzke, T. (1997) The chemistry of benzothiadiazole plant activators. *Pesticide Science*, **50**, 275-282.

Kurtz, A., Schouten, A. and Sikora, R. (2007) Phylogenetic analysis of endophytic Fusarium oxysporum strains inducing systemic resistance against the burrowing nematode Radopholus similis. *IOBC/WPRS Bulletin*.

Latch, G.C.M. (1993) Physiological Interactions of Endophytic Fungi and Their Hosts - Biotic Stress Tolerance Imparted to Grasses by Endophytes. *Agriculture Ecosystems & Environment*, **44**, 143-156.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S. and Ryals, J. (1995) Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. *Mol Plant Microbe Interact*, **8**, 863-870.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996) Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant Journal*, **10**, 71-82.

Lawton, K.A., Potter, S.L., Uknes, S. and Ryals, J. (1994) Acquired-Resistance Signal-Transduction in Arabidopsis Is Ethylene Independent. *Plant Cell*, **6**, 581-588.

Lay, F.T. and Anderson, M.A. (2005) Defensins - Components of the innate immune system in plants. *Current Protein & Peptide Science*, **6**, 85-101.

Lee, S.B. and Taylor, J.W. (1992) Phylogeny of 5 Fungus-Like Protoctistan Phytophthora Species, Inferred from the Internal Transcribed Spacers of Ribosomal DNA. *Molecular Biology and Evolution*, **9**, 636-653.

Lehmann, P.F., Lin, D. and Lasker, B.A. (1992) Genotypic identification and characterization of species and strains within the genus Candida by using random amplified polymorphic DNA. *J Clin Microbiol*, **30**, 3249-3254.

Leslie, J. and Summerell, B. (2006) The Fusarium laboratory manual. Blackwell Publishing-Wiley, Hoboken, NJ.

Lewis, D.H. (1985) Symbiosis an mutualism: Crisp concepts and soggy semantics. In: Boucher DH, ed. The biology of mutualism. London, UK: Croom-Helm Ltd., 29-39.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-408.

Lobuglio, K.F., Pitt, J.I. and Taylor, J.W. (1993) Phylogenetic Analysis of 2 Ribosomal DNA Regions Indicates Multiple Independent Losses of a Sexual Talaromyces State among Asexual Penicillium Species in Subgenus Biverticillium. *Mycologia*, **85**, 592-604.

Macpherson, J.M., Eckstein, P.E., Scoles, G.J. and Gajadhar, A.A. (1993) Variability of the Random Amplified Polymorphic DNA Assay among Thermal Cyclers, and Effects of Primer and DNA Concentration. *Molecular and Cellular Probes*, **7**, 293-299.

Malnoy, M., Jin, Q., Borejsza-Wysocka, E.E., He, S.Y. and Aldwinckle, H.S. (2007) Overexpression of the apple MpNPR1 gene confers increased disease resistance in Malus x domestica. *Mol Plant Microbe Interact*, **20**, 1568-1580.

Manulis, S., Kogan, N., Reuven, M. and Benyephet, Y. (1994) Use of the Rapd Technique for Identification of Fusarium-Oxysporum F Sp Dianthi from Carnation. *Phytopathology*, **84**, 98-101.

Mason, H.S., Dewald, D.B., Creelman, R.A. and Mullet, J.E. (1992) Coregulation of Soybean Vegetative Storage Protein Gene-Expression by Methyl Jasmonate and Soluble Sugars. *Plant Physiology*, **98**, 859-867.

Mbofung, G.Y., Hong, S.G. and Pryor, B.M. (2007) Phylogeny of Fusarium oxysporum f. sp lactucae inferred from mitochondrial small subunit, elongation factor 1-alpha, and nuclear ribosomal intergenic spacer sequence data. *Phytopathology*, **97**, 87-98.

Mbwana, A.S.S. and Rukazambuga, N.D.T.M. (1999) anana IPM in Tanzania. Pages 237-245. In: Frison, E.A., Gold, C.S., Karamura, E.B. and Sikora, R.A. (eds). Mobilizing IPM for sustainable bnanana production in Africa. INIBAP, Montpellier, France.

McSarlay, R. and Parrado, J. (1986) *Helicotylenchus multicintus* on bananas: An international problem. *Nematropica*, **16**, 73-91.

Menjivar Barahona, R.D. (2005) Estudio del potencial antagonistica de hongos endophiticos para el control biologico del nematode barrendador Radopholus similis en plantaciones de banano en Costa Rica. M.Sc. Thesis, CATIE, Turrialba, Costa Rica.

Metlen, K.L., Aschehoug, E.T. and Callaway, R.M. (2009) Plant behavioural ecology: dynamic plasticity in secondary metabolites. *Plant Cell and Environment*, **32**, 641-653.

Métraux, J.P., Ahl-Goy, P., Staub, T., J., S., Steinemann, A., Ryals, J. and Ward, E. (1991) Induced systemic resistance in cucumber in response to 2,6-dichloro-isonicotinic acid and pathogens. In: Hennecke H and Verma DPS (eds). Advances in Molecular Genetics of Plant-Microbe Interactions Vol 1 (pp 432-439). Kluwer Academic Puplishers, Dortrecht.

Meunier, J.R. and Grimont, P.A.D. (1993) Factors Affecting Reproducibility of Random Amplified Polymorphic DNA-Fingerprinting. *Research in Microbiology*, **144**, 373-379.

Mitsuhara, I., Iwai, T., Seo, S., Yanagawa, Y., Kawahigasi, H., Hirose, S., Ohkawa, Y. and Ohashi, Y. (2008) Characteristic expression of twelve rice PRI family genes in response to pathogen infection, wounding, and defense-related signal compounds (121/180). *Molecular Genetics and Genomics*, **279**, 415-427.

Morris, S.W., Vernooij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C.C., Frederiksen, R.A., Bhandhufalck, A., Hulbert, S. and Uknes, S. (1998) Induced resistance responses in maize. *Molecular Plant-Microbe Interactions*, **11**, 643-658.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. (1986) Specific Enzymatic Amplification of DNA Invitro - the Polymerase Chain-Reaction. *Cold Spring Harbor Symposia on Quantitative Biology*, **51**, 263-273.

Mullis, K.B. and Faloona, F.A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*, **155**, 335-350.

Nasution, R. (1991) A taxonomic study of the species *Musa accuminata* Colla with its intraspecific taxa in Indonesia. *Mem. Tokyo Univ. Agric.*, **32**, 1-122.

Nicholson, R.L. and Hammerschmidt, R. (1992) Phenolic-Compounds and Their Role in Disease Resistance. *Annual Review of Phytopathology*, **30**, 369-389.

Niderman, T., Genetet, I., Bruyère, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B. and Mösinger, E. (1995) Pathogenesis-related PR-1 Proteins Are Antifungal *Plant Physiology*, **108**, 17-27.

Niere, B.I. (2001) Significance of non-pathogenic isolates of Fusarium oxysporum Schlecht.: Fries for the biological control of the burrowing nematode Radopholus similis (Cobb) Thorne on tissue cultured banana. Ph.D. Thesis. University of Bonn.

Niere, B.I., Speijer, P.R., Gold, C.S. and Sikora, R.A. (1999) Fungal endophytes from banana for the control of *R. similis*. Pages 313-318 in: Frison, E.A., Gold, C.S., Karamura, E.B. and Sikora, R.A. (eds.).Mobilizing IPM for suitable banana production in Africa.INIBAP, Montpellier, France. .

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N. and Ohashi, Y. (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant and Cell Physiology*, **39**, 500-507.

Nurnberger, T., Brunner, F., Kemmerling, B. and Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev*, **198**, 249-266.

Nylander, J.A.A. (2004) MrModeltest 2.2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University. .

O'Donnell, K. (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete Fusarium sambucinum (Gibberella pulicaris). *Curr Genet*, **22**, 213-220.

O'Donnell, K., Cigelnik, E. and Nirenberg, H.I. (1998a) Molecular systematics and phylogeography of the Gibberella fujikuroi species complex. *Mycologia*, **90**, 465-493.

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

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Magnon, K.C., Cox, P.A., Revankar, S.G., Sanche, S., Geiser, D.M., Juba, J.H., van Burik, J.A.H., Padhye, A., Anaissie, E.J., Francesconi, A., Walsh, T.J. and Robinson, J.S. (2004) Genetic diversity of human pathogenic members of the Fusarium oxysporum complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: Evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. *Journal of Clinical Microbiology*, **42**, 5109-5120.

O'Bannon, J.H. (1977) Worldwide distribution of *R. similis* and its importance for crop production. *Journal of Nematology*, **9**, 16-25.

O'Bannon, J.H. and Esser, R.P. (1985) Citrus declines caused by nematodes in Florida. I Soil Factors. Nematology Circular No. 114, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL, USA. .

Oerke, E. (2005) Crop losses to pests. *The Journal of Agricultural Science, Cambridge Univ Press*.

Oostendorp, M., Kunz, W., Dietrich, B. and Staub, T. (2001) Induced disease resistance in plants by chemicals. *European Journal of Plant Pathology*, **107**, 19-28.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. (1989) Detection of Polymorphisms of Human DNA by Gel-Electrophoresis as Single-Strand Conformation Polymorphisms. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 2766-2770.

Ortiz, R. (1997) Morphological variation in Musa germplasm. *Genetic Resources and Crop Evolution*, **44**, 393-404.

Osbourn, A.E. (1996) Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell*, **8**, 1821-1831.

Ou, L.-T., Thomas, J. and Dickson, D. (1994) Degradation of fenamiphos in soil with a history of continuous fenamiphos applications. *Soil Sci Soc Am J* **58**, 1139-1147.

Page, R.D.M. (1996) TreeView: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357-358.

Paparu, P., Dubois, T., Coyne, D. and Viljoen, A. (2007) Defense-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.

Paparu, P., Dubois, T., Gold, C.S., Niere, B., Adipala, E. and Coyne, D. (2006) Colonisation pattern of nonpathogenic Fusarium oxysporum, a potential biological control agent, in roots and rhizomes of tissue cultured Musa plantlets. *Annals of Applied Biology*, **149**, 1-8.

Paparu, P., Macleod, A., Dubois, T., Coyne, D. and Viljoen, A. (2009) Efficacy of chemical and fluorescent protein markers in studying plant colonization by

endophytic non-pathogenic Fusarium oxysporum isolates. *Biocontrol*, **54**, 709-722.

Park, S.-C., Lee, J., Kim, J., Hwang, I., Nah, J.-W., H, C., Park, Y. and Hahm, K.-S. (2009) Pr-1, a novel antifungal protein from pumpkin rinds. *Biotechnology Letter*, **32**, 125-130.

Parmesan, C. and Yohe, G. (2003) A globally coherent fingerprint of climate change impacts across natural systems. *Nature*, **421**, 37-42.

Patrick, G., Chiri, A., Randall, D., Libelo, L. and Jones, J. (2001) Fenamophos environmental risk assessment. US Environmental protection Agency.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., DeSamblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M. and Broekaert, W.F. (1996) Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell*, **8**, 2309-2323.

Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Metraux, J.P. and Broekaert, W.F. (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell*, **10**, 2103-2113.

Petrini, O. (1986) Taxonomy of endophytic fungi of areal plant parts. In: Fokkema NJJ, Van den Heuvel J, eds. Microbiology of the phyllosphere. Cambridge Universtiy Press p. 175-187.

Petrini, O. (1991) Fungal endophytes of tree leaves. Pages 179-197 in: Andrews, J.H. and Hirano, S.S. (eds.). Microbial ecology of leaves. Springer-Verlag, New York, NY, USA.

Pierce-Shimomura, J.T., Morse, T.M. and Lockery, S.R. (1999) The fundamental role of pirouettes in Caenorhabditis elegans chemotaxis. *Journal of Neuroscience*, **19**, 9557-9569.

Pieterse, C.M. and Van Loon, L. (2004) NPR1: the spider in the web of induced resistance signaling pathways. *Current Opinion in Plant Biology*, **7**, 456-464.

Pieterse, C.M.J., Van Pelt, J.A., Ton, J., Parchmann, S., Mueller, M.J., Buchala, A.J., Metraux, J.P. and Van Loon, L.C. (2000) Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiological and Molecular Plant Pathology*, **57**, 123-134.

Pieterse, C.M.J., Van Wees, S.C.M., Ton, J., Van Pelt, J.A. and Van Loon, L.C. (2002) Signaling in rhizobacteria-induced systemic resistance in Arabidopsis thaliana. *Plant Biology*, **4**, 535-544.

Pieterse, C.M.J., van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, N., Weisbeek, P.J. and van Loon, L.C. (1998) A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell*, **10**, 1571-1580.

Pieterse, C.M.J., vanWees, S.C.M., Hoffland, E., vanPelt, J.A. and vanLoon, L.C. (1996) Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell*, **8**, 1225-1237.

Pikaard, C. (2002) Transcription and Tyranny in the Nucleolus: The Organization, Activation, Dominance and Repression of Ribosomal RNA Genes. *The Arabidopsis Book, ed. CR Somerville, MD: Am. Soc. Plant. Biol.*

Pline, M. and Dusenbery, D.B. (1987) Responses of Plant-Parasitic Nematode Meloidogyne-Incognita to Carbon-Dioxide Determined by Video Camera-Computer Tracking. *Journal of Chemical Ecology*, **13**, 873-888.

Ploetz, R.C. (1994) Panama-Disease - Return of the First Banana Menace. *International Journal of Pest Management*, **40**, 326-336.

Ploetz, R.C. and Correll, J.C. (1981) Vegetative compatebility among races of Fusarium oxysporum f. sp. cubense. *Plant Dis.*, **72**, 328-328.

Ploetz, R.C. and Pegg, K. (1997) Fusarium wilt of banana and Wallace's line: Was the disease originally restricted to his Indo-Malayan region? . *Australasian Plant Pathology*, **26**, pp. 239-249.

Plowright, R.A., Grayer, R.J., Gill, J.R., Rahman, M.L. and Harborne, J.B. (1996) The induction of phenolic compounds in rice after infection by the stem nematode Ditylenchus angustus. *Nematologica*, **42**, 564-578.

Pocasangre, L. (2000) Biological enhancement of banana tissue culture plantlets with endophytic fungi for the control of burrowing nematods Radopholus similis and the Panama disease (Fusarium oxysporum f.sp. cubense). Ph.D: Thesis, University of Bonn, Germany.

Pocasangre, L., Menjivar Barahona, R.D., Zum Felde , A., Riveros, A.S., Rosales, F.E. and Sikora, R.A. (2006) Endophytic fungi as biological control agents of plant parasitic nematodes in banana. *International Meeting ACROBAT*.

Price, N. (1995) Banana morphology - part 1:roots and rhizomes. Pp. 179-189 in: Gowen SR (ed.). Bananas and Plantains. Chapman and Hall, London, UK.

Prot, J.C. (1980) Migration of plant-parasitic nematodes towards plant roots. *Revue de Nematolgie*, **3**, 305-318.

Puhalla, J.E. (1985) Classification of Strains of Fusarium-Oxysporum on the Basis of Vegetative Compatibility. *Canadian Journal of Botany-Revue Canadienne De Botanique*, **63**, 179-183.

Rambaut, A. and Charleston, M. (2001) TreeEdit. *Dep. Of Zoology, University of Oxford, South Parks Road, Oxford, 0X1 3PS, UK*.

Rambaut, A. and Drummond, A. (2003-2005) Tracer. MCMC Trace Analysis Tool, Version 1.3. University of Oxford.

Rao, V.B. (1994) Direct sequencing of polymerase chain reaction-amplified DNA. *Anal Biochem*, **216**, 1-14.

Read, J.C. (1999) Mycorrhiza-the state of the art. In: Varma A., Hock B., ed. Mycorrhiza. Berlin, Germany: Springer-Verlag, 3-34

Read, J.C. and Camp, B.J. (1986) The Effect of the Fungal Endophyte Acremonium-Coenophialum in Tall Fescue on Animal Performance, Toxicity, and Stand Maintenance. *Agronomy Journal*, **78**, 848-850.

Redman, R.S., Freeman, S., Clifton, D.R., Morrel, J., Brown, G. and Rodriguez, R.J. (1999) Biochemical analysis of plant protection afforded by a nonpathogenic endophytic mutant of Colletotrichum magna. *Plant Physiology*, **119**, 795-804.

Rehner, S.A. and Samuels, G.J. (1994) Taxonomy and Phylogeny of Gliocladium Analyzed from Nuclear Large Subunit Ribosomal DNA-Sequences. *Mycological Research*, **98**, 625-634.

Rice, E.L. (1979) Allelopathy - Update. *Botanical Review*, **45**, 15-109.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487-491.

Sambrook, J. and Russell, D. (2000) Molecular Cloning a Laboratory Manual. Edition: 3rd edition Binding: Paperback Publisher: COLD SPRING HARBOR LABORATORY PRESS.

Sarah, J.L. (1989) Banana nematodes and their control in Africa. *Nematropica*, **19**, 199-217.

Sarah, J.L. (2000) Burrowing nematode. Pp. 295-303 in: Diseases of bananas, abacá and enset. Jones, DR (ed.) CAB International, Wallingford, UK. .

Sarah, J.L., Oinochet, J. and Stanton, J. (1996) The burrowing nematode of bananas, Radopholus similis Cobb. Musa Pest Fact Sheet No.1. INIBAP, Montpellier; France.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, X., Ohta, H. and Tabata, S. (2001) Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: Self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. *DNA Research*, **8**, 153-161.

Sasser, J.N. and Freckman, D.W. (1986) A World Perspective on Nematology - the Role of the Society. *Journal of Nematology*, **18**, 596-596.

Schouten, A. (2009) Plants and Microorganisms: Complex checks and balances in tritrophic interactions. Habilitation Thesis, Agricultural Faculty, University of Bonn.

Schouten, A., van den Berg, G., Edel-Hermann, V., Steinberg, C., Gautheron, N., Alabouvette, C., de Vos, C.H., Lemanceau, P. and Raaijmakers, J.M. (2004) Defense responses of Fusarium oxysporum to 2,4-diacetylphloroglucinol, a broad-spectrum antibiotic produced by Pseudomonas fluorescens. *Molecular Plant-Microbe Interactions*, **17**, 1201-1211.

Schulz, B., Rommert, A.K., Dammann, U., Aust, H.J. and Strack, D. (1999) The endophyte-host interaction: a balanced antagonism? *Mycological Research*, **103**, 1275-1283.

Selitrennikoff, C.P. (2001) Antifungal proteins. *Applied and Environmental Microbiology*, **67**, 2883-2894.

Seo, S., Seto, H., Yamakawa, H. and Ohashi, Y. (2001) Transient accumulation of jasmonic acid during the synchronized hypersensitive cell death in Tobacco mosaic virus-infected tobacco leaves. *Molecular Plant-Microbe Interactions*, **14**, 261-264.

Sikora, R. (1992a) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. *Annual Review of Phytopathology*, **30**, 245-270.

Sikora, R. and Schuster, R.P. (2000) Handbuch der Phytonematologie. Shaker Verlag, Aachen, Germany.

Sikora, R.A. (1992b) Management of the antagonistic potential in agricultural ecosystems for the control of plant parasitic nematodes. *Annual Revue of Phythopathology*, **30**, 245-270.

Sikora, R.A., Bafokuara, N.D., Mbwana, A.S.S., Oloo, G.W., Uronu, B. and Sehsu Reddy, K.V. (1989) Interrelationship between banana weevil, root lesion nematode and agronomic practices and their performance for banana decline in the United Republic of Tanzania. FAO Plant Protection Bulletin 37: 593-598.

Sikora, R.A., Pocasangre, L., Zum Felde , A., Niere, B.I., Vu, T. and Dababat, A.A. (2008) Mutalistic endophytic fungi an *in-planta* suppressiveness to plant parasitic nematodes. *Biological Control*, **46**, 15-23.

Sikora, R.A. and Schuster, R.P. (1999) Novel approaches to nematode IPM. P. 127-136 in E.A. Frison, C.S. Gold, E.B. Karamura and Sikora R.A. (eds.): Mobilizing IPM for sustainable banana production iin Africa. INIBAP, Montpellier, France.

Sikora, R.A., Schuster, R.P. and Griesbach, M. (1999) Improved plant health through enhancement of planting material with mutualistic endophytes. *Acta Horticulturea*, **540**, 409-413.

Simmonds, N.W. (1962) Evolution of the bananas. Longman, London, UK.

Simmonds, N.W. (1966) Bananas (Longmans, London), 2nd Ed.

Smith, M.C., Rutter, J., Burt, P.J.A., Ramirez, F. and Gonzalez, E.H. (1997) Black Sigatoka disease of banana: spatial and temporal variability in disease development. *Annals of Applied Biology*, **131**, 63-77.

Sneath, P. and Sokal, R. (1973) Numerical Taxonomy. Freeman, San Francisco.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol*, **98**, 503-517.

Speijer, P.R. and De Waele, D. (1997) INIBAP technical guidelines. 1. Screening of Musa germplasm for resistance and tolerance to nematodes. INIBAP, Montpellier, Fance.

Speijer, P.R., Kajumba, C. and Ssango, F. (1999a) East African highland banana production as influenced by nematodes and crop management in Uganda. *International Journal of Pest Management*, **45**, 41-49.

Speijer, P.R., Kajumba, C. and Tushemereirwe, W. (1999b) Dissemination and adaptation of a banana clean planting material technology in Uganda. *InfoMusa*, **8**, 11-13.

Spoel, S.H., Mou, Z.L., Tada, Y., Spivey, N.W., Genschik, P. and Dong, X.N.A. (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell*, **137**, 860-872.

Sticher, L., MauchMani, B. and Metraux, J.P. (1997) Systemic acquired resistance. *Annual Review of Phytopathology*, **35**, 235-270.

Stinson, K.A., Campbell, S.A., Powell, J.R., Wolfe, B.E., Callaway, R.M., Thelen, G.C., Hallett, S.G., Prati, D. and Klironomos, J.N. (2006) Invasive plant suppresses the growth of native tree seedlings by disrupting belowground mutualisms. *Plos Biology*, **4**, 727-731.

Stintzi, A., Heitz, T., Prasad, V., Wiedemannmerdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M. and Fritig, B. (1993) Plant Pathogenesis-Related Proteins and Their Role in Defense against Pathogens. *Biochimie*, **75**, 687-706.

Stirling, G.R. and Pattison, A.B. (2008) Beyond chemical dependency for managing plant-parasitic nematodes: Examples from the banana, pineapple and vegetable industries of tropical and subtropical Australia. CSIRO Publishing.

Stover, R. and Simmonds, N. (1987) Bananas, 3rd ed. Longmans, London, United Kingdom.

Stover, R.H. (1962) Fusarium Wilt (Panama Disease) of Bananas and other Musa species. Phytopathological Paper No. 14, Commonwealth Mycological Institute, Kew, UK.

Suett, D., Fournier, J.-C., Papadopoulou-Mourkidou, E., Pussemier, L. and Smelt, J. (1999) Accelerated degradation: The European dimension *Soil Biology and Biochemistry*, **28**, 1741-1748.

Swofford, D.L. (2003) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. *Sinauer Associates, Sunderland, Massachusetts.*

Tahirialaoui, A., Dumasgaudot, E., Gianinazzi, S. and Antoniw, J.F. (1993) Expression of the Pr-B(1)'' Gene in Roots of 2 Nicotiana Species and Their Amphidiploid Hybrid Infected with Virulent and Avirulent Races of Chalara-Elegans. *Plant Pathology*, **42**, 728-736.

Thomma, B.P.H.J., Penninckx, I.A.M.A., Broekaert, W.F. and Cammue, B.P.A. (2001) The complexity of disease signaling in Arabidopsis. *Current Opinion in Immunology*, **13**, 63-68.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876-4882.

Tornero, P., Gadea, J., Conejero, V. and Vera, P. (1997) Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Molecular Plant-Microbe Interactions*, **10**, 624-634.

Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C. and Grant, M. (2007) Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc Natl Acad Sci U S A*, **104**, 1075-1080.

Valette, C., Andary, C., Geiger, J.P., Sarah, J.L. and Nicole, M. (1998) Histochemical and cytochemical investigations of phenols in roots of banana infected by the burrowing nematode Radopholus similis. *Phytopathology*, **88**, 1141-1148.

Vallad, G.E. and Goodman, R.M. (2004) Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science*, **44**, 1920-1934.

van Belkum, A. (1994) DNA fingerprinting of medically important microorganisms by use of PCR. *Clin Microbiol Rev*, **7**, 174-184.

Van den Berg, N., Berger, D.K., Hein, I., Birch, P.R.J., Wingfield, M.J. and Viljoen, A. (2007) Tolerance in banana to Fusarium wilt is associated with early up-regulation of cell wall-strengthening genes in the roots. *Molecular Plant Pathology*, **8**, 333-341.

Van den Berg, N., Berger, D.K., Hein, I., Birch, P.R.J., Wingfield, M.J. and Viljoen, A. (2009) Genes Up-Regulated in Tolerant Cavendish Banana Roots in Response to Fusarium oxysporum f. sp. cubense Infection. Proceedings.

ISHS Acta Horticulturae 828: International Symposium on Recent Advances in Banana Crop Protection for Sustainable Production and Improved Livelihoods. Eds.: D. Jones and I. van den Berg.

Van Loon, L. (2000) Systemic induced resistance. In: Slusarenko AJ, Fraser RSS, Van Loon LC, editors. Mechanisms of Resistance to Plant Diseases. Dordrecht: Kluwer Academic Publishers. pp. 521-574.

Van Loon, L., Pierpoint, W., Boller, T. and Conejero, V. (1994) Recommendations for naming plant pathogenesis-related proteins. *Plant Molecular Biology Report*, **12**, 245-264.

Van Loon, L.C. (1985) Pathogenesis-Related Proteins. *Plant Molecular Biology*, **4**, 111-116.

Van Loon, L.C. (2001) The families of pathogenesis-related proteins. 6th International Workshop on PR-Proteins. May 20-24, 2001, Spa Belgium. Book of Abstracts, p. 9.

Van Loon, L.C., Bakker, P.A. and Pieterse, C.M. (1998) Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol*, **36**, 453-483.

Van Loon, L.C., Rep, M. and Pieterse, C.M.J. (2006) Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology*, **44**, 135-162.

Van Loon, L.C. and van Kammen, A. (1970) Polyacrylamide disc electrophoresis of the soluble leaf proteins from Nicotiana tabacum var. "Samsun" and "Samsun NN". II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology*, **40**, 190-211.

Van Loon, L.C. and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology*, **55**, 85-97.

Van Wees, S.C., Luijendijk, M., Smoorenburg, I., van Loon, L.C. and Pieterse, C.M. (1999) Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. *Plant Mol Biol*, **41**, 537-549.

Van Wees, S.C.M., de Swart, E.A.M., van Pelt, J.A., van Loon, L.C. and Pieterse, C.M.J. (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 8711-8716.

VanEtten, H.D., Mansfield, J.W., Bailey, J.A. and Farmer, E.E. (1994) 2 Classes of Plant Antibiotics - Phytoalexins Versus Phytoanticipins. *Plant Cell*, **6**, 1191-1192.

VanWees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., VantWestende, Y.A.M., Hartog, F. and VanLoon, L.C. (1997) Differential induction of systemic resistance in Arabidopsis by biocontrol bacteria. *Molecular Plant-Microbe Interactions*, **10**, 716-724.

Verberne, M.C., Hoekstra, J., Bol, J.F. and Linthorst, H.J.M. (2003) Signaling of systemic acquired resistance in tobacco depends on ethylene perception. *Plant Journal*, **35**, 27-32.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditzjawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994) Salicylic-Acid Is Not the Translocated Signal Responsible for Inducing Systemic Acquired-Resistance but Is Required in Signal Transduction. *Plant Cell*, **6**, 959-965.

Vigers, A.J., Wiedemann, S., Roberts, W.K., Legrand, M., Selitrennikoff, C.P. and Fritig, B. (1992) Thaumatin-Like Pathogenesis-Related Proteins Are Antifungal. *Plant Science*, **83**, 155-161.

Vu, T., Hauschild, R. and Sikora, R.A. (2006) Fusarium oxysporum endophytes induced systemic resistance against Radopholus similis on banana. *Nematology*, **8**, 847-852.

Vu, T.T.T. (2005) *Modes of action of non-pathogenic Fusarium oxysporum endophytes for bio-enhancement of banana toward Radopholus similis. Ph.D. Thesis. University of Bonn.* Cuvillier, Göttingen.

Waalwijk, C., deKoning, J.R.A., Baayen, R.P. and Gams, W. (1996) Discordant groupings of Fusarium spp from sections Elegans, Liseola and Dlaminia based on ribosomal ITS1 and ITS2 sequences. *Mycologia*, **88**, 361-368.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Huckelhoven, R., Neumann, C., von Wettstein, D., Franken, P. and Kogel, K.H. (2005) The endophytic fungus Piriformospora indica reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 13386-13391.

White, J.F., Sullivan, R., Moy, M., Patel, R. and Duncan, R. (2000) An overview of problems in the classification of plant-parasitic Clavicipitaceae. *Studies in Mycology*, 95-105.

White, T.J., Bruns, T., Lee, S. and Tailor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics *In: Innis MA,*

Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: A guide to methodes and applications. San Diego: Academic Press, 315-322.

Whitehead, A.W. (1998) Plant nematode control. *CAB International Wallingford, Oxford UK*, pp. 394.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA Polymorphisms Amplified by Arbitrary Primers Are Useful as Genetic-Markers. *Nucleic Acids Research*, **18**, 6531-6535.

Wuyts, N., Lognay, G., Verscheure, M., Marlier, M., De Waele, D. and Swennen, R. (2007) Potential physical and chemical barriers to infection by the burrowing nematode Radopholus similis in roots of susceptible and resistant banana (Musa spp.). *Plant Pathology*, **56**, 878-890.

Wuyts, N., Swennen, R. and De Waele, D. (2006) Effects of plant phenylpropanoid pathway products and selected terpenoids and alkaloids on the behaviour of the plant-parasitic nematodes Radopholus similis, Pratylenchus penetrans and Meloidogyne incognita. *Nematology*, **8**, 89-101.

Wyss, P. and Bonfante, P. (1993) Amplification of Genomic DNA of Arbuscular Mycorrhizal (Am) Fungi by Pcr Using Short Arbitrary Primers. *Mycological Research*, **97**, 1351-1357.

Yu, J.Q., Shou, S.Y., Qian, Y.R., Zhu, Z.J. and Hu, W.H. (2000) Autotoxic potential of cucurbit crops. *Plant and Soil*, **223**, 147-151.

Yuan, Y., Zhong, S., Li, Q., Zhu, Z., Lou, Y., Wang, L., Wang, J., Wang, M., Li, Q., Yang, D. and He, Z. (2007) Functional analysis of rice NPR1-like genes reveals that OsNPR1/NH1 is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. *Plant Biotechnol J*, **5**, 313-324.

Zambino, P.J. and Szabo, L.J. (1993) Phylogenetic-Relationships of Selected Cereal and Grass Rusts Based on Rdna Sequence-Analysis. *Mycologia*, **85**, 401-414.

zum Felde , A. (2008) Studies on the Characteristics of the Antagonistic Relationship between Radopholus similis (Cobb) Thorne and Mutualistic Endophytic Fungi in Nematode-Suppressive Banana Plants (Musa AAA), Dissertation.

zum Felde, A. (2008) Studies on the Characteristics of the Antagonistic Relationship between Radopholus similis (Cobb) Thorne and Mutualistic Endophytic Fungi in Nematode-Suppressive Banana Plants (Musa AAA), Dissertation.

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12 Appendix

Partial IGS sequences of the fungal isolates used for the phylogenetic analysis (fasta file format).

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13 Glossary

Accession: Plant sample, strain or population held in a genebank or breeding programme for conservation or use.

Akaike Information Criterion (AIC): AIC can be interpreted as the amount of information lost when we use a particular model to approximate the real process of nucleotide substitution; thus, the model with the smallest AIC is favored. Given equal priors for each of the competing models, the model with the smallest BIC is equivalent to the model with the maximum posterior probability.

Allele: One of the (usually two) alternative forms of a gene, found at the same place on a chromosome.

ANalysis Of VAriance between groups (ANOVA): ANOVA tests whether several means (for different conditions or groups) are equal across one or more variables.

Anamorph: fungus with asexual reproductive stage.

Bayesian inference is a statistical inference in which evidence or observations are used to update or to newly infer the probability that a hypothesis may be true.

BLAST search: The Basic Local Alignment Search Tool finds regions of local similarity between sequences.

Centre of diversity: Geographic region in which the greatest variability of a crop occurs. A primary centre of diversity, where the highest diversity occurs, including wild relatives, is interpreted as the region the crop originated from and where its domestication and cultivation first began. Secondary centres are regions of lesser diversity which have developed as a result of the subsequent spread of a crop.

Character: An attribute of an organism resulting from the interaction of a gene or genes with the environment.

Consistency Index: Index for the fit of an individual character on a phylogenetic tree, calculated by dividing the minimum number of parsimony steps by the number of steps observed.

Characterization: Determination of the structural or functional attributes of a plant in order to distinguish between accessions.

Clone: A group of cells, tissues or plants descended by mitosis from a common ancestor (cell, tissue or plant).

Convergence: An evolutionary event which occurs in two independent sequences.

Cultivar: A cultivated variety of a domesticated crop plant. Synonym: Variety.

Distribution: Geographical area where a species grows naturally or has been cultivated by smallholders for many years.

Delta Rn: Normalized reporter signal minus the baseline signal (7500 Quantifast).

Denaturing gradient gel electrophorisis (DGGE): Method to separate DNA fragments of identical size, but different nucleotide sequences.

Dice: Coefficient to measure the similarity amongst related taxa.

Endophyte: ‘Organism that, at some time of its life cycle, lives symptomless within plant tissue’, Petrini 1991.

Ex situ conservation: Literally conservation ‘off-site’. Conservation of a plant outside of its original or natural habitat, e.g. in a genebank, botanical garden or field genebank and stored as seed, tissue, entire plant or pollen.

Gene: The functional unit of heredity. A gene is a section of DNA that codes for a specific biochemical function in a living organism.

Genebank: Facility where germplasm is stored in the form of seeds, pollen or *in vitro* cultures, or in the case of a field genebank, as plants growing in the field.

Genepool: All the genes and their different alleles present in an interbreeding population.

Gene expression: The process in which a gene`s coded information is translated into RNAs or proteins.

Genetic diversity: The variation present in a group of individuals, populations or species that is due to genetic differences (as opposed to the expression of the same genetic background in different environments).

Genetic erosion: Loss of genetic diversity between and within populations of the same species or the loss of entire species (e.g. wild relatives) over time, or reduction of the genetic base of a species due to human intervention, environmental change etc.

Genetic resources: Genetic materials of plants, animals and other organisms which is of value as a resource from a genetic point of view for present and future generations of people.

Genome: 1. The genetic material of an organism. 2. A set of chromosomes corresponding to the haploid set of a species.

Genotype: 1. The entire genetic constitution of an organism. 2. A group of organisms with the same genetic constitutions.

Germplasm: A set of genotypes that may be conserved or used; synonymous with genetic resource.

Habitat: A specific place that is occupied by an organism or community, and where interactions with other organisms and the environment occur.

Holotype: A specimen commonly recognize as representative of the species or group.

Milli-Q water: Rrefers to water that has been purified and deionized to a high degree by a water purification systems manufactured by Millipore Corporation.

Mutualist: In this work mutualist is defined as endophyte that is able to systemically induce enhanced resistance against *R. similis* penetration.

In situ conservation: Literally 'on-site' conservation. Conservation of plants and animals in the areas where they developed their distinctive properties, i.e. in the wild or in farmers' fields.

Indigenous knowledge: Knowledge that develops in a particular area and accumulates over time through being handed down from generation to generation.

Jackknife: A method of resampling phylogenetc data in an effort to assess confidence in the hypothesised relationships between taxa, (Very similar to bootstrapping).

Landraces: Farmer-developed varieties of crop plants that are heterogenous, adapted to local environmental conditions and have their own local names. Landraces are farmers' varieties that have not been improved by formal breeding programmes.

Modern variety: The product of formal, institutional (including NGO) and scientific plant breeding applying modern techniques of selection and technology and resulting in mostly homogenous varieties/cultivars.

Morphotaxonomic description: Description of the plant made by observing the morphological characters. The description is always related to the environment in which the plant is growing because some of the characters are expressed differently according to the environment.

Parthenocarpy: The development of fruit without pollination or fertilization.

Pathogen: In this work defined as endophitic growing *F. oxysporum* with uncharacterized properties in therms of inducing *R. similis* resistance in banana.

Phenotype: 1. Physical or external appearance of an organism as contrasted with its genetic constitution (genotype). 2. A group of organism with similar physical or external makeup.

Plantain (true): A particular type of cooking banana belonging to the AAB genome group characterised by the yellow-orange colour of the compound tepal. The fruits are generally only palatable after cooking. The male axis is sometimes absent or degenerated. If present, it is clothed with persistent male bracts and flower relicts.

Ploidy: The number of basic sets (x) of chromosomes in a cell, tissue or plant. Polyploid: A plant with other than two basic sets of chromosomes in somatic cells. That is triploid (2n=3x), tetraploid (2n=4x) and various aneuploids (2n is not a multiple of x).

Pseudostem (banana): The ‘trunk’ (or ‘false stem’) of a banana plant which, prior to flowering, is composed only of overlapping leaf bases. It contains no woody tissues and therefore the banana is classified as an herb.

Relative Quantification: A process which determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. The calibrator sample can be an untreated control or a sample at time zero in a time-course study (Livak and Schmittgen, 2001).

Rn (Normalized Reporter Signal): The cycle-by-cycle ratio of the fluorescence of the reporter dye to the fluorescence of the passive reference dye in a given well. During PCR, Rn increases as the amplification copy number increases, as measured by the fluorescence of the reporter dye, until the reaction approaches a plateau. When using the instrument in its plate read mode (end-point analysis), the

fluorescence signal is read at a single point in time after the completion of PCR rather than at intervals during the course of PCR.

RQ Min/Max Confidence: Specifies the confidence value that 7500 Fast System software will use to calculate the standard error of the mean expression level (RQMax and RQMin values) for samples used in ddCT-studies.

Single Nucleotide Polymorphism (SNP): A single base pair of DNA in the genome that differs between individuals.

Somaclonal variation: Variation observed in plants regenerated from *in vitro* culture and which do not conform to the original plant.

Somatic mutation: Variation in the gene or chromosome structure of a plant that occurs during vegetative reproduction, and is heritable even through sexual reproduction. These are normally due to accidents which occur during cell division and produce a permanent change in a clone.

Species: A group of organisms capable of interbreeding freely with each other but not with members of other species. In taxonomic classification a subdivision of a genus. A group of closely related individuals descended from the same stock.

Suckers (banana): Shoots appearing at the base of the mother plant. The mother plant dies when the bunch is harvested and the oldest sucker becomes the next mother plant.

Sword sucker (banana): A sucker bearing narrow ‘sword’ leaves and attached to the mother rhizome.

Teleomorph: Sexual reproductive stage (Fungi: typically producing fruiting bodies).

UPGMA: Unweighted Pair-Group Method with Arithmathic Averaging. This is a method of producing a dendrogram from a distance matrix.

Variety: In classical botany a variety is a subdivision of a species. An agricultural variety is a group of similar plants that by structural features and performance can be identified from other varieties of the same species. Synonom: Cultivar.

Vegetative propagation: Asexual propagation by which a plant is produced which is identical in genotype with the source (mother) plant.

Water sucker (banana): A sucker of superficial origin bearing broad leaves. Water suckers are not subjected to growth control by the mother plant and lack resources provided by the mother plant.

Wild relative: Non-cultivated species which is more or less closely related to a crop species (usually in the same genus) and has genetically contributed to the genome of the cultivated species.