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Biological control of *Fusarium oxysporum* f.sp. *cubense* using non-pathogenic *F. oxysporum* endophytes

by

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Declaration

I, the undersigned, declare that the work contained in this thesis is my own and original work and that it has not previously in its entirety or part submitted for a degree to any other university.

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PREFACE

Fusarium oxysporum f.sp. *cubense* Schlecht (*Foc*), causal agent of Fusarium wilt of banana (Panama disease), is considered to be one of the most serious threats to banana production in the world. There is no effective control measure for Fusarium wilt, except for the replacement of susceptible with resistant banana varieties. However, resistant varieties are not always acceptable to producers and local consumer markets. A greater awareness of the detrimental effect of chemicals on the environment has stimulated research on biological control of plant pathogens. The use of indigenous microorganims, such as non-pathogenic *F. oxysporum* and the bacterium *Pseudomonas fluorescens*, therefore, offers not only an environmentally safe but also an economical approach to combat Fusarium wilt of banana as part of an integrated disease management strategy.

Non-pathogenic *F. oxysporum* and *P. fluorescens* isolates have previously been isolated from the root rhizosphere in disease suppressive soils. These isolates have the ability to reduce the incidence of Fusarium wilt in greenhouse pathogenicity trials. In this study we had hoped to expand on existing knowledge on the biological control of Fusarium wilt of banana with non-pathogenic endophytic *F. oxysporum* and *P. fluorescens*. Isolates that significantly suppress disease development in greenhouse trials were tested under field conditions. Physiological and histological studies were also performed to understand the modes of action of putative biological control agents. For the histological investigations, non-pathogenic *F. oxysporum* isolates were modified with green and red fluorescent proteins.

Chapter 1 depicts a general overview of the biological control of Fusarium wilt diseases of agricultural crops. This chapter addresses the biology and pathogenesis of *F. oxysporum*, before strategies to control Fusarium wilt are discussed. The application of biological control organisms was analysed in terms of potentially useful organisms, where they can be isolated, and their possible modes of action. Finally, factors that influence biological control of Fusarium wilt diseases are discussed.

A good source of prospective biocontrol agents is suppressive soils. In **Chapter 2,** non-pathogenic *F. oxysporum* isolates were collected from healthy banana roots in

disease suppressive soil. Random Fragment Length Polymorphisms of the intergenic spacer region were then applied to group the non-pathogenic *F. oxysporum* isolates into genotypes, from which candidates were selected for biological control studies. The selected endophytes were then inoculated onto banana roots to determine their ability to act as biocontrol agents against *Foc*. The isolates that protected banana best against Fusarium wilt in the greenhouse, together with *P. fluorescens* WCS 417, were tested in the field to determine whether these isolates could effectively reduce disease incidence in an uncontrolled environment.

The ability of non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 to induce systemic resistance in Cavendish banana plants against *Foc* was investigated in **Chapter 3** with the use of a split-root technique. The putative biocontrol agents were inoculated, separately and in combination, on one half of the roots in a split-root experiment, while the other half was challenged by a pathogenic isolate of *Foc*. Five different phenolic acids were assayed which included total soluble phenolic acids, non-conjugated (free acids) phenolic acids, ester-bound phenolic acids, glycoside-bound phenolic acids and cell wall-bound phenolic acids. The knowledge gained will contribute to the understanding of how the biocontrol agents may induce defense responses in banana roots against *Foc*.

Non-pathogenic isolates of *F. oxysporum* were transformed with the green fluorescent protein (*GFP*) and *DsRed-Express* genes in **Chapter 4**. These isolates were used to visualise their interactions with a GFP-transformed *Foc* isolate on the banana root in a non-destructive manner by means of confocal laser scanning microscopy (CLSM) in **Chapter 5**. The ability of non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 to induce structural changes was also investigated with a split-root system using the CLSM. Antibioses as a mode of action of the two potential biocontrol agents was tested *in vitro*. Understanding the modes of action of non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 are important when considering strategies for the implementation of these isolates in an integrated disease management strategy against Fusarium wilt of banana.

Chapter 1

Biological control of Fusarium wilt diseases

ABSTRACT

Fusarium wilt is a destructive disease of many economically important crops caused by the soil-borne fungus *Fusarium oxysporum*. *Fusarium oxysporum* consists of pathogenic and non-pathogenic strains that are morphologically indistinguishable. Fusarium wilt is difficult to control, and little success has been achieved using chemical and cultural control methods. The use of disease resistant plants is the most effective means to combat Fusarium wilt, but resistant varieties are sometimes not acceptable to consumer markets. Biological control offers an environmentally safe means to limit the damage caused to crops by *F. oxysporum*. Potential biological control candidates, such as non-pathogenic *F. oxysporum* and *Pseudomonas fluorescens*, can be isolated from Fusarium wilt suppressive soils. These microbes, whether they live inside plant tissue as endophytes or in the rhizosphere, have the advantage that they are adapted to the same environmental conditions as the wilt pathogen. Modes of action whereby biocontrol agents inhibit Fusarium wilt pathogens include antibioses, competition and induced resistance. It is important to consider these mechanisms when strategies are developed to use biological control agents in an integrated disease management program against Fusarium wilt.

INTRODUCTION

Fusarium wilt causes highly destructive diseases in many economically important agricultural crops. The disease almost destroyed the international banana trade in Central America in the 1950's, (Stover, 1962), resulting in losses estimated at approximately US$ 400 million (US$ 2.3 billion in 2000-value) (Ploetz, 2005). In the United States, Fusarium wilt severely limited the production of cotton, causing losses of over 109,000 bales in 2004 (Blasingame and Patel, 2005). Tomato producers also suffered immense losses due to the disease in many countries of the world (Walker, 1971; Volin and Jones, 1982), and although resistant cultivars are known to exist, the occurrence and development of new races is a continuing problem (Borrero *et al*., 2006). It is not only commercial farmers that suffer because of intense cultivation of crops. The pathogen responsible for Fusarium wilt can survive in soil and infected plant rests for decades (Di Pietro *et al*., 2003; Ulloa *et al*., 2006), and often heralds the end of crop production in infested fields. In Marocco, for instance, Fusarium wilt of date palm (*Phoenix dactylifera*), a disease also known as "Bayoud" disease, caused the death of more than 12 million palm trees over a period of one century (Djerbi, 1983). There is also a progressive disappearance of high-quality cultivars with poor-yielding date palm seedling trees (Djerbi *et al*., 1986).

Fusarium wilt diseases are caused by *Fusarium oxysporum* Schlecht, a most ubiquitous and adaptable soil microorganism. *Fusarium oxysporum* can be divided into many *formae speciales*, most of which attack a single crop system (Kistler, 1997). Apart from their ability to cause disease to plants, they also colonize roots as harmless endophytes, and as saprophytes the soil, organic debris and non-host plant roots (Gordon and Martyn, 1997). Non-pathogenic *F. oxysporum* strains may even protect plants against pathogenic forms of the fungus (Fravel *et al*., 2003), and can thus be considered as potential biological control organisms. The most sustainable means of controlling Fusarium wilt diseases, however, remains the introduction of resistance in susceptible plants.

The objective of this review is to summarize the knowledge available for the biological control of Fusarium wilt diseases. In the first section, the Fusarium wilt pathogen and the disease it causes is introduced. Means to control Fusarium wilt is

then reviewed, before biological control of *F. oxysporum* is discussed in detail. The review also introduces concepts such as suppressive soils, from where potential biological control agents can be isolated, and the use of biocontrol in integrated disease management programmes.

THE FUSARIUM WILT PATHOGEN

Fusarium oxysporum is a cosmopolitan fungus that can be found in soils in all parts of the world. The fungus is known to produce sparse to abundant aerial mycelium, and white, pink, salmon and purple pigmentation on the reverse side of the colony in culture (Gerlach and Nirenberg, 1982; Nelson *et al*., 1983). *Fusarium oxysporum* appears to rely solely on asexual reproduction and produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Kistler and Miao, 1992). Micro- and macroconidia are produced on branched and unbranched monophialides (Nelson *et al*., 1983). The microconidia are one- or two-celled, oval to kidney shaped, and are borne in false heads (Nelson *et al*., 1983). The macroconidia are four- to eight-celled, sickle-shaped, thin-walled and delicate, with foot-shaped basal and attenuated apical cells (Gerlach and Nirenberg, 1982). Chlamydospores are globose, thick-walled resting spores that are formed singly or in pairs terminally and intercalary in hyphae or in conidia (Ploetz and Pegg, 2000). A teleomorph (sexual stage) for *F. oxysporum* has not been found.

Fusarium oxysporum includes pathogenic and non-pathogenic members that cannot be distinguished morphologically (Snyder and Smith, 1981). The pathogenic forms are divided into approximately 120 different *formae speciales* according to the host plant that they cause disease to (Armstrong and Armstrong, 1981). Non-pathogenic forms of the pathogen are even more diverse (Gordon and Okamoto, 1992; Lori *et al*., 2004; Nel *et al*., 2006). Both pathogenic and non-pathogenic *F. oxysporum* infect plant roots. While the non-pathogen is most often limited to the cortex where they survive as endophytes, the pathogen enters the vascular tissue to cause wilting of their hosts (Olivain and Alabouvette, 1997; 1999; Ito *et al*., 2005). Other than living as endophytic fungi in plant roots, non-pathogenic fungi can survive as saprophytes in the root rhizosphere or in soil organic matter (Beckman, 1990; Gordon and Martyn, 1997; Di Pietro *et al*., 2003).

Formae speciales of *F. oxysporum* are named according to the specific host that they attack. For instance, isolates of the pathogen that attack bananas are called *F. oxysporum* f. sp. *cubense*, those attacking carnation are named *F. oxysporum* f. sp. *dianthi*, and *F. oxysporum* f.sp. *lini* and *F. oxysporum* f.sp. *lycopersici* are pathogenic to flax and tomato, respectively (Booth, 1971; Armstrong and Armstrong, 1981). *Formae speciales* can be further subdivided into races. Races include individuals within a *formae specialis* that attack a specific cultivar within a crop (Kuninaga and Yokosawa, 1992). A gene-for-gene relationship has been proposed to mediate the interaction between *F. oxysporum* races and host cultivars, based on dominant monogenic resistance traits against known races. Simons *et al*. (1998) have confirmed this gene-for-gene relationship by cloning the tomato resistance gene *I2* that confers resistance to *F. oxysporum* f.sp. *lycopersici* race 2. In the *F. oxysporum* complex only the tomato pathogen can be described this way. New pathogenic races of *F. oxysporum* f.sp. *lycopersici* continue to be discovered, since a single mutation can give rise to a new race (Borrero *et al*., 2006). Races of other *formae speciales* that attack crops other than tomato are defined by a multigene resistance and do not form that easily because a sequence of mutations is necessary. Race designation in *F. oxysporum* is determined either in the glasshouse using a set of differential cultivars, or in the field. Problems can occur with race identification in the field because host resistance to the pathogen is influenced by environmental interaction, as is the situation for *F. oxysporum* f.sp. *cubense* (Moore *et al*., 1991; Moore, 1994).

Formae speciales and pathogenic races in *F. oxysporum* are subdivided into vegetative compatibility groups (VCGs). Vegetative compatibility is based on the formation of a stable heterokaryon between compatible mutants, and in *F. oxysporum* is considered homogenic, implying that two strains are vegetatively compatible if the alleles at each of the corresponding *vic* loci are identical (Correll, 1991). Vegetative compatibility can serve as a method for identifying and differentiating *formae speciales* and races in *F. oxysporum* (Correll, 1991). Some *formae speciales*, however, have a complex relationship where more than one race can occur in a single VCG or where isolates of a single race belong to different VCGs (Correl *et al*., 1985; Ploetz *et al*., 1990). Vegetative compatibility can also be useful in distinguishing pathogens from non-pathogens, as well as characterizing genetic diversity within the

population (Correll, 1991). Some *F. oxysporum* pathogens have a high degree of VCG diversity, such as *F. oxysporum* f.sp. *lycopersici* (Elias and Schneider, 1991; Leslie, 1993; Katan and Di Primo, 1999). Analysis of non-pathogenic *F. oxysporum* populations has also resulted in the identification of a large number of VCG's (Correll *et al*., 1986; Gordon and Okamoto, 1992; Lori *et al*., 2004). The high degree of diversity in non-pathogenic strains of *F. oxysporum* may be useful in studies where non-pathogenic strains have been used as biological control agents (Schneider, 1984; Correll *et al*., 1986).

THE DISEASE

Penetration of the host plant by *F. oxysporum* involves germination of spores, adhesion of the pathogen to the host surface, and orientation of pathogen growth to a suitable infection site (Deacon, 1996). Isolates of *F. oxysporum* remain dormant as chlamydospores in decayed host tissue or in the soil until stimulated by host roots, root exudates of non-hosts, or when they come into contact with pieces of fresh non-colonized plant remains (Stover, 1962, Beckman, 1990). After germination, hyphae are produced that adhere to the host root surface before fungal infection commences (Bishop and Cooper, 1983). The root tips of tap and lateral roots are the natural and initial sites of infection (Beckman, 1990), but the fungus can also penetrate root hairs or epidermal cells near the root cap, behind the root tip or within the zone of elongation (MacHardy and Beckman, 1981). The pathogen then move inter- and intracellularly through the root parenchyma tissue until they reach the protoxylem vessels (Mai and Abawi, 1987) from where they invade the large reticulate vessels and spread from vessel to vessel through the pits in the vessel wall. Wounds that expose the vascular elements greatly enhance infection and disease incidence and severity (Stover, 1972). In banana, direct penetration occurs infrequently or not at all, and wounds are essential for vascular infection (MacHardy and Beckman, 1981).

Wilting symptoms are the result of fungal spores and mycelium that block the xylem, toxin production, and host defence responses such as tyloses, gums and gels (Beckman, 1987). External symptoms include vein clearing, leaf epinasty, wilting, chlorosis, necrosis and abscission, and internal symptoms involve vascular browning (MacHardy and Beckman, 1981). As long as the host plant is alive, *F. oxysporum* will

remain in the xylem tissue. Severely infected plants eventually wilt and die, and the chlamydospores are released back to the soil in the infected and decaying host tissue (Nash *et al*., 1961; Di Pietro *et al*., 2003) where they remain viable for several years (Ploetz and Pegg, 2000). The disease cycle is repeated when the chlamydospores germinate and invade a new host plant (Stover, 1962).

CONTROL OF FUSARIUM WILT

Fusarium wilt diseases are difficult to control (Borrero *et al*., 2006; Elmer, 2006). Control methods that were investigated against Fusarium wilt include chemical, biological and cultural control methods, and the use of disease resistant varieties. Of these methods, the use of resistant planting material is the most effective means of reducing disease, while a limited amount of success had been achieved by means of chemical and cultural control. In recent years, the use of biological control agents became popular as an environmentally friendly approach to Fusarium wilt control.

Chemical control

Fungicides used to minimise Fusarium wilt severity include the benzimidazole fungicides such as benomyl, carbendazim, fuberidazole, thiabendazole, thiophanate and thiophanate-methyl. They all generate methyl benzimidazole carbamate (MBC), either as the principal active ingredient, or as a breakdown compound formed on mixing with water. Benzimidazoles have a common mode of action that involves interfering with cell division and hyphal growth of sensitive fungi (Uesugi, 1998). They are apically systemic with a broad range of activity against ascomycetes, fungi imperfecti and basidiomycetes. Muskmelon plants treated with benomyl as a soil drench reduced infection by *F. oxysporum* f.sp. *melonis* (Maraite and Meyer, 1971). Benomyl was also partly effective as a root dip treatment and soil drench against *F. oxysporum* f.sp. *cubense* (Nel *et al*., 2007). Benomyl, followed by carbendazim, was effective in reducing *F. oxysporum* f.sp. *gladioli* (Ram *et al*., 2004). A root dip treatment with carbendazim against Fusarium wilt of tomato seedlings increased the yield by 24% (Khan and Khan, 2002). Seed treatment of chickpea with carbendazim (Bacistin^{TM}) is used to minimise effect of *F. oxysporum* f.sp. *ciceri* in infected fields (Dubey *et al*., 2007).

There is a constant threat that pathogens may become resistant to fungicide treatment. As various pathogens became resistant to methyl benzimidazole (Baldwin and Rathmell, 1988), other classes of fungicides were tested against *F. oxysporum*. The demethylation-inhibiting (DMI) fungicides (prochloraz, propiconazole and cyproconazole/propiconazole) act by inhibiting the demethylation step in the biosynthesis of sterols needed in fungal walls. Prochloraz proved to be the most effective fungicide against the Fusarium wilt pathogens of banana and tomato (Song *et al*., 2004; Nel *et al*., 2007). Strobilurins such as azoxystrobin, kresoxym-methyl and trifloxystrobin effectively controlled Fusarium wilt of carnation and azoxystrobin reduced Fusarium wilt on cyclamen and Paris Daisy (Gullino *et al*., 2002; Elmer and McGovern, 2004). *Fusarium oxysporum* f.sp. *cubense* and *F. oxysporum* f.sp. *dianthi* were inhibited by a phosphonate fungicide *in vitro* (Davis *et al*., 1994). Although there is great success with chemical control of Fusarium wilt in some crops, effective soil fungicide treatments for crops such as basil are unavailable (Reuveni *et al*., 2002; Borrero *et al*., 2006).

Apart from the use of fungicides, chemical treatment can also include the use of surface sterilants, fumigants and plant activators. Nel *et al*. (2007) showed that certain quaternary ammonium compounds were effective as sterilants against *F. oxysporum* f.sp. *cubense*. Other sterilants that had been used successfully against Fusarium wilt diseases include formaldehyde, copper sulphate and copper oxychloride (Weststeijn, 1973; Moore *et al*., 1999). Soil fumigation with methyl bromide (Herbert and Marx, 1990) showed that the Fusarium wilt pathogen of banana reinvaded the soil within 3 years. A lot of research has gone into finding an alternative to replace methyl bromide, since it was banned in 2005 (Cebolla *et al*., 2000; Tamietti and Valentino, 2006). Fumigants such as a combination of 1,3-dichloropropene and chloropicrin were proposed as replacements of methyl bromide in the control of *F. oxysporum* f.sp. *lycopersici* (Gilreath and Santos, 2004). Soil solarization reduced Fusarium wilt incidence in melon by 82-90% and also provided good control of tomato wilt (Katan and DeVay, 1991; Sivan and Chet, 1993; Tamietti and Valentino, 2006).

Plant activators such as 2,6-dichloroisonicotinic acid (INA) and benzo-(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), commercially known as

Bion®, are the best studied chemical elicitors available (Oostendorp *et al*., 2001). Both are functional analogs of salicylic acid, and elicit a systemic form of induced resistance across a broad range of plant–pathogen interactions (Friedrich *et al*., 1996; Vallad and Goodman, 2004). One of the requirements of plant activators is that they do not display any antimicrobial activity (Kessmann *et al*., 1994). For example, INA showed no antagonistic activity against *F. oxysporum* f.sp. *cucumerinum* and validamycin A (VMA) and validoxylamine A (VAA) were also not antifungal against *F. oxysporum* f.sp. *lycopersici* in *in vitro* tests (Métraux *et al*., 1991; Ishikawa *et al*., 2005). Another plant activator, ß-Aminobutyric Acid (BABA), has been shown to induce resistance in melon and watermelon against *F. oxysporum* f.sp. *melonis* and *F. oxysporum* f.sp. *niveum*, respectively, when applied as a soil and root drench (Cohen, 1996; Ovadia *et al*., 2000). Chitin and chitosan amendments reduced Fusarium wilt of radish and celery when small amounts were added to the soil (Mitchell and Alexander, 1962; Bell *et al*., 1998). Chitosan and chitin are known to be potential elicitors in plant defense responses, and have proved to stimulate chitinases and formation of wall appositions in tomato plants (Benhamou and Theriault, 1992).

Cultural control

Soil amendments have been used to reduce the severity of Fusarium wilt diseases. The application of calcium as $CaCO_3$ or $Ca(OH)_2$ to Fusarium wilt conducive soils significantly decreased the germination of chlamydospores of *F. oxysporum* f.sp. *cubense* (Chuang, 1991; Peng *et al*., 1999). The composition of nitrogen fertiliser added to the soil may also influence pH and disease severity in the field. Nitrogen fertilisers containing nitrate nitrogen (NO_3-N) generally leads to less Fusarium wilt than fertilisers containing ammonium nitrogen (NH_4-N). It has been found that NH_4-N is necessary for germtube growth of *F. oxysporum* (Ciotola *et al*., 2000) and NO_3-N supports saprophytic growth (Papavizas, 1969; Huber and Watson, 1974). Also, NO_3-N (such as liming) increases the pH of the soil, which leads to low levels of micronutrients. Micronutrients such as zink, copper, iron, phosphate, magnesium and manganese are necessary for growth and sporulation of the pathogen (Scher and Baker, 1982; Handreck and Black, 2002). Higher soil pH levels reduce Fusarium wilt development in crops by enhancing bacterial activity (Domínguez *et al*., 2001) and reducing germination of *F. oxysporum* chlamydospores (Woltz and Jones, 1981;

Chuang, 1991). Increased phosphate rates above the level needed to grow the crop can increase the severity of Fusarium wilt in cotton and muskmelon (Jones *et al*., 1989). Woltz and Jones (1981) demonstrated that Fusarium wilt of tomato was reduced in low phosphate soils making the pathogen more vulnerable than the host.

Cultural control strategies that were used successfully to reduce the impact of Fusarium wilt diseases include crop rotation, flood fallowing, sterilants and the use of clean planting material. In Taiwan, crop rotation of paddy rice with banana for 3 years reduced the disease incidence from 40 to 3.6% (Su *et al*., 1986). Crop rotation of cotton in China, also with rice, is followed as part of an integrated management programme for Fusarium wilt (Shen, 1985). Extensive watermelon production was increased with crop rotation with crops such as peppers and tomatoes in Spain (Miguel *et al*., 2004). Flooding is detrimental for the survival of race 4 of *F. oxysporum* f.sp. *cubense* in soil due to the creation of an anaerobic environment (Sun, 1977) and fallow has been used to control fusarial wilt of bananas in tropical America (Stover, 1962). Sterilants are used to disinfect equipment, vehicles and other implements to prevent the spread of Fusarium wilt in the field or from one area to another. In Australia "Farmcleanse" is used and in South Africa copper oxychloride has been replaced by a quaternary ammonium compound called 'Sporekill' as disinfectant in order to control the spread of *F. oxysporum* f.sp. *cubense* (Moore *et al*., 1999; Nel *et al*., 2007). Formaldehyde was used on glasshouse structures to kill macroconidia of the Fusarium wilt pathogen of tomato (Weststeijn, 1973). Micropropagated plants are free of fungal and bacterial pathogens and can help to prevent the spread of Fusarium wilt in an uninfected field. Micropropagated plantlets are the most reliable source of clean material for planting banana plants in the field (Ploetz and Pegg, 2000). To avoid Fusarium wilt in basil, certified *F. oxysporum* f.sp. *basilici*-free basil seeds must be used whenever possible (Garibaldi *et al*., 1997). Disease-free ginger clones performed well under field conditions and well-developed rhizomes did not rot during storage for up to 6 months (Sharma and Singh, 1997).

Disease resistance

Resistance to Fusarium wilt diseases can be introduced into crops by means of conventional and unconventional breeding. By means of conventional breeding,

resistance is introduced from parent plants to the offspring when either one parent contributes characteristics such as improved yield, nutrition and shorter cycle time through the process of sexual recombination (Hwang, 1999). Using conventional breeding, resistance have been developed to Fusarium wilt in chickpea (Haware *et al*., 1992), cotton (Ulloa *et al*., 2006), cowpea (Rigert and Foster, 1987) and date palm (Djerbi *et al*., 1986) have been developed. Where propagation of planting material relies on clonal propagation, as is the case with banana, unconventional breeding strategies have to be used for crop improvement. Such unconventional strategies include the use of somaclonal variation, induced mutations, protoplast culture and genetic transformation (Crouch *et al*., 1998).

Somaclonal variation in crops is achieved by prolonged *in vitro* culture due to nuclear chromosomal re-arrangement, gene amplification, non-reciprocal mitotic recombination, transposable element activation, point mutations and reactivation of silent genes (Jain, 2001). By means of somaclonal variation, Cavendish banana selections were made in Taiwan with good tolerance to Fusarium wilt (Hwang and Ko, 2004). Mutations for plant improvement can be induced by chemical treatment or gamma irradiation (Bhagwat and Duncan, 1998). The dosage and time of exposure during gamma irradiation determines the mutation rate (Bhagwat and Duncan, 1998). Chemicals such as ethyl methane sulphonate and diepoxybutane induce mutations in banana, rice and tomato plants (Van den Bulk *et al*., 1990; Bhagwat and Duncan, 1998; Wu *et al*., 2005). In protoplast culture, the genetic pool of plants can be widened by means of protoplast fusion (Davey *et al*., 2005). This method is employed for the production of normal hybrid plants where sexual recombination is not possible (Marshall, 1993). Protoplast-derived tomato plants showed resistance against *F. oxysporum* f.sp. *lycopersici* (Shahin and Spivey, 1986).

Genetic modification of plants is achieved by introducing foreign genes into plant genomes by means of *Agrobacterium*-mediated transformation and particle bombardment (Ságí *et al*., 1995; Ganapathi *et al*., 2001; Van Bel *et al*., 2001). *Agrobacterium*-mediated transformation was used to introduce a human lysozyme (HL) gene under the control of the constitutive cauliflower mosaic virus 35S promoter into banana (Pei *et al*., 2005). It has been shown that HL inhibits *F. oxysporum* f.sp. *cubense in vitro*. After 60 days, 24 transgenic banana plants showed no Fusarium wilt

symptoms in the greenhouse, and two transgenic plants remained healthy following field testing. In particle bombardment, DNA is coated on microcarriers and transferred to the cytoplasm of cells by force (Gasser and Fraley, 1989). Particle bombardment was used to successfully introduce resistance against Fusarium wilt in asparagus, banana and cotton (Cabrera-Ponce *et al*., 1997; Côte *et al*., 1997; Becker *et al*., 2000; Zhang *et al*., 2000). Genetically modified bananas with resistance to Fusarium wilt are not yet commercially available.

Biological control

Biological control agents are used to manage Fusarium wilt because of environmental and economical constraints associated with other control strategies. Biological control can be used as sole disease management approach, or combined with other control methods in an integrated disease management strategy.

BIOLOGICAL CONTROL OF FUSARIUM WILT

Difficulties in controlling Fusarium wilt diseases without the excessive use of chemicals has stimulated renewed interest in biological control as a disease management alternative (Borrero *et al*., 2006). Suppressive soils are good sources of potential biocontrol agents. Once a putative biological control agent has been identified, it becomes important to find the mechanisms whereby it controls the pathogen in order to find efficient ways to apply and manage *F. oxysporum*. The biocontrol agent must also be safe to humans and plants so that it can be used in the field.

Suppressive soils

Soils where high levels of production can be maintained despite the presence of the pathogen, a susceptible host plant, and climatic conditions favourable for disease development are referred to as suppressive soils (Alabouvette *et al*., 1993; Hoitink *et al*., 1993). Soil may exert its influence through its physiochemical characteristics, its biological characteristics, or both (Alabouvette *et al*., 1996). The physical and chemical characteristics include soil texture and structure, soil water, clay type, pH,

micronutrients and organic matter (Louvet *et al*., 1981; Alabouvette *et al*., 1996). Microorganisms and their metabolites represent the biological component of suppressive soils (Alabouvette *et al*., 1996). For instance, the fluorescent Pseudomonads produce several types of metabolites such as siderophores and antibiotics that can compete and are toxic to Fusarium wilt pathogens, respectively (Leeman *et al*., 1996; Schouten *et al*., 2004).

Mechanisms of biological control

Biological control agents reduce disease severity through direct or indirect antagonism against the pathogen (Alabouvette and Lemanceau, 2000). Direct antagonism implies the interaction between two microorganisms that share the same ecological niche, and includes competition for nutrients and antibiosis (Alabouvette and Lemanceau, 2000). Indirect antagonism involves a reduced disease severity by means of induced disease resistance in plants (Olivain *et al*., 1995; Fuchs *et al*., 1997). A single strain of the biocontrol organism may express one or several modes of action (Whipps, 2001). The modes of action by which a disease can be reduced may not necessarily be exclusive, and may involve the complementary effect of microbial antagonism and induced resistance (Duijff *et al*., 1998, 1999; Alabouvette and Lemanceau, 2000). In most cases, the mechanisms of control have been demonstrated *in vitro* or under controlled greenhouse conditions, but have not been investigated in the field.

Antibiosis

Some microorganims can produce secondary metabolites that are toxic to other microorganisms (Lorito *et al*., 1993; Milner *et al*., 1995; Keel *et al*., 1996). The broad-spectrum antibiotic 2,4 – diacetylphloroglucinol has been shown to play a key role in biological control of various plant pathogens, including *F. oxysporum* (Duffy *et al*., 2004). The enzyme ß-1,3-glucanase, produced by *Streptomyces* sp. strain 385, can lyse the cell walls of *F. oxysporum* f.sp. *cucumerinum*, the Fusarium wilt pathogen of cucumber (Singh *et al*., 1999), while the endophytic *Streptomyces* strain NRRL 30562 inhibited *F. oxysporum in vitro* (Castillo *et al*., 2002). *In vitro* studies performed by Suárez-Estrell *et al*. (2007) showed that *Trichoderma harzianum* Rifai inhibited the growth of *F. oxysporum* f.sp. *melonis*, and *Bacillus subtillis* and *T.*

harzianum inhibited fungal growth of *F. oxysporum* f.sp. *ciceris* (Hervás *et al*., 1998). In most cases it is bacteria such as *Pseudomonas* spp., *Bacillus* spp. and *Streptomyces* spp. that consistently show antibiosis as mode of action against *F. oxysporum* pathogens (Landa *et al*., 1997; Sturz *et al*., 1999; Getha and Vikineswary, 2002; Taechowisan *et al*., 2005). To determine whether a specific metabolite is responsible for antagonistic behaviour, mutants are produced from the biocontrol agent that are unable to synthesize that specific metabolite, and these mutants are then tested to see if they lost their ability to reduce disease in plants (Weller and Thomashow, 1993).

Competition

Competition for carbon (C) is one of the primary mechanisms involved in soils suppressive to Fusarium wilts (Sivan and Chet, 1989; Alabouvette and Lemanceau, 2000). Alabouvette and Couteaudier (1992) showed that some non-pathogenic *F. oxysporum* strains competed more efficiently for C than the other non-pathogenic strains. Non-pathogenic *F. oxysporum* strains also compete with the pathogenic strain for C and reduced disease severity of Fusarium wilt of flax much better (Alabouvette and Couteaudier, 1992). Competition for the minor element iron is another way whereby especially fluorescent Pseudomonads can inhibit pathogens (Leong, 1986). Siderophores are low molecular weight molecules that are secreted by *P. fluorescens* to take up iron from the environment (Höfte, 1993). These siderophores effectively compete for iron with microorganisms that produce siderophores in lower concentrations or with a lower affinity for iron, and that are unable to use the siderophore produced by the suppressing strain (Bakker *et al*., 1987). Siderophores produced by fluorescent Pseudomonads enhance the microbial acquisition of iron in an iron-deficient environment (Neilands, 1973). High iron availibility and the addition of siderophore-producing *Pseudomonas* spp. reduced Fusarium wilt incidence of radish, flax and cucumber (Scher and Baker, 1982; Leeman *et al*., 1996).

Competition for root area plays a role in reducing Fusarium wilt. An experiment conducted by Olivain *et al*. (2006) showed that the non-pathogenic and pathogenic strains of *F. oxysporum* compete for infection sites behind the apex of the growing root. *Pseudomonas* spp. and other plant growth-promoting rhizobacteria (PGPR) compete for root nutrients rich in carbon sinks (sugars) (Rovira, 1965), amino acids (Simons *et al*., 1997) and organic acids (Welbaum *et al*., 2004), which the PGPR

utilize (Lugtenberg *et al*., 1999). The potential biocontrol agent must have the ability to establish effective root colonization and the ability to survive on the plant roots for a considerable time period in the presence of indigenous microflora to achieve rhizosphere competence (Lugtenberg and Dekkers, 1999). Bolwerk *et al*. (2003) used confocal laser scanning microscope analyses to show that *P. fluorescens* and *Pseudomonas chlororaphis* effectively competed for the same niche and for root exudates on the tomato root against *F. oxysporum* f.sp. *lycopersici*.

Induced resistance

Induced resistance is the process whereby the detrimental effect of a pathogen on a plant is reduced by prior treatment with an elicitor (Van Loon, 1997;Van Loon *et al*., 1998). Thereafter, when the host plant is challenged by the pathogen, the plant triggers a cascade of events that leads to the induction of chemical and structural defense responses such as accumulation of reactive oxygen species, phenolics, hydrolytic enzymes and phytoalexins (Niemann *et al*., 1990). There are two types of induced resistance, namely locally acquired resistance (LAR) and systemic resistance (SR). In cases where resistance is not translocated and leads to a hypersensitive response, the form of resistance is referred to as LAR (Siegrist *et al*., 2000). In this instance, necrotic or dying cells are visible at the area of infection (Van Loon, 1997). Systemic resistance is transferred to tissue distant from the infection site, and can be divided into systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is effective against a wide range of pathogens (Vallad and Goodman, 2004), and is usually induced by chemicals and non-pathogenic organisms and triggers the accumulation of salicylic acid and pathogenesis-related (PR) proteins (Sticher *et al*., 1997; Van Loon *et al*., 1998). Bacteria such as PGPR stimulate ISR (Van Loon *et al*., 1998) by triggering the ethylene and jasmonic acid-regulated pathways (Pieterse *et al*., 1996).

Several biological control agents are known to induce SR in plants. These include PGPR such as *Pseudomonas* spp. and fungi such as non-pathogenic *F. oxysporum*. It has been demonstrated that non-pathogenic *F. oxysporum* reduced Fusarium wilt through ISR of banana (Gerlach *et al*., 1999), cucumber (Mandeel and Baker, 1991), watermelon (Larkin *et al*., 1996) and tomato (Olivain *et al*. 1995; Fuchs *et al*. 1997). *Pseudomonas fluorescens* is also able to induce resistance against Fusarium wilt

pathogens of carnation (Van Peer *et al*., 1991), watermelon (Larkin *et al*., 1996) and tomato (Duijff *et al*., 1998).

Biological control agents

Root-colonizing plant-beneficial bacteria and fungi are important in protecting plants from root pathogens (Haas and Défago, 2005). The principal groups of plant-beneficial organisms controlling Fusarium wilt diseases consist of bacterial species belonging to *Pseudomonas* and *Bacillus*, and non-pathogenic *F. oxysporum* (Fravel *et al*., 2003; Haas and Défago, 2005). Several other microbes have been reported to reduce Fusarium wilt incidence. These include the actinomycetes (Meredith, 1943; Cao *et al*., 2005), and fungi such as *Trichoderma* spp. (Harman *et al*., 2004) and *Gliocladium* spp. (Sivan and Chet, 1986). Biocontrol organisms alone have the ability to reduce disease incidence, but often perform more efficiently when used in combination with other biocontrol agents and different integrated disease management strategies.

Pseudomonas and *Bacillus* spp.

Pseudomonas fluorescens can be isolated from the root rhizosphere as PGPR or from inside plant tissue as an endophyte (Gray and Smith, 2005). PGPR competitively colonize plant roots, and stimulate plant growth or reduce the incidence of plant disease (Kloepper and Schroth, 1978). Endophytic and PGPR *P. fluorescens* control Fusarium wilt with mechanisms that include production of antifungal compounds, siderophore production, nutrient competition, niche exclusion, and induction of systemic resistance (Cook and Baker, 1983; Chen *et al*., 1995).

Pseudomonas fluorescens produces the broad-spectrum antibiotic 2,4-diacetylphloroglucinol (Keel *et al*., 1996) that inhibits mycelial growth of *F. oxysporum* (Schouten *et al*., 2004). *Bacillus* spp. produce the antibiotic zwitermicin A to help them establish in the rhizosphere (Milner *et al*., 1995). Under iron-limiting conditions, *P. fluorescens* produces low molecular weight compounds called siderophores to acquire iron (Whipps, 2001) and this leads to natural suppressiveness in soil and competition for root niches (Scher and Baker, 1982; Compant *et al*., 2005). Leeman *et al*. (1996) showed that siderophores produced by *P. fluorescens* at low iron

availability were involved in the induction of systemic resistance against Fusarium wilt in radish. *Pseudomonas putida* strain B10 suppressed Fusarium wilt in iron-limiting conditions in the soil (Kloepper *et al*., 1980). De Weert *et al*. (2002) and Bolwerk *et al*. (2003) found that *P. fluorescens* and *P. chlororaphis* multiplied and reached the tomato root much faster than the pathogen, thus competing for root exudates and root niches. On tomato roots, *Pseudomonas* spp. reduced the density of the *F. oxysporum* hyphae at day seven, and Bolwerk *et al*. (2003) hypothesised that the tomato root might have leaked exudates which the bacteria were utilizing more effectively, thus preventing the pathogen from colonizing and penetrating the roots.

Fluorescent *Pseudomonas* isolated from disease suppressive soil can reduce Fusarium wilt by inducing disease resistance that is systemically transferred to all plant roots (Van Loon *et al*., 1998; Pieterse *et al*., 2001). The *P. fluorescens* strain WCS 417 induced resistance in carnation against Fusarium wilt in cultivars ranging from resistant to susceptible (Van Loon *et al*., 1998), and strain 63-28 increased resistance of tomato plants against *F. oxysporum* f.sp. *radicis-lycopersici* (M'Piga *et al*., 1997). Leeman *et al*. (1995) showed that the lipopolysaccharides of *P. fluorescens* induced resistance against Fusarium wilt of radish, and Van Peer *et al*. (1991) demonstrated that phytoalexin production in the carnation plant increased after root colonization of *P. fluorescens* and inoculation with *F. oxysporum* f.sp. *dianthi*. Thangavelu *et al*. (2003) showed that phenolic content of banana plants inoculated with *P. fluorescens* increased steeply upon inoculation with *F. oxysporum* f.sp. *cubense*. Tomato plants treated with *Bacillus pumilus* strain SE34 had an increase in cell wall density and accumulation of polymorphic deposits, which reduced the severity of wilt caused by *F. oxysporum* f.sp. r*adici-lycopersici* (Benhamou *et al*., 1998).

Non-pathogenic Fusarium oxysporum

Non-pathogenic *F. oxysporum* occurs naturally in almost all agricultural soils (Alabouvette *et al*., 2001), and spend part of their life cycle inside plant tissues as endophytes without causing visible symptoms (Wilson, 1995; Ito *et al*., 2005). Non-pathogenic *F. oxysporum* have the ability to control the population of pathogenic *F. oxysporum* by competition for infection sites (Olivain *et al*., 2006) and nutrients (Couteaudier and Alabouvette, 1990), as well as to induce systemic resistance (Edel *et al*., 1997; Fuchs *et al*., 1997; He *et al*., 2002). The production of specific metabolites

has not been demonstrated in non-pathogenic *F. oxysporum* (Alabouvette *et al*., 1996), while reports on the antifungal effect that endophytes have on plants and other fungi are rare (Schardl *et al*., 2004). The advantage of using non-pathogenic strains of the same or closely related species as the pathogen is that these biocontrol agents have similar environmental requirements (Larkin and Fravel, 2002).

A large number of non-pathogenic and pathogenic *F. oxysporum* strains in the soil can lead to competition for nutrients and fungistasis (inhibition of chlamydospores germination) (Mandeel and Baker, 1991). Competition for infection sites is another method by which disease incidence can be reduced (Mandeel and Baker, 1991; Freeman *et al*., 2002). Mandeel and Baker (1991) found that competition for C was the reason for fungistasis of *F. oxysporum* f.sp. *cucumerinum*. Freeman *et al*. (2002) generated *F. oxysporum* f.sp. *melonis* mutants by UV mutagenesis, and demonstrated that the non-pathogenic strains reduced Fusarium wilt incidence of muskmelon and watermelon under controlled environmental conditions. No parasitism, hyphal interference or toxin production was observed, suggesting that the non-pathogenic strains competed more efficiently than the pathogen for infection sites and nutrients (Freeman *et al*., 2002). Different non-pathogenic *F. oxysporum* strains may also vary in their ability to utilize C and, thus, the reduction of disease severity by the different strains may be inconsistent (Couteaudier and Alabouvette, 1990). When the non-pathogenic *F. oxysporum* isolate Fo47 was applied at a higher concentration than the Fusarium wilt pathogen of tomato, they attached to more sites on the roots and their spores germinated faster, thus limiting the attachment sites for the pathogen (Bolwerk *et al*., 2005). Olivain *et al*. (2006), however, showed that there are no real infection sites, and that pathogenic and non-pathogenic *F. oxysporum* isolates colonise the tomato root at random. For the non-pathogenic *F. oxysporum* to prevent infection by the pathogen, the non-pathogen therefore has to cover the entire root surface.

Non-pathogenic isolates of *F. oxysporum* have demonstrated an ability to induce a resistance response to pathogenic isolates on several agricultural crops. When applied to tomato roots, Fo47 was able to increased chitinase, β-1,3-glucanase, and β-1,4-glucosidase activity, thereby reducing attack by *F. oxysporum* f.sp. *lycopersici* (Fuchs *et al*., 1997). Non-pathogenic *F. oxysporum* also increased the activities of peroxidases, phenylalanine ammonia-lyase and lignin content in asparagus after

inoculation with *F. oxysporum* f.sp. *asparagi* (He *et al*., 2002). Biles and Martyn (1989) treated watermelon with non-pathogenic *F. oxysporum* isolates 24 and 72 hours before inoculation with the pathogen. The plants inoculated 3 days later showed enhanced resistance to the pathogen (Biles and Martyn, 1989).

Other microorganisms reducing Fusarium wilt

Actinomycetes are gram-positive bacteria with a fungal-like growth habit. *Actinomyces, Mycobacterium* and *Streptomyces* are some of the representative genera in this phylum. The genus *Streptomyces* in the family *Streptomycetaceae* is active in the rhizosphere, and their modes of action include antibiotic production, lysis of fungal cell walls, competition and hyperparasitism (Mohammadi and Lahdenperä, 1992; Minuto *et al*., 2006). *Streptomyces* spp. produce the antibiotic oligomycin A that have inhibitory activity against filamentous fungi and this aid in the colonization of the rhizosphere (Kim *et al*., 1999). Extracellular chitinases produced by *Streptomyces* spp. strain 385 suppressed *F. oxysporum* f.sp. *cucumerinum* (Compant *et al*., 2005). *Streptomyces griseoviridis* reduced the spread of Fusarium wilt of carnations and increased the yield (Lahdenperä, 1987). Endophytic *Actinomycetes*, which were identified as *Streptomyces griseorubiginosus*, were isolated from healthy and wilting banana roots and leaves and showed antagonistic behaviour towards *F. oxysporum* f.sp. *cubense* (Cao *et al*., 2004; 2005).

Fungi such as *Trichoderma* and *Gliocladium* spp. have also been studied for their ability to reduce disease severity (Paulitz and Bélanger, 2001). *Gliocladium virens* (=*Trichoderma virens*) Miller, Giddens & Foster, produces toxic substances such as gliotoxin and gliovirin that are released into the soil (Howell and Stipanovic, 1995). *Trichoderma harzianum* T-22 was tested against *F. oxysporum* f.sp. *asparagi* on asparagus but could only control the pathogen at low inoculum levels (Reid *et al*., 2002). This strain has also been shown to colonize all parts of the tomato root system, to persist for long periods in the soil, and to reduce Fusarium crown and root rot of tomato (Datnoff *et al*., 1995). The mechanisms of action of *G. virens* and *Trichoderma* spp. in cucumber and cotton are mycoparasitism and the production of chitinases, β-1,3-glucanases, cellulases and peroxidases (Yedidia *et al*., 2000, Soresh *et al*., 2005), antibiotics (Howell and Stipanovic, 1995; Zhang *et al*., 1996) and induced resistance (Yedidia *et al*., 1999; Viterbo *et al*., 2005). *Trichoderma* spp. also

reduce disease severity by pathogenic *F. oxysporum* by competing for C (Sivan and Chet, 1989). The germination rate of *F. oxysporum* f.sp. *melonis* and *F. oxysporum* f.sp. *vasinfectum* chalmydospores was significantly reduced in soil amended with 0.4 mg glucose/g soil and conidia of *T. harzianum* (t-35) (Sivan and Chet, 1989). *Aspergillus* spp. isolated from mature compost inhibited the growth of *F. oxysporum* f.sp. *melonis in vitro* and *in vivo* (Suárez-Estrella *et al*., 2007).

Mycorrhizae

Roots of most plants form a symbiotic relationship with certain kinds of zygomycete, ascomycete and basidiomycete fungi and the infected roots are transformed into unique morphological structures called mycorrhizae (Azcón-Aguilar and Barea, 1997; Agrios, 2005). The way the hyphae of the fungi are arranged within the cortical tissues of the roots determines the type of mycorrhizae, namely ectomycorrhizae (intercellularly) or endomycorrhizae (intracellularly) (Agrios, 2005). Endomycorrhizae are the most common mycorrhizae and their fungal hyphae grow in the cortical cells of the feeder roots with specialised feeding hyphae, called arbuscules, or food-storing hyphal swellings called vesicles (Agrios, 2005). Some endomycorrhizae contain both these hyphae and are called vesicular-arbuscular mycorrhizae (VAM). The mycorrhizae benefit from gaining organic nutrients from the plant, and in turn, the plant benefits by enhanced water and nutrient uptake, increased growth and yield and protection against soilborne pathogens (Harley and Smith, 1983; Linderman, 1994; Smith and Read, 1997; Dakora, 2003).

Reduced Fusarium wilt severity of alfalfa, banana, cucumber and strawberry can be achieved using mycorrhizae fungi. In alfalfa, *Glomus fasciculatus* (Thax.) Gerd. & Trappe and *Glomus mosseae* (Nicolaj & Gerd.) Gerd. & Trappe increased the shoot weights and reduced Fusarium wilt incidence (Hwang, 1992). In cucumber seedlings, arbuscular mycorrhizae (AM) inoculation lead to higher levels of secondary metabolites and phosphate levels resulting in increased resistance to wilt diseases (Zhipeng *et al*., 2005). Strawberry plants were inoculated with five different AM *Glomus* spp. and Fusarium wilt incidence was 22.2% compared to the 100% in non-AM plots (Matsubara *et al*., 2004). Jaizme-Vega *et al*. (1998) applied two AM fungi (*Glomus* spp.) to micro-propagated banana plantlets (Grand Naine) in the greenhouse to enhance plant development and nutrient uptake. The AM fungi reduced both

internal and external symptoms of Fusarium wilt, but long-term protection of banana by AM fungi against Fusarium wilt of banana has not yet been demonstrated (Ploetz *et al*., 2003).

Endophytic biological control organisms

Endophytes may alter the physiological, developmental and morphological properties of host plants by enhancing their competitiveness, especially in stressful environments (Bacon, 1993; Malinowski and Belesky, 1999). Cook (1993) also stated that microorganisms isolated from roots of the target host plant are better candidates for selection of effective agents because they are already associated with that plant species and with the physical environment under which they must operate. These endophytes can be isolated from roots, stems rhizomes and leaves and the presence of endophytes has been demonstrated in all plants investigated including important crops such as banana (Photita *et al*., 2001), rice (Fisher and Petrini, 1992) and tomato (Hallman and Sikora, 1994).

Endophyte-plant relationships are diverse, with numerous bacterial species found within virtually every plant part in a multitude of plant species (McInroy, 1993). Endophytic bacteria survive within cortical or vascular tissues (Patriquin and Dödereiner, 1978) of plants and are provided with a protected environment when compared with the rhizosphere and the phylloplane where they must compete for nutrients and endure environmental fluctuations (Chen *et al*., 1995). Endophytic bacteria can be established as pre-selected beneficial organisms and may overcome the failure of certain biocontrol agents to efficiently control a disease due to poor rhizosphere competence (Sturz and Nowak, 2000). Chen *et al*. (1995) found that the endophytic bacteria *B. pumilus* and *P. putida* from the internal tissue of cotton reduced the disease severity of vascular wilt of cotton caused by *F. oxysporum* f.sp. *vasinfectum*. Nejad and Johnson (2000) showed that *Pseudomonas* spp. isolated from inside the roots of tomato were able to improve growth of tomato seedlings and reduced Fusarium wilt severity.

Non-pathogenic *F. oxysporum* can spend part of their life cycle inside plant tissues without causing visible symptoms (Wilson, 1995). Non-pathogenic *F. oxysporum* isolates benefit their plant host by acquisition of limiting nutrients and increasing

competitive abilities (Paracer and Ahmandjian, 2000). Dhingra *et al*. (2006) found that the suppressive effect of endophytic *F. oxysporum* to suppress Fusarium wilt of beans was due to saprophytic competitiveness and that it reduced the availability of infection sites. Gerlach *et al*. (1999) and Nel *et al*. (2006) both demonstrated that non-pathogenic endophytic *F. oxysporum* isolated from disease suppressive soils significantly protected banana plants against Fusarium wilt in greenhouse trials. Also, endophytic *F. oxysporum* has been isolated and shown to protect crops such as cowpea and tomato against Fusarium wilt in the greenhouse (Ito *et al*., 2005; Rodrigues and Menezes, 2005).

Combining different biological control agents

Biological control of pathogenic *F. oxysporum* in the root rhizosphere can be enhanced by using combinations of biocontrol agents, particularly if they exhibit different or complementary modes of actions (Whipps, 2001). Combining different strains of *P. fluorescens* enhanced disease suppression of Fusarium wilt of radish more than when using one strain alone (De Boer *et al*., 1999). The combination of *P. fluorescens, T. harzianum* and *Trichoderma viride* Pers.:Fr performed well against *F. oxysporum* f.sp. c*ubense* to reduce wilt incidence in banana plants (Saravanan *et al*., 2003). Olivain *et al*. (2004) combined non-pathogenic *F. oxysporum* strain Fo47 with the C7 strain of *P. fluorescens* to enhance disease suppression of Fusarium wilt of tomato. The combination of non-pathogenic *F. oxysporum* and the *P. fluorescens* also reduced Fusarium wilt in flax (Éparvier *et al*., 1991), watermelon (Larkin *et al*., 1996) and tomato (Lemanceau and Alabouvette, 1991; Duijff *et al*., 1998) more effectively together than alone. Duijff *et al*. (1999) found that the combination of *P. putida* and the non-pathogenic *F. oxysporum* inhibited *F. oxysporum* f.sp. *lini*.

The combination of different biocontrol agents does not always provide greater protection against Fusarium wilt diseases. In such cases the individual non-pathogenic *F. oxysporum* strains reduce Fusarium wilt diseases more effectively. Larkin and Fravel (1998) performed an experiment on tomato and found that no improvement in disease control of Fusarium wilt above that obtained by non-pathogenic *F. oxysporum* alone was detected with the use of combinations with bacteria such as *P. fluorescens* and *Burkholderia cepacia* and other fungi such as *Fusarium* spp., *Trichoderma* spp. and *G. virens*. In another experiment, non-pathogenic *F. oxysporum* in combination

with *B. subtillis* or *T. harzianum* did not reduce Fusarium wilt of chickpea better than it did alone (Hervás *et al*., 1998).

Combining biological control agents with other control strategies

The combination of a chemical control agent and a biocontrol agent can lead to better Fusarium wilt control (Dubey *et al*., 2007). The chemical weakens the pathogen and other microflora in the soil, thus the inoculated biocontrol agent can flourish and provide better control of the disease (Henis and Papavizas, 1982). Minuto *et al*. (1995) and Elmer and McGovern (2004) combined fungicides with beneficial microorganisms and found that it reduced Fusarium wilt of cyclamen. Dubey *et al*. (2007) found that combining *Trichoderma* spp. with fungicide-treated seed reduced Fusarium wilt of chickpea better than the individual treatments.

Soil solarization has proven to reduce Fusarium wilt of cotton and watermelon (Katan *et al*., 1983; Ioannou *et al*., 1998). Growers are sceptical about using soil solarization, as soil needs to be free of cultivation for at least 4 weeks (Minuto *et al*., 2006). In combination with a biocontrol agent, the time period needed to solarize the soil is reduced and cultivation of the crop can start earlier (Minuto *et al*., 2006). Fusarium wilt of tomato has been reduced with a combination of *S. griseoviridis* and solarization (White *et al*., 1990; Minuto *et al*., 2006). Saravanan *et al*. (2003) showed that a combination of neem cake and *P. fluorescens* reduced *F. oxysporum* f.sp *cubense* race 1 infection of banana in the greenhouse and in the field. The use of sewage sludge compost and *Trichoderma asperellum* (=*T. atroviride*) Karsten reduced Fusarium wilt on tomato (Cotxarrera *et al*., 2002). In the production of high-quality basil, careful irrigation, fertility management, soil disinfestations and the application of antagonistic *Fusarium* spp. are applied (Garibaldi *et al*., 1997). In chickpea, Fusarium wilt severity was reduced by changing the sowing date in combination with *Bacillus* spp., *P. fluorescens* and non-pathogenic *F. oxysporum* application (Landa *et al*., 2004).

Factors affecting biological control

Biological control of Fusarium wilt diseases is often inconsistent, particularly under varying environmental conditions (Larkin and Fravel, 2002). Temperature, and soil

physical and chemical characteristics can affect the physiology of the host, disease development, and the interactions between pathogen and biocontrol agent (Larkin and Fravel, 2002). Biocontrol agents, therefore, may be introduced into environments in which they are ecologically unsuited (Deacon, 1991).

Temperature, soil pH and soil texture can influence the activity of *P. fluorescens* and its ability to produce siderophores that compete for soil iron (Leeman *et al*., 1996). Temperatures above 33°C suppress the growth of fluorescent *Pseudomonas*, and the optimal temperature for siderophore production is 28°C (Mattar and Digat, 1991). Clay soils favour bacterial activity and are less favourable to fungal growth (Stotzky and Rem, 1967). In such soils, limited amounts of the microelement iron induce *Pseudomonas* spp. to produce siderophores and inhibit the growth of the pathogen (Scher and Baker, 1982). Iron is also available at lower concentrations in alkaline or neutral soils, which favour siderophore production and iron competition by *P. fluorescens* (Lindsay and Schwab, 1991; Alabouvette *et al*., 1996).

Fluctuating pH and the composition of soils can influence the interaction between soil microbiota. Compost usually shows high bacterial activity and is antagonistic towards the Fusarium wilt pathogen. The compost used in a study by Cotxarrera *et al*. (2002) contained sewage sludge as raw material and contained a high C:N ratio. This high ratio leads to low levels of available ammonium (NH_4), which, in turn, reduced Fusarium wilt of tomato. The same compost also had an increased pH that lowered the availability of iron, zinc and copper. Copper reduces disease development by stimulating plant growth in combination with *Pseudomonas* spp. (Duffy and Défago, 1997). Zinc stabilizes the regulatory genes necessary for antibiotic production in fluorescent pseudomonads (Duffy and Défago, 1995). Organic matter may further contain growth regulators and antibiotics that can influence the microbial balance in the soil (Alabouvette *et al*., 1996, Steinberg *et al*., 2004). Williams and Vickers (1986) showed that humus soils and soils with a high clay content inactivate antibiotics and may influence potential biocontrol microorganisms whose main mode of action is antibiosis.

Non-pathogenic *F. oxysporum* has the same environmental requirements as pathogenic *F. oxysporum*, and is able to suppress the Fusarium wilt pathogen at its

optimum growth temperature of 10-35°C (Fravel *et al*., 1996). When kept in a controlled environment and at the optimum temperature of 27°C under intense disease pressure, however, a breakdown in biocontrol potential against *F. oxysporum* f.sp. *lycopersici* has been reported (Larkin and Fravel, 2002). Reapplication of the non-pathogenic strain to the field environment might be essential for the non-pathogen to keep optimum biocontrol activity if disease pressure is high.

CONCLUSION

Fusarium wilt is a highly destructive disease of many plants (Green, 1981) and is difficult to control. No effective control measure for Fusarium wilts of crops such as banana, basil, beans and tomato has been found other than the use of resistant varieties (Jones *et al*., 1991; Ploetz and Pegg, 2000; Reuveni *et al*., 2002; Dhingra *et al*., 2006). Although resistance is available, consumers often prefer the susceptible cultivar due to its taste and because of questions related to the use of genetically modified crops (Ploetz and Pegg, 2000; Malarkey, 2003; Dhingra *et al*., 2006). While tomato wilt-resistant cultivars are available and provide some degree of control, there is a constant threat that new races of the pathogen may develop (Borrero *et al*., 2006).

To minimise new infections and suppress *F. oxysporum* in soil, methods other than conventional disease management strategies must be investigated. While fungicides, sterilants and plant activators do provide some relief (Maraite and Meyer, 1971; Gullino *et al*., 2002; Khan and Khan, 2002), public concern about food safety and the use of chemicals must be recognized (Alabouvette and Lemanceau, 2000). Cultural control can be used to limit dissemination of the pathogen, especially when pathogen-free planting material is used. Biocontrol, however, seemed to have become one of the more favoured methods to control Fusarium wilt pathogens as part of an integrated management strategy. Biocontrol is environmentally safe option and involves the use of living microorganisms that are well-adapted to the environment from where they were isolated.

Two microorganisms have been particularly successful in control of Fusarium wilt diseases; the bacterium *P. fluorescens* WCS 417 and the non-pathogenic *F. oxysporum* isolate Fo 47. WCS 417 was isolated from the rhizosphere of wheat grown

in a field suppressive to take-all disease of wheat (Lamers *et al*., 1988), and Fo 47 was found in soil naturally suppressive to Fusarium wilt of tomato and melon at Châteaurenard, France (Alabouvette, 1986). In subsequent trials, WCS 417 has proved to significantly reduce Fusarium wilt of banana, carnation and tomato (Duijff *et al*., 1998; Van Loon *et al*., 1998; Nel *et al*., 2006), and non-pathogenic *F. oxysporum* reduced Fusarium wilt of beans, chickpea, tomato and watermelon in the greenhouse (Larkin *et al*., 1996; Hervás *et al*., 1998; Larkin and Fravel, 2002; Dhingra *et al*., 2006). For large-scale application, it is important that microorganisms be selected in the countries where they will be applied, as their introduction into foreign countries is often not feasible or desirable (Dhingra *et al*., 2006). It is also important to demonstrate that the biological control agent can render disease suppression under fluctuating environmental conditions. As combinations of microorganisms are often more useful for disease control than when applied separately, their modes of action should be properly understood to optimise their implementation application and management (Larkin and Fravel, 1998).

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Chapter 2

Evaluation of non-pathogenic *Fusarium oxysporum* endophytes from banana for biological control of *Fusarium oxysporum* f.sp. *cubense*

ABSTRACT

Fusarium oxysporum f.sp. *cubense* (*Foc*) causes Fusarium wilt, a highly destructive disease of banana. No control options other than disease prevention and the use of resistant planting material exist for the management of this disease. Fusarium wilt of crops other than banana, however, has been successfully controlled using micro-organisms isolated from suppressive soils in the past. Non-pathogenic isolates of *F. oxysporum*, particularly, were involved in disease suppression in such soils. This study, therefore, investigated the potential of non-pathogenic endophytic isolates of *F. oxysporum* from banana roots as potential biological control agents of Fusarium wilt of banana. Endophytes were isolated from the roots of banana plants grown in suppressive soils in Kiepersol, South Africa, and isolates of *F. oxysporum* were identified using morphological characteristics and species-specific primers. These isolates were first divided into genotypes by means of PCR-based restriction fragment length polymorphism (RFLP) analysis of the intergenic spacer region. Representatives of each genotype were chosen and their pathogenicity determined. Non-pathogenic isolates were then evaluated for their potential to suppress *Foc* in the greenhouse and in the field. The *F. oxysporum* endophytes found in suppressive soils in Kiepersol, South Africa were highly diverse, and could be divided into fourteen genotypes. One of the non-pathogenic isolates of *F. oxysporum* was highly effective in reducing Fusarium wilt in the greenhouse. The field trial, however, had to be terminated after 6 months because of severe frost damage. The field trial will be repeated with the isolate that was effective in reducing disease severity in the greenhouse, as well as other potential biological control agents.

INTRODUCTION

Fusarium wilt, commonly known as Panama disease, is considered as one of the most destructive diseases of bananas (Ploetz and Pegg, 2000). Fusarium wilt was first discovered in 1876 (Ploetz and Pegg, 2000), and by 1950 the disease had been disseminated to most banana-growing countries of the world (Stover, 1962). By 1960 Fusarium wilt had almost destroyed the banana export industry in Central America that was entirely based on the highly susceptible cultivar 'Gros Michel'. Only the conversion to resistant cultivars in the Cavendish subgroup saved the export industry from complete collapse. A new race of the Fusarium wilt pathogen *Fusarium oxysporum* f.sp. *cubense* (E. F. Smith) Snyd. & Hans (*Foc*), called *Foc* race 4, today threatens Cavendish cultivars in the tropical and subtropical countries of the world (Ploetz and Pegg, 2000; Viljoen, 2002). In South Africa *Foc* "subtropical" race 4 is a major threat to the local banana industry that consists entirely of Cavendish cultivars (Viljoen, 2002). Similarly, Cavendish banana cultivars are threatened in several Southeast Asian countries by *Foc* "tropical" race 4 (Ploetz, 1990). Since there is no effective control measure for Fusarium wilt of banana apart from exclusion of the pathogen from fields and the use of disease resistant plants, it is important to consider the use of alternative control strategies (Ploetz and Pegg, 2000; Viljoen, 2002). One such an alternative is biological control, a disease management strategy that provides an opportunity to control soil-borne diseases of agricultural crops in an environmentally friendly way (Wardlaw, 1961).

In some agricultural soils the incidence of Fusarium wilt is reduced despite the presence of a susceptible host, virulent pathogen and favourable environmental conditions. Such soils are known as Fusarium wilt suppressive (Stover, 1962). Suppressiveness is due to the actions of various factors, both biotic and abiotic (Louvet *et al.*, 1981; Alabouvette *et al.,* 2004). Peng *et al.* (1999) found that manipulation of soil amendments, soil pH and soil water supply can aid in suppressing banana wilt caused by *Foc.* Soil suppressiveness, however, is primarily biological in nature (Alabouvette, 1986; Larkin and Fravel, 2002; Alabouvette *et al.,* 2004). Many microorganisms, such as bacteria, actinomycetes and fungi have been associated with soil suppressiveness. Non-pathogenic *F. oxysporum*, along with fluorescent *Pseudomonas* were, however, most frequently shown as the cause of

Fusarium wilt suppression (Scher and Baker, 1982; Alabouvette, 1990; Duiff *et al.,* 1998; 1999). Non-pathogenic *F. oxysporum* reduced Fusarium wilt of watermelon and tomato (Larkin *et al.,* 1996; Larkin and Fravel, 1998), while *Pseudomonas* spp. have proved to inhibit Fusarium wilt of flax and banana (Sivamani and Gnanamanickam, 1987; Duijff *et al.,* 1999).

Non-pathogenic isolates of *F. oxysporum* can be isolated from the root rhizosphere or from inside symptomless banana roots (Larkin and Fravel, 1998; Gerlach *et al.*, 1999). Fungi that live for all, or at least part, of their life cycle inside asymptomic plant parts are called endophytes (Saikkonen *et al.,* 1998). It is thought that endophytic fungi can interact mutualistically with their host plants, mainly by increasing host resistance to pathogens, pests and environmental stresses (Caroll, 1988; Faeth and Fagan, 2002). Potent fungal toxins produced by endophytes had been shown to deter herbivores that showed a preference for uninfected plants (Carroll, 1988). Systemic endophytes from grasses also increased host competitive abilities by increasing germination success and resistance to drought and water stress (Clay, 1988; Faeth and Fagan, 2002). Non-pathogenic endophytic isolates of *F. oxysporum* were shown to increase the Cavendish banana cultivar Williams's resistance to *Foc* and to reduce disease incidence in the greenhouse (Gerlach *et al.,* 1999; Nel *et al*., 2006b). The mechanism of induced systemic resistance was proposed for non-pathogenic *F. oxysporum* that persist in banana root vascular tissue (Gerlach *et al*., 1999).

Pathogenic strains of *F. oxysporum* are not distinguishable from non-pathogens by means of traditional agar plating techniques and comparison of morphological characters (Konstantinova and Yli-Mattila, 2004). Thus, host specificity is required to classify pathogenic strains into one of approximately 120 *formae speciales*, and cultivar specificity to further divide these *formae speciales* into races (Armstrong and Armstrong, 1981). PCR-restriction fragment length polymorphism (RFLP) analysis of the ribosomal (r)DNA is useful for differentiating closely related strains within *F. oxysporum*, and to estimate the genetic relationship between these groups (Edel *et al.,* 1995; 1997a). The sequences of the intergenic spacer region (IGS) have been used for RFLP analysis of pathogenic and non-pathogenic strains of *F. oxysporum* before (Appel and Gordon, 1995). Molecular techniques have also enhanced our ability to accurately identify morphologically closely related *Fusarium* species (Mishra *et al*.,

2002). In this respect, Edel *et al.* (1997a) developed a PCR-based RFLP for the differentiation of *Fusarium* strains at species level, while Abd-Elsalam *et al*. (2003) and Mishra *et al.* (2002) successfully used the internal transcribed spacer (ITS) region to generate species-specific primers. Edel *et al.* (2000) also developed a rDNA-targeted oligonucleotide probe and PCR assay specific for the identification of *F. oxysporum.*

The aim of this study was to isolate non-pathogenic *F. oxysporum* endophytes from banana roots in Fusarium wilt suppressive soils in the Kiepersol area of South Africa, to determine their genetic relatedness by means of PCR-RFLP analysis of the IGS region, and to evaluate them as potential biological control organisms of Fusarium wilt of banana, both in the greenhouse and in the field.

MATERIAL AND METHODS

Isolation of endophytes:

Fungal endophytes were isolated from the roots of symptomless Cavendish banana plants grown in three Fusarium wilt suppressive soil sites in Kiepersol, South Africa. In total, ten banana plants were selected for sampling. Three plants were selected from each of the three disease suppressive sites, while the tenth plant was found in a greenhouse (3 m long x 3 m wide x 5 m high) erected in a banana field that was severely affected with Fusarium wilt. From each banana plant, five roots were randomly sampled.

Banana roots collected in the field were washed to remove all excess dirt, and transported in McCartney bottles placed on ice to the laboratories at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria. At the laboratory, the roots were cleaned of any remaining soil by first washing them with sterile water. The roots were then surface sterilized with 75% ethanol for 1 minute, 1% sodium hypochloride for 3 minutes and 95% ethanol for 30 seconds. Each root was cut into five pieces, and pieces of the same root placed apart from each other onto modified Komada medium in the same Petri dish (Komada, 1975). The Petri dishes were incubated at 25°C and were checked daily for fungal growth. Once colonies of

Fusarium developed sufficiently, single-spore isolates were prepared (Nelson *et al*., 1983) and plated onto half strength Potato dextrose agar (PDA) (39 g of Difco PDA powder, 1000 ml H_2O) containing 0.02 $g.l^{-1}$ Novobiocin (Sigma-Aldrich, Steinheim, Germany). Representative isolates of each of the banana roots were then preserved in 15% glycerol, freeze-dried, and maintained at the culture collection at FABI.

Identification of the isolates:

Morphological identification:

Each single-spore isolate of *Fusarium* was plated onto PDA and carnation leaf agar (CLA) (20 g of Biolab agar, 1000 ml of H_2O, one or two 5-mm sterilized carnation leaves) for cultural and morphological characterization, respectively. All PDA plates were then incubated at 25 and 30°C with a 12-hour day/night light cycle under cool-white and near-ultraviolet fluorescent lights, while cultures grown on CLA were grown at 25°C only. After 7 days, the colony diameter of each isolate grown on PDA was measured with a digimatic electronic calliper (Mitutoyo, Andover, Hampshire, UK), and the colour of the colonies recorded. Dependent on their growth rate and colony colour, isolates were tentatively divided into groups. Isolates grown on CLA were studied under both stereo and light microscopes, and the presence of microconidia, macroconidia and conidiophores was recorded, along with the presence of chlamydospores (Nelson *et al*., 1983).

Molecular identification:

DNA extraction: DNA was extracted from all the *Fusarium* isolates using the method described by Sambrook *et al*. (1989). Cultures were homogenized with a pestle in 300 µl DNA extraction buffer in an eppendorf tube, freeze-dried in liquid nitrogen and boiled in water for 5 minutes. After adding 700 µl phenol-chloroform (1:1), samples were vortexed and centrifuged for 7 minutes at 14000 rpm. The upper aqueous layer was transferred to a new tube and the phenol-chloroform step was repeated until the white interface disappeared. The rest of the procedure was performed similar to that described by Sambrook *et al*. (1989), with the exception that the tubes were centrifuged for 10 minutes after the precipitation step. DNA was dried under vacuum, after which the resulting pellet was resuspended in 100-200 µl SABAX water.

RnaseA (10 µg/µl) was added to the DNA samples, and the samples incubated at 37°C for 3 to 4 hours to digest any residual protein or RNA. DNA was visualized on a 1% agarose gel (wt/v) (Roche Molecular Diagnostics, Mannheim, Germany) stained with ethidium bromide, and viewed under an ultra-violet light. Lambda DNA marker (marker III) (Roche Molecular Diagnostics) was used to determine size and concentration of the DNA.

The ITS region of the rDNA of the isolates was amplified using the *F. oxysporum*-specific primers FOF1 (5' – ACA TAC CAC TTG TTG CCT CG – 3') and FOR1 (5' – CGC CAA TCA ATT TGA GGA ACG – 3') (Mishra *et al*., 2002). The primer pair was synthesized by Inqaba Biotechnical industries (Pretoria, South Africa). Reactions were carried out in a 20µl reaction volume containing PCR buffer (10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCL, pH 8.3) (Roche Molecular Diagnostics), 0.2 mM of each dNTP (Roche Molecular Diagnostics), 0.3 µM of each primer, and 1 U Taq DNA polymerase (Roche Molecular Diagnostics). SABAX water was used to achieve the final volume. The amplifications were performed in an Eppendorf Mastercycler gradient PCR machine (Eppendorf Scientific, Hamburg, Germany). The following conditions were used: An initial denaturation temperature of 94°C for 60 seconds, followed by 25 cycles of 94°C for 60 seconds, 58°C for 30 seconds and 72°C for 60 seconds, and a final extension of 7 minutes at 72°C. Negative and positive controls were included in each reaction, containing SABAX water with no DNA template, and DNA of a known *F. oxysporum* isolate, respectively. The PCR products were visualized by running them on a 1% agarose gel stained with ethidium bromide in 1X Tris acetic acid EDTA (TAE, pH 8.3) buffer, and visualized under ultra-violet light. A 100-bp molecular weight marker XIV (Roche Molecular Diagnostics) was used to determine the size of the PCR products.

Characterization of *F. oxysporum* isolates:

A forward Primer 1 PNFo (5'-CCCGCCTGGCTGCGTCCGACTC- 3') and reverse Primer 2 PN22 (5'-CAAGCATATGACTACTGGC - 3') were designed at Inqaba Biotechnical Industries to amplify the IGS region of the *F. oxysporum* isolates (Edel *et al*., 1995). Reactions were carried out in 50-µl reaction volumes containing PCR

buffer, 0.25 mM of each dNTP, 0.2 µM of each primer, 2 U of Taq DNA polymerase and SABAX water. The amplifications were performed in an Eppendorf Mastercycler gradient PCR machine, with 30 cycles of 90 seconds at 95°C, 60 seconds at 50°C, and 90 seconds at 72°C. Negative and positive controls were included in each reaction, containing SABAX water with no DNA template, and DNA of a known *F. oxysporum* isolate, respectively. The PCR-products were visualized by running a 1% agarose gel stained with ethidium bromide in 1X Tris acetic acid EDTA (TAE, pH 8.3) buffer, and viewed under ultra-violet light. The lambda DNA marker was used to determine the size of the fungal DNA fragments.

Aliquotes of 10 µl of PCR products were digested with 0.5 µl restriction endonuclease (2 Units). The five restriction enzymes used were *Msp*I, *Rsa*I, *Hae*III, *Hindf*I and *Scr* FI (Roche Molecular Diagnostics). Restriction buffer (1X) and SABAX water were added to the PCR products to achieve an end reaction volume of 20 µl, and the restriction enzyme mixtures incubated at 37°C for 4 hours. The restriction fragment patterns were visualized by running the restriction enzyme mixture on a 3 to 4% agarose gel stained with ethidium bromide at 60 V for 2 hours. The 100-bp molecular weight marker XIV was used to determine the size of the restriction fragments. The fragments on the gel were visualized under ultra-violet light.

Pathogenicity testing:

The pathogenic status of all the isolates of *F. oxysporum* from banana roots was determined on 10-cm Cavendish banana plantlets (cv Chinese Cavendish). The plantlets were micropropagated at Du Roi Laboratories in Letsitele, South Africa. Before inoculation the plantlets were replanted to 250-ml plastic cups filled with water (Nel *et al*., 2006b), and fertilized every 2 weeks with a hydroponic nutrient mixture (0.6 $g.l^{-1}$ $Ca(NO_3)_2H_2O$, 0.9 $g.l^{-1}$ Agrasol, and 3 $g.l^{-1}$ Micromax). The plants were then kept in the cups until sufficient root development occurred.

The inoculum for the pathogenicity tests was grown in Armstrong *Fusarium* medium (Booth, 1977) in 500-ml Erlenmeyer flasks (100 ml in each). The flasks were placed on a rotary shake incubator (Labotec, Midrand, South Africa) with a rotation speed of

177 rounds per minute at 25°C. After 7 days the sporulation medium was poured through cheesecloth, and the spore concentrations adjusted to 1 x 10^6 spores.ml^{-1} with the aid of a hemacytometer (Laboratory & Scientific Equipment Company (Pty) Ltd. (LASEC), Randburg, South Africa). Five ml of the respective suspensions were then added per cup to achieve a final spore concentration of 1 x 10^5 spores/ml. Two sets of control plants were included in the trial. The one set of control plants received water only and the other set of control plants was inoculated with *Foc* (CAV 092) at a final spore concentration of 1 x 10^5 spores/ml. Roots of all the banana plantlets were slightly damaged by hand, by squeezing the rootball to ensure infection. Six replicate plants were used for each treatment, and the trial was repeated.

Inoculated plants were kept in a phytotron with a 12-hour day/night illumination cycle, with the "day" temperature set at 28°C and the "night" temperature at 20°C. After 3 to 4 weeks the rhizomes of plants were cut open to see whether internal symptoms developed. Severity of symptoms was rated according to the INIBAP rating scale (Carlier *et al*., 2002). No discolouration of the rhizome was rated as a 0, 1 to 25% discolouration as 1, 26 to 50% discolouration as 2, 51 to 75% discolouration as 3 and 76 to 100% discolouration of the rhizome as 4. Disease severity was calculated using the formula of Sherwood and Hagedorn (1958): Disease severity (%) = Σ [(number of plants in disease scale category) x (specific disease scale category) / (total number of plants) x (maximum disease scale category)] x 100. The pathogen was re-isolated from diseased rhizome tissue to prove Koch's postulates.

Biological control of *Foc:*

Greenhouse testing:

Seventeen isolates of *F. oxysporum*, representative of all RFLP genotypes, were evaluated in the greenhouse as potential biological control organisms of *Foc*. Ten-cm tissue culture Cavendish banana plants were obtained from Du Roi Laboratories and prepared for greenhouse testing as described above. Once sufficient root development was obtained, the plantlets were inoculated with each of the putative biological control isolates at a concentration of 1 x 10^5 spores.ml^{-1}. After 1 week the endophyte-infested plants were replanted to pots filled with 500 g of *Foc*-infested soil. *Foc*

(CAV 092) was established in this soil by first cultivating it on millet seeds (Strauss and Labuschagne, 1995), and then mixing the pathogen-colonized millet seed with sandy soil to a concentration of 3%. Control plants were not treated with any potential biocontrol agent, and were planted in both *Foc*-infested and -uninfested soil. For each treatment, six pots were used, and the experiment was repeated. The banana plants received 12 hours of illumination daily at a temperature of 28°C, while the temperature in the dark was set at 20°C. After 7 weeks the plants were uprooted, cut open and symptoms rated according to the INIBAP rating scale presented earlier (Carlier *et al*., 2002).

Field testing:

A non-pathogenic *F. oxysporum* isolate, selected after greenhouse evaluation of endophytes in this study (CAV 553), a non-pathogenic *F. oxysporum* isolate from the banana root rhizosphere, CAV 255 (Nel *et al*., 2006b), and a well-known biocontrol agent, the bacterium *Pseudomonas fluorescens* WCS 417, provided by Prof. L. C. van Loon (University of Utrecht, The Netherlands), were selected for biological control testing of Fusarium wilt of banana in the field. Both *F. oxysporum* isolates were grown in Armstrong *Fusarium* medium (Booth, 1977) to enhance sporulation as described before. After 7 days, the conidia were harvested by filtering through cheesecloth, and centrifugation at 5000 x *g* at 15ºC for 20 minutes. The spores were then washed three times in sterile distilled water and adjusted to a final concentration of 1 x 10^6 spores.ml^{-1}. The *P. fluorescens* isolate was grown on *Pseudomonas* selective agar at 37°C in the dark for 2 days (King *et al*., 1954). The bacteria were then scraped from the medium, suspended in sterile distilled water, and adjusted to a final cell concentration of 1 x 10^8 cfu.ml^{-1}.

Field-ready (20-cm) pathogen-free tissue culture banana plantlets of the Cavendish cultivar Chinese Cavendish were obtained from Du Roi Laboratories. These plants were each treated with 100 ml of the putative biological control organisms 1 week before field planting. Four different treatments were used that include the three different organisms, separately, and combined. Before planting, the roots of plants were again dipped into the different spore and cell suspensions. Two control treatments were also applied at field planting. These include a root drench of banana plants with propiconazole (Tilt), a fungicide that proved to reduce the incidence of

Foc in vitro and Fusarium wilt in the greenhouse (Nel *et al*., 2006b) at 25 ppm (a.i.), as well as tap water. There were 30 plants per treatment, planted in a completely randomised block design in an *Foc*-infested field site. In the Kiepersol area where the trial was set up large areas have been forced out of production due to Fusarium wilt, thus no artificial inoculation with *Foc* were required (Viljoen, 2002). Each block contained ten plants, and there were three blocks per treatment. Guard rows of Cavendish banana plants were planted between the blocks to prevent cross-contamination upon root contact between plants. The trial site was managed according to standard farmer practices.

Statistical analysis:

Statistical analysis was conducted using Statgraphics Version 5.0. Experiments were analyzed using multifactor analysis of variance (ANOVA). Significance was evaluated at $P<0.05$ for all tests. The data of the *in vivo* biological control testing was pooled for the two repeat experiments if the experiment x treatment interaction was not significant.

RESULTS

Identification of isolates:

Endophytic fungi were isolated from the roots of all banana plants collected at the three suppressive soil sites and the greenhouse. These isolates include species of *Fusarium*, but also of known mycoparasites such as *Trichoderma* and *Gliocladium*. In total, 70 isolates of *Fusarium* were collected based on colony colour and spore morphology. Isolates of *F. oxysporum* developed as dark purple colonies on PDA that grew more rapidly at 25 than at 30^{o}C. They were separated from other species of *Fusarium* by the production of large numbers of non- and 1-septated microconidia in false heads on short monophialides (Fig. 1A). Terminal and intercalary chlamydospores were formed singly or in pairs in hyphae or conidia (Fig. 1B). Only a few large, hyaline, pedicellate, sickle-shaped macroconidia were produced with attenuated apical and foot-shaped basal cells (Nelson *et al.,* 1983). In total, 43 isolates were identified as *F. oxysporum.*

Amplification of *F. oxysporum* isolates with species-specific primers designed by Mishra *et al*. (2002) permitted the formation of a single 340-bp PCR fragment (Fig. 2). Isolates identified as other *Fusarium* spp. did not produce this fragment. Of the *F. oxysporum* isolates, six proved to be pathogenic to banana, and could be re-isolated from the diseased plants. Only isolates of *F. oxysporum* that proved to be non-pathogens were selected for further biological studies.

Characterization of *F. oxysporum* isolates:

The primer set PNFo and PN22 amplified a single DNA fragment that represents the IGS region of approximately 1700 bp for all 43 *F. oxysporum* isolates (Fig. 3). When digested with restriction enzymes, a number of different banding patterns were produced. For *Msp*I and *ScrF*1, three distinct RFLP fingerprinting patterns were recognized, for *Rsa*I four and for *Hinf*I, and *Hae*III, five patterns were recognized (Fig. 4). When a different alphabetical letter was assigned to each unique fragment pattern generated by a restriction enzyme, and each isolate was designated a five-letter code, the 43 isolates were divided into 14 groups (Table 1). The largest IGS genotype was ACCBA (group 6), which contained eight non-pathogenic *F. oxysporum* isolates. The pathogenic *Foc* isolate (CAV 092) isolate grouped with the six *F. oxysporum* isolates that proved to be pathogenic to banana, in the IGS genotype ACDCC.

Several of the genotypes were found at all three sites (Table 2). In site A, for instance, five genotypes were present, while eight genotypes were present in both sites B and C. In the greenhouse, four genotypes were found associated with the healthy roots of a single banana plant. Some genotypes, such as ACABA and ACCBA were found at all three field sites (A, B and C), while four of the genotypes were found at two of the field sites. Five groups were unique to the respective sites, three of which came from site C.

Biological control of *Foc:*

Greenhouse testing:

Fusarium wilt of banana was reduced in greenhouse trials by several of the non-pathogenic *F. oxysporum* isolates obtained from disease suppressive soils (Fig. 5). Ten isolates reduced the disease significantly ($P \leq 0.05$). When compared to the control treatment, three isolates CAV 553, CAV 552 and CAV 563 reduced disease incidence the best by 69.23%, 65.38% and 57.69% respectively.

Field testing:

No Fusarium wilt symptoms were visible in any banana plant 3 months after planting (March 2006). When the trial site was visited after 4 more months (July 2006), the plants were severely affected by frost damage. Since many of the plants were killed, a decision was made to terminate the trial.

DISCUSSION

Non-pathogenic *F. oxysporum* endophytes, isolated from Fusarium wilt suppressive soils in Kiepersol, were able to substantially reduce the incidence of the disease in the greenhouse. This is consistent to previous reports by Gerlach *et al*. (1999) and Nel *et al*. (2006b) who suggested that non-pathogenic *F. oxysporum* be considered as biological control agents for Fusarium wilt of banana. The one limitation of the former studies was that non-pathogenic *F. oxysporum* controlling Fusarium wilt in the greenhouse were never evaluated in the field. Non-pathogenic strains of *F. oxysporum* were previously shown to be highly effective in controlling Fusarium wilt of sweet potatoes caused by *F. oxysporum* f. sp. *batatas* in the field when applied to cuttings (Sneh, 1998). Preliminary field tests with non-pathogenic isolates of *F. oxysporum* and *F. solani* on tomato seedlings suppressed Fusarium wilt of tomato by between 50 to 80% (Larkin and Fravel, 1998), and again under a variety of environmental conditions (Larkin and Fravel, 2002). In South Africa Fusarium wilt symptoms on bananas are most severe after winter (Viljoen, 2002). In the current study, field evaluation was attempted, but was suspended after 7 months because of adverse environmental conditions that severely damaged the inoculated plants. The field study is currently being repeated to ascertain the true potential of these organisms.

The non-pathogenic isolates of *F. oxysporum* from Fusarium wilt suppressive soils consist of several different genotypic groups when analysed by PCR-RFLPs. This is

an indication of the great diversity that exists in non-pathogenic *F. oxysporum* populations. Since non-pathogenic *F. oxysporum* endophytes from the three field sites sometimes grouped in the same IGS genotype, one can expect that these genotypes are either widely distributed, or that a great movement of genotypes occurred in the area. Another possibility is that banana root exudates might favour their selectiveness (Stover, 1961; Edel *et al.,* 1997b; Nel *et al*., 2006a). Gordon and Okamoto (1992), Appel and Gordon (1995) and Lori *et al.* (2004) also reported that non-pathogenic isolates of *F. oxysporum* were very diverse, while diversity was absent from pathogenic strains. The IGS genotype grouping of six endophytic *F. oxysporum* isolates pathogenic to banana with a known *Foc* isolates is indicative of the stability and clonal nature of the Fusarium wilt pathogen in South Africa. The diversity of the saprophytic isolates of *F. oxysporum* might be due to the fact that they also grow easily in disease suppressive soils, while pathogenic *F. oxysporum* established with difficulty in such soils (Smith and Snyder, 1972).

The non-pathogenic *F. oxysporum* isolates investigated in this study were collected from two of the same sites where Nel *et al*. (2006a) collected their isolates from the root rhizosphere. Yet, none of the IGS genotypes collected in these two independent studies were identical. This might be co-incidental, but could also be due to a change in the population structure of the non-pathogens over time, as the endophytes were collected 12 months later. A study by Edel *et al*. (2001) found that two soils having the same degree of suppressiveness could, in fact, harbour different soil-borne *F. oxysporum* populations with different population structures. This means that the same sites in Kiepersol could have retained their suppressiveness despite a change in the population structure. It might also explain why the two sites in Kiepersol with different population structures were both suppressive to Fusarium wilt of banana.

Another explanation for the difference in IGS genotype composition found in this study and that of Nel *et al*. (2006a) may be because of the material that the isolates were collected from (roots vs. rhizosphere). Edel *et al*. (1997b) found that the structure of populations associated with the roots of wheat and tomato differed from the structure of populations isolated from soil. Because root and soil isolates were collected from different fields by these authors, it would be an oversimplification to conclude that roots and the root rhizosphere of plants harbour different populations of

F. oxysporum. Whether plants have a definite selective effect on populations of *F. oxysporum* colonizing their root systems as endophytes (Edel *et al*., 1997b; Alabouvette *et al*., 2001), however, should also be further investigated.

Greenhouse screening of candidate organisms, supported by proper field screening is currently the only means to find non-pathogenic *F. oxysporum* isolates that can be considered as biological control agents. This method can be rewarding, as the well-known biological control isolate Fo47 was discovered this way in soils suppressive to Fusarium wilt of flax in Châteaurenard, France (Alabouvette and Couteaudier, 1992; Alabouvette, 1986), but the process is time and space consuming. If mass screening of non-pathogenic *F. oxysporum* isolates for biological of Fusarium wilt diseases needs to be done, a screening technique that is more time and cost-effective than pathogenicity testing needs to be developed. Such a technique might either involve the development of molecular markers for the screening of large numbers of candidate organisms, or the use of an *in vitro* technique that is dependent on the mechanism of control. Whether the PCR-RFLP method is useful to rapidly select isolates with greater biological control activity is not entirely clear, but it seems to be unlikely. The ten non-pathogenic *F. oxysporum* isolates that reduced Fusarium wilt severity significantly in this study were all placed in separate IGS genotype groups, except for CAV 563 and CAV 565 that grouped together. In contrast, Nel *et al*. (2006b) found that the rhizosphere isolates that suppressed the disease the most were grouped in the same genotype. It might be interesting to investigate whether other molecular fingerprinting techniques or vegetative compatibility group (VCG) testing could be used to rapidly identify putative biological control agents.

Understanding the mode of action of biological control organisms is not only important to develop a rapid screening technique for candidate biological control organisms, but also to determine the best application procedures for effective disease control (Sneh, 1998). Non-pathogenic *F. oxysporum* strains can reduce disease incidence through competition for nutrients (Alabouvette and Couteaudier, 1992) or infection sites (Schneider, 1984) and by inducing systemically acquired resistance in plants (Fuchs *et al*., 1997; Larkin and Fravel, 2002). Since non-pathogens in the rhizosphere are known to be good colonizers of the soil and root area (Smith and Snyder, 1972; Alabouvette *et al*., 1993), they most likely compete well for nutrients

such as carbon with the pathogen (Couteaudier and Alabouvette, 1990). Endophytes, in contrast, might spend some energy colonising the tissue inside the plant (Wilson, 1995) and induce the plant's own defence responses (Mandeel and Baker, 1991; Olivain *et al*., 1995, He *et al*., 2002). It is, therefore, expected that the simultaneous use of rhizosphere non-pathogens (Nel *et al*., 2006b) and endophytes together might be more effective than the use of these isolates alone. The biocontrol agents must also be able to successfully establish themselves in or on the plant roots, and survive for extended periods in the soil and in plant roots (Alabouvette *et al*., 1993).

The application of non-pathogenic *F. oxysporum* isolates to banana roots promises to be a cost effective and environmentally friendly approach to Fusarium wilt control. Most commercial banana plantations are now being established with micropropagated plants that are disease and pest free. Once taken from *in vitro* culture, the rooted banana plantlets are transplanted into seedling trays, hardened off, and prepared for field planting in the nursery. At any of these stages the biological control agent can be established on banana roots before field planting. Micropropagated grape plantlets inoculated with arbuscular mycorrhizal (AM) fungi at the hardening-off stage led to higher survival rates under greenhouse and field conditions (Krishna *et al*., 2005), while AM fungi enhanced the percentage survival and improved tolerance of cassava to transplanting stress (Azcón-Aguilar *et al*., 1997). Banana plantlets treated with non-pathogenic *F. oxysporum* before field planting also significantly increased the survival rate of Cavendish bananas in nematode-infested fields in Costa Rica (Viljoen, personal communication). Once a potential biocontrol agent has been identified it can be used alone or in combination with other biological, chemical or cultural control practices for better reduction of disease severity.

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Table 1. Intergenic spacer region (IGS) genotype groups obtained with restriction fragment length polymorphism analysis of *Fusarium oxysporum* isolates collected from healthy banana roots in Fusarium wilt suppressive soils, and their pathogenicity status.

					IGS genotype[1]				
Group	Isolate	Collection site	Date isolated	Pathogenicity	*Msp*I	*Rsa*I	*Hae*III	*Hindf*I	*Scr* FI
1	CAV 526	Kiepersol, Site A, SA	03/03	Non-pathogen	A	A	A	A	A
2	CAV 527	Kiepersol, Site A, SA	03/03	Non-pathogen	A	B	E	B	A
	CAV 551	Kiepersol, Site C, SA	03/03	Non-pathogen	A	B	E	B	A
	CAV 564	Kiepersol, Site GH, SA	03/03	Non-pathogen	A	B	E	B	A
3	CAV 529	Kiepersol, Site A, SA	03/03	Non-pathogen	A	C	A	B	A
	CAV 531	Kiepersol, Site A, SA	03/03	Non-pathogen	A	C	A	B	A
	CAV 532	Kiepersol, Site A, SA	03/03	Non-pathogen	A	C	A	B	A
	CAV 533	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	A	B	A
	CAV 535	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	A	B	A
	CAV 560	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	A	B	A
4	CAV 536	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	A	B	B
	CAV 563	Kiepersol, Site GH, SA	03/03	Non-pathogen	A	C	A	B	B
	CAV 565	Kiepersol, Site GH, SA	03/03	Non-pathogen	A	C	A	B	B
5	CAV 566	Kiepersol, Site GH, SA	03/03	Non-pathogen	A	C	A	C	A
6	CAV 530	Kiepersol, Site A, SA	03/03	Non-pathogen	A	C	C	B	A
	CAV 534	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	C	B	A
	CAV 537	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	C	B	A
	CAV 540	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	C	B	A
	CAV 545	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	C	B	A
	CAV 547	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	D	B	A
	CAV 557	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	C	B	A
	CAV 558	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	C	B	A
7	CAV 554	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	D	B	A
	CAV 555	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	D	B	A
	CAV 556	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	D	B	A
	CAV 548	Kiepersol, Site B, SA	03/03	Non-pathogen	A	C	D	B	A
	CAV 562	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	D	B	A
8	CAV 092[2]	Kiepersol, SA		Pathogen	A	C	D	C	C
	CAV 538	Kiepersol, Site B, SA	03/03	Pathogen	A	C	D	C	C
	CAV 539	Kiepersol, Site B, SA	03/03	Pathogen	A	C	D	C	C
	CAV 542	Kiepersol, Site B, SA	03/03	Pathogen	A	C	D	C	C
	CAV 550	Kiepersol, Site B, SA	03/03	Pathogen	A	C	D	C	C
	CAV 635	Kiepersol, Site B, SA	03/03	Pathogen	A	C	D	C	C
	CAV 633	Kiepersol, SiteB, SA	03/03	Pathogen	A	C	D	C	C

					IGS genotype[1]				
Group	Isolate	Collection site	Date isolated	Pathogenicity	*Msp*I	*Rsa*I	*Hae*III	*Hindf*I	*Scr* FI
9	CAV 552	Kiepersol, Site C, SA	03/03	Non-pathogen	A	C	A	A	A
10	CAV 559	Kiepersol, Site C, SA	03/03	Non-pathogen	A	D	A	B	A
11	CAV 528	Kiepersol, Site A, SA	03/03	Non-pathogen	B	A	B	E	A
	CAV 549	Kiepersol, Site B, SA	03/03	Non-pathogen	B	A	B	E	A
12	CAV 546	Kiepersol, Site B, SA	03/03	Non-pathogen	B	C	A	D	B
13	CAV 541	Kiepersol, Site B, SA	03/03	Non-pathogen	C	A	A	A	A
	CAV 543	Kiepersol, Site B, SA	03/03	Non-pathogen	C	A	A	A	A
	CAV 544	Kiepersol, Site B, SA	03/03	Non-pathogen	C	A	A	A	A
	CAV 561	Kiepersol, Site C, SA	03/03	Non-pathogen	C	A	A	A	A
14	CAV 553	Kiepersol, Site C, SA	03/03	Non-pathogen	C	A	A	B	A

[1]Restriction fragment patterns obtained for each enzyme were designated a letter. The five-letter code represents the restriction fragment pattern obtained for the five restriction fragment enzymes.

[2]Pathogenic isolate of *Fusarium oxysporum* f.sp. *cubense* from Kiepersol, South Africa.

Table 2. The number of *Fusarium oxysporum* isolates obtained from banana roots planted in Fusarium wilt suppressive soils in Kiepersol, South Africa. The isolates were grouped according to their PCR-restriction fragment length polymorphisms of the intergenic spacer region.

	Number of isolates from			
IGS Genotype	Site A	Site B	Site C	Greenhouse (GH)
AAAAA	1			
ABEBA	1		1	1
ACABA	3	2	1	
ACABB		1		2
ACACA				1
ACCBA	1	5	2	
ACDBA		1	4	
ACDCC		6		
ACAAA			1	
ADABA			1	
BABEA	1	1		
BCADB		1		
CAAAA		3	1	
CAABA			1	
Total	7	20	12	4

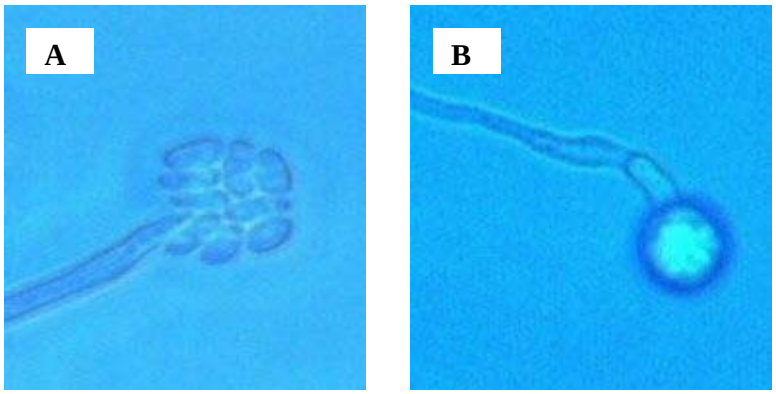


Figure 1. Morphological characteristics of *Fusarium oxysporum*: **A)** Microconidia borne in false head, and **B)** A single chlamydospore produced apically on a fungal hyphae.

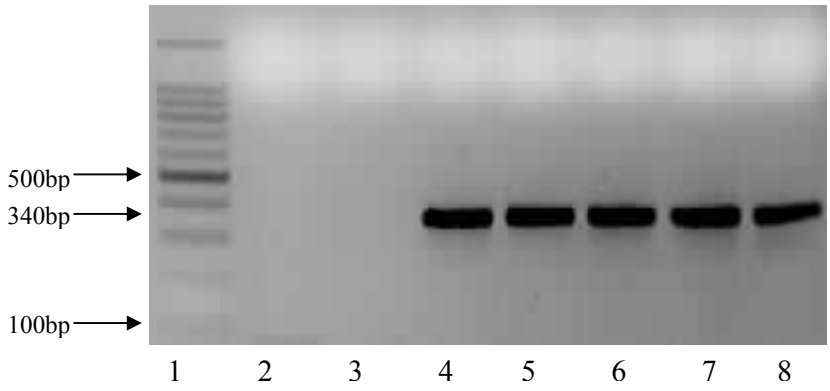


Figure 2. Identification of *Fusarium oxysporum* isolates by using PCR primers FOF1 and FOR1. Lanes 1: 100bp DNA marker; 2: Water used as negative control; 3: *Fusarium solani*; 4: *Fusarium oxysporum* f.sp. *cubense*; 5-8: Endophytic *F. oxysporum* isolates CAV 552, 553, 557 and 563. The sizes of the molecular weight marker and the size of the band are indicated to the left of the figure.

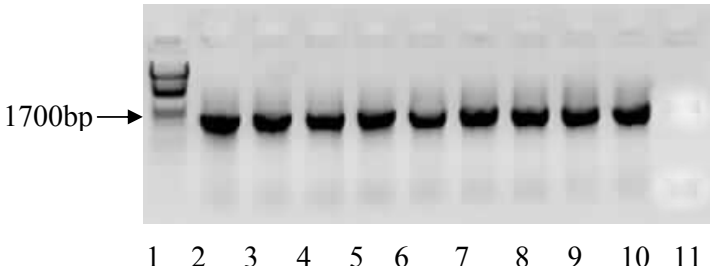
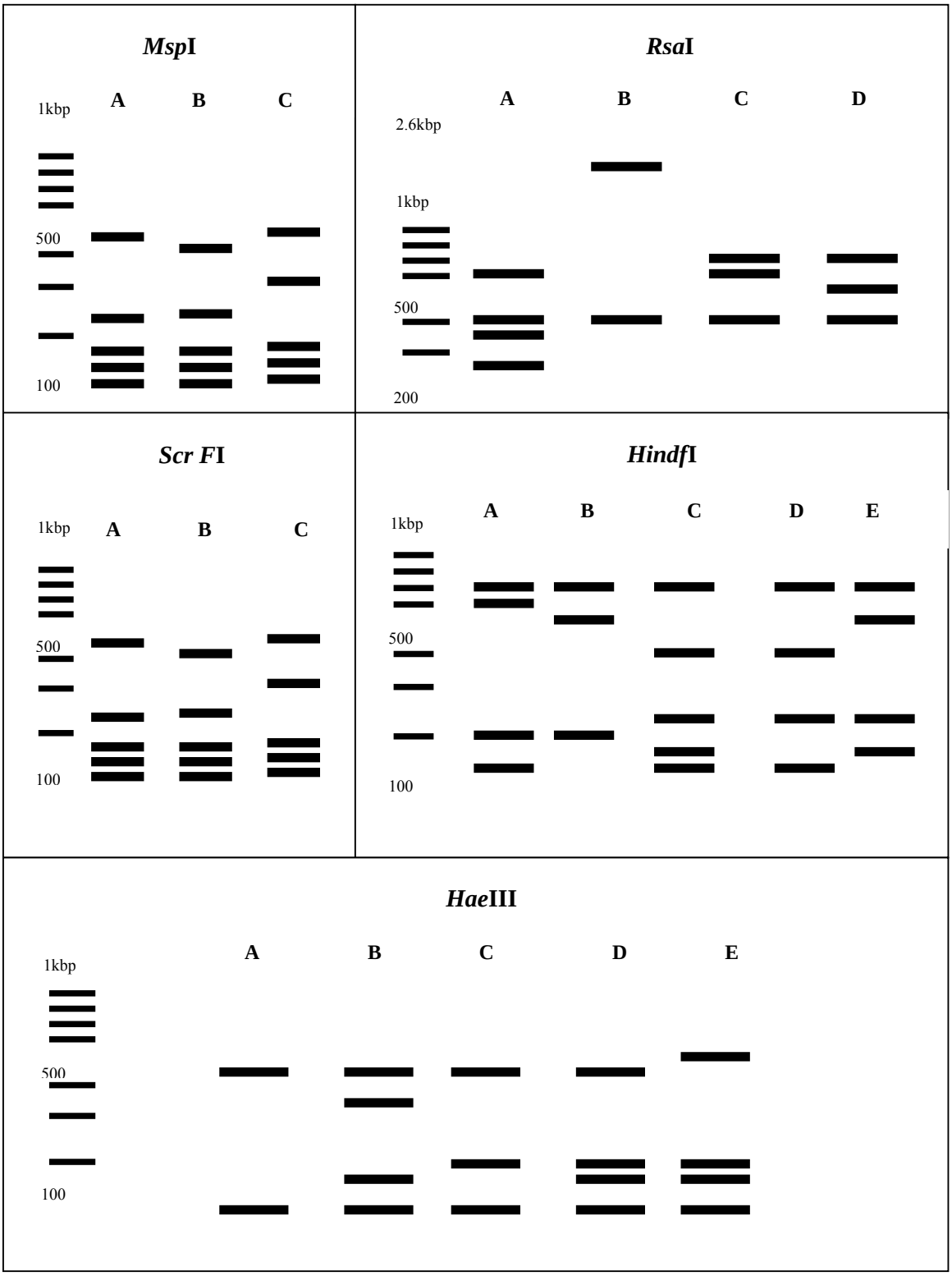


Figure 3. PCR amplification products of the intergenic spacer region of the ribosomal DNA of *Fusarium oxysporum* isolates. PCR products were visualized on a 0.8% agarose gel stained with ethidium bromide. Lanes 1: λ molecular weight marker; 2-10: *Fusarium oxysporum* isolates; and 11: water control.

Figure 4. Restriction fragment length polymorphism (RFLP) patterns obtained for *Fusarium oxysporum* isolates from healthy banana roots. Each of the illustrations represent the RFLP pattern produced when the intergenic spacer region of the ribosomal DNA was digested with the restriction enzymes *Msp*I, *Rsa*I, *ScrF*I, *Hindf*I, and *Hae*III.


*Msp*I
1kbp
A
B
C
500
100
*Rsa*I
A
B
C
D
2.6kbp
1kbp
500
200
*Scr F*I
1kbp
A
B
C
500
100
*Hindf*I
A
B
C
D
E
1kbp
500
100
*Hae*III
A
B
C
D
E
1kbp
500
100

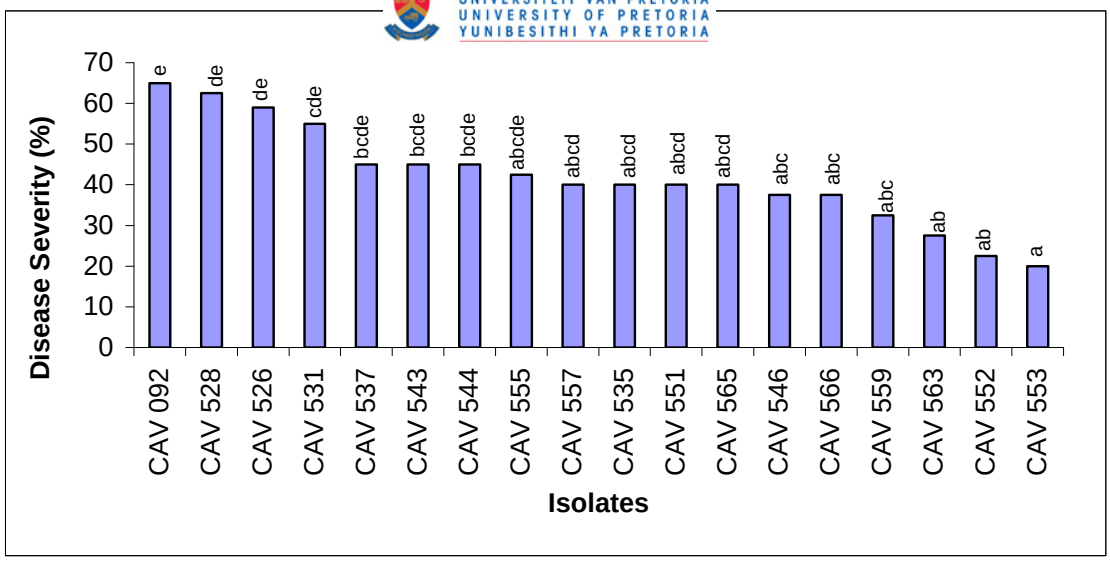


Figure 5: The mean incidence of Fusarium wilt of banana caused by the pathogen *Fusarium oxysporum* f. sp. *cubense* in the greenhouse, as affected by treatments with various isolates of non-pathogenic *F. oxysporum*. The control treatment is CAV 092 and received water only. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

Chapter 3

Phenolic acid production in Cavendish banana roots following colonization by non-pathogenic *Fusarium oxysporum* and *Pseudomonas fluorescens*

ABSTRACT

Fusarium wilt is a most destructive disease of banana caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*). No control measure is effective for the disease other than the use of disease resistant cultivars. As a result of the successful control of other Fusarium wilt diseases using microorganisms isolated from disease suppressive soils, there has been an increased interest in the potential biological control of *Foc*. Non-pathogenic *F. oxysporum* and *Pseudomonas fluorescens* are two of the microorganisms most often associated with Fusarium wilt suppressive soils. In this study, the ability of endophytic non-pathogenic *F. oxysporum* and *P. fluorescens* to induce systemic resistance and defense responses in Cavendish bananas against *Foc*, was investigated. The putative biocontrol agents were inoculated, separately and in combination, on one half of the roots in a split-root experiment, while the other half was challenged by a pathogenic isolate of *Foc.* The induction of total soluble phenolic acids, non-conjugated (free acids) phenolic acids, ester-bound phenolic acids, glycoside-bound phenolic acids and cell wall-bound phenolic acids was then determined. All applications of the putative biocontrol agents induced a resistance response against *Foc*. There was a significant induction of glycoside-bound phenolic acids and free phenolic acids by non-pathogenic *F. oxysporum* and *P. fluorescens*, but it decreased after 24 hours. High levels of total and cell wall-bound phenolic acids were produced following inoculation by non-pathogenic *F. oxysporum* and cell wall-bound phenolics acids were the highest at 48 hours. High levels of total phenolic acids were produced in response to *P. fluorescens* both locally and systemically. This suggests that the putative biocontrol microorganisms are able to stimulate the production of precursors of antimicrobial substances that may be toxic to the pathogen and aid in the strengthening of the cell wall, thus inhibiting pathogen infection of the banana roots.

INTRODUCTION

Plants defend themselves against pathogen attack through preformed and induced resistance responses (Agrios, 2005; Vallad and Goodman, 2004). For non-specific pre-formed resistance, structural barriers and antimicrobial compounds are formed that protect plants against a range of pathogens (Thatcher *et al*., 2005). When pathogens overcome these barriers, they are subjected to an induced resistance response that relies on pathogen recognition. Recognition triggers a series of signalling cascades that activate numerous defence pathways to prevent the pathogen from causing disease (Yang *et al*., 1997). The first plant response usually involves an oxidative burst that gives rise to the development of a hypersensitive response (HR) (Durner *et* al., 1998; Thatcher *et al*., 2005). This hypersensitive response prevents further progress of pathogens by means of local cell necrosis (Cameron *et al*., 1994; Van Loon, 1997). This activation of plant defence responses in primary infected parts is called locally acquired resistance (LAR). If, however, the pathogen proceeds past this first line of defence, tissue spatially separated from the primary invader becomes more resistant in a process called systemically acquired resistance (SAR) (De Meyer and Höfte, 1997). In this instance, structural barriers can be induced that include cell wall lignification, papillae formation, production of glycoproteins and vascular occlusions (Yang *et al*., 1997). In addition, antifungal compounds such as pathogenesis-related (PR) proteins, phytoalexins, peroxidases (POX) and antimicrobial secondary metabolites are produced (Van Loon *et al*., 1998; Thatcher *et al*., 2005). Enzymes involved in the biosynthesis of compounds with biocidal activity like glycosides, flavonoids and phenolic acids are also induced (Cowan, 1999). An increase in phenolic content is regarded as an early response to pathogen attack, and contributes to biotic and abiotic stress resistance by forming oxidation compounds (polymeric products) that are toxic (Lewis and Yamamoto, 1990).

Phenolic compounds in plants are formed by way of the shikimate and phenylpropanoid metabolic pathways (Hahlbrock and Scheel, 1989; Nicholson and Hammerschmidt, 1992; Boudet *et al*., 1995). The shikimate pathway is the biosynthetic route to aromatic amino acids such as tryptophan, tyrosine and phenylalanine (Herrmann, 1995). In higher plants, these amino acids are also used as precursors for a number of secondary metabolites for the plant to defend itself (Dixon

and Paiva, 1995). In the phenylpropanoid pathway, phenylalanine is deaminated to cinnamic acid by phenylalanine ammonia lyase (PAL) (Koukol and Conn, 1961; Dixon and Paiva, 1995), that is then hydroxylated to ρ-coumaric acid (Dixon *et al*., 2002; Jiang *et al*., 2005). ρ-Coumaric acid serves as precursor to three monolignols, ρ-coumaryl, coniferyl and sinapyl alcohol, (Schnablová *et al*., 2006) which can later be polymerised to form lignin (Higuchi, 1985).

Phenolic acids are the most widespread class of plant secondary metabolites, and are of great significance in plant soil systems (Siqueria *et al*., 1991). They may function as part of the structural plant matrix (Siqueria *et al*., 1991), act as constitutive protection against invading organisms (Vidhyasekaran, 1988), affect cell and plant growth (Rice, 1984), and are structural and functional components of soil organic matter (Haider *et al*., 1975). Phenolic compounds can exist as free or bound molecules since they can form complexes with other macromolecules such as proteins and cellular components (Luthria *et al*., 2006). Cell wall-bound phenolic esters may act directly as defense compounds, or may serve as precursors for the synthesis of lignin, suberin, and other wound-induced polyphenolic barriers (Hahlbrock and Scheel, 1989).

Phenolic acids can occur in multiple conjugated forms with sugars, acids and other phenolic compound (Robbins, 2003). Some linkages between polymers, however, are not regarded as true links. These include the glycosidic linkages that form between single monosaccharides and short oligosaccharides (non-toxic glycosides), and the terminal phenolic and side chain hydroxyls on lignin (Bacic *et al*., 1988; Lam *et al*., 1992). Once phenolic glycosides are cleaved by fungal glycosidase, they become toxic to the pathogen (Agrios, 2005). When the phenolic glycosides, such as the cinnamates, coumarines, caffeic acids, ferulic acids and sinapic acids (Dixon *et al*., 2002) are released and diffuse out of storage, they become hydrolysed to free phenolics, which then become oxidized and eventually polymerized with the host and pathogen structures (Beckman, 1987) to form lignin and suberin. Lignin is a complex phenolic polymer that is responsible for mechanical support, water transport and defence in vascular plants (Campbell and Sederoff, 1996). Different covalent cross-links occur in lignified cell walls, which include an ester link between uronic acids (Iiyama *et al*., 1994), an ether linkage between polysaccharides and lignins (Iiyama *et*

al., 1994), and hydroxycinnamic acids esterified or etherified to lignin surfaces (Bacic *et al*., 1988; Lam *et al*., 1992). Cross-linking of cell wall polymers would reduce accessibility of the pathogen's hydrolytic enzymes to their substrates and contribute to cell wall strengthening and blocking ingress of pathogens (Iiyama *et al*., 1994).

Lignin production and phenolic accumulation in banana roots play an important role in disease resistance to *Fusarium oxysporum* f.sp. *cubense* (*Foc*), causal agent of Fusarium wilt of banana (Mace, 1963; Beckman, 1969; 1987; 1990; Van den Berg, 2006) and elicitors thereof (De Ascensao and Dubery, 2000; 2003). Regrettably, banana varieties resistant to *Foc* are not always acceptable to local markets (Viljoen, 2002). Other means to reduce the impact of the Fusarium wilt of banana, therefore, have to be found. In recent years, Fusarium wilt diseases of several agricultural crops have been managed effectively by using microbial biological control agents (Scher and Baker, 1980; Alabouvette, 1990; Duijff *et al*., 1998, 1999). Non-pathogenic *F. oxysporum* and *P. fluorescens*, in particular, were identified as micro-organisms able to suppress Fusarium wilt incidence by means of competition for nutrients, competition for infection sites, and induced resistance (Larkin *et al*., 1996). In this study, the active induction of phenolic compounds by non-pathogenic *F. oxysporum* and *P. fluorescens*, locally and systemically, was investigated. To ascertain that these secondary metabolites were of primary importance, their production was studied over time in the presence and absence of the Fusarium wilt pathogen.

MATERIALS AND METHODS

Preparation of isolates:

Two putative biological control agents were used in this study: a non-pathogenic endophytic *F. oxysporum* isolate (CAV 553) from banana roots (Chapter 2), and a bacterial isolate of *P. fluorescens* (WCS 417) provided by Prof. L.C. van Loon, University of Utrecht, The Netherlands. Both these isolates proved to reduce the incidence of Fusarium wilt of banana significantly in the greenhouse (Chapter 2; Nel *et al*., 2006). The pathogenic *Foc* isolate used (CAV 092) was obtained from a diseased Cavendish banana plant in Kiepersol, South Africa. All three isolates are maintained at the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

The pathogenic and non-pathogenic *F. oxysporum* isolates were grown on half strength Potato Dextrose Agar (PDA) (19.5 g of Difco PDA powder, 19.5 g of Biolab Agar powder, 1000 ml H_2O) at 25°C with a 12-hour day/night light cycle under cool-white and near-ultraviolet (UV) fluorescent lights, for 7-10 days. The mycelia of the isolates were then scraped from the PDA plates and suspended separately in sterile water in 1-L Erlenmeyer flasks, shaken and passed through sterile cheesecloth. The spore concentration was determined with a haemacytometer (Laboratory & Scientific Equipment Company (Pty) Ltd. (LASEC), Randburg, South Africa) and adjusted to 1 x 10^6 spores.ml^{-1}. The *P. fluorescens* isolate was streaked onto *Pseudomonas* selective agar 2 days before inoculation (King *et al*., 1954), and grown at 37°C in the dark. The bacteria were then scraped from the agar medium and suspended in sterile distilled water, and adjusted to a final concentration of 1 x 10^8 colony forming units (cfu).ml^{-1} using a spectrophotometer.

Plant material:

Micropropagated Cavendish banana plantlets (cultivar Williams) were obtained from DuRoi Laboratories in Letsitele, South Africa. Williams bananas are known to be highly susceptible to *Foc* race 4. The plantlets were removed from their seedling trays, and transplanted into a plastic cup system as described earlier (Chapter 2). Banana plantlets were fertilized weekly with a nutrient solution (Chapter 2) and kept in a greenhouse until sufficient root development has taken place for inoculation.

Greenhouse testing for induced resistance:

Approximately 2 weeks after replanting, the roots of each plant were divided into two halves for a split-root experiment (Fuchs *et al*., 1997). Each half was placed in a 250-ml plastic cup containing water. A plastic lid was placed around the stem, using sponge to support the plants, and masking tape to secure the lid to the two cups (Figure 1). Wet cotton wool was wrapped around the pseudostem to prevent it from drying out. The cotton wool was kept damp by covering it with a black plastic bag. The plants were fertilised weekly with a hydroponic mixture (Chapter 2).

One half of the roots in the split-root experiment was treated with either sterile water (control), the non-pathogen (CAV 553), *P. fluorescens* (WCS 417), or a combination

of CAV 553 and WCS 417. These roots were slightly wounded to ensure penetration by the putative biological control agents. The non-pathogenic *F. oxysporum* was applied to achieve a final spore concentration of 1 x 10^5 spores.ml^{-1}, and the bacterium cell suspension to achieve a final concentration of 1 x 10^7 cfu.ml^{-1} in the water surrounding the banana roots. The other half of the banana roots were inoculated 3 days later with *Foc* with a final spore concentration of 1 x 10^5 spores.ml^{-1}. The roots were again slightly damaged to ensure penetration by the pathogen. Five plants were used for each treatment, and the experiment was repeated three times. Inoculated plants were kept in a phytotron set at a 12-hour day/night illumination cycle, with the daytime temperature set at 28°C, and the night temperature at 20°C.

The banana plants were evaluated for internal symptom development 4 weeks after inoculation. Severity of symptoms was rated according to the INIBAP rating scale (Carlier *et al*., 2002). No discolouration of the rhizome was rated 0, 1-25% discolouration as 1, 26-50% discolouration as 2, 51-75% discolouration as 3 and 76-100% discolouration of the rhizome as 4. Disease severity was calculated using the formula of Sherwood and Hagedorn (1958): Disease severity (%) = ∑[(number of plants in disease scale category) x (specific disease scale category)/ (total number of plants) x (maximum disease scale category)] x 100.

Phenolic assays:

For the phenolic assays, a split-root experiment was set up as described above. One half of the roots was treated with sterile distilled water (control), the non-pathogenic *F. oxysporum* isolate, *P. fluorescens*, or the pathogenic *Foc* isolate. Roots were slightly wounded to ensure penetration by the inoculated organisms. The other half of the roots were not inoculated with any of the isolates, and these roots were also not wounded. To determine the effect of wounding on phenol production, plants without wounding and splitted into two halves were also included in the study.

Roots of the non-inoculated half of banana plants in split-root assays were collected for phenolic analysis at 0, 6, 24 and 48 hours after inoculation. Three root samples were taken at each time interval from each of five plants. The roots were then placed into 50-ml Falcon tubes (Greiner bio-one, Frickenhausen, Germany), and the opening

of the tubes was sealed with tissue paper and an elastic band. All the tubes were stored at -70°C, where after the root material was freeze-dried.

Phenolics were extracted from banana roots using a modification to the method described by De Ascensao and Dubery (2003). The dried root material was ground with an electric homogeniser (IKA A111 basic analytical mill, United Scientific (Pty) Ltd., San Diego, USA). Phenolics were extracted in duplicate from 0.05 g root material with a 1 ml solution of methanol (MeOH) (AnalaR, Wadeville, Gauteng, South Africa) : acetone (Merck, Darmstadt, Germany) : distilled water at a ratio of 7:7:1 (v:v:v). The mixture was homogenised for 1 hour on a rotary shake incubator (Labotec, Midrand, South Africa) at 25°C, and centrifuged for 10 minutes at 12 000 x g. After centrifugation, the supernatant was saved and poured into a 2-ml eppendorf tube (Merck, Darmstadt, Germany). The remaining precipitate was re-homogenised and centrifuged as above. The second supernatant was combined with the first and the procedure was repeated. The three combined supernatants were concentrated to 1 ml. Sterile water was added to the concentrated supernatant to make up 2 ml. This was done to ensure the separation of the layers when anhydrous diethyl ether (Saarchem, Merck Laboratories, Darmstadt, Germany) was added. Aliquots of 0.5 ml were made into four 2-ml eppendorf tubes in order to determine total soluble phenolic acids, free phenolic acids, MeOH-soluble ester-bound phenolic acids and MeOH-soluble glycoside-bound phenolic acids. The remaining precipitate was dried at 70°C for 24 hours. The resulting alcohol insoluble residue (AIR) yielded the cell wall material that was used to extract the ester-bound cell wall phenolic acids.

Total soluble phenolic acids

Total soluble phenolic content was determined by the reduction of the phospho-molybdene/phospho-tungstate that is present in the Folin-Ciocalteau reagent (Swain and Hills, 1959). Five µl of the concentrated phenolics supernatant was diluted to 175 µl with water and mixed with 25 µl of 20% (v/v) Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, Missouri, USA) in ELISA plates. After 3 minutes, 50 µl of saturated aqueous sodium carbonate ($NaCO_3$) (Glassworld, Roodepoort, South Africa) was added, and the suspension mixed and incubated at 40°C for 30 minutes. A blank of water was used as control. The absorbance was read using an ELISA reader

(Multiskan Ascent V1.24354 – 50973, Version 1.3.1). Gallic acid was used as a phenolic standard to construct a standard curve ranging from 0 to 40 mg (y=1.3527x – 0.0109, R^2 = 0.9986). The concentration of the phenols in the various extracts was calculated from the standard curve and expressed as mg gallic acid.g^{-1} dry weight.

Non-conjugated phenolic acids

Fifty µl of 1 M Trifluoroacetic acid (TFA) (Sigma) was added to 500 µl of the aliquoted phenolics supernatant to acidify the solution prior to extraction with 1 ml of anhydrous diethyl ether (Saarchem, Merck Laborotories) (Cvikrová *et al.*, 1993). The extraction process was repeated three times. The diethyl ether extract was dried overnight in the laminar flow and the resulting precipitate was re-suspended in 250 µl of 50% MeOH. This solution was used to determine the free phenolic content with the Folin-Ciocalteau reagent as described above.

Glycoside-bound phenolics

The MeOH soluble glycoside-bound phenolic content was determined by hydrolysing 500 µl of the aliquoted phenolics supernatant with 50 µl concentrated pure HCL (Merck) at 96°C for 1 hour. It was then placed on ice for 10 minutes and the glycoside-bound phenolics extracted thereafter with 1 ml anhydrous diethyl ether (Saarchem, Merck Laboratories). The ether extract was dried overnight in a laminar flow cabinet, and the remaining precipitate was re-suspended in 250 µl 50% MeOH. This solution was used to determine the glycoside phenolic content with the Folin-Ciocalteau reagent.

Ester-bound phenolics

Soluble ester-bound phenolics were extracted after alkaline hydrolysis of the measured root powder samples under mild conditions (Cvikrová *et al*., 1993). To determine the phenolic ester content of the aliquoted sample, 125 µl of a 2 M NaOH (Merck, Midrand, South Africa) was added to the sample and the tubes were left to stand at room temperature for 3 hours. After hydrolysis, 150 µl 1 M HCL was added and the phenolics extracted with 1 ml anhydrous diethyl ether (Saarchem, Merck Laboratories) as described above. This solution was used to determine the phenolic ester content using the Folin-Ciocalteau reagent.

Cell wall-bound phenolic acids

Ester-bound phenols incorporated in the cell wall were extracted from the 0.05 g root sample following alkaline hydrolysis (Campbell and Ellis, 1992). Dry cell wall material (AIR) was weighed (10 mg) and re-suspended in 1 ml 0.5 M NaOH for 1 hour at 96°C. Under these mild saponification conditions, cell wall-esterified hydroxycinnamic acid derivatives were selectively released. After saponification the tubes were cooled on ice for 10 minutes and the supernatant was acidified to pH 2 with 40 µl concentrated HCl, centrifuged at 12 000 x g for 10 minutes, and then extracted with 1 ml anhydrous diethyl ether (Saarchem, Merck Laboratories). The extract was dried overnight in a laminar flow and the precipitate was re-suspended in 250 µl 50% MeOH. This solution was used to determine the cell wall-esterified phenolic acids content with the Folin-Ciocalteau reagent.

Statistical analysis:

For the split root experiment the General Linear Models (GLM) procedure of *Statistica*, version 7 (STATSOFT Inc. 2004) was used. Experiments were analyzed using one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Significance was evaluated at $P < 0.05$ for all tests.

RESULTS

Greenhouse testing for induced resistance:

When applied to one half of banana roots in a split root system, the non-pathogenic *F. oxysporum* isolate reduced the disease severity of Fusarium wilt significantly ($P < 0.05$) (Fig. 2). The non-pathogen and *P. fluorescens* WCS 417 reduced disease severities by 62.5% and 45.8%, respectively. The combined application of the non-pathogen and bacterium reduced disease severity by 37.5% (Fig. 2).

Phenolic assays:

Total soluble phenolics

The pathogenic and non-pathogenic *F. oxysporum* isolates, as well as the *P. fluorescens* isolate, significantly ($P \leq 0.05$) increased production of total phenolics in

inoculated banana roots 6 hours after inoculation (Fig. 3A). A significant increase in total phenolic content continued at 48 hours in the roots treated with *P. fluorescens* and *Foc,* but decreased in roots treated with the non-pathogen after 24 hours. The amount of total soluble phenolics for *P. fluorescens* and *Foc* treated roots induced after 24 hours, however, did not differ significantly from the phenolics produced at 0 hours, except where CAV 553 was inoculated. There was no significant difference in production between the pathogen and the putative biological control agents. Total phenolics did not increase in the non-wounded control treatments, while a minor, non-significant increase was observed in the wounded control plants (Fig. 3A).

When measured in non-inoculated roots, *P. fluorescens* increased total phenolic production significantly within 6 hours, while the pathogenic and non-pathogenic *F. oxysporum* only induced significant systemic production of total phenolics after 48 hours (Fig. 3B). There was a significant increase in phenolic production in the banana roots of the wounded control treatments after 6 hours, which became non-significant after 24 hours. No increase was found in the non-wounded control treatments. The increase of total phenolics in wounded banana roots of the control treatment was not significantly different to those induced by the *F. oxysporum* isolates. However, the bacterium induced the production of significantly more total phenolics than the wounded roots of the control treatment after 24 hours (Fig. 3B).

Total phenolic content induced locally (infected roots) and systemically (non-infected roots) was similar for all treatments, with a few exceptions (Fig. 3A and B). For *P. fluorescens*, phenolic production increased significantly in roots distant from the inoculated roots after 24 hours. Similarly, total soluble phenolic production was significantly higher in non-treated roots of the wounded control 6 hours after inoculation.

Non-conjugated phenolic acids (*Free acids*)

Pseudomonas fluorescens, the non-pathogenic *F. oxysporum* isolate and *Foc* increased the levels of free phenolics in the inoculated banana root, but not significantly (Fig. 4A). The exception was a significantly higher production of free phenolic acids 24 hours after infection with the non-pathogenic *F. oxysporum* isolate and following wounding. This increased production was reduced significantly after 48

hours when infected by the non-pathogen, but not in the case of wounding. *Foc* also elevated the level of free phenolic production, but not significantly. No difference in phenolic production was observed in roots inoculated with *P. fluorescens* over time.

Free phenolic acid production in non-inoculated roots of plants treated with the non-pathogenic *F. oxysporum* isolate and *Foc* was significant induced after 6 hours, but decreased again after 24 hours (Fig. 4B). Production of free phenolic acids in plants wounded and treated with the bacterium did not differ from the control treatment over time.

Free phenolic acids produced locally in wounded roots of the wounded control treatment were significantly higher than in the non-wounded side of the roots of the same treatment after 24 hours. No difference, however, was found after 48 hours. On the contrary, free phenolics induced systemically by pathogenic and non-pathogenic *F. oxysporum* was significantly higher after 6 hours than that induced locally.

Glycoside-bound phenolics

The non-pathogenic *F. oxysporum* isolate produced significantly more glycoside-bound phenolics 6 hours after root inoculation with non-pathogenic *F. oxysporum* when compared to wounding and treatment with *P. fluorsescens* (Fig. 5). This production, however, was reduced to levels similar to the other treatments after 24 hours. Glycoside-bound phenolics were not induced significantly in non-inoculated roots following wounding or treatment with *F. oxysporum* or *P. fluorescens* in the split-root experiment. The local response of roots also did not differ from the systemic response (Fig. 5).

Ester-bound phenolics

The ester-bound phenolics produced in banana roots showed an increase 6 and 24 hours after inoculation with non-pathogenic *F. oxysporum*, *P. fluorescens* and *Foc* (Fig. 6). This increase was more substantial when compared to the amount of phenolics produced in non-wounded control roots. However, none of the treatments produced significantly more phenolics than the non-wounded control treatment, apart from *Foc* 6 hours after inoculation. Ester-bound phenolics produced locally and systemically following wounding and treatment with putative biological control

agents also did not differ significantly from each other (Fig. 6). In the systemic response, phenol production was also increased after 6 and 24 hours in the wounded control and following treatment of roots with pathogenic and non-pathogenic *F. oxysporum*. Significantly more phenolics, however, were only produced in the plants with wounded roots after 6 hours, when compared to the control treatments (Fig. 6).

Cell wall-bound phenolics

The non-pathogenic *F. oxysporum* isolate produced higher levels of cell wall-bound phenolics in banana roots when compared to non-wounded roots and roots inoculated with *P. fluorescens* and *Foc*, but not when compared to wounded control roots (Fig. 7). The highest production occurred after 48 hours. Phenolics production in roots wounded and infected with the non-pathogen differed significantly from non-wounded roots and roots treated with the bacterium 24 hours after inoculation, and from non-wounded roots and treatment with *Foc* 48 hours after inoculation. Similarly, the systemic induction of cell-wall bound phenolics was most substantial in plants where roots were wounded and in those treated with the non-pathogenic *F. oxysporum*, with significantly more phenolic production in these treatments after 24 and 48 hours. No significant differences in the amount of phenolic acids produced locally and systemically were observed following wounding and treatment with *F. oxysporum* and *P. fluorescens*, except for treatment with the bacterium after 6 hours.

DISCUSSION

Induced resistance was demonstrated as an important mode of action whereby non-pathogenic *F. oxysporum* significantly reduced Fusarium wilt in banana in this study. Due to the separation of the roots using the split-root technique, the hypotheses of competition for nutrients or for infection sites between the biocontrol candidates and *Foc* must be excluded as means of control. Induced resistance is a common mechanism whereby microbial agents such as non-pathogenic *F. oxysporum* and *P. fluorescens* protect agricultural crops against Fusarium wilt diseases (Scher and Baker, 1980; Alabouvette, 1990; Leeman *et al.*, 1995a, b; Leeman *et al.*, 1996; Fuchs *et al.*, 1997; Duijff *et al.*, 1998, 1999; Thangavelu *et al.*, 2003).

Non-pathogenic *F. oxysporum* reduced Fusarium wilt by means of induced resistance of cucumber (Mandeel and Baker, 1991), chickpea (Hervás *et al*., 1995) and tomato (Fuchs *et al*., 1997). Treatment of cucumber plants with non-pathogenic *F. oxysporum* Fo47 showed the elaboration of newly formed barriers, a phenomenon that was not seen in Fo47-free plants, and the occlusion of intercellular spaces with a dense material probably enriched in phenolics (Benhamou *et al*., 2002). The accumulation of PR proteins including chitinases and ß-1,3-glucanses were shown to be involved in induced resistance against Fusarium wilt of tomato by non-pathogenic *F. oxysporum* (Fuchs *et al*., 1997; Duijff *et al*., 1998). *Pseudomonas fluorescens* has been shown to make plants more sensitive to pathogen infection, leading to suppression of Fusarium wilt in radish (De Boer *et al*., 1999), cucumber (Jeun *et al*., 2004) and tomato (Duijff *et al*., 1998). In tomato plants, *P. fluorescens* Pf1 increased accumulation of phenolics and activities of PAL, peroxidase (POX) and PPO in treated root tissue (Ramamoorthy *et al*., 2002). Thangavelu *et al*. (2003) found that *P. fluorescens* Pf10 increased PAL, POX, chitinase, ß-1,3-glucanase and accumulated phenolics after root treatment in banana plants.

Despite a reduction of 45.8 and 37.5%, *P. fluorescens*, alone or in combination with non-pathogenic *F. oxysporum*, respectively, did not reduce Fusarium wilt significantly. This is consistent with the findings of Larkin *et al*. (1996) who demonstrated that non-pathogenic *F. oxysporum* induced systemic resistance in watermelons, and that other *Fusarium* spp. and bacterial isolates (including fluorescent pseudomonads) were unable to significantly reduce Fusarium wilt. Olivain *et al*. (2004), however, found that non-pathogenic *F. oxysporum* Fo47 in combination with *P. fluorescens* C7 reduced Fusarium wilt of flax better than Fo47 alone, while enhanced disease suppression was shown when non-pathogenic *F. oxysporum* and *Pseudomonas* spp. were combined to treat Fusarium wilt of carnation (Lemanceau *et al*., 1992) and cucumber (Park *et al*., 1988). The use of combinations of biological control agents, therefore, might either enhance or reduce efficiency, and should be tested before application.

Induced resistance is defined as enhancement of plant defense responses activated by exogenous stimuli (Sticher *et al*., 1997) not only in the primary infected plant parts, but also in non-infected, spatially separated tissues (Van Loon *et al*., 1998). In this

investigation, it was demonstrated that pathogenic (*Foc*), non-pathogenic *F. oxysporum* and *P. fluorescens* induced resistance in banana roots both locally and systemically. SAR and LAR are effective across a wide range of plant species (Van Loon *et al*., 1998). Biles and Martyn (1989) found that *F. oxysporum* f.sp. *cumerinum* and avirulent races of *F. oxysporum* f. sp. *niveum* induced local and systemic resistance to Fusarium wilt in watermelon cultivars. Split root experiments performed by He *et al*. (2002) also resulted in a hypersensitive response and induced the systemic production of peroxidase, PAL and lignin in the asparagus (*Asparagus officinalis* L.) root system when inoculated with non-pathogenic *F. oxysporum* strains. When non-pathogenic *F. oxysporum* were inoculated on tissue culture banana plantlets they intensely colonised the rhizome, but their numbers were reduced drastically after field planting (Paparu, 2005). Sikora *et al*. (2007), however, found evidence that the endophytes initially inoculated on the mother plant were able to induce resistance to nematodes in banana suckers

Non-pathogenic *F. oxysporum* significantly induced the local production of glycoside-bound phenolics 6 hours after *Foc* challenge, followed by its significant reduction after 24 hours. Free phenolic production was significantly more 24 hours after *Foc* challenge, before it was reduced after 48 hours. Cell wall bound phenolics were highest after 48 hours. This sequence of events suggests that the glycoside-bound phenolic acids were possibly first released and then polymerised to the cell wall, thereby strengthening the cell wall (Higuchi, 1985). The role of these changes in phenolic composition of banana roots, induced by non-pathogenic *F. oxysporum*, should be further demonstrated in histochemical studies. *Foc*-tolerant and resistant varieties might also show more definite progression in phenolic expression in banana roots, as they were previously shown to produce more phenolics than susceptible varieties (De Ascensao and Dubery, 2000; 2003; Van den Berg *et al*., 2007). Systemic induction of the respective phenolic acids by non-pathogenic *F. oxysporum* did not follow the same pattern as locally induced phenolic production. In systemic induction, free phenolic acid production was highest after 6 hours, and was potentially lignified in cell walls thereafter.

Defense responses in plants are characterized by the early accumulation of phenolic compounds at the infection site that slow the development of the pathogen as a result

of rapid cell death (Mace, 1963; Fernandez and Heath, 1989). This probably explains the significant induction of free and glycoside-bound phenolic acids in roots following infection with non-pathogenic *F. oxysporum* and *P. fluorescens*. Both the phenolic acids decreased after 24 hours, as they most likely become oxidized and eventually polymerized to form structural components of plant cell walls (Bidlack *et al*., 1992; Higuchi, 1985). This probability is reflected in the rise of cell wall-bound phenolics, both locally and systemically, in the case of non-pathogenic *F. oxysporum*. A significant rise in total phenolic content was found in distant roots of plants treated with *P. fluorescens*. *Pseudomonas fluorescens* is known to help sensitise the roots and stimulate production of secondary metabolites, which may aid in defence response (Van Loon *et al*., 1998). It has been suggested that plants have flexible detection systems, probably employing several recognition and signal transduction pathways to activate their defence mechanisms (Johal *et al*., 1995).

The only known mechanism to act as defense response against *Foc* in banana plants, is the build-up of mechanical barriers, vascular occluding gels and tyloses that may prevent the spread of the pathogen to the vascular system (Beckman, 1987; 1990). In this study, *Foc* substantially induced the production of ester-bound phenolics in inoculated roots after 6 hours, which could suggest cell wall strengthening in tissue challenged by the Fusarium wilt pathogen. Production of ester-bound phenolics, however, was reduced to normal after 24 hours. None of the other phenolics, apart from free phenolic acids in distant roots were significantly induced by the Fusarium wilt pathogen. Free phenolic acids are known for their antifungal properties and ability to serve as precursors for cell wall strengthening, and their production might be the result of cell wall-bound phenolics that were marginally reduced. Success or failure of resistance may depend on the relative rate and extent of the host's lignification response (De Ascensao and Dubery, 2000). Cavendish bananas are not resistant to *Foc* race 4 (Viljoen, 2002), and one would expect either the pathogen to suppress plant response, or the plant not to respond to pathogen attack (Agrios, 2005). When the banana cultivar Goldfinger, that is tolerant to *Foc* race 4, was challenged with cell wall components of the pathogen, the cell wall polysaccharides were esterified with hydroxycinnamic acids to resist the lytic enzymes produced by the pathogen (De Ascensao and Dubery, 2000). Similarly, when the non-pathogenic *F. oxysporum* and *P. fluorescens* were applied to Cavendish banana roots in this study,

the ester-bound phenolics increased both locally and systemically, although the increase was non-significant.

Cell wall-bound phenolic acids were induced rapidly at significant levels, locally and systemically, by non-pathogenic *F. oxysporum* in banana roots, but not by *Foc*. The cell wall-bound phenolics can be lignified in cell walls and act as an effective barrier to pathogen entrance and spread (Ride, 1983). It might also aid in the formation of tyloses, gums and pappilae, blocking the pathogen from further invasion. This might affect the outcome of the host's response to *Foc* race 4 and may contribute to resistance in the otherwise susceptible Cavendish banana variety. When inoculated with *Foc*, Williams was unable to produce similar quantities of cell wall-bound phenolics that could inhibit progress of the pathogen. Reasons for the inability to respond to *Foc* infection is outside the scope of this study, but might involve an inability of receptors in the plant to recognise the pathogen, or the suppression of the plant's defence responses by the pathogen (Di Pietro *et al*., 2003; Recorbet *et al*., 2003; Agrios, 2005).

It was clear from our results that wounding had an effect on phenolic acid production in bananas. Phenolic acids were induced locally and systemically, from 6 to 48 hours after damaging of the roots, as had been indicated by León *et al*. (2001) before. The substantial increase in cell wall-bound phenolics in this study strongly suggests repair of structural damage in the part of the banana roots that were wounded, and the strengthening of the part of the roots of the same banana plant that were not wounded (León *et al*., 2001). The accumulation of phenolic compounds after wounding had been demonstrated on purple flesh potatoes (Reyes and Cisneros-Zevallos, 2003) and Romaine lettuce (Kang and Saltveit, 2003) before. Farmer and Ryan (1992) proposed a model for expression of defense-related genes in tomato leaves in response to wounding. Systemin initiates a cascade of intracellular events resulting in the activation of cytoplasmic phospholipase that releases linolenic acid from membranes. Linolenic acid is converted to jasmonic acid, which is a powerful activator of genes coding for both signal pathway enzymes and defensive proteinase inhibitors and polyphenol oxidase (Farmer and Ryan, 1992; Orozco-Cárdenas *et al*., 2001). Polyphenol oxidase catalyses the oxidation of hydroxyphenols to their quinone derivatives, which then spontaneously polymerize (Shi *et al*., 2001). This

polymerisation may lead to increased levels of cell wall-bound phenolics, as was demonstrated in this study, which may become lignified and inhibit pathogen ingress. If the viability of a plant part is limited due to wounding that is severe it will be more advantageous for the plant if abscission of the plant part affected occurs (León *et al*., 2001).

In this study, it seemed likely that the non-pathogenic *F. oxysporum* isolate stimulated banana roots to produce high levels of antimicrobial phenolic compounds that eventually diffused out of storage, became polymerised and increased cell wall-bound phenolics. This had possibly led to a more impermeable cell wall layer in the roots that inhibited *Foc* infection. Our results, therefore, indicate that non-pathogenic *F. oxysporum* might be considered a biological control agent to help reduce infection by *Foc* race 4 to Cavendish bananas. Since no control strategy has yet been developed to protect Cavendish bananas against Fusarium wilt, an integrated strategy, which involves the use of non-pathogenic *F. oxysporum*, might provide a temporary means to limit the impact of the disease until alternative strategies are developed to provide more sustainable protection of Cavendish bananas.

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Figure 1: Roots of banana were split into two parts, and each half of the roots was planted in a 250-ml cup in a split-root experiment. The cups were filled with 150 ml water to prevent overflow of water into the other cup. Strips of sponge were wrapped around the stems to ensure that the lids did not damage the stems. The exposed stems were covered with wet cotton wool, and the cup system was enclosed in a black plastic bag to prevent the roots from drying out.

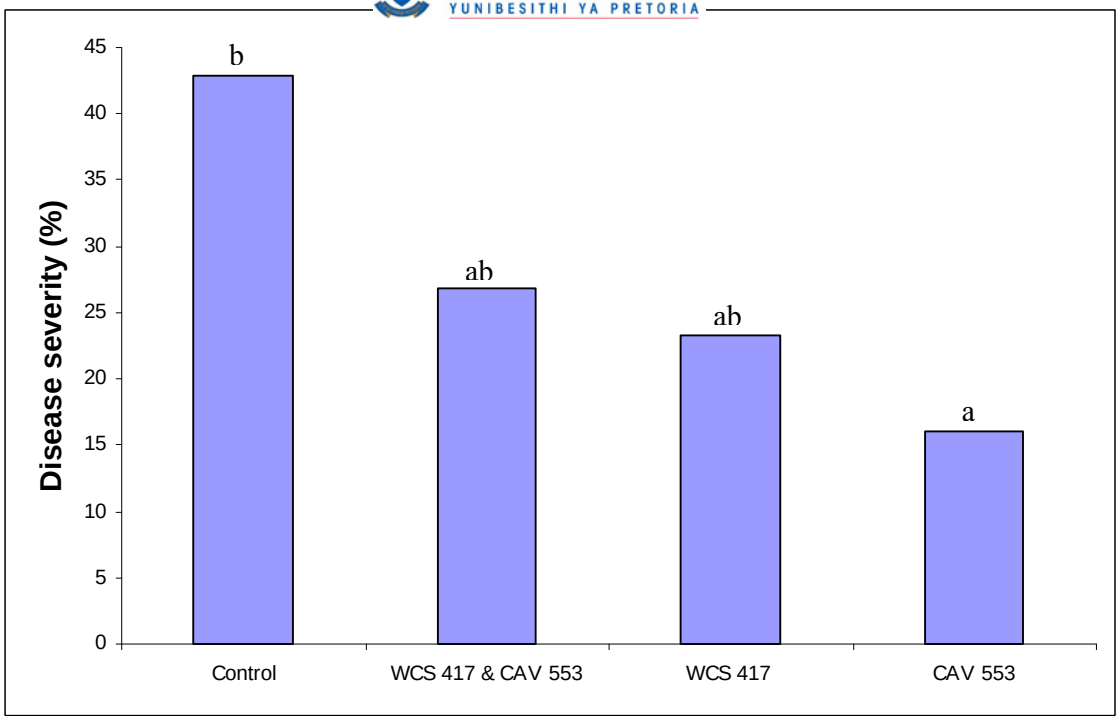

Figure 2: Mean Fusarium wilt disease severity in banana roots inoculated with *Fusarium oxysporum* f.sp. *cubense* following a split-root treatment with sterile water (control), non-pathogenic *F. oxysporum* (CAV 553), *Pseudomonas fluorescens* (WCS 417) and a combination of the two. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

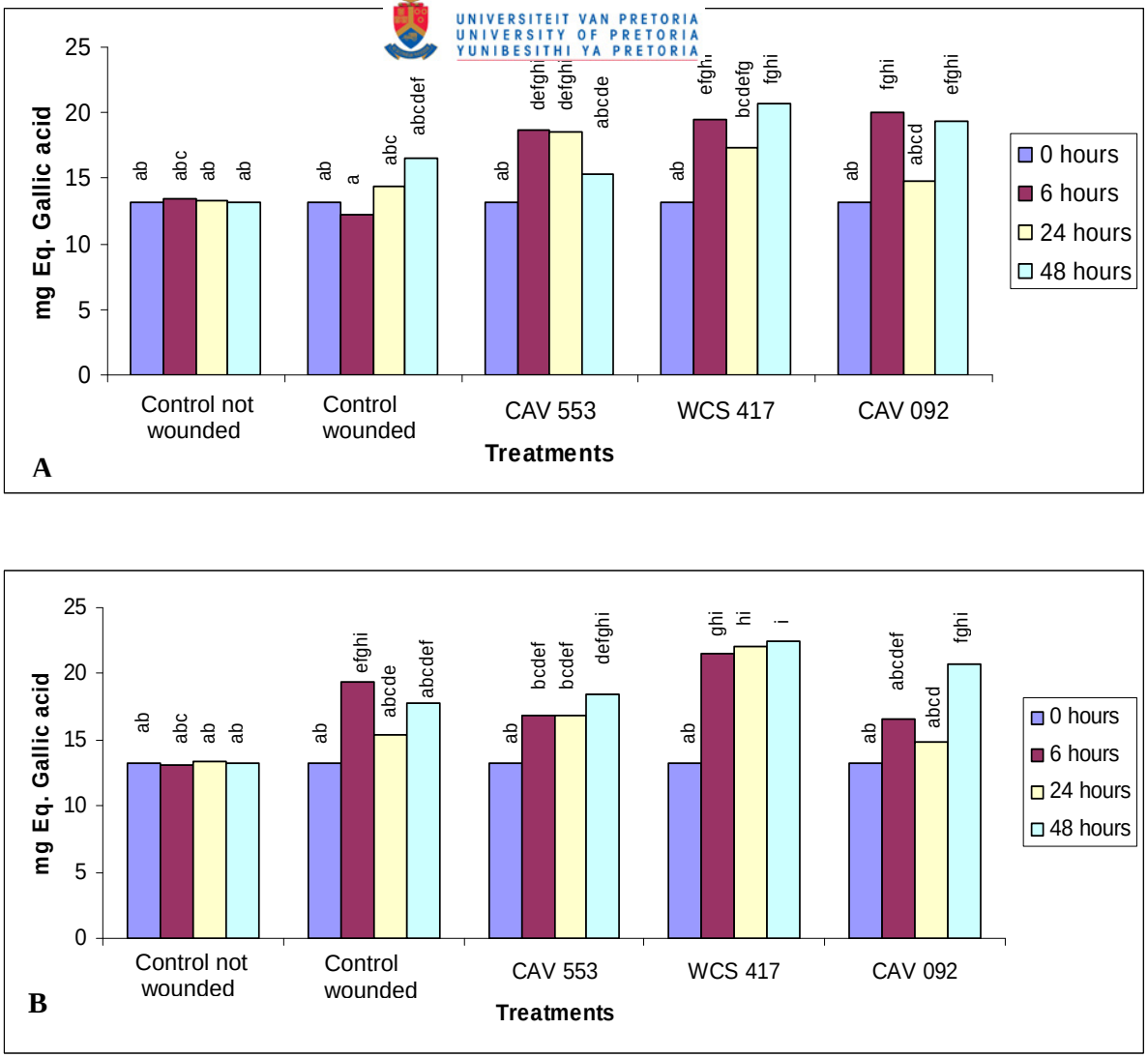

Figure 3: Total soluble phenolic content in Williams banana plants at 0, 6, 24 and 48 hours after inoculation with non-pathogenic *Fusarium oxysporum* (CAV 553), *Pseudomonas fluorescens* (WCS 417) and the pathogenic *F. oxysporum* f.sp. *cubense* (CAV 092). The banana root ball was split into two parts. **A** represents the side of the banana roots that was treated with the different microorganisms. **B** represents that half of the banana roots that was treated with sterile water only. Phenolics were determined with the Folin reagent in milligrams of Gallic acid/g dry weight. Experiments were analysed using one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

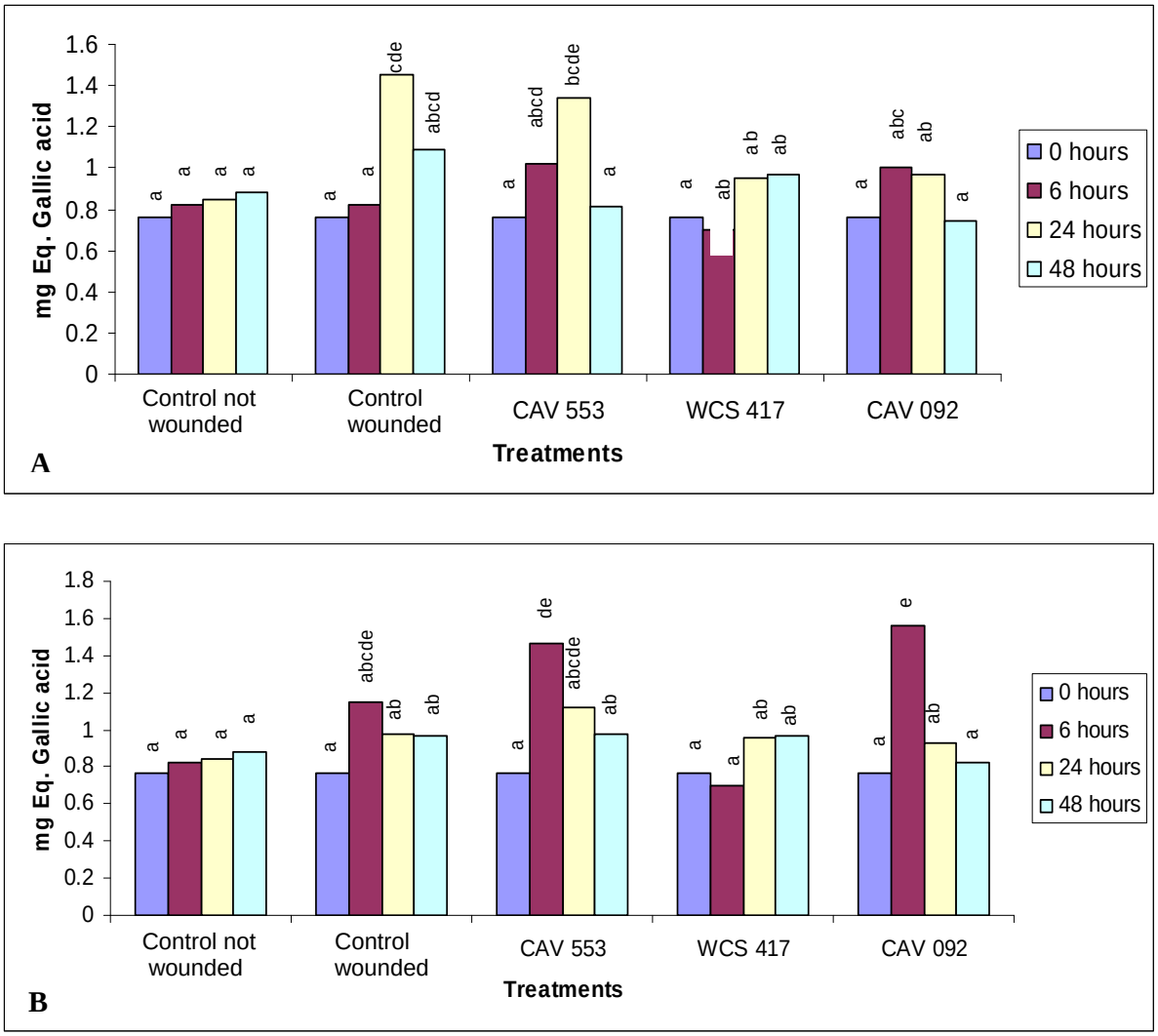

Figure 4: Free phenolic content in Williams banana plants at 0, 6, 24 and 48 hours after inoculation with non-pathogenic *Fusarium oxysporum* (CAV 553), *Pseudomonas fluorescens* (WCS 417) and the pathogenic *F. oxysporum* f.sp. *cubense* (CAV 092). The banana root ball was split into two parts. **A** represents the side of the banana roots that was treated with the different microorganisms. **B** represents that half of the banana roots that was treated with sterile water only. Phenolics were determined with the Folin reagent in milligrams of Gallic acid/g dry weight. Experiments were analysed using one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

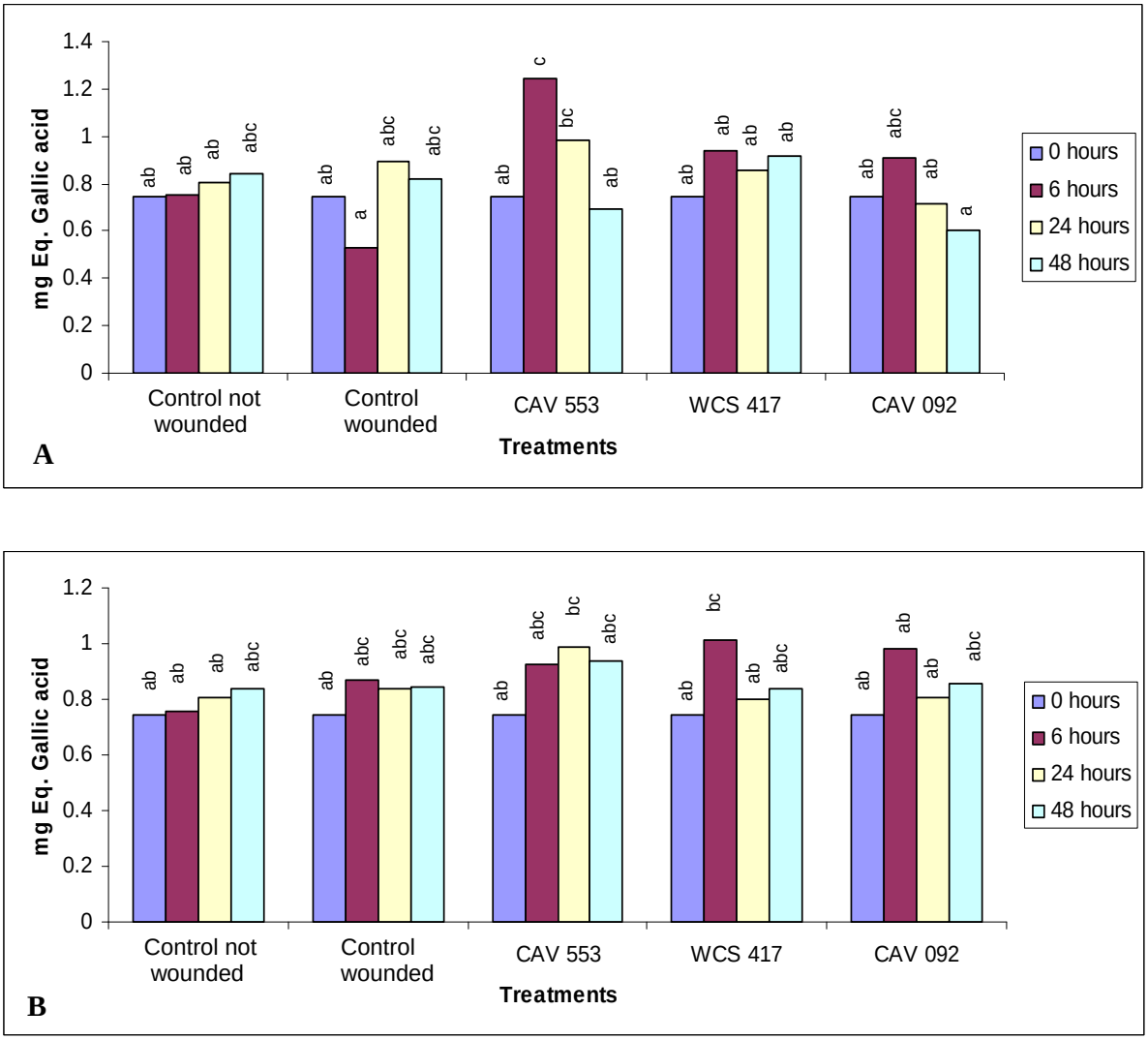

Figure 5: Glycoside-bound phenolic content in Williams banana plants at 0, 6, 24 and 48 hours after inoculation with non-pathogenic *Fusarium oxysporum* (CAV 553), *Pseudomonas fluorescens* (WCS 417) and the pathogenic *F. oxysporum* f.sp. *cubense* (CAV 092). The banana root ball was split into two parts. **A** represents the side of the banana roots that was treated with the different microorganisms. **B** represents that half of the banana roots that was treated with sterile water only. Phenolics were determined with the Folin reagent in milligrams of Gallic acid/g dry weight.

Experiments were analysed using one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

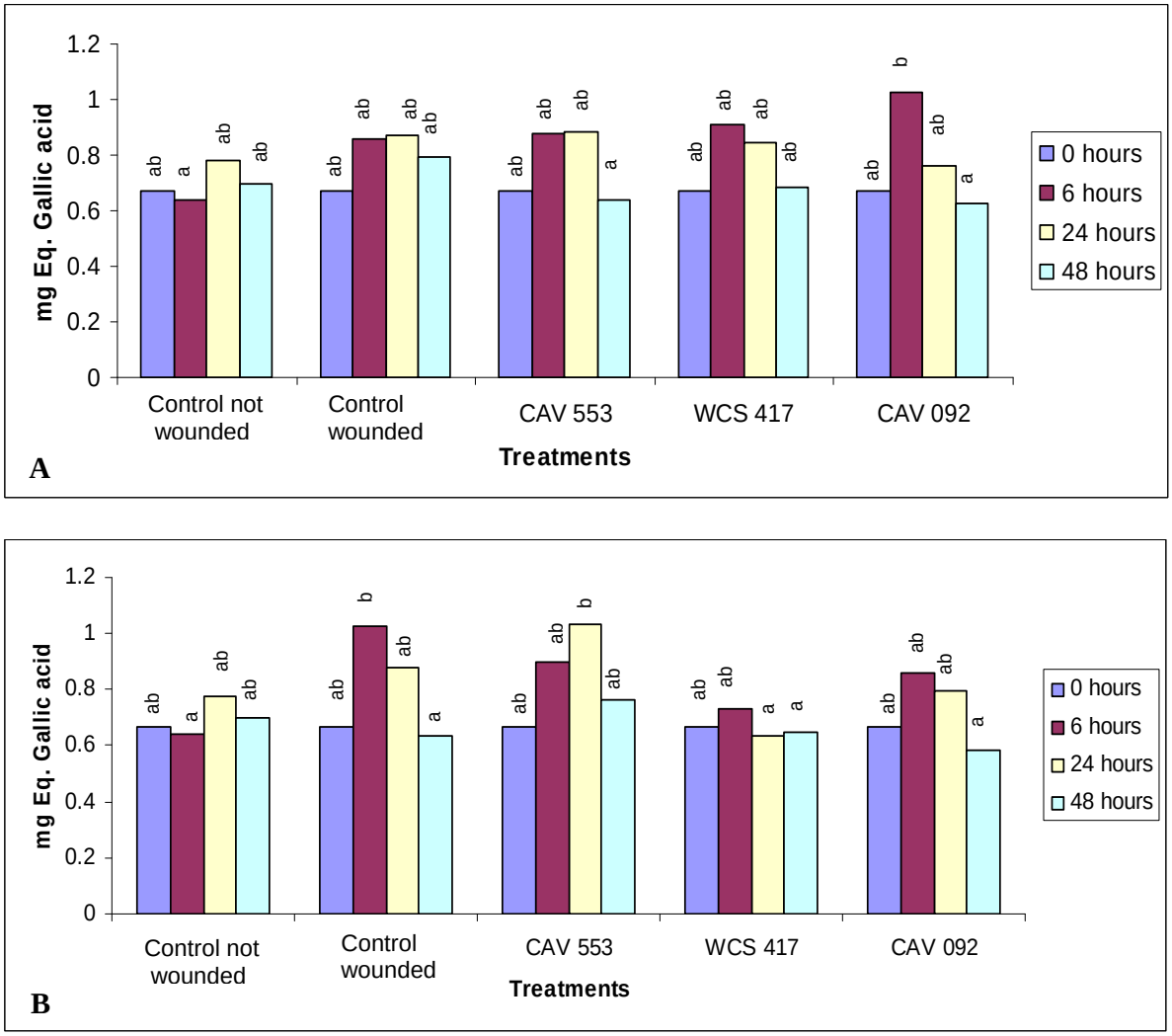

Figure 6: Ester-bound phenolic content in Williams banana plants at 0, 6, 24 and 48 hours after inoculation with non-pathogenic *Fusarium oxysporum* (CAV 553), *Pseudomonas fluorescens* (WCS 417) and the pathogenic *F. oxysporum* f.sp. *cubense* (CAV 092). The banana root ball was split into two parts. **A** represents the side of the banana roots that was treated with the different microorganisms. **B** represents that half of the banana roots that was treated with sterile water only. Phenolics were determined with the Folin reagent in milligrams of Gallic acid/g dry weight. Experiments were analysed using one-way analysis of variance (ANOVA) and the

Tukey Honest Significant Difference (HSD) test. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

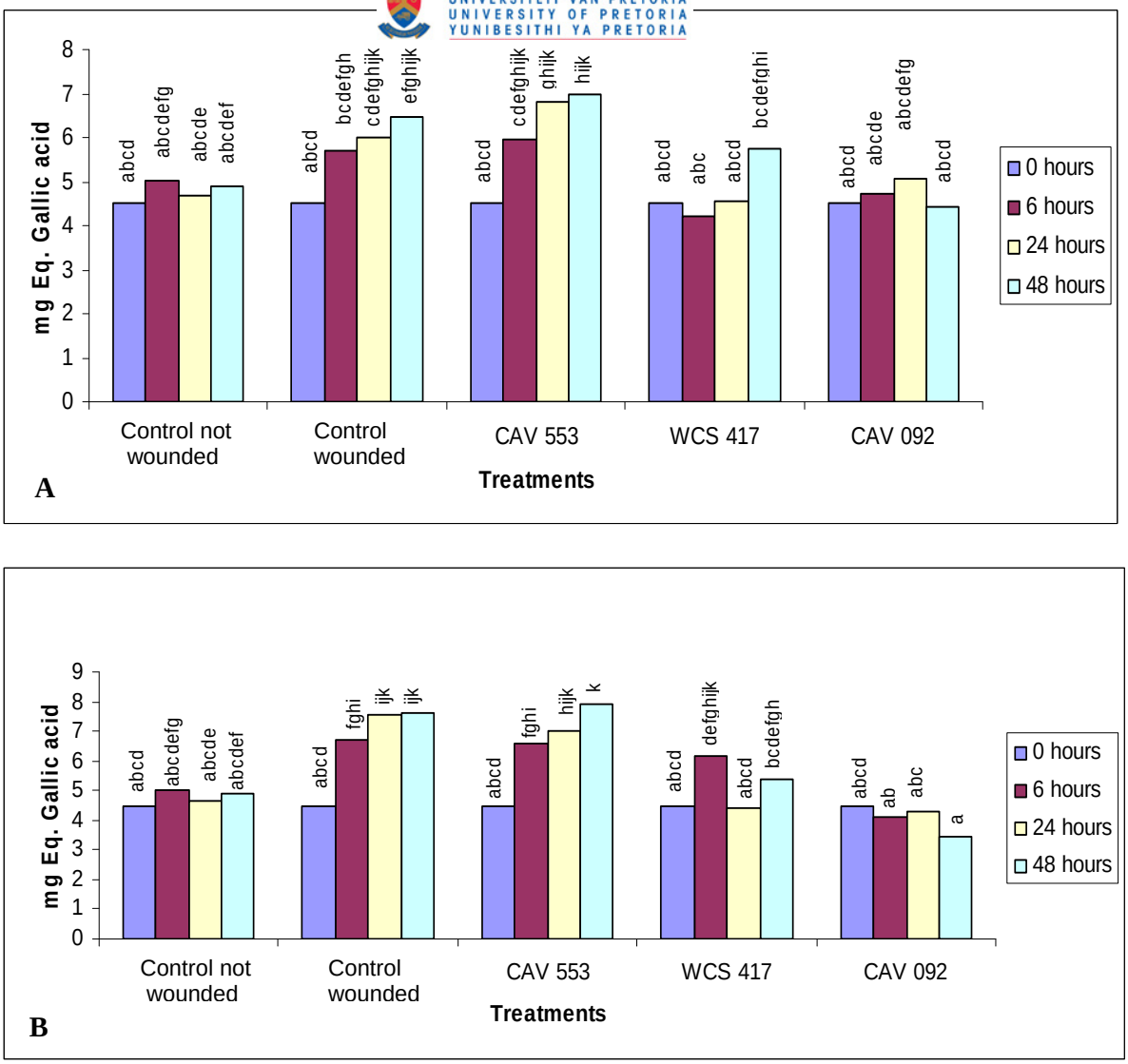

Figure 7: Cell wall-bound phenolic content in Williams banana plants at 0, 6, 24 and 48 hours after inoculation with non-pathogenic *Fusarium oxysporum* (CAV 553), *Pseudomonas fluorescens* (WCS 417) and the pathogenic *F. oxysporum* f.sp. *cubense* (CAV 092). The banana root ball was split into two parts. **A** represents the side of the banana roots that was treated with the different microorganisms. **B** represents that half of the banana roots that was treated with sterile water only. Phenolics were determined with the Folin reagent in milligrams of Gallic acid/g dry weight. Experiments were analysed using one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

Chapter 4

Transformation of a non-pathogenic *Fusarium oxysporum* endophyte with the green (GFP) and red (DsRed-Express) fluorescent protein genes

ABSTRACT

The green fluorescent protein (*GFP*) and *DsRed-Express* genes emit green and red fluorescence, respectively, when exited by UV light using the appropriate filters. These reporter genes are useful tools for studying gene expression, labelling pathogenic fungi, and following the development of labelled fungi in their plant hosts. In this study, a non-pathogenic *F. oxysporum* isolate that was previously shown to be superior in reducing Fusarium wilt of banana, was transformed with the two reporter genes using hygromycin as a selectable marker. Fluorescence microscopy revealed expression of the *GFP* and *DsRed-Express* genes in all the fungal structures. PCR analysis of the transformed isolates that were sub-cultured to non-selective media for a prolonged period of time confirmed that the *GFP* and *DsRed-Express* genes were present in genomic DNA. The transformed isolates did not differ from the wild type in growth and morphological cultural characteristics. Non-pathogenic *F. oxysporum* isolates can reduce the severity of Fusarium wilt diseases by competing for infection sites and nutrients in the soil, and by inducing systemic resistance in the plant. The non-pathogenic *F. oxysporum* isolates transformed in this study will be screened to determine their efficiency to colonize banana roots and suppress the Fusarium wilt pathogen *Fusarium oxysporum* f. sp. *cubense* infection in a non-invasive manner.

INTRODUCTION

The green fluorescent protein (GFP) was first isolated from the jellyfish *Aequorea Victoria* (Murbach and Shearer) in 1992 (Lorang *et al*., 2001). Its GFP homologue, DsRed (Wall *et al*., 2000), was then isolated from *Discoma*, which is a reef coral species (Matz *et al*., 1999). Both GFP and DsRed have been expressed in plants (Stewart, 2001; Jach *et al.*, 2001), mammals (Pines, 1995; Marsh-Armstrong *et al*., 1999; Lauf *et al*., 2001), yeasts (Niedenthal *et al*., 1996; Rodrigues *et al*., 2001) and fungi. These fluorescent protein genes are used as reporters of gene expression (Prasher, 1992; Yeh *et al*., 1995), to label pathogenic fungi (Sheen *et al*., 1995; Visser, 2003; Nahalkova and Fatehi, 2003), and to follow the development of labelled fungi in their plant hosts (Bolwerk *et al*., 2005; Olivain *et al*., 2006). Filamentous fungi labelled with the GFP gene include *Ustilago maydis* (de Candolle) Corda (Spellig *et al*., 1996), *Aureobasidium pullulans* (de Barry) G. Amaud (Van den Wymelenberg *et al*., 1997), *Colletotrichum lindemuthianum* (Saccardo and Magnus) Briosi (Dumas *et al*., 1999), *Cochliobolus heterostrophus* (Drechsler) (Maor *et al*., 1998), *Aspergillus flavus* (Link) (Du *et al*., 1999), *Aspergillus niger* (von Tiegham) (Santerre Henriksen *et al*., 1999), *Trichoderma harzianum* (Rifai) (Bae and Knudson, 2000) and *Fusarium oxysporum* f. sp. *cubense* (E. F. Smith) Snyd. & Hans (*Foc*) (Visser, 2003), while those transformed with DsRed-Express include *Penicillium paxilli* (Bainier), *Trichoderma harzianum* (Rifai), *Trichoderma virens* (Miller, Giddens and Foster) von Arx (Mikkelsen *et al*., 2003), and *Neurospora crassa* (Schear and Dodge) (Freitag and Selker, 2005). Non-pathogenic as well as pathogenic forms of *F. oxysporum* have been transformed with the GFP and DsRed variants (Bolwerk *et al*., 2005; Olivain *et al*., 2006).

The pathogen *Foc* causes a highly destructive vascular wilt disease of banana plants (Stover, 1962). This fungus is a soil inhabitant and extremely difficult to control. More knowledge on the *in vivo* interactions between the pathogenic fungus and the plant could lead to the discovery of more efficient ways to control the disease. Details of these interactions can be essential in studies of biocontrol of the fungus by beneficial antagonistic microorganisms, such as non-pathogenic *F. oxysporum*, that colonize the banana pseudostem or the banana rhizosphere (Chapter 2; Gerlach *et al*., 1999; Nel *et al*., 2006). It has been found that non-pathogenic *F. oxysporum* isolates

can reduce infection by pathogenic *F. oxysporum* isolates through competition for infection sites and nutrients, as well as by inducing systemic resistance in the plant host (Couteaudier and Alabouvette, 1990; Mandeel and Baker, 1991; Fuchs *et al*., 1997; Fravel *et al*., 2003 and Alabouvette *et al*., 2004). Dual labelling of pathogenic and non-pathogenic *F. oxysporum* isolates with different autofluorescent proteins will allow the in depth analysis of direct interactions between the biocontrol agent and *Foc* on the banana root.

The objective of this study was to develop stable green- and red fluorescent transformants of a non-pathogenic *F. oxysporum* isolate (CAV 553) through transformation with the reporter genes GFP and DsRed-Express. The DsRed-Express gene is a variant from the wild type *Discoma* sp. red fluorescent protein (BD Living Colors™.User Manual Volume II. BD Biosciences. 2003). DsRed-Express has a reduced level of residual green emission and allows for complete separation of red-emitting and true green emitting populations (Bevis and Glick, 2002; BD Living Colors™.User Manual Volume II. BD Biosciences. 2003). The transformants that showed the highest levels of fluorescence were compared to the wild-type isolate to determine whether the transformants retained their wild-type morphological characteristics. The reporter gene-labelled non-pathogenic transformants will be used in future microscopy studies to investigate their mode of interactions with a GFP-labelled *Foc* isolate (Visser *et al*., 2004) when suppressing disease development.

MATERIALS AND METHODS

Fungal isolates and culture conditions

A non-pathogenic isolate of *Fusarium oxysporum* (CAV 553) that has previously been shown to suppress Fusarium wilt of bananas (Chapter 2), was selected for transformation with the *GFP* and *DsRed-Express* genes. CAV 553 was isolated from non-symptomatic banana roots in Fusarium wilt suppressive soils in Kiepersol, South Africa. This isolate is maintained in 15% glycerol at -80ºC and as freeze-dried stocks in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Transformation vectors

The transformation vector pCT74 (Lorang *et al*., 2001), kindly provided by J.M. Lorang (Oregon State University, Oregon, USA), was used to express GFP in CAV 553. pCT74 contains both the synthetic (s)*GFP* gene driven by the *Tox*A promotor of *Pyrenophora tritici-repentis* (Died.) Drechsler, and the *hyg*B gene under control of the *trpC* promoter of *Aspergillus nidulans* (Eidem).

Expression of the red fluorescent protein gene *DsRed-Express* in isolate CAV 553 was obtained using vector pPgpd-DsRed, kindly provided by L. Mikkelson (Royal Veterinary and Agricultural University, Frederiksberg, Denmark). The *DsRed-Express* gene is expressed under control of the constitutive *A. nidulans* glyceraldehydes 3-phophate promoter (*PgpdA*), with expression being terminated by the *A. nidulans trpC* transcriptional terminator (Mikkelsen *et al*., 2003). *pPgpd-DsRed* does not carry the *hyg*B resistance gene. Protoplasts transformed with this vector were, therefore, co-transformed with plasmid pHyg8 containing the *Escherichia coli hyg*B resistance gene. Vector pHyg8, donated by Dr. A. Mcleod (Stellenbosch University), was constructed by cloning the blunt-ended *Sal*I fragment from pCT74 into pBluescript (Stratagene, La Jolla, CA). pCT74 contained the *hyg*B gene driven by the *A. nidulans trp*C promoter, and Bluescript was digested with *Eco*RV before cloning.

Preparation of fungal protoplasts

Isolate CAV 553 was transformed using a protoplast-based polyethylene glycol/calcium chloride method (Lu *et al*., 1994). The isolate was first grown for 10 days on Potato Dextrose Agar (PDA) (39 g of Difco PDA powder, 1000 ml H_2O) (Biolab Diagnostics, Wadeville, South Africa). The culture plates were then flooded with sterile distilled water, and the spores harvested by filtering the suspension through miracloth (Calbiochem, EMB Biosciences, Inc., Merck KGa, Darmstadt, Germany). The spore suspension was adjusted to a final concentration of 2 x 10^6 spores.ml^{-1} and an equal volume of 2x 2YEG broth (0.8 g Yeast Extract, 4 g Glucose and 200 ml water) was added. One hundred millilitres of this suspension was transferred to an Erlenmeyer flask that was rotated at 50 rpm for 5 to 6.5 hours at 30ºC, until at least 1% germinating spores could be detected. The spore suspension was then centrifuged at 250 rpm for 10 minutes to collect the germinated spores. The

top solution was decanted, and the spore pellet washed in 30 ml 0.7 NaCl by spinning it again at 2500 rpm for 10 minutes.

An enzyme solution was added to the washed spore pellet. This enzyme solution consisted of 0.00142 g chitinase (Sigma-Aldrich, St. Louis, Missouri, USA), 0.67 g driselase (Sigma-Aldrich), 1 g lysing enzyme (Sigma-Aldrich), 0.8 g ß-1,3-glucanase (InterSpex Products, Inc., San Mateo, CA, USA) and 0.15 g cellulose (Yakult Pharmaceuticals, LTD, Minato-KU, Tokyo, Japan) in a total volume of 20 ml 0.7 M NaCl. Driselase was added to the NaCl first, and the suspension placed on ice for 15 minutes. It was then spun at 1800 rpm for 5 minutes before the supernatant was decanted into a clean tube. This process removed the starch that was present in the driselase formulation. The germinating spore suspension was then digested with the remaining enzymes in the solution by spinning at 50 rpm for 1.5 to 2 hours at 30ºC. When enough protoplasts have formed, the solution was spun at 2500 rpm for 10 minutes at 5ºC. The pellet was washed with 30 ml 0.7 M NaCl by being spun again at 2500 rpm at 5ºC for 10 minutes. The pellet was washed twice in 30 ml cold STC buffer (54.65 g Sorbitol, 1.84 g $CaCl_2$, 5 ml of 500 mM Tic-HCl pH 7.5 and 250 ml water), and spun for 10 more minutes at 2500 rpm at 5ºC. After the second wash the protoplast pellet was carefully re-suspended in the STC buffer.

Transformation of fungal protoplasts

Fungal protoplasts were transformed by mixing 100 µl (1 x 10^8 protoplasts.ml^{-1}) of protoplasts with the plasmid vectors. To insert the *GFP* gene into *Foc*, the protoplasts were mixed with 20 µl of pCT74. For expression of the *DsRed-Express* gene in transformants, protoplasts were co-transformed with 10 µl of pHyg8 and 20 µl of pPgpd-Ds-Red. The protoplast and vector mixtures were incubated for 10 minutes on ice, whereafter three aliquots (200, 200 and 800 µl) of PEG/Tris (12 g PEG, 400 µl 500 mM Tris-HCl pH 7.5, 1 ml 1 M $CaCl_2$ and 250 ml water) were carefully added to each tube. The protoplast and plasmid mixtures were then incubated at room temperature for 10 minutes, followed by the addition of 2 ml of STC to each tube.

Transformed spheroplasts were plated after mixing 400 µl of the protoplast suspension per 20 ml of molten regeneration media agar that was pre-cooled to 50ºC. The regeneration medium was prepared by dissolving 12 g water agar powder

(Biolab) in 337.5 ml of water. In a separate bottle, 256.5 g of sucrose was dissolved in 375 ml of water. A third flask was also prepared with 750 mg yeast extract, 750 mg casein hydrolysate and 37.5 ml water. The contents of the bottles were then autoclaved and mixed while the temperature was still above 50ºC. The transformed spheroplasts were then poured into 90-mm Petri dishes and incubated at 25^{o}C overnight. The following morning, each plate was overlaid with 1% water agar containing 150 µg/ml hygromycin (Calbiochem). After the overlaid agar has solidified, plates were incubated right side up at room temperature. Transformed isolates grew through the overlay in the presence of hygromycin-B within 2 to 7 days.

Putative transformants on the primary transformation plates were transferred to PDA amended with hygromycin (Calbiochem) at a final concentration of 150 µg/ml. The putative transformants were examined for reporter gene expression under ultra-violet (UV) light using an epi-fluorescence microscope (Carl Zeiss Ltd, Mannheim, Germany) equipped with filter set 10 (488010-0000) and filter set 15 (488015-0000), with spectral properties matching those of the GFP (450-490 nm excitation, 515-565 nm emission) and the DsRed-Express proteins (546/12 nm excitation, 590 nm emission). Images were captured with an AxioCam HR camera (Carl Zeiss Ltd, Mannheim, Germany) and processed with Adobe Photoshop 7.0. Transformants that showed strong fluorescence were single-spored twice on water agar (19 g Difco water agar powder, 1000 ml H_2O) amended with hygromycin (150 µg/ml), in order to obtain homokaryotic transformants. The single-spored isolates were again checked for fluorescence after a week of growth on non-selective media (PDA without hygromycin).

Stable transformation of the transformants was examined through successive transfers on non-selective media. The isolates were grown on PDA without hygromycin for a week and then sub-cultured weekly onto fresh PDA media (without hygromycin) over a period of 6 weeks. The fluorescence of each isolate was observed between transfers using epifluorescence microscopy. Following the sixth transfer onto non-selective PDA, a polymerase chain reaction (PCR) was performed to confirm the presence of either the *GFP* or the *DsRed- Express* gene.

Detection of *GFP* and *Ds-Red Express* genes using gene-specific PCR primers

Transformed isolates were grown on non-selective PDA plates for 7 to 10 days. Total DNA from each isolate was extracted using a slightly modified phenol-chloroform-based extraction method described by Sambrook *et al*. (1989). Cultures were homogenized with a pestle in 300 µl DNA extraction buffer in an eppendorf tube, frozen in liquid nitrogen and boiled in water for 5 min. Subsequently, 700 µl phenol-chloroform (1:1) was added and the samples were vortexed and centrifuged for 7 min at 14000 rpm. The upper aqueous layer was transferred to a new tube and the phenol-chloroform step was repeated until the white interface was no longer visible. The rest of the procedure was performed as described by Sambrook *et al*. (1989), with the exception that the tubes were centrifuged for 10 min after the precipitation step. DNA was dried under vacuum, followed by re-suspension of the resulting pellet in 100-200 µl SABAX water. RnaseA (10 µg/µl) was added to the DNA samples, and incubated at 37°C for 3 to 4 hours to digest any residual RNA. DNA was visualized by running a 1% agarose gel (wt/v) (Roche Molecular Diagnostics, Mannheim, Germany) stained with ethidium bromide, and viewed under UV light. Lambda DNA marker (marker III) (Roche Molecular Diagnostics) was used to determine size and concentration of the DNA.

The presence of the *GFP* gene in transformants derived from the non-pathogenic *F. oxysporum* isolate CAV 553 was detected using *GFP*-gene specific PCR primers designed by Lorang *et al*. (2001). The GFP-specific primers were GFP1 (5' TAG TGG ACT GAT TGG AAT GCA TGG AGG AGT 3') and GFP2 (5' GAT AGA ACC CAT GGC CTA TAT TCA TTC TTC 3'). The primer pair was synthesized by Inqaba Biotec (Pretoria, South Africa). Reactions were carried out in 25 µl reaction volumes containing PCR buffer (10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCL, pH 8.3) (Roche Molecular Diagnostics), 0.4 mM dNTPs each (Roche Molecular Diagnostics), 10 pmole of each primer, 0.25 U Taq DNA polymerase (Roche Molecular Diagnostics), and 2 ng of DNA. Amplifications were performed in an Eppendorf Mastercycler gradient PCR machine (Eppendorf Scientific, Hamburg, Germany). The PCR amplification conditions consisted of an initial denaturation temperature of 96°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 45 s and a final extension of 7 min at 72°C. A negative control

consisting of SABAX water and no template DNA, as well as a positive control consisting of DNA of plasmid pCT74 (containing the GFP gene), were included in each amplification step. The PCR- products were visualized by running a 1% agarose gel in 1 x Tris acetic acid EDTA (TAE, pH 8.3) buffer stained with ethidium bromide, and visualized under UV light. A 100-bp molecular weight marker XIV (Roche Molecular Diagnostics) was used to determine the size of the PCR products.

The presence of the *DsRed-Express* gene in transformants derived from the non-pathogenic *F. oxysporum* isolate CAV 553 was detected through PCR amplification of a fragment of the *DsRed-Express* gene. A fragment of the *DsRed-Express* gene was amplified with primers DsF (5' ATG GCC TCC TCC GAG GAC 3') and DsSeq (5' GTA CTG GAA CTG GGG GGA CAG 3') that were designed based on the vector sequence of plasmid pDsRed-Express (Clonetech Laboratories, Palo Alto, CA, USA). The primer pair was synthesized by Inqaba Biotechnical Industries. Reactions were carried out using the same protocol as for the *GFP* gene apart from the amplification conditions, which were for the *DsRed-Express* gene: an initial denaturation temperature of 96°C for 2 min, followed by 36 cycles of 94°C for 30 s, 65°C for 45 s and 72°C for 45 s, and a final extension of 7 min at 72°C. In each amplification step, a negative control containing no template DNA, as well as a positive control containing plasmid DNA (pPgpd-DsRed) carrying the DsRed-Express gene, were included. The PCR- products were visualized as previously described for the *GFP* gene.

Morphological and cultural characteristics

The morphological and cultural characteristics of the non-pathogenic *F. oxysporum* transformants were compared to that of the wild-type isolate. The transformed isolates as well as the wild type isolate CAV 553 were transferred to carnation leaf agar (CLA) (20 g of Biolab agar, 1000 ml of H_2O, one or two 5-mm sterilized carnation leaves per Petri dish) and PDA, and incubated at 25°C with a 12-hour day/night light cycle under cool-white and near-UV fluorescent lights. Slide preparations of 7-day-old transformant and wild-type cultures were made in sterile water, and strands of hyphae and spores were studied under the microscope using white and UV light. The colony diameter of each isolate grown on PDA without hygromycin was measured with the aid of the digimatic electronic callipers (Mitutoyo, Andover, Hampshire, UK)

after 7 days. Six PDA plates per transformant were used for the measurement of the colony diameter, and the experiment was repeated.

Statistical analysis

Data obtained for the measurement of the colony diameter of the transformants were analysed using the Statgraphics Version 5.0. Experiments were analyzed using multifactor analysis of variance (ANOVA). Significance was evaluated at $P<0.05$ for all tests.

RESULTS

Transformation of fungal protoplasts

The majority of the putative transformants (99%) that were transferred from the primary transformation plates (plates containing transformed protoplasts, overlaid with hygromycin selective media) grew when transferred onto new selective media. These transformants further proved to be stable since they retained hygromycin resistance after six successive transfers onto non-selective media. The transformation efficiency for isolate CAV 553 was low (0.5-2 transformants/µg vector DNA). All of the spores transformed with pCT74 showed varied levels of green fluorescence, with the exception of one isolate that did not fluoresce when viewed with epifluorescence microscopy. Eighty percent of transformants that were co-transformed with vector pPgpd-DsRed and pHyg8 showed various levels of red fluorescence when viewed with epifluorescence microscopy.

Detection of *GFP* and *Ds-Red Express* genes using gene-specific PCR primers

The PCR analyses of GFP transformants that were transferred six times onto non-selective media showed that the GFP gene was most likely integrated into the genomic DNA of these transformants. PCR amplification with the GFP-specific primers only yielded a 417-base pair product in transformed GFP isolates as well as in the positive plasmid (pCT74) control, whereas no product was observed in the wild-type CAV 553 isolate (Fig. 1A).

PCR analyses of DsRed-Express transformants that were transferred six times onto non-selective media showed that the DsRed-Express gene was most likely present

within the genomic DNA of the isolates. Amplification with the DsRed-Express-specific primers only yielded a 200 base-pair product with the DsRed-Express transformants and the positive plasmid (pPgpd-DsRed) control, but no amplification product with the wild-type CAV553 isolate (Fig. 1B).

Morphological and cultural characteristics

No morphological changes in size and shape of the vegetative structures of transformants were observed. The mycelial growth of the transformants did not significantly differ in growth from CAV 553 (Fig. 2). It was observed, however, that CAV 1777 (43.76 mm) and CAV 1780 (44.10 mm) grew significantly slower than the transformant CAV 1778 (51.45 mm). No differences were noticed in sporulation and colony appearance. The transformed isolates still retained the typical wild type colony morphology of cottony growth of aerial mycelium and purple pigmentation. The fungal hyphae, micro– and macroconidia of the transformed isolates showed constitutive expression of the *GFP* and *DsRed-express* genes when viewed with epifluorescence microscopy (Fig. 3). The wild type isolate did not show any fluorescence when viewed with epifluorescence microscopy.

DISCUSSION

In this study, a non-pathogenic *Fusarium oxysporum* strain (CAV 553) that reduces Fusarium wilt severity in banana plants was successfully transformed with the reporter genes *GFP* and *DsRed-Express*. The transformed isolates proved to be stable, since they retained the reporter genes and fluoresced after successive transfers on non-selective media. Similar results have been obtained for other non-pathogenic fungi as well as pathogenic fungi (Mikkelson *et al*., 2003; Nahalkova and Fatehi, 2003; Olivain *et al*., 2006; Sarrocco *et al*., 2006). Differences were observed in the intensity of fluorescence of the transformed isolates. This may be attributed to the integration of the plasmid into different chromosomal sites as well as difference is copy number (Lorang *et al*., 2001; Visser, 2003).

The morphological characteristics of the transformed isolates did not differ from the wild-type isolate, although growth rate was slower. Visser (2003) also found no differences between wild-type *Foc* and GFP-transformed *Foc*. Fluorescent

microscopy showed that only the transformed isolates fluorescence, and that fluorescence was present in all the fungal structures. All the micro- and macroconidia of a transformed fungal colony that were observed showed the same level of brightness. Nahalkova and Fatehi (2003) found that the intensity of the DsRed-expression among the microconidia varied and found that it might be due to the different age of the spores.

The transformation efficiency for isolate CAV 553 was low (0.5-2 transformants/µg vector DNA). The process of generating protoplasts and optimising the process of transformation can be laborious and success is not always guaranteed (Covert *et al*., 2001). In the transformation of *Foc* by Visser *et al*. (2004) the transformation efficiency depended on mycelium age, the choice of enzymes, and the temperature and duration of incubation. The transformation of non-pathogenic *F. oxysporum* in the current study needed some optimisation with removing the cell wall of the spores using cell wall-degrading enzymes.

The GFP and DsRed-Express-labelled transformants will enable studies on the infection and colonization process of non-pathogenic *F. oxysporum* in bananas using fluorescence microscopy. Furthermore, these isolates can be used to study the interaction between a GFP-labelled pathogenic *Foc* (Visser *et al.*, 2004) and a non-pathogenic *F. oxysporum* isolate in banana. For example, Olivain *et al*. (2006) studied the infection of *F. oxysporum* f. sp. *lycopersici*, a tomato pathogen, together with non-pathogenic *F. oxysporum* by labelling the fungi with GFP and DsRed2, respectively. In the current study only epifluorescence was used in microscopy studies. Future studies will aim to investigate the infection and colonization of isolates using a confocal laser-scanning microscope, which yields three-dimensional images with better resolution than images obtained with epifluorescence microscopy (Sorrocco *et al*., 2006).

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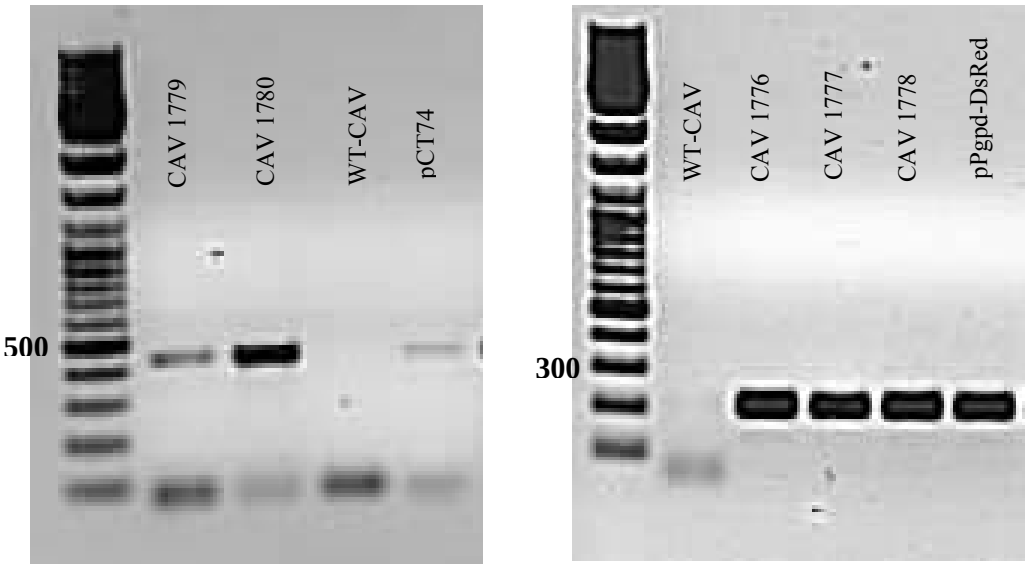

Figure 1: PCR analyses of *Fusarium oxysporum* transformants confirming the presence of the (a) *GFP* and (b) *DsRed-Express* gene. Transformants were derived from endophytic *F. oxysporum* isolate CAV 553 (WT-CAV). PCR using primers specific to the *GFP* gene or *DsRed-Express* gene, yielded a 417-bp (GFP) or 200-bp (DsRed-Express) fragment. A positive plasmid control was loaded in the last lane of each gel a) pCT74 for the *GFP* and b) pPgpd-DsRed for the *DsRed-Express* gene. Transformants were analyzed after being transferred six times onto non-selective media.

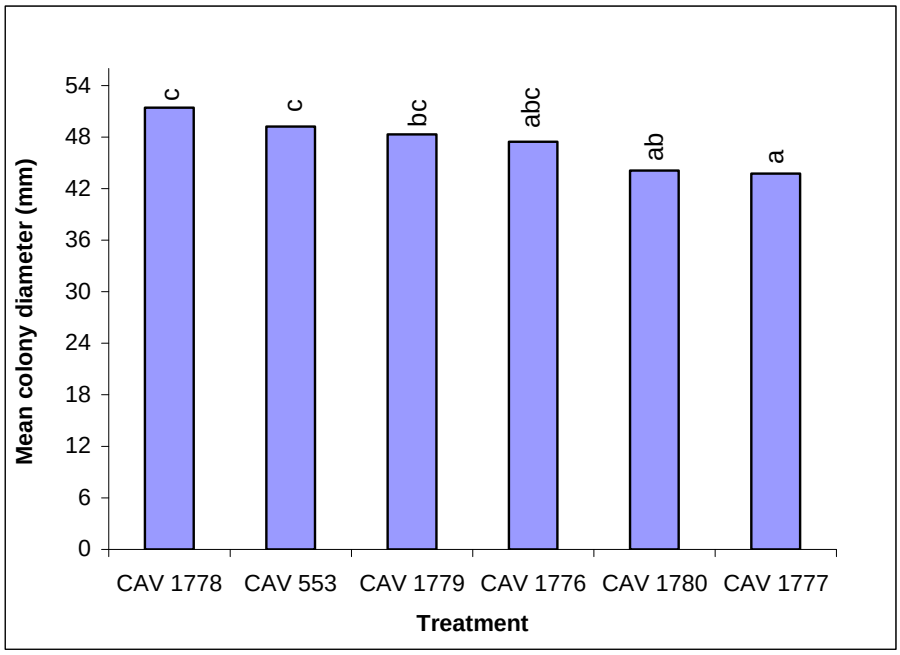

Figure 2: The mycelial growth rates of *Fusarium oxysporum* transformants CAV 1776, CAV 1777, CAV 1778, CAV 1779 and CAV 1780 compared to the wild-type non-pathogenic *F. oxysporum* isolate CAV 553. Bars presented with the same letter are not significantly different at $P \leq 0.05$.

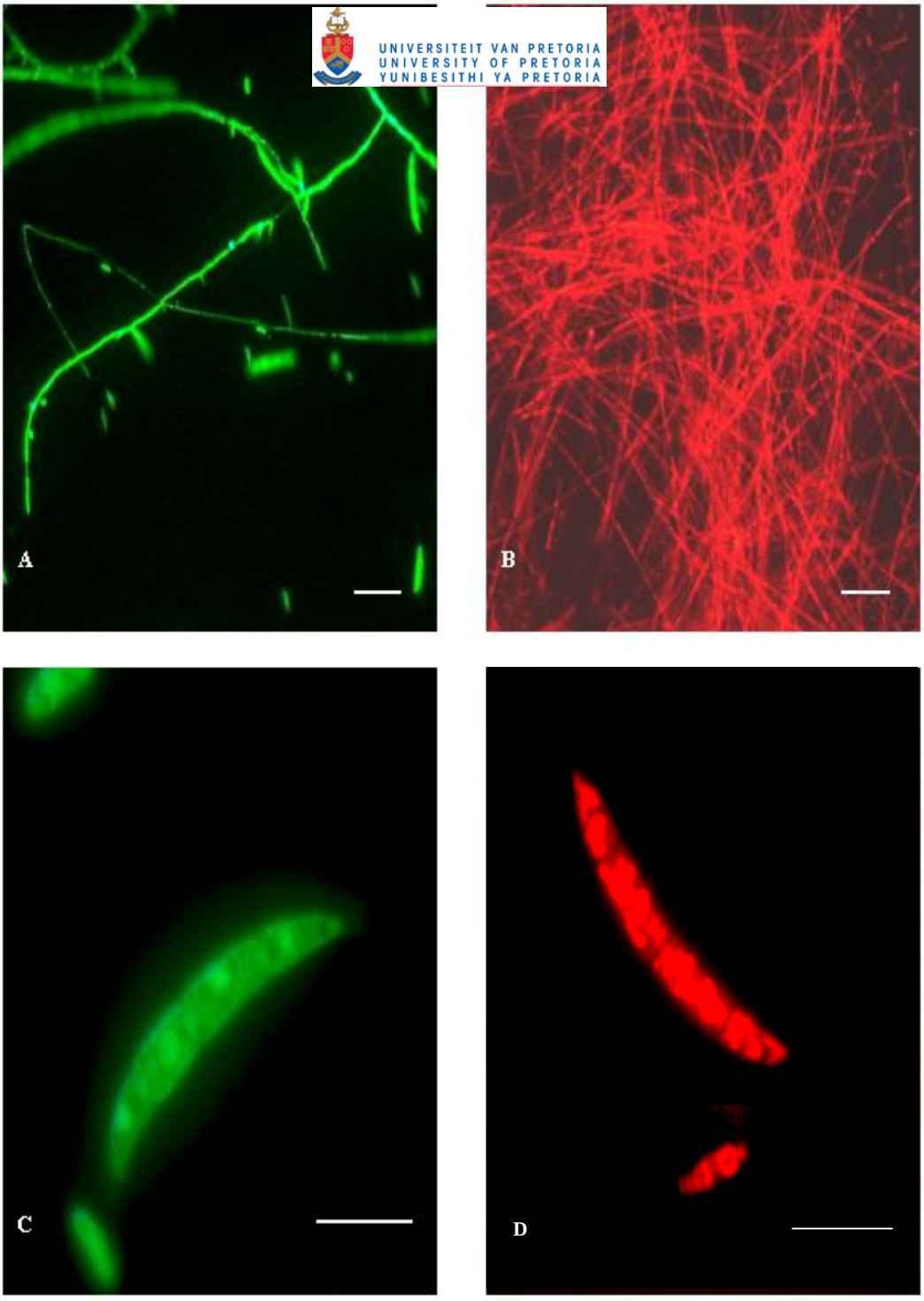

Figure 3: Structures of transformed isolates of *Fusarium oxysporum* fluorescing bright green (GFP-transformed) and bright red (DsRed-Express transformed). A and B) Fluorescing hyphal mass (x10, scale bar = 60µm). C and D) Typical size and shape of microconidia and macroconidia of non-pathogenic *F. oxysporum* (x63, Scale bar = 20µm).

Chapter 5

Histological investigation of the interaction between pathogenic and non-pathogenic isolates of *Fusarium oxysporum*, and banana roots

ABSTRACT

Fusarium oxysporum contains pathogenic and non-pathogenic isolates that cannot be distinguished morphologically. The pathogenic *F. oxysporum* isolates penetrate plant roots, spread into the xylem vessels, block water transport and cause a lethal wilt of economically important crops. Most isolates of *F. oxysporum*, however, are non-pathogenic soil inhabitants that do not cause disease. Non-pathogenic *F. oxysporum*, alone or in combination with *Pseudomonas fluorescens*, are found in disease suppressive soils, and are responsible for suppression of diseases caused by pathogenic isolates of *F. oxysporum*. Disease suppression can take place by means of competition and/or by induced resistance in the host plant. Competition between GFP-transformed *F. oxysporum* f.sp. *cubense* (*Foc*) and DsRed-transformed non-pathogenic *F. oxysporum* isolates, inoculated simultaneously on banana roots, was investigated in a hydroponic system. To test whether *F. oxysporum* and *P. fluorescens* induce structural changes in banana roots, distant roots were inoculated with GFP-transformed *Foc* isolates in a split-root system. Root samples were collected 1, 2, 4, 7 and 14 days after inoculation. Transvers and longitudinal hand cuts were made of root samples taken 0-1, 4-5, and 9-10 cm from the root tips, and studied under a Confocal Laser Scanning Microscope (CLSM). Antibioses as a mode of action against *Foc* was tested for both microorganisms *in vitro*, but no inhibition zone between pathogen and any of the putative biological control agents was observed. Studies with CLSM revealed that *Foc* and non-pathogenic *F. oxysporum* colonised the root surface within 1 and 2 days. After 4 days, germination tubes and hyphae of both organisms became invisible, and the fungi began to form chlamydospores after 7-14 days. No penetration of banana roots occurred, not even in the control treatments. No competition was observed between non-pathogenic *F. oxysporum* and *Foc* when inoculated at equal concentrations and at the same time. Factors such as the time of inoculation, and the ratio of the non-pathogen to the pathogen need to be further examined, as that might influence the biocontrol potential of the non-pathogen. Further investigation is also needed to study systemically induced resistance in wounded banana roots against *Foc* following inoculation with non-pathogenic *F. oxysporum* and *P. fluorescens*.

INTRODUCTION

Fusarium oxysporum Schlecht. is a common, widespread fungus found in soil around the world (Kistler, 1997). The species contains both pathogenic and non-pathogenic isolates that cannot be distinguished from each other morphologically (Ploetz *et al*., 2003). The pathogenic isolates of *F. oxysporum* are best known for causing Fusarium wilt diseases of important agricultural crops (Davis, 1968; Alabouvette and Couteaudier, 1992; Recorbet and Alabouvette, 1997). The fungus penetrates the roots directly, but in some instances requires wounds for infection to occur (MacHardy and Beckman, 1981). Once the roots have been entered, the pathogen spreads through the xylem vessels into the tracheary elements of the stem or pseudostem of plants (MacHardy and Beckman, 1981). Microcondidia of *F. oxysporum* eventually block the vascular sieve cells, thereby causing a lethal wilting of the plant to occur (Di Pietro *et al*., 2003). In resistant plants, however, the progress of *F. oxysporum* is blocked in the roots by cell wall strengthening and the formation of occlusive gels (Beckman and Halmos, 1962).

Pathogenic members of *F. oxysporum* are recognised on a sub-specific level as *forma speciales*. Based on the host plants that they attack (Kuninaga and Yokosawa, 1992), more than 120 *formae speciales* are known for *F. oxysporum* (Armstrong and Armstrong, 1981). A *forma specialis* can further be subdivided into races on the basis of their differential pathogenicity to host cultivars (Kuninaga and Yokosawa, 1992). For instance, isolates of *F. oxysporum* causing Fusarium wilt of banana are known as *F. oxysporum* f.sp. *cubense* (*Foc*), and consist of three races (Ploetz and Pegg, 2000). Race 1 of *Foc* attacks "Gros Michel" bananas, race 2 attacks the "Bluggoe" variety, and race 4 attacks the "Cavendish" bananas and all varieties susceptible to races 1 and 2 (Viljoen, 2000; Ploetz, 2006). *Foc* race 4 is further subdivided into 'tropical' and 'subtropical' strains, dependent on the environmental conditions under which they cause disease. *Foc* race 1 became notorious when it almost led to the demise of the banana export industry in Central America during the 1950's, and *Foc* race 4 is currently destroying Cavendish plantations in many Southeast Asian countries (Ploetz, 2006).

Most individuals belonging to *F. oxysporum* are non-pathogenic, saprophytic soil inhabitants (Fravel *et al*., 2003). These non-pathogens are efficient colonisers of the plant rhizosphere and the root cortex (Olivain and Alabouvette, 1997), but do not induce any symptoms in plants (Elias *et al*., 1991). Alone, or in combination with *Pseudomonas fluorescence*, non-pathogenic *F. oxysporum* is the main organism responsible for the reduced incidence of Fusarium wilt in disease suppressive soils (Alabouvette *et al*., 1993). Suppressive soils are defined as those soils where the incidence of Fusarium wilt remains low despite the presence of the pathogen, susceptible host and favourable environmental conditions (Alabouvette *et al*., 2004).

The mechanisms whereby non-pathogenic *F. oxysporum* protect plant roots against pathogenic forms of the fungus include competition for nutrients in the soil and for infection sites on and in the root (Bao and Lazarovits, 2001; Olivain *et al*., 2006). *Pseudomonas fluorescens* is known to reduce the pathogen through competition for carbon and iron (Duijff *et al*., 1999). It was demonstrated that both non-pathogenic *F. oxysporum* and *Pseudomonas* spp. can also protect plant roots by inducing the production of biochemical substances and by the formation of mechanical barriers that prevent further ingress by pathogenic *F. oxysporum* (Olivain *et al*., 1995; Fuchs *et al*., 1997; Duijff *et al*., 1998; He *et al*., 2002). In tomato roots, non-pathogenic *F. oxysporum* isolates lead to cell death, thereby limiting colonisation by the pathogen to a few cells only (Olivain and Alabouvette, 1997). Wall appositions and thickenings, intercellular plugging, intracellular deposits and hypertrophied cells were also observed in the tomato root cells infected with non-pathogenic isolates of *F. oxysporum* (Olivain and Alabouvette, 1997). Biochemical investigations of bacterized plants showed host metabolic changes and a number of structural changes such as accumulation of callose and lignin (Kloepper *et al*., 1993). *Pseudomonas fluorescens* strain 63-28 induced callose-enriched wall appositions at sites of attempted penetration by pathogenic *F. oxysporum* (M'Piga *et al*., 1997), while inoculation of tomato with WCS 417r lead to thickening of the cortical cell walls when the epidermal cells were colonised (Duijff *et al*., 1997).

The interactions between pathogenic and non-pathogenic isolates of *F. oxysporum* can be studied in a non-invasive and non-destructive way using isolates modified with green (GFP) and red (DsRed-Express) fluorescent protein genes (Lorang *et al*., 2001;

Mikkelsen *et al*., 2003; Olivain *et al*., 2006). GFP- and DsRed-transformed isolates of *F. oxysporum* had been used to study colonization and infection rate of tomato roots by *F. oxysporum* f.sp. *radicis-lycopersici* (Lagopodi *et al*., 2002; Nahalkova and Fatehi, 2003), as well as the interaction between the pathogen and the non-pathogenic isolate Fo47 in the root zone (Bolwerk *et al*., 2005). In this study, the protection of banana roots by non-pathogenic *F. oxysporum* and *P. fluorescens* isolates against *Foc* will be studied. Antibiosis and competition as mechanisms of action during root colonization will be investigated *in vitro* and *in planta*, respectively, while a split-root inoculation experiment will be used to investigate systemically acquired resistance. For confocal laser scanning microscope (CLSM) studies, a non-pathogenic *F. oxysporum* isolate was transformed with DsRed-Express (Chapter 4), and an isolate of *Foc* transformed with GFP (Visser *et al*., 2004).

MATERIALS AND METHODS

Isolates used:

Pathogenic (*Foc*) and non-pathogenic *F. oxysporum* isolates were colleted from Kiepersol, South Africa. The *Foc* isolate was sampled from a diseased Cavendish banana plant, while the non-pathogenic *F. oxysporum* isolates were all collected from banana roots in a disease suppressive field (Chapter 2). The pathogenic (CAV 092) and one of the non-pathogenic isolates (CAV 553) of *F. oxysporum* were then transformed with the *GFP* (Visser *et al*., 2004) (CAV 666) and *DsRed*-Express (Chapter 4) (CAV 1776) genes, respectively. *Pseudomonas fluorescens* WCS 417, known for its ability to suppress Fusarium wilt diseases (Van Loon *et al*., 1998), was included in the study. This isolate, kindly provided by Prof. L.C. van Loon (University of Utrecht, Netherlands), was originally isolated from the rhizosphere of wheat grown in a field suppressive to take-all disease of wheat (Lamers *et al*., 1988). These isolates are all maintained at the culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria in Pretoria, South Africa.

In vitro testing:

Foc and non-pathogenic *F. oxysporum* isolates were cultivated on Potato Dextrose Agar (PDA) (39 g of Difco PDA powder, 1000 ml H_2O) amended with hygromycin

(2.4 ml (50 mg/ml) per 800 ml of PDA) (Sigma-Aldrich, Steinheim, Germany), and grown at 25ºC for 7 days. Of each non-pathogenic culture, a 5-mm-diameter mycelial plug was dislodged and placed on one side of a 90-mm Petri dish. Mycelial plugs of *Foc* were placed on the opposite side of each of the non-pathogens. In addition, the *P. fluorescens* isolate was streaked onto PDA, and a mycelial plug of *Foc* placed on its opposite side. The *in vitro* experiment with the bacterium was also tested on *Pseudomonas* selective agar (King *et al*., 1954). For each isolate, five Petri dishes were used, and the plates were studied for signs of fungal inhibtion over a period of 2 weeks.

Inoculum preparation:

Mycelium of each *F. oxysporum* isolate grown on PDA plates was inoculated into 100 ml Armstrong's *Fusarium* medium (Booth, 1977) prepared in 500-ml Erlenmeyer flasks to enhance sporulation. The flasks were then rotated on a shaker (Labotec, Midrand, South Africa) set at a rotation speed of 177 rounds per minute (rpm) at 25°C. Spores were collected after 7 days, poured through cheesecloth, and adjusted to a final concentration of 1 x 10^6 spores.ml^{-1} using a haemacytometer (Laboratory & Scientific Equipment Company (Pty) Ltd. (LASEC), Randburg, South Africa). The isolate of *P. fluorescens* was streaked onto *Pseudomonas*-selective agar and grown at 37°C in the dark for 2 days before inoculation (King *et al*., 1954). The bacterium was then scraped from the agar medium and suspended in sterile distilled water, and its concentration adjusted to 1 x 10^8 cfu.ml^{-1} using a spectrophotometer.

Plant cultivation and inoculation

Pathogen-free tissue culture banana plantlets (of the Cavendish cultivar Chinese Cavendish) were obtained from Du Roi Laboratories in Letsitele, South Africa. The plantlets were transplanted to 250-ml plastic cups filled with water (Chapter 2), and fertilised weekly with a hydroponic nutrient mixture (Chapter 2) until the roots were approximately 10 cm long. The banana plantlets were kept in a greenhouse set at 12 hours of daily illumination, with a daytime temperature of 28°C and a night temperature set at 20°C.

To study competition between the genetically modified pathogenic and non-pathogenic *F. oxysporum* isolates on banana roots, isolates CAV 1776 and CAV 666

were simultaneously inoculated in the plastic cups. Of each isolate, 2.5 ml of the fungal spore suspension was added to the water in the cups to achieve a final concentration of 1 x 10^5 spores.ml^{-1}. For the controls, banana plants were inoculated with sterile water, or with either the pathogen or the non-pathogen. Three plants were used for each treatment, and the experiment was repeated.

To determine whether non-pathogenic *F. oxysporum* and *P. fluorescens* induced systemic resistance in banana roots, a split-root system was set up as described in Chapter 3. One half of the roots were inoculated with the putative biological control agent (either the non-pathogenic *F. oxysporum* or the *P. fluorescens* isolate). Considering the results obtained with the phenolic assays (Chapter 3), it was decided to inoculate the other half of the roots with *Foc* after 2 days. For the control treatment, the one half of the roots was inoculated with water before the pathogen was added to the other half 2 days later. The pathogen and non-pathogens were added to water in the cups to result in final concentrations of 1 x 10^5 spores.ml^{-1}, while the concentration of the bacterium was adjusted to a final concentration of 1 x 10^7 cfu.ml^{-1}. Roots were not wounded during the inoculation process. The reason for this was to prevent easy access for the pathogen to the plant vascular system. Three plants were used for each treatment and the experiment was repeated three times.

Confocal laser scanning microscopy (CLSM)

Banana roots were sampled 1, 2, 4, 7 and 14 days after inoculation with *Foc* for CLSM analysis. Two roots of 10 cm or longer were selected from each plant. Material for microscopy was prepared from root segments taken 0-1, 4-5, and 9-10 cm from the root tip. One root was used to make transverse cuts, while the other root was used to make longitudinal cuts using a blade. The root sections were then mounted onto a slide with an artificial well that was prepared by using Vaseline, also known as Petroleum jelly. The well was made after filling a syringe with Vaseline, and applying the Vaseline to the slide through a needle (Figs. 1A and B). The root sections were immersed in sterile de-ionized water and examined immediately under white and ultra-violet (UV) light using the CLSM (Zeiss Ltd, Mannheim, Germany). Digital images were acquired by scanning with optimal settings for GFP excitation with the 488 Argon laser and detection of emitted light at 490 nm (autofluorescence detection

505 Long Pass), and for DsRed-Express excitation with the 543 Argon laser and detection of emitted light at 545 nm (autofluorescence detection 560 Long Pass).

RESULTS

In vitro testing

Non-pathogenic *F. oxysporum* isolates did not inhibit growth of the pathogenic *Foc* isolate in culture (Fig. 2). When placed on opposite sides of the Petri dish, the colonies grew towards each other, with the formation of only a thin barrier between the two cultures. When *P. fluorescens* isolate WCS 417 was plated out opposite *Foc*, no inhibition zone was formed. Mycelial growth of *Foc* reached the bacterium within 1 week and, thereafter, would begin to overgrow *P. fluorescens*.

Root colonization by pathogenic and non-pathogenic *F. oxysporum*

Spores of the fluorescent non-pathogenic *F. oxysporum* and *Foc* isolates germinated within 24 hours and colonised the banana root area extensively within 2 days (Fig. 3). A hyphal mat was formed on all parts of the root surface and at the very tip of the roots. The hyphal networks on the root surface began to merge after 2 days. No differences were observed in the pattern whereby roots were colonized by the non-pathogenic *F. oxysporum* isolate and *Foc*. *Foc* produced haustorium-like structures and infection pegs (Fig. 4), but these structures were not observed for the non-pathogenic *F. oxysporum* isolate. Neither the pathogen nor the non-pathogen, however, penetrated the cortical cells or the cambium. Germ tubes of *Foc* and the non-pathogenic *F. oxysporum* became less visible from day 4. From days 7-14, no germ tubes were visible anymore, and chlamydospores were prominent, especially on the root hairs (Fig. 3).

With combined inoculation, the pathogen and non-pathogen appeared to be equally distributed on banana roots in the first 48 hours. No difference in colonization pattern was observed when the pathogen and non-pathogen were applied separately or in combination. After 4 days, however, the pathogen was appeared to be more prominent than the non-pathogen. Two weeks later the pathogen and the non-pathogen were visible in structures reminiscent of chlamydospores (Fig. 3).

Induced resistance by non-pathogenic *F. oxysporum* and *P. fluorescens*

Germination and colonization of banana roots by *Foc*, following treatment with non-pathogenic *F. oxysporum* and *P. fluorescens* in a split-root experiment, followed the same order of events as described above. Long germtubes were formed within 2 days, and substantial fungal growth was observed, especially on the root hairs (Fig. 5). After 4 days the hyphae became invisible and completely disappeared after 1 week. Haustoria and penetration pegs were not observed, and *Foc* did not penetrate the roots at any stage. No difference in colonization pattern was observed in banana roots treated with the non-pathogenic *F. oxysporum*, *P. fluorescens* or water (control). After 14 days, structures that appeared to be chlamydospores were formed on the root hairs.

DISCUSSION

Non-pathogenic isolates of *F. oxysporum* and the bacterial isolate *P. fluorescens* WCS 417 are known to reduce the incidence of Fusarium wilt of banana in greenhouse inoculation studies (Gerlach *et al*., 1999; Nel *et al*., 2006; Chapter 2). The mode of protection has previously been suggested and may involve systemically acquired resistance (Chapter 3) but competition was not excluded as an additional means of protection. In the current study, the interaction between the putative biological control organisms and *Foc* on banana roots was investigated for the first time using confocal laser microscopy. Our results demonstrated that competition for infection sites is an unlikely mode of protection, but failed to demonstrate that induced resistance resulted in reduced infection of banana roots by the pathogen.

Competition for infection sites and nutrients appeared to be an unlikely mechanism of control of *Foc* on banana roots by the non-pathogenic *F. oxysporum* isolate. This consideration is supported by results of the *in vitro* tests, where the non-pathogenic *F. oxysporum* isolate was not able to inhibit the growth of *Foc*. Bolwerk *et al*. (2005), however, showed that the non-pathogenic *F. oxysporum* isolate Fo47 competed for niches and nutrients with *F. oxysporum* f.sp. *radicis-lycopersici* on tomato roots. After 4 days, Fo47 became less aggressive and grew slower than the pathogen (Bolwerk *et al*., 2005). In this study, the density of non-pathogenic *F. oxysporum* was also reduced on banana roots when compared to *Foc* after 4 days. Surprisingly, antibiosis had been shown as the mode of protection in culture when the non-

pathogenic *F. oxysporum* isolate Fo47 was tested against *Pythium ultimum* Trow (Benhamou *et al*., 2002). *Pseudomonas fluorescens* did not inhibit *Foc* growth *in vitro*, but when tomato roots were inoculated with *P. fluorescens* and *Pseudomonas chlororaphis*, the density of *F. oxysporum* f.sp. *radicis-lycopersici* was reduced five times after 7 days (Bolwerk *et al*., 2003). The authors hypothesized that the bacteria could have utilized or degraded a signal required for colonization of the epidermis by the fungus.

Microscopic analyses indicated that the non-pathogenic *F. oxysporum* and *P. fluorescens* isolates did not induce a systemic response that prevented banana roots from becoming colonised by *Foc*. Yet, earlier pathogenicity tests clearly demonstrated that these isolates reduced Fusarium wilt incidence by more than 65% (Chapter 2, Nel *et al*. 2006). One can possibly explain this apparent inconsistency by arguing that a biochemical, rather than a structural response, prevented infection of distant banana roots from taking place. The inability of *Foc* to infect non-wounded banana roots in control treatments throughout this study, unfortunately, prevents this hypothesis from any further exploitation. Yet, if the assumption was accurate that induced resistance was the primary means of protection against *Foc*, one would expect that the non-pathogen does not need to compete with the pathogen for longer than 4 days.

Extensive early colonization of banana roots by *Foc* and the non-pathogenic *F. oxysporum* isolate was observed in this study. The fungal spores germinated within 1 day, and the most significant colonization occurred in the regions of root hair development. Olivain and Alabouvette (1997, 1999) believe that the root hairs provide the fungus with a source of carbon to support their growth (Olivain *et al*., 2006). Root hairs are, thus, expected to be a primary site where colonization and infection begins (Lagopodi *et al*., 2002). In a hydroponic system, Olivain and Alabouvette (1999) showed that penetration of tomato roots occurred within 24 hours, and that the pathogen reached the stele of the tomato root after 7 days. In support of this finding, Lagopodi *et al*. (2002) demonstrated that *F. oxysporum* f.sp. *radicis-lycopersici* surrounded tomato roots in the soil within 2 days, and that penetration occurred in 4 days.

In this study, neither the *Foc* nor the non-pathogenic *F. oxysporum* isolate was able to infect the banana roots, even after 14 days. This might indicate that the pathogen needed a wound to penetrate banana roots efficiently. MacHardy and Beckman (1981) and Beckman *et al*. (1989) reported that direct penetration of banana roots occurs infrequently or not at all, and that wounds are essential for vascular infection. Yet, Lagopodi *et al*. (2002), Bolwerk *et al*. (2005) and Olivain *et al*. (2006) were able to demonstrate that both pathogenic and non-pathogenic isolates of *F. oxysporum* were able to infect roots in the absence of wounds in tomato. Their trials, however, involved inoculation of plant roots in soil, which might have damaged roots more than the hydroponic system used in this investigation. Another possible explanation why banana plants in this study were not infected could involve the *Foc* isolate that was used. *Foc* 'subtropical' race 4, the group to which this isolate belongs, is known to attack Cavendish bananas under abiotic stress conditions only (Viljoen, 2000). The greenhouse conditions used in the current investigation might not have stressed the plants sufficiently for infection to occur. Whether the non-pathogenic *F. oxysporum* and *P. fluorescens* isolates used would still protect bananas by means of induced resistance after wounding and following abiotic stress conditions needs to be further investigated.

Pathogenic and non-pathogenic isolates of *F. oxysporum* were applied simultaneously and at equal concentrations on banana roots in this study. Whether timing and concentration of the non-pathogen is important to compete with *Foc* on banana roots is not clear. When watermelon roots were inoculated with non-pathogenic *F. oxysporum* 24 and 72 hours before it was inoculated with *F. oxysporum* f.sp. *melonis*, plants proved to be more resistant to the pathogen following the 72 hour interval (Biles and Martyn, 1989). Bolwerk *et al*. (2005) also found that Fo47 used competition as a mode of action when introduced at a 50-fold higher inoculum concentration than the tomato pathogen *F. oxysporum* f.sp. *radicis-lycopersici*. However, in an investigation on the colonization of tomato roots by pathogenic and non-pathogenic *F. oxysporum*, Olivain *et al*. (2006) showed that the non-pathogen Fo47 performed better than the pathogen, despite any differences in the concentrations of the two microorganisms.

Fluorescence in the DsRed-transformed non-pathogenic *F. oxysporum* and GFP-transformed *Foc* isolates were bright on days 1 and 2, but became less visible 4 days after inoculation in the non-pathogen. Nahalkova and Fatehi (2003) showed that the intensity of DsRed-expression, under control of the *gdp* promotor, varied in the microconidia of *F. oxysporum* f.sp. *lycopersici* due to the different ages of the spores. The *gpd* promotor that drives the expression of DsRed-Express is metabolically regulated, which might result in reduced transcriptional levels in older cultures (Olivain *et al*., 2006). The fungal promoter used for GFP, in contrast, drives strong constitutive expression (Lorang *et al*., 2001), which might explain why GFP-transformed isolates were still visible after 14 days. The strong autofluorescence of the banana root tissue could also have made viewing of the DsRed-Express transformed isolate difficult.

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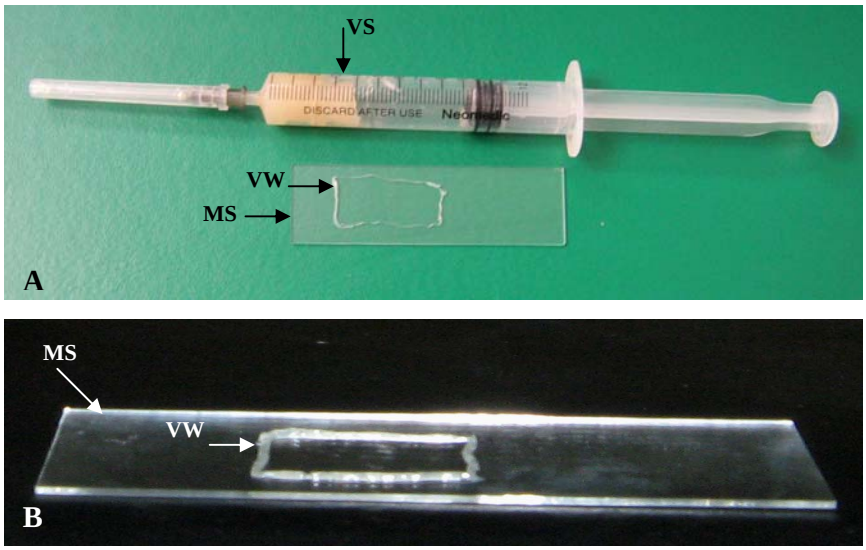


Figure 1: A syringe filled with Vaseline (also known as Petroleum jelly) was used to make wells so that the hand-cut samples of banana roots could be mounted in distilled water in a handmade well, and viewed under confocal laser microscope. A) Syringe filled with Vaseline (VS) and a microscope slide (MS) with a well made of Vaseline (VW). B) Close-up photo of a microscope slide with a Vaseline well.

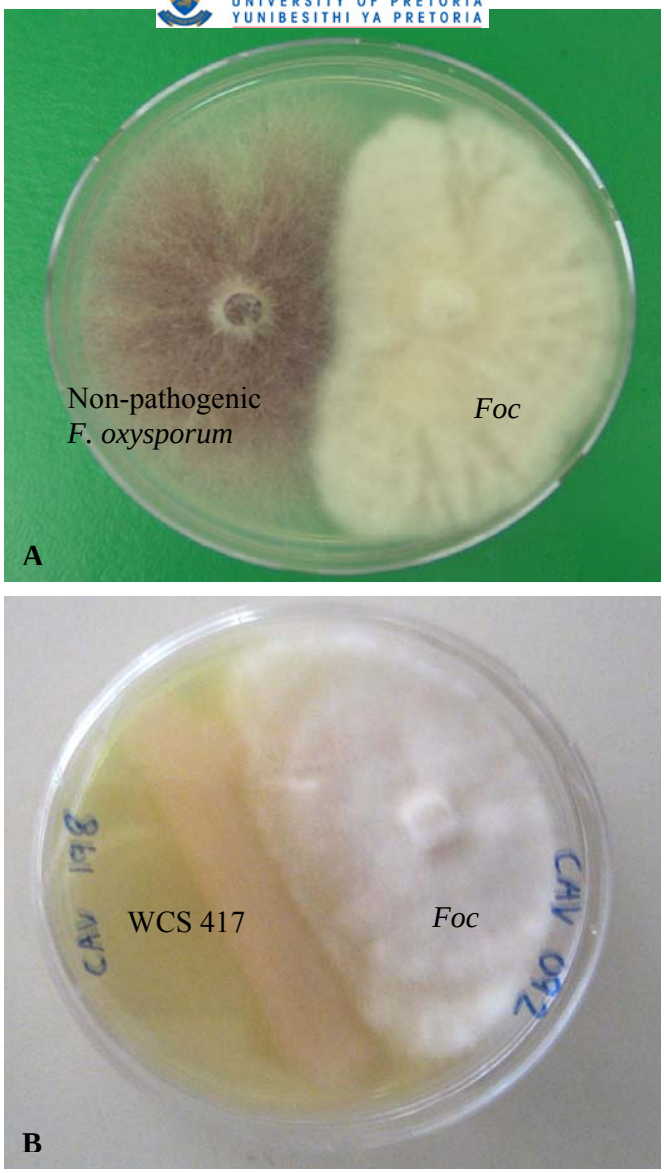
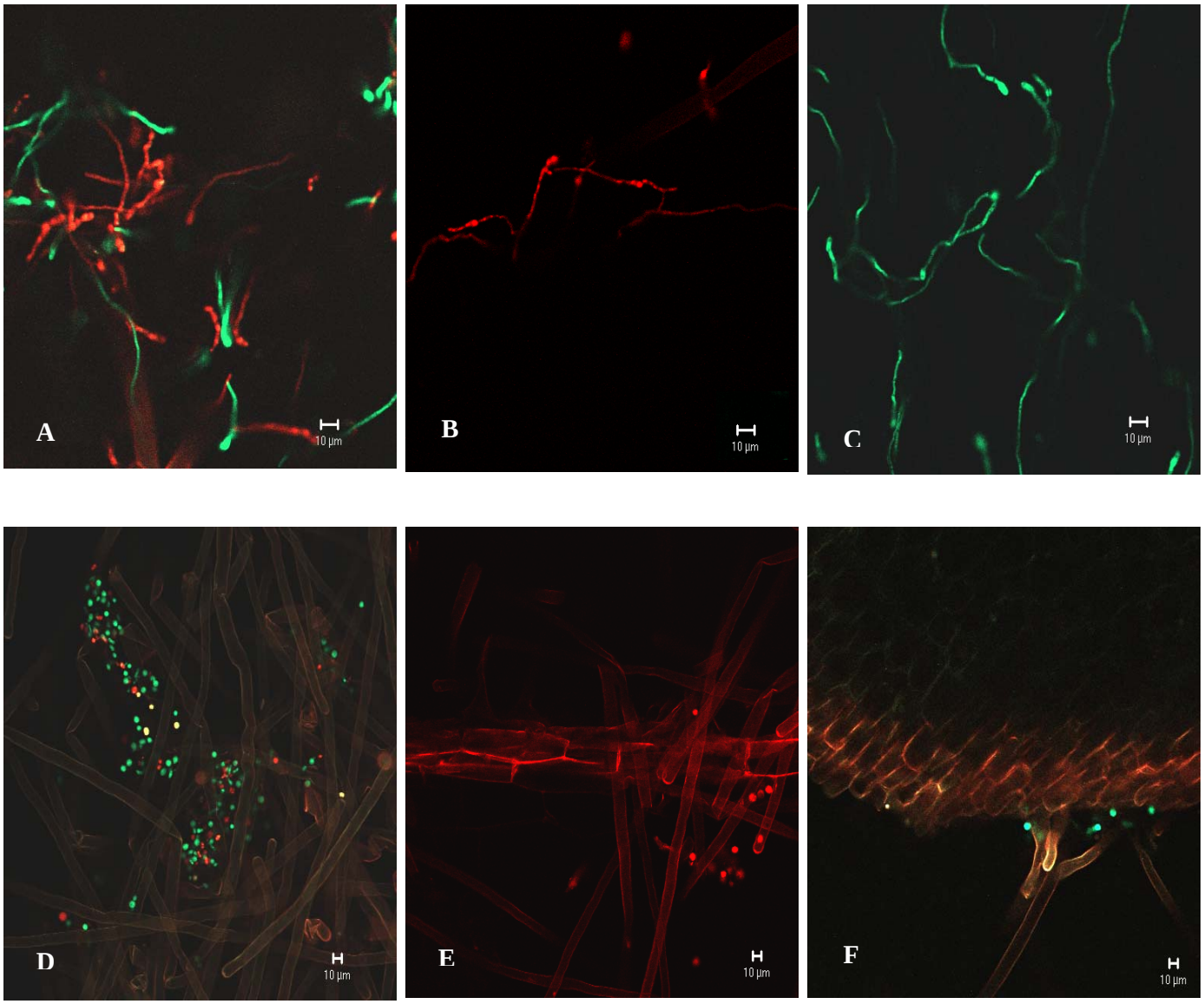


Figure 2: A) A non-pathogenic, endophytic *Fusarium oxysporum* isolate and a *F. oxysporum* f.sp. *cubense* (*Foc*) isolate plated out on opposite sides of a Petri dish containing potato dextrose agar. **B)** *Pseudomonas fluorescens* WCS 417 was streaked out opposite *Foc* on *Pseudomonas*-selective agar medium. No inhibition zones were observed on either of the plates.

Figure 3: Cavendish banana roots inoculated with a combination of non-pathogenic *Fusarium oxysporum* (DsRed-Express transformed) and *F. oxysporum* f.sp. *cubense* (*Foc*) (GFP transformed). The pictures on the left (A and D) illustrate the combined inoculation of roots with both organisms, the pictures in the middle (B and E) represent roots inoculated with the non-pathogenic *F. oxysporum*, and the pictures on the right (C and F) depict roots inoculated with *Foc*. The pictures at the top (A, B and C) were taken 2 days after inoculation, and those on the bottom (D, E and F) 14 days after inoculation. All pictures were photographed using a confocal laser microscope (Zeiss Ltd, Mannheim, Germany). Pictures A, B, C, D and E show roots that were cut longitudinally, and picture F shows a transverse section of the roots. The scale bar = 10 µm. The root hair and the root surface is visible in D, E and F.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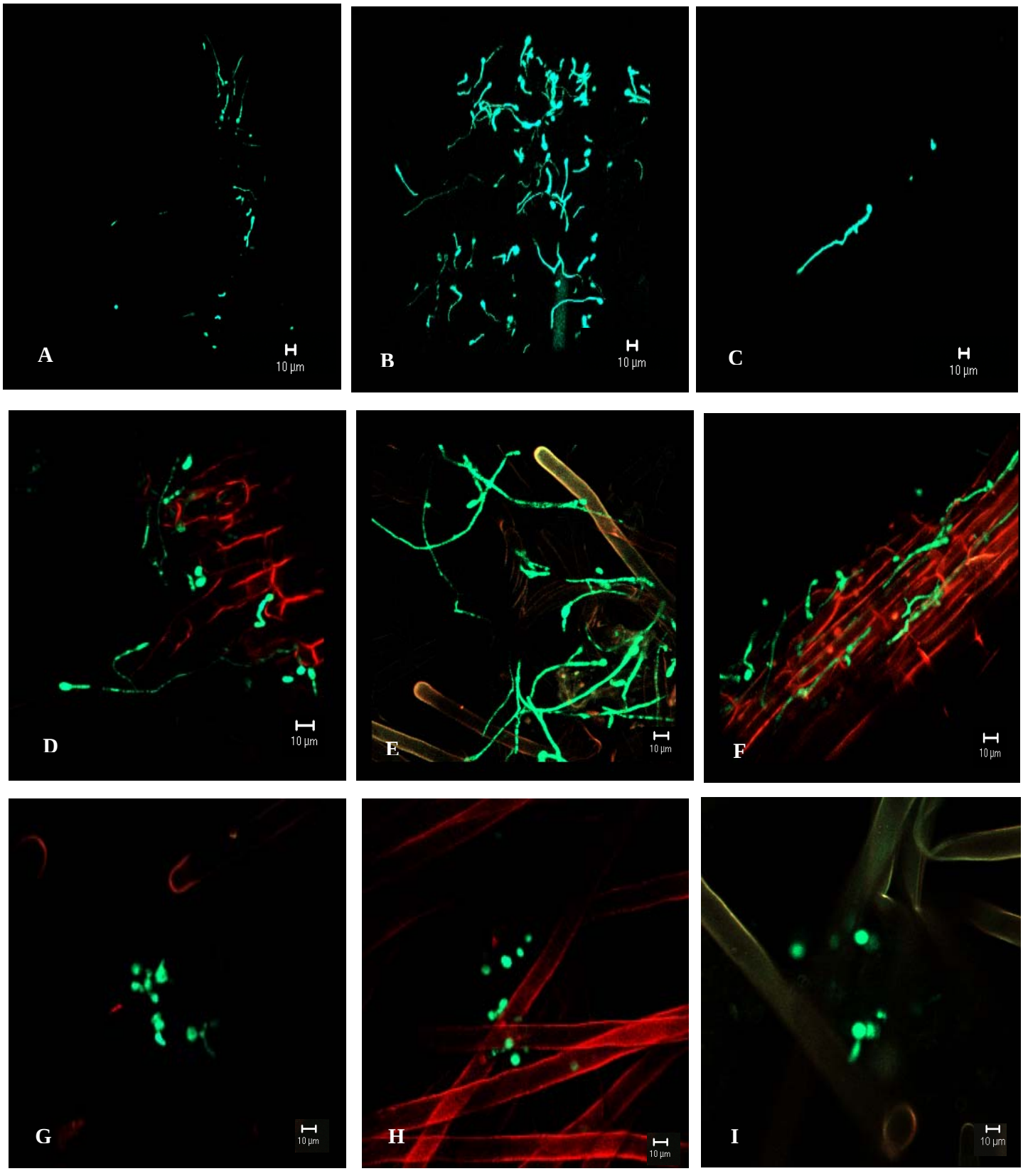
F
10 µm

Figure 4: Cavendish banana roots in a split-root experiment inoculated with sterile water (A, D and G), non-pathogenic *Fusarium oxysporum* (CAV 553, non-transformed) (B, E and H) and *Pseudomonas fluorescens* (WCS 417, non-transformed) (C, F and I), and challenged with *F. oxysporum* f.sp. *cubense* (*Foc*, GFP transformed) 2 days later. Pictures presented were only taken from the side of the banana roots that were treated with *Foc* (GFP transformed) and green structures were observed. Photos D – I were taken with both the 488 Argon laser (GFP excitation) and 543 Argon laser (DsRed-Express) in order to visualise the root structures. The red structures represent the root surface (D and F) and the root hairs of the banana roots (E, G, H and I). The pictures at the top (A, B and C) were taken with only the 488 Argon laser, 2 days after inoculation with *Foc*, the pictures in the middle (D, E and F) 4 days after inoculation, and those at the bottom (G, H and I) 14 days after inoculation. All pictures were photographed using a confocal laser microscope (Zeiss Ltd, Mannheim, Germany). Pictures A, C and D show transverse sections of the roots, and pictures B, E, F, G, H and I show roots that were cut longitudinally. The scale bar = 10 µm.


A
10 µm
B
10 µm
C
10 µm
D
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E
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F
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10 µm

SUMMARY

Fusarium oxysporum Schlecht is a cosmopolitan fungus and causes Fusarium wilt of economically important crops. The species complex contains pathogenic and non-pathogenic strains that cannot be distinguished morphologically. The pathogenic *F. oxysporum* can penetrate the root, spread in the xylem vessels and block water transport, thereby causing a lethal vascular wilt. Most individuals belonging to *F. oxysporum* are non-pathogenic, saprophytic soil inhabitants. These non-pathogens are efficient colonisers of the plant rhizosphere and the root cortex but do not induce any symptoms in plants. Pathogenic strains of *F. oxysporum* can survive in the soil for long periods of time without a host, making it impossible to eradicate them from infested agricultural fields.

Fusarium oxysporum f.sp. *cubense* (*Foc*) is the Fusarium wilt pathogen of banana. Chemical and cultural control has been used with little success to control this disease. Since Fusarium wilt is significantly influenced by host genotype, the best means of controlling this disease is by using disease resistant planting material. Resistance breeding can be difficult when no dominant gene is known. In recent years, the use of biological control agents such as *Pseudomonas fluorescens* and non-pathogenic *F. oxysporum* has resulted in a reduction in Fusarium wilt incidence. Suppressive soils generally host potential biocontrol agents. In this thesis, the control of *Foc* using biocontrol agents such as non-pathogenic *F. oxysporum* isolated from suppressive soils and *P. fluorescens* WCS 417 was investigated. Non-pathogenic *F. oxysporum* isolates from disease suppressive soils were subjected to Restiction Fragment Length Polymorphisms analyses of the intergenic spacer region. Great diversity exists in the non-pathogenic strains, which might suggest that the genotypes are widely distributed, or that great movement of these genotypes occurred. The clonal nature and stability of *Foc* was confirmed when all the pathogenic isolates grouped into a single genotype. The selected non-pathogenic *F. oxysporum* isolates reduced Fusarium wilt of banana effectively in the greenhouse, but the field trial failed due to unfavourable environmental conditions.

In this study, it was demonstrated that non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 induced disease resistance in banana roots both locally and

systemically. *Pseudomonas fluorescens* WCS 417 induced significant higher levels of total phenolic content in the one half on the banana roots that were not wounded than in the roots other half of the same rootball of the banana plant that were wounded and inoculated. Non-pathogenic *F. oxysporum* stimulated banana roots to produce high levels of antimicrobial phenolic compounds that possibly diffused out of storage, became polymerised and increased cell wall-bound phenolics. The cell wall-bound phenolics can be lignified in cell walls or could aid in the formation of tyloses, gums and pappilae, blocking the pathogen from further invasion. The role of these changes in phenolic composition of banana roots, induced by non-pathogenic *F. oxysporum*, should be further demonstrated in histochemical studies.

A non-pathogenic *F. oxysporum* isolate was successfully transformed with the *GFP*- and *DsRed-Express* genes. Fluorescent microscopy showed that all the structures of the fungus fluoresced brightly, and successive transfers to non-selective media proved that the transformation was stable. The transformed isolates were then used for infection studies on the banana root in a non-invasive and non-destructive manner. In this study, the interaction between the putative biological control organisms and a GFP-transformed *Foc* isolate was investigated by using a confocal laser scanning microscope. Our results demonstrated that competition for infection sites is an unlikely mode of protection. When applied simultaneously and at equal concentrations, the non-pathogenic *F. oxysporum* isolate and *Foc* extensively colonised the banana root in the first few days. The density of the non-pathogenic *F. oxysporum* decreased from day 4. Whether timing and concentration of the non-pathogenic *F. oxysporum* is important to compete with *Foc* on banana roots is not clear. No inhibition of *Foc* by non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 was observed *in vitro*, suggesting that antibioses does not play a role in the reduction of Fusarium wilt disease.

A split-root technique was used to study whether induced systemic resistance may influence infection of banana roots by the GFP-transformed *Foc* isolate. The one side of the banana root system was inoculated with non-pathogenic *F. oxysporum* or *P. fluorescens* WCS 417 2 days before inoculation of the other side with *Foc*. Microscopic analyses suggested that the non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 isolates did not induce a systemic response that prevented

banana roots from becoming colonised by *Foc*. One can argue that a biochemical, rather than a structural response, prevented infection of distant banana roots from taking place, since induced resistance was suggested as a mode of action of non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 in earlier greenhouse pathogenicity trials. The inability of *Foc* to infect non-wounded banana roots in control treatments, unfortunately, prevents this hypothesis from any further exploitation. None of the roots were wounded in this experiment, thus the results indicate that wounding might be essential for *Foc* penetration. The *Foc* isolate used in this study is known to attack Cavendish bananas under abiotic stress conditions only. Whether the non-pathogenic *F. oxysporum* and *P. fluorescens* WCS 417 isolates used would still protect bananas by means of induced resistance after wounding and following abiotic stressful conditions also needs to be further investigated.