Biology, pathogenicity and diversity of *Fusarium oxysporum* f.sp. *cubense*

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 is a ubiquitous soil-borne fungus that includes pathogenic and non-pathogenic members. The pathogenic members are best known for causing Fusarium wilt diseases of many economically important agricultural crops. One such a crop is banana (*Musa* spp.), which is affected by a special form of the fungus known as *F. oxysporum* f.sp. *cubense* (*Foc*). Fusarium wilt was responsible for devastating losses of Gros Michel export bananas in Central America during the first half of the 20th century, and is now, once again, threatening world banana production that is primarily based on the sweet Cavendish varieties, both in the tropics and subtropics.

To effectively manage Fusarium wilt, adequate knowledge of the pathogen, plant and environment is required. With this thesis I hope to contribute to the current knowledge available on the pathogen. Previous studies investigated the phenotypic and genotypic diversity, the spread and distribution, and the phylogeny of *Foc*. Some aspects related to the biology, physiology, diversity and pathogenicity of *Foc*, however, appeared to be unresolved. These aspects are important in order to develop a sustainable management strategy for Fusarium wilt to ensure continued banana production.

Chapter 1 depicts a general review on *F. oxysporum* as the causal agent of Fusarium wilt of various fundamental crops, and gives a broad overview of the biology, taxonomy, physiology and pathogenicity of the pathogen. Through the application of modern molecular genetic techniques, a lot of progress has been made in the identification of genes and processes involved in the biology and pathogenesis of Fusarium wilt pathogens. The review concludes that some work, however, still needs to be conducted before topics such as race designation and pathogenesis in *Foc* are fully understood.

Temperature, pH and nutrition are all factors contributing to the pathogenesis of *F. oxysporum*. The different factors can either favour or suppress the pathogen, or they can have a stimulating or inhibiting effect on the host plant. In **Chapter 2** the pathogenicity and phenotypic characteristics of a genotypically uniform population of *Foc* was investigated. Physiological studies included determining the minimum,

maximum and optimum temperatures and pH at which *Foc* grows *in vitro*, and what nitrogen sources stimulate and inhibit growth of *Foc*. Knowledge on these aspects could contribute to the management of the pathogen in the field.

Differentiation among species of *Fusarium* can be problematic. To resolve questions related to the nomenclature in *Fusarium*, our research focus has shifted to the use of molecular tools for identification and determination of evolutionary relationships among and within species. In the past, phylogenetic studies on *Foc* were conducted using molecular tools such as sequencing, Restriction Fragment Length Polymorphisms, Random Amplified Polymorhic DNA and DNA Amplification Fingerprinting, with varying amounts of success. In **Chapter 3** the usefulness of Amplified Fragment Length Polymorhism (AFLP) analysis to study diversity in *Foc* isolates was investigated.

Of the 21 vegetative compatibility groups (VCGs) of *Foc* identified around the world, only VCG 0120 is found in South Africa. **Chapter 4** aimed to identify an AFLP polymorphic DNA fragment unique to VCG 0120, and to develop a molecular marker of this fragment. Such a marker would be extremely valuable to distinguish between VCG 0120 and other isolates of *F. oxysporum* in terms of identification and confirmation of Fusarium wilt of banana in South Africa.

Several pathogenicity-related genes have been identified in *F. oxysporum*. In **Chapter 5**, the presence of three pathogenicity-related genes (*fmk1*, *pg1* and *xyl3*) in *F. oxysporum* isolates pathogenic and non-pathogenic to banana were verified by means of PCR amplification. The value of pathogenicity genes such as *fmk1* and *pg1* in comparative phylogenetic analysis was further substantiated.

CHAPTER 1

The biology and Pathogenesis of *Fusarium oxysporum*, causal agent of Fusarium wilt of higher plants – a review

INTRODUCTION

The fungal kingdom constitutes of a highly versatile group of eukaryotic carbon-heterotrophic organisms that have successfully occupied most natural habitats (Knogge 1996). Most known fungal species are strictly saprophytic, with less than 10% of the more or less 100 000 known fungal species able to colonize plants. Even a smaller fraction of these are capable of causing disease (Knogge 1996, Agrios 1997). Plant parasitic fungi have conquered the living plant as an abundant source of nutrients (Mendgen *et al*. 1996). Scheffer (1991) observed different levels of specialization in plant-fungal interactions. The first group includes the opportunistic parasites which enter plants through wounds or require otherwise weakened plants for colonisation. These fungal species are usually characterised by a broad host range but a relatively low virulence. The second group involves true pathogens that rely on living plants to grow, but can survive outside of their hosts. Most of these pathogens are highly virulent on only a limited number of host species. The third group contains obligate pathogens, where the living plant is an absolute requirement to fulfil their complete life cycle. During the course of infection obligate fungal parasites engage in many sophisticated but poorly understood activities that redirect nutrient flow in plant tissues and alter growth and morphology of the plant (Jackson & Taylor 1996).

Fusarium oxysporum Schlecht. causes vascular wilt diseases in a wide variety of economically important crops (Beckman 1987). Vascular wilt has been a major limiting factor in the production of many agricultural and horticultural crops, including banana (*Musa spp*.) (*F. oxysporum* f. sp. *cubense*), cabbage (*Brassica spp.*) (*F. oxysporum* f. sp. *conglutinans*), cotton (*Gossypium spp.*) (*F. oxysporum* f. sp. *vasinfectum*), flax (*Linum spp.*) (*F. oxysporum* f.sp *lini*), muskmelon (*Cucumis spp.*) (*F. oxysporum* f. sp. *melonis*), onion (*Allium spp.*) (*F. oxysporum* f. sp. *cepae*), pea (*Pisum spp.*) (*F. oxysporum* f. sp. *pisi*), tomato (*Lycopersicon spp.*) (*F. oxysporum* f. sp. *lycopersici*), watermelon (*Citrullus spp.*) (*F. oxysporum* f. sp. *niveum*), china aster (*Calistephus spp.*) (*F. oxysporum* f. sp. *callistephi*), carnation (*Dianthus spp.)* (*F. oxysporum* f. sp. *dianthi*), chrysanthemum (*Chrysanthemum spp*.) (*F. oxysporum* f. sp. *chrysanthemi*), gladioli (*Gladiolus spp*.) (*F. oxysporum* f. sp. *gladioli*) and tulip (*Tulipa*

spp.) (*F. oxysporum* f. sp. *tulipae*) (Armstrong & Armstrong 1981, MacHardy & Beckman 1981).

Management of Fusarium wilt is achieved mainly through chemical soil fumigation and resistant cultivars. The broad-spectrum biocides used to fumigate soil before planting, particularly methyl bromide, are environmentally damaging. The most cost effective, environmentally safe method of control is the use of resistant cultivars, when they are available (Fravel *et al*. 2003). Resistant tomato and melon cultivars are successful in conferring resistance to races of *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *melonis*, respectively (Ori *et al*. 1997, Joobeur *et al*. 2004). Unfortunately, resistance breeding can be very difficult when no dominant gene is known, e.g. in bananas (Stover & Buddenhagen 1986), or if the host is dioecious (eg. palm trees) (Fravel *et al*. 2003). To complicate the matter further, new races of the pathogen can develop which might overcome plant resistance. Virulent races of *F. oxysporum* f.sp. *ciceris* have undermined the importance of resistant cultivars of chickpea in recent years (Haware & Nene 1982, Jiménez-Díaz *et al*. 1993). In cases where there is no treatment for Fusarium wilt, the disease is controlled by preventing the introduction of the pathogen, the destruction of diseased plants, and the isolation of susceptible plants from infested sites (Simone & Cashion 1996).

BIOLOGY OF *Fusarium oxysporum*

Taxonomy

Based on the structure in or on which conidiogenous hyphae are borne, *Fusarium spp.* are classified under the Hyphomycetidae subclass of the Deuteromycetes. *Fusarium oxysporum*, as emended by Snyder & Hansen (1940), comprises all the species, varieties and forms recognised by Wollenweber & Reinking (1935) within an intragenic grouping called section Elegans. Booth (1971) described *F. oxysporum* as a cosmopolitan soil-borne filamentous fungus. It is an anamorphic species that includes numerous plant pathogenic strains causing wilt diseases of a broad range of agricultural and ornamental host plant species (Appel & Gordon 1996).

Fusarium oxysporum produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Nelson *et al*. 1983). Conidia are produced on monophialides and in sporodochia, and are scattered loosely over the surface of a mycelium (Griffin 1994). Microconidia are predominantly uninucleate and germinate poorly and variably, with germination efficiency ranging from 1 - 20% (Ebbole & Sachs 1990). The macroconidia are produced abundantly, are multinucleate, and germinate rapidly, thereby reproducing the fungus efficiently. Chlamydospores are viable, asexually produced accessory spores resulting from the structural modification of a vegetative hyphal segment(s) or conidial cell possessing a thick wall, mainly consisting of newly synthesized cell wall material (Schippers & van Eck 1981). Its function is primarily survival in soil. Morphological characterization of *F. oxysporum* is based on the shape of macroconidia, the structure of microconidiophores, and the formation and disposition of chlamydospores (Beckman 1987). Asexual reproduction in *F. oxysporum* is accomplished by macroconidia and microconidia, while a sexual state of the fungus has never been observed (Booth 1971).

Plant pathogenic forms of *F. oxysporum* are divided into *formae speciales* based on the hosts they attack (Armstrong & Armstrong 1981). Further subdivisions of *formae speciales* into races are often made based on their virulence to a set of differential host cultivars (Correll 1991). The genetic basis of host specificity (*formae speciales*) and cultivar specificity (pathogenic races) of *F. oxysporum* is unknown (Baayen *et al*. 2000). These pathogenic fungi are morphologically indistinguishable from each other, as well as from non-pathogens.

Somatic fusion and heterokaryon formation between individuals can occur independently of sexual reproduction, but usually only amongst strains with similar genotypes (Kistler 1997). These exclusive networks of strains capable of heterokaryosis are called vegetative compatible groups (VCGs) (Puhalla 1985). Puhalla (1985) proposed a classification system for strains of *F. oxysporum* based on their vegetative compatibility, and described a method based on pairing nitrate non-utilising mutants to determine the VCG of each strain. Some *formae speciales* correspond to a

single VCG, while others include several VCGs. Katan (1999) reported 59 VCGs for 38 *formae speciales*. The determination of VCGs can, therefore, not be used as a universal tool to identify *formae speciales* (Fravel *et al*. 2003).

Due to shortcomings of morphological characters for delineating species and subgeneric groupings of *Fusarium* the research focus has shifted to molecular tools for identification and determination of evolutionary relationships among species. These molecular tools include sequencing, Restriction Fragment Length Polymorphism (RFLP), and Random Amplified Polymorphic DNA (RAPD) (Visser 2003). Determining *formae speciales* in *F. oxysporum*, unfortunately, still relies on the time-consuming procedure of testing the fungus for pathogenicity to various plant species (Fravel *et al*. 2003).

Life cycle

The life cycle of *F. oxysporum* commences with a saprophytic phase when the fungus survives in soil as chlamydospores (Beckman & Roberts 1995). Chlamydospores remain dormant and immobile in the remains of decayed plant tissue until stimulated to germinate by utilising nutrients that are released from extending roots of a variety of plants (Stover 1962 a,b, Beckman & Roberts 1995). Following germination, a thallus is produced from which conidia form in 6-8 hours, and chlamydospores in 2-3 days if conditions are favourable. Invasion of the roots is followed by the penetration of the epidermal cells of a host or a non-host (Beckman & Roberts 1995) and the development of a systemic vascular disease in host plants (Stover 1970). In the advanced stages of the disease, the fungus grows out of the vascular system into adjacent parenchyma cells, producing vast quantities of conidia and chlamydospores. The pathogen survives in infected plant debris in the soil as mycelium and in all its spore forms, but most commonly as chlamydospores in the cooler temperate regions (Agrios 1997).

Formation and germination of spores

Chlamydospore formation in pathogenic *Fusarium* species commonly takes place in hyphae in the infected and decaying host tissue (Nash *et al*. 1961, Christou & Snyder

1962). They may also be formed abundantly from macroconidia that originate from sporodochia on lesions at the soil level (Nash *et al*. 1961, Christou & Snyder 1962). Schippers & van Eck (1981) proposed that chlamydospore formation depends on the nutrient status of the inoculum. Under field conditions, fungal inoculum may be subjected to much lower nutrient levels when compared to the 'well-fed' macroconidia produced on rich agar media. Once carbohydrates are released from decaying plant tissue or from roots, chlamydospore germination is stimulated (Schippers & van Eck 1981). Qureshi & Page (1970) further suggested that chlamydospores are formed with the addition of organic or inorganic carbon sources. From the close resemblance of chlamydospore formation in weak salt solutions to that on soil and in soil extracts, Hsu & Lockwood (1973) concluded that an environment deficient in energy, but with an appropriate weak salt solution, may be required for chlamydospore formation.

Chlamydospore germination in nature appears to be dependent on exogenous energy sources (e.g. carbon and nitrogen) (Cook & Schroth 1965, Griffin 1969). Spore density is the single most important factor affecting the nutritional requirements for germination of conidia and chlamydospores in pure culture (Griffin 1981). Exogenous carbon and nitrogen were required for high or complete chlamydospore germination at high spore densities in axenic culture (but not at low spore density) and in soil (Cook & Schroth 1965, Griffin 1969, Griffin 1970). At high conidial densities macroconidia do not germinate, but every conidium is converted into a chlamydospore. At low conidial densities, the conidia germinate but do not convert into chlamydospores (Schneider & Seaman 1974). According to Griffin (1970, 1981), the inability of macroconidia to germinate at high conidial densities resulted from the presence of a self-inhibitor. Self-inhibitors are substances accumulating in growth medium suppressing germination of macroconidia at higher spore densities in the soil (Robinson & Park 1966, Griffin 1969, Robinson & Garett 1969, Griffin 1970).

Infection

The process of vascular infection by *F. oxysporum* is complex and requires a series of highly regulated processes:

Adhesion: Fungal infection commences when infection hyphae adhere to the host root surface (Bishop & Cooper 1983a). Adhesion of fungi to the host surface is not a specific process, as they can adhere to the surface of both host and non-hosts (Vidhyasekaran 1997). Site-specific binding may be important in anchoring the propagules at the root surface, after which other processes required for colonization can proceed (Recorbet & Alabouvette 1997).

Penetration: Penetration is likely to be controlled by a combination of different factors that include fungal compounds, plant surface structures, activators or inhibitors of fungal spore germination, and germ tube formation (Mengden *et al*. 1996). The means whereby wilt pathogens penetrate roots may differ, but there are two distinct types. Some pathogenic forms penetrate roots directly, whereas others must enter indirectly through wounds (Lucas 1998). The most common sites of direct penetration are located at or near the root tip of both taproots and lateral roots (Lucas 1998). The pathogen enters the apical region of the root where the endodermis is not fully differentiated and fungi are able to grow through and reach the developing protoxylem. *Fusarium oxysporum* has been found to penetrate the root cap and zone of elongation intercellularly in the root of banana (Brandes 1919), china aster (Ullstrup 1937), radish and cabbage (Smith & Walker 1930), while *F. oxysporum* f.sp. *dianthi* probably enters carnation roots through the zone of elongation (Pennypacker & Nelson 1972). The muskmelon wilt organism penetrated a susceptible host variety between cells in the region of elongation (Reid 1958). Although mechanical wounding increases infection it is not essential for lateral root infection (Stover 1962a).

Colonization

During colonization, the mycelium advances intercellularly through the root cortex until it reaches the xylem vessels and enters them through the pits (Bishop & Cooper 1983b). The fungus then remains exclusively within the xylem vessels, using them to colonize the host (Bishop & Cooper 1983b). Fungal colonization of the host's vascular system is often rapid and frequently facilitated by the formation of microconidia within the xylem vessel elements (Beckman *et al*. 1961) that are detached and carried upward in the sap stream (Bishop & Cooper 1983b). Once the perforation plates stop the spores, they

eventually germinate and germ tubes penetrate the perforation plates. Hyphae and subsequently conidiophores and conidia are formed (Beckman *et al.* 1961, Beckman *et al*. 1962).

Disease development

Wilting is most likely caused by a combination of pathogen activities. These include accumulation of fungal mycelium in the xylem and/or toxin production, host defence responses, including production of gels, gums and tyloses, and vessel crushing by proliferation of adjacent parenchyma cells (Beckman 1987). The wilting symptoms appear to be a result of severe water stress, mainly due to vessel occlusion. Symptoms are quite variable, but include combinations of vein clearing, leaf epinasty, wilting, chlorosis, necrosis, and abscission. Severely infected plants wilt and die, while plants affected to a lesser degree may become stunted and unproductive (MacHardy & Beckman 1981). The most prominent internal symptom is vascular browning (MacHardy & Beckman 1981).

RESISTANCE IN HIGHER PLANTS AGAINST *Fusarium oxysporum*

The host plants react to infection by wilt *Fusarium* species in a variety of ways. Elicitors from the pathogen and host synergistically act as signalling molecules for the activation of defence mechanisms (Vidhyasekaran 1997). The most prominent defence mechanisms in Fusarium wilt diseases include structural and chemical defences.

***Structural host defence*:**

Some structural defences are present in the plant even before the pathogen comes in contact with the plant. The surface of a plant constitutes its first line of defence that pathogens must penetrate before it can cause infection (Agrios 1997). The structure of the epidermal cell walls, and the presence in the plant of tissues composed of thick-walled cells, further hinder the advance of the pathogen. Cell walls consist of celluloses, hemicelluloses, pectins, structural proteins, and the middle lamella, which consist primarily of pectins (Fig.1). Cellulose is a polysaccharide consisting of chains of

glucose molecules (Agrios 1997). Hemicelluloses are major constituents of the primary cell wall and may also make up a varying proportion of the middle lamella and secondary cell wall. Hemicellulosic polymers include xyloglucan, glucomannans, galactomannans, arabinogalactans and others. Pectic substances are polysaccharides consisting mostly of chains of galacturonan molecules interspersed with a much smaller number of rhamnose molecules and small side chains of galacturonan and some other five-carbon sugars. Pectic substances constitute the main components of the middle lamella, and a large portion of the primary cell wall, in which they form an amorphous gel filling the spaces between the cellulose microfibrils (Agrios 1997).

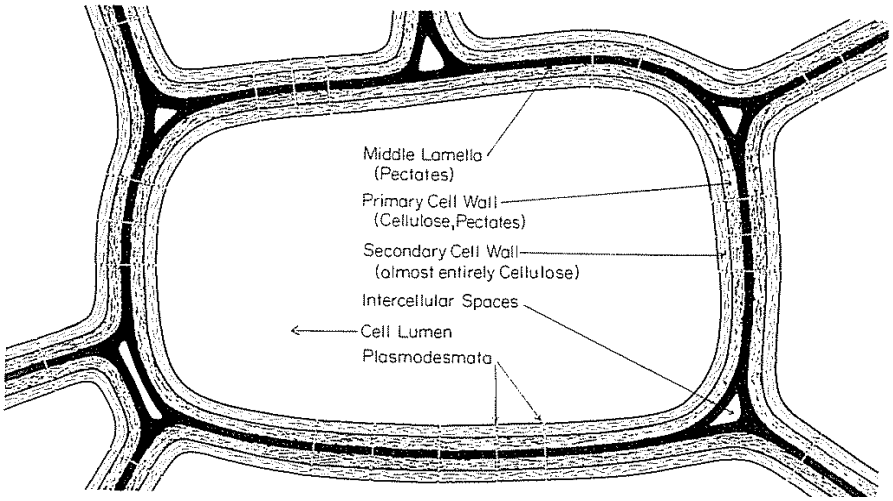

Figure 1. A schematic presentation of the structure and composition of plant cell walls (Agrios 1997).

Vascular wilt pathogens and non-pathogens can both trigger the main structural defences of hosts, including gels, tyloses and lignification that leads to vascular

occlusion (Beckman *et al*. 1962, Davis 1966) (Fig. 2). Cell wall thickening and the formation of callose were observed in tomato plants when treated with *Pseudomonas fluorescens* against *F. oxysporum* f.sp. *radicis-lycopersici* (M'Piga *et al*. 1997). Gels and gums play an important role in localizing the pathogen in hosts by trapping conidia in the vessel elements (Beckman *et al*. 1962). If gels persist long enough to allow tyloses to form, as frequently occurs in banana, the pathogen is successfully contained (Beckman *et al*. 1962). Beckman (1964) found that, if the gels are short-lived or formation of tyloses is delayed or absent, conidia spread ahead of the vascular occlusion. According to Pennypacker (1981), the formation of hypertrophied, hyperplastic cells in response to invasion by *F. oxysporum* is limited to a few host species including banana, (Wardlaw 1930), tomato (Chambers & Corden 1963) and carnation (Pennypacker & Nelson 1972).

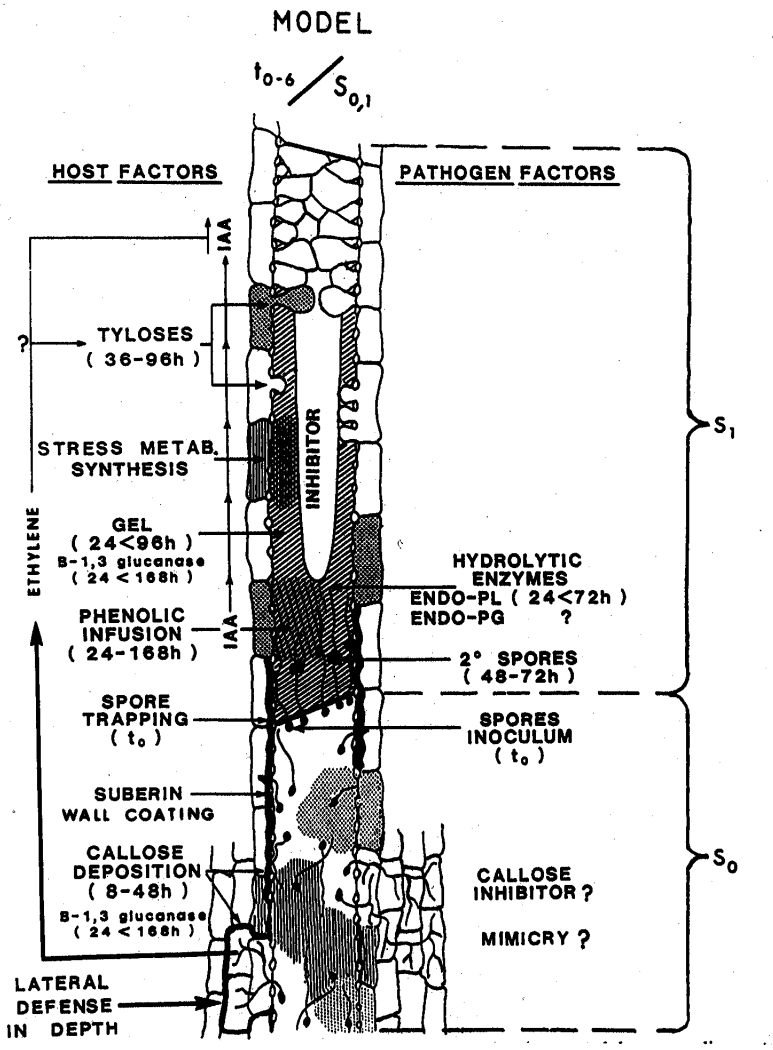

Figure 2. Diagrammatic presentation of the interactions between a host and *Fusarium oxysporum* within an initially infected vessel that delimits $Space_0$ (S_0) and the next vessel above (S_1) during a time 0 to 6 days (t_{0-6}) after inoculation (Beckman 1989).

Biochemical host defence

During induced biochemical defence, the host recognizes the pathogen and produces enzymes that act on the fungal cell surface, releasing fungal elicitors. These enzymes

include chitinases (Pegg & Vessey 1973) and β-1,3-glucanases (Keen & Yoshikawo 1983). It is well-established that the β-1,3-glucanases can inhibit fungal growth by degrading cell walls, usually in concert with chitinases (Mauch *et al*. 1988, Sela-Buurlage *et al*. 1993, Stintzi *et al*. 1993). Fungal elicitors then induce the production of phenylalanine ammonia lyase (PAL) and peroxidase (PER), both key enzymes involved in the synthesis and depolymerisation of lignin precursors. A more rapid increase in and higher levels of PAL and PER activity were found in resistant than susceptible interactions (Aguilar *et al*. 2000). For example, a significantly higher PER activity was reported for resistant banana cultivars against *F. oxysporum* f.sp. *cubense* (Morpugo *et al*. 1994, Aguilar *et al*. 2000) and resistant tomato cultivars against *F. oxysporum* f.sp. *lycopersici* (de Vecchi & Matta 1988), when compared with susceptible cultivars.

Enhanced production of phenolic derivatives through the stimulation of the Shikimic acid pathway has long been associated with disease resistance mechanisms in plants. These phenolics may either function directly as phytoalexins or be incorporated into structural barriers such as phenol-conjugated, lignified or suberised cell walls of appositions (Aist 1983). Phytoalexins are low molecular weight antimicrobial compounds that are toxic to and inhibit the growth of fungi pathogenic to plants (Agrios 1997). Stevenson *et al*. (1997) concluded that phytoalexins are fundamental components of the resistance mechanism of chickpea to Fusarium wilt. Another response to pathogen attack commonly observed is the production of so-called pathogenesis-related (PR) proteins, many of which have antimicrobial activity (Kitajima & Sato 1999, Van Loon & Van Strien 1999). In a study by Rep *et al*. (2002) a new member of the PR-5 family (PR-5x) was identified that accumulated in both compatible and incompatible reactions upon infection of tomato plants with *F. oxysporum*. Some PR-5 proteins have been shown to be active *in vitro* against *F. oxysporum*. These include AF24 and NP24, which are very similar to PR-5x (Rodrigo *et al*. 1993, Abad *et al*. 1996, Hu & Reddy 1997, Rep *et al*. 2002). Leeman *et al*. (1996) suggested that resistance induced by *P. fluorescens* strains against Fusarium wilt of radish was due to the production of salicylic acid (SA). It was further demonstrated that the production of SA at the site of infection might be involved in the induction of *F. oxysporum* f.sp. *asparagi* resistance in asparagus roots (He & Wolyn 2005).

PATHOGENESIS IN *Fusarium oxysporum*

Pathogenesis describes the complete process of disease development in the host, from initial infection to production of symptoms (Lucas 1998). During the initial stages of the interaction, fungal pathogens must sense stimuli from the plant and respond with appropriate morphogenic and biochemical changes (Roncero *et al*. 2003). The signalling process represents the first and most critical step in defining the outcome of fungal infection (Roncero *et al*. 2003). Griffin (1969) proposed that soil-borne fungal pathogens, including *F. oxysporum*, are able to sense the presence of the plant even before physical contact, most likely through compounds present in root exudates.

Effects caused by pathogens on plants are almost entirely the result of biochemical reactions taking place between substances secreted by the pathogen and those present in or produced by the plant. During pathogenesis the fungus penetrates the complex defense barriers that plant cell walls comprise of (Mengden *et al*. 1996). To gain entrance to plant cells, fungi generally secrete a mixture of hydrolytic enzymes including cutinases, cellulases, pectinases and proteases (Knogge 1996). After penetration, the fungus often secretes toxins or plant hormone-like compounds that manipulate the plants' physiology to the benefit of the pathogen (Knogge 1996). This is often achieved through the production of phytotoxins with varying degrees of specificity toward different plants (Walton 1994).

Enzymes

Fusarium oxysporum produces several enzymes that act upon the pectic and cellulose components of cell walls (Agrios 1997).

Pectinases

Pectinases catalyse the degradation of pectic polysaccharides, the main component of the middle lamella, that is, the intercellular cement that holds in place the cells of plant tissues (Rombouts & Pilnik 1980). Pectic enzymes consist primarily of pectin methylesterase, polygalacturonase and pectate lyase (Kawano *et al*. 1999, Verlent *et al*. 2004).

Pectin methylesterase: The capacity to produce pectin methylesterase (PME) is a general feature among the vascular wilt *Fusarium* species (MacHardy & Beckman 1981). PME is a pectin-degrading enzyme and its enzymatic reaction results in partially demethoxylated pectin chains and methanol (Verlent *et al*. 2004). In a study to test the hypothesis that pectic enzymes could reproduce disease symptoms, pectic enzyme preparations from several sources were assayed for PME. A correlation between the severity of vascular browning in tomato plants and PME activity was observed in seven different enzyme preparations (pectinol 100D, pectin metyl esterase, pectinase, hemicellulase, pectinol M129 B, pectinol M 137B and a preparation from the tomato wilt fungus) (Gothoskar *et al*. 1953). Significance of the correlation between vascular discoloration and PME is not readily understood. Although vascular discoloration is probably caused by the plant polyphenol oxidase system, the source of the substrate for this enzyme system still remains undetermined. The observation that PME free of polygalacturonase or depolymerase cannot produce wilting is an indication that the pectin in the cell wall is broken down (Gothoskar *et al*. 1955). An increase of PME is found in diseased plants. This makes it more susceptible to attack by the pectin chain-splitting enzyme, pectic depolymerase. Thus, according to Deese & Stahman (1962), pectic depolymerase coupled with PME are important biochemical factors involved in producing the symptoms of Fusarium wilt in tomatoes.

Polygalacturonase: Polygalacturonases (PGs) are important pectolytic enzymes produced by phytopathogenic fungi during the process of infection and colonisation of host plants (Posada *et al*. 2001). PGs (poly[1,4-α-D-galacturonide] glucanhydrolases) are the first pectinases produced by pathogens when cultured on isolated plant cell walls or during infection (Jones *et al*. 1972, Di Pietro & Roncero 1998). PG is a highly polymorphic enzyme and exhibits either an endo- or exo- mode of action (Posada *et al*.

2001). The role of endo-PGs in pathogenesis is tissue maceration and cell death (Bateman & Bashman 1976) and the generation of oligogalacturonides that could act as elicitors of plant defense responses (Walton 1994). Exo-PGs are responsible for the release of soluble low molecular weight oligogalacturonides from highly polymeric substrates which can enter into the cell where they are catabolised and act as inducers of other pectic enzymes (Cooper 1983). Oligogalacturonides produced by endo-PGs could in turn be degraded to monomers by the action of exo-PG enzymes, thereby suppressing their function as elicitors (Posada *et al*. 2001).

The large increase in depolymerase activity in diseased tomato plants may cause wilting by clogging of the vessels with degradation products caused by fungal pectic enzymes acting on constituents of the cell walls (Deese & Stahman 1962). According to Baayen *et al*. (1997), development of wilt symptoms in inoculated carnations was accompanied by a quadratic increase in PG activity. Although the fungal pectin degrading enzymes clearly contribute to degradation of the xylem of infected plants, PG activity in itself does not appear to be necessary for development of disease symptoms. Regardless of their role in pathogenesis, fungal PGs provide a reliable and rapid biochemical factor for monitoring fungal growth and quantifying partial resistance of carnation cultivars to Fusarium wilt (Baayen *et al.* 1997). *Pg1* and *pg5* encoding an endo-PG, and *pgx4* encoding an exo-PG, were identified in *F. oxysporum* f.sp. *lycopersici*, but it has been found that targeted inactivation of all of these genes (individually) had no effect on virulence (Di Pietro & Roncero 1996a, 1998, García-Maceira *et al*. 2000).

Pectate lyases: Pectate lyases (PLs) catalyse the trans-elimination of pectate (Linhardt *et al*. 1986) and have been suggested to play an important role in the development of vascular wilt (Beckman 1987). A specific PL (designated PL1) has been purified and characterised from *F. oxysporum* f.sp. *lycopersici*, and was found in infected tomato root and stem tissue (Di Pietro & Roncero 1996b). However, according to Di Pietro *et al*. (2003), knockout of the encoding gene *pl1* had no effect on the virulence of the pathogen to tomato plants. The pea pathogen *F. solani* (Mart.) f.sp. *pisi* (F.R. Jones) W.C. Snyder & H.N. Hans has at least four different PL genes, and inactivation of these genes individually also had no detectable decrease in virulence (Rogers *et al*. 2000).

Nevertheless, the simultaneous disruption of two PL genes, *pelA* and *pelD*, drastically reduced virulence, and the subsequent restoring of either gene caused recovery in virulence. These results indicate that PL can be considered a virulence factor in *Fusarium*. Thus, disruption of all functionally redundant genes is required to demonstrate the role of cell wall degrading enzymes (CWDE) in pathogenesis (Rogers *et al*. 2000).

Cellulase enzymes:

Since cellulose could serve as a carbohydrate source for *F. oxysporum* following partial decomposition by cellulase enzymes, several studies have explored a possible role for cellulase in wilt pathogenesis. There is, however, little evidence to support this idea (MacHardy & Beckman 1981). Cellulase enzymes, designated C1, C2 and Cx, are required to degrade cellulose. The C1 and C2 enzymes act upon native, insoluble cellulose to produce linear chains that are attacked by the Cx enzyme to produce cellobiose and glucose (MacHardy & Beckman 1981). According to Husain & Dimond (1960) *Fusarium* produces both the Cx and C1 enzymes. No cellulase activity was detected in diseased stems of tomato plants during experiments performed by Matta & Dimond (1963). Results of the investigations done by Fisher (1965) have shown that cellulytic enzyme production is induced in surface rot and Fusarium wilt of sweet potato. There was also no direct relationship between cellulytic enzyme production and the pathogenic reaction of *F. oxysporum* f.sp. *callistephi* on china aster, but isolates with no cellulytic activity were non-pathogenic (Horst 1965). Husain & Dimond (1960) reported that the action of cellulase produced by the tomato wilt pathogen *F. oxysporum* f.sp. *lycopersici* may be conceived to act in pathogenesis in three ways. Firstly, it is involved in wilt induction. Secondly, hydrolytic products of cellulase activity may provide *Fusarium* with carbohydrates for its continued development in the host, and thirdly, it is involved in the escape of the pathogen from vascular tissue in advanced stages of disease when the host is in dying condition.

Hemicellulase:

Few studies have explored the involvement of hemicellulases in Fusarium wilt pathogenesis. These enzymes, however, could be of greater significance than

pectinases and cellulase enzymes in that the greater part of the amorphous matrix in which cellulase microfibrils are embedded is composed of hemicellulose-type materials, such as glucans, xylans and mannans (Zaroogian & Beckman 1968, MacHardy & Beckman 1981). Hemicellulose, which was found in greater amounts than pectinaceous materials in tissue of four banana varieties, was fractionated into components A and B. The pectin-free and lignin-free tissue filtrates were extracted and precipitated with glacial acetic acid. This precipitate was designated hemicellulose A. To the remaining filtrate, an equal volume of acetone was added, and this was called component B. No correlation was apparent between resistance to *F. oxysporum* f.sp. *cubense* and the amount of hemicellulose A in all varieties, but the resistant varieties had significantly more hemicellulose B than susceptible varieties (Zaroogian & Beckman 1968). Several hemicellulases seem to be produced by many plant pathogenic fungi. Depending on the monomer released from the polymer on which they act, the particular enzymes are called xylanase, galactanase, glucanase, arabinase, mannase and so on (Agrios 1997).

Xylanases: Xylan is a heterogenous carbohydrate composed of β-1,4-xylopyranosyl residues and makes up a significant part of the hemicellulose fraction of the plant cell walls (Ruiz-Roldán *et al*. 1999, Di Pietro *et al*. 2003). Conversion of xylan to soluble products requires an array of enzymes, such as endo-β-1,4-xylanases, β-xylosidases, α-L-arabinofuranosidases and α-glucoronidases to cleave different bonds in the xylan molecule (Biely 1985). Xylanases have been isolated from a wide variety of bacterial and fungal pathogens (Walton 1994). Studies were performed in order to determine the role of xylan degradation in virulence of *F. oxysporum* f.sp. *lycopersici*. It was found that four different endoxylanase genes (*xyl2*, *xyl3*, *xyl4* and *xyl5*) were expressed during different stages of infection, but targeted inactivation of *xyl3*, *xyl4* and *xyl5* individually had no detectable affect on virulence. All of these mutants still had the ability to cause disease in tomato plants (Ruiz-Roldán *et al*. 1999, Gómez- Gómez *et al*. 2001, 2002). Therefore, in *F. oxysporum* and other fungi such as *Cochliobolus carbonum* R.R. Nelson and *Magnaporthe grisea* Herbert, xylanase genes were not found to be essential for pathogenesis, probably due to the presence of additional xylanase genes (Apel-Birkhold & Walton 1996, Wu *et al*. 1997, Gómez- Gómez *et al*. 2001, 2002).

Other hemicellulases: In previous studies the presence of xylanase, arabinase, mannanase and galactanase in culture filtrates of *F. oxysporum* f.sp. *vasinfectum* and *F. oxysporum* f.sp. *lycopersici*, was revealed (Ismail *et al*. 1989, Abdel-Rahman 1992).

Chitin synthases

One of the major structural components of the fungal cell wall is chitin, a β-1,4-linked polysaccharide made of *N*-acetylglucosamine (Bartnicki-Garcìa 1968). Polymerisation of *N*-acetylglucosamine from the substrate UDP-*N*- acetylglucosamine is catalysed by chitin synthases (EC 2.4.1.16) (Madrid *et al*. 2003). Fungal chitin synthases have been divided into five classes on the basis of their structure in conserved regions (Bowen *et al*. 1992, Din *et al*. 1996, Specht *et al*. 1996, Munro & Gow 2001). In studies done by Madrid *et al*. (2003) and Ortoneda *et al*. (2004), it was demonstrated that class V chitin synthase was required for host infection (both tomato and mice) by the pathogen *F. oxysporum* f.sp. *lycopersici*. The *chsV* gene was identified in an insertional mutagenesis screen for pathogenicity mutants. These studies suggest that *F. oxysporum* requires a specific class V chitin synthase for pathogenesis, most probably to protect itself against plant defence mechanisms.

Toxins

The secretion of plant hormone-like compounds that manipulate the plants' physiology to the benefit of the pathogen is often achieved through the production of phytotoxins with varying degrees of specificity toward different plants (Knogge 1996, Walton 1996). Despite the production of numerous toxins by other *Fusarium* spp., *F. oxysporum* is known to produce a limited number of toxins (Nelson *et al*. 1981). The most well known toxin produced by *F. oxysporum* is fusaric acid, while some isolates have been reported to produce enniatins, moniliformin, naptazarins and sambutoxin (Kern 1972, Rabie *et al*. 1982, Marasas *et al*. 1984, Bottalico *et al*. 1989, Kim *et al*. 1995, Herrmann *et al*. 1996). There has also been a report on fumonisin production by a strain of *F. oxysporum*. (Seo *et al*. 1996).

Fusaric acid

Fusaric acid (FA) was first isolated by Yabuta *et al*. (1934) from *F. heterosporum* Nees:Fr as a compound that inhibited the growth of rice seedlings. FA is a secondary metabolite that is synthesized by strains of the *F. moniliforme* J. Sheld, *F. crookwellense* Burgess Nelson and Toussoun, *F. subglutinans* (Wollenweb. & Reinking) Nelson, Toussoun & Marasas, *F. sambucinum* Fuckel, *F. napiforme* (Marasas, Nelson & Rabie), *F. heterosporum, F. oxysporum*, *F. solani* (Martius) Saccardo and *F. proliferatum* (Matsushima) Nirenberg (Bacon *et al*. 1996). Notz *et al*. (2002) found that FA production by *F. oxysporum* is strain and media dependent. A sucrose-based medium favours FA production by *F. oxysporum*. The *in planta* production of FA by several *formae speciales* of *F. oxysporum* occurs in watermelon, tomato, flax, cabbage and carnation (Davis 1969). Page (1959) reported the isolation of FA from cultures of *F. oxysporum* f.sp. *cubense*, and from rhizomes of Gros Michel banana plants infected with this fungus. FA was also isolated from filtrates of *F. oxysporum* f.sp. *lycopersici*. The FA-producing abilities and the virulence of a number of UV-induced mutants of *F. oxysporum* f.sp. *cubense* were compared. Some mutants that produced at most a trace of FA had little or no pathogenicity, while other low producers were highly pathogenic. High FA-producing strains varied similarly. This evidence suggests that FA does not have a key role in disease development caused by *F. oxysporum* f.sp. *cubense*. It was concluded that FA does not have a direct role in symptom development and may not be essential for pathogenicity (Kuo & Scheffer 1964).

A toxin may promote disease by predisposing the host plant to injury by other fungal products that are produced simultaneously or in close sequence with the toxin. FA production in several plants, and its correlation with pathogenicity, was investigated by Davis (1969). In both watermelon (Davis 1969) and tomato (Tamari & Kaji 1954, Gaumann 1957), FA is known to play an important role in the plant disease process, but no correlation between plant toxicity and the amount of FA produced by the infecting isolate has been made (Bacon *et al*. 1996). Results indicated that the low and unchanging level of FA, as disease progresses in flax and tomato, indicate that in these

plants FA is, at most, a secondary factor promoting pathogenesis. Only in the watermelon wilt disease is FA implicated in selective pathogenicity. This was demonstrated by the fact that 1) the concentration of FA in seedlings increases as the disease progressed, 2) more than traces of FA was produced in both living and non-living seedlings and 3) there was a positive correlation between pathogenicity of six isolates of *F. oxysporum* f.sp. *niveum* and the FA content of diseased watermelon plants.

Growth regulators

Plant growth is regulated by a small number of naturally occurring compounds that act as hormones and that are generally called growth regulators (Agrios 1997). The most important growth regulators are auxins, gibberellins and cytokinins, but other compounds, such as ethylene and growth inhibitors, play important regulatory roles in the life of the plant. Growth regulators act in very small concentrations and even slight deviations from the normal concentration may bring about strikingly different plant growth patterns (Agrios 1997). Auxins and gibberellins are most frequently associated with enhanced cell elongation, and cytokinins with cell division (Manners 1982).

Plant growth regulators play a key role in resistance responses to infection instead of in pathogenesis (MacHardy & Beckman 1981). It has been suggested that auxins (e.g. indole acetic acid (IAA)) induce tylose formation and other overgrowth phenomena in wilt-diseased plants (Gordon & Paleg 1961, Beckman & Halmos 1962). Tylose formation is the primary type of abnormal growth in *Fusarium*-infected roots (Beckman *et al*. 1962) and is stimulated by IAA. According to Mace & Solit (1966), IAA and phenols appear to play key roles in Fusarium wilt of banana. Phenols appear to be responsible for the vascular discoloration in wilt-diseased plants (Dimond 1955, Beckman 1964). It has been found that Dopamine or its oxidation products do not interfere with the auxin activity of IAA in stimulating other tylose formation (Mace & Solit 1966).

Pathogens can affect growth and development of the plant by producing growth hormones, or by affecting production of plant growth hormones by the host or degradation of hormones in the tissues. If production of growth hormones by the pathogen is involved, the nature and rate of production of growth hormones may be different from that produced *in vitro*. Therefore, information concerning the production of growth hormones by pathogens in culture may give little insight concerning the situation within the diseased plant (Manners 1982). It is not clear what the role of growth regulators is in the virulence of Fusarium wilt pathogens, but it was found that the auxin IAA is produced by *F. oxysporum* f.sp. *cubense* (Mace 1965).

Polysaccharides

Polysaccharides are polymers made up of many monosaccharides that are joined together by glycosidic linkages (wikipedia). Polysaccharide involvement in the blockage of vascular vessels of plants is possible when macromolecular substances are released in the vessels through the breakdown of host substances by pathogens (Agrios 1997). Large polysaccharide molecules released by the pathogen in the xylem may be sufficient to cause a mechanical blockage of vascular bundles and thus initiate wilting (Agrios 1997).

THE GENETICS OF VIRULENCE IN *Fusarium oxysporum*

The size of the *F. oxysporum* genome has been estimated to range from 18.1 to 51.5 Mb (Migheli *et al*. 1993), including linear mitochondrial plasmids (Kistler 1997), with chromosome numbers varying between 7 and 14 (Migheli *et al*. 1993, O' Donnell *et al*. 1998). Approximately 5% of the genome appears to be constituted by different families of transposable elements (Daboussi & Langin 1994).

Host specificity in Fusarium oxysporum

A gene-for-gene relationship has been proposed for the interaction between *F. oxysporum* races and host cultivars, since monogenic, dominant resistant traits against

known races have been described for *F. oxysporum* f.sp. *lycopersici* (Roncero *et al*. 2003). In tomato, resistance gene *I-2* confers resistance to *F. oxysporum* f.sp. *lycopersici* race 2 (Ori *et al*. 1997) by expressing the putative avirulence gene *avrI-2* (Mes *et al*. 1999). The gene-for-gene theory, however, does not include all *formae speciales* of *F. oxysporum*. For example, in the banana wilt pathogen, grouping of *F.oxysporum* f.sp. *cubense* isolates into races is determined by their pathogenicity to a limited number of banana differentials under field conditions (Moore *et al*. 1993).

Several important findings regarding the phylogeny and classification of the *F. oxysporum* complex were obtained from a study conducted by O' Donnell *et al*. (1998). This complex is strongly supported as monophyletic, despite many *formae speciales* being polyphyletic, suggesting that host pathogenicity has evolved convergently. The evidence for polyphyly in *F. oxysporum* f.sp. *cubense* and *F. oxysporum* f.sp. *melonis*, however, challenges the *forma specialis* naming system. O' Donnell *et al*. (1998) concluded that this potentially obscures communication of critical information concerning the genetic diversity of pathogens that might be needed for effective breeding programs and disease control efforts.

The role of signal transduction in pathogenesis

Fusarium oxysporum possesses signalling mechanisms that enable the fungus to sense environmental cues and respond to those in order to infect plants (Di Pietro *et al*. 2003). Studies from a wide range of fungi have converged to define two conserved signal transduction cascades regulating fungal development and virulence: a cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA) signalling system (cAMP-PKA) cascade, and a mitogen-activated protein kinase (MAPK) cascade (Lengeler *et al*. 2000). Both pathways are required for pathogenesis, and play a crucial role in the formation of specialized infection structures, such as appressoria, that are produced by most pathogens that attack aerial parts of the plant (Lengeler *et al*. 2000).

The role of the cAMP-PKA and MAPK pathways are not well understood in soil-borne pathogens, despite recent evidence that both pathways operate in *F. oxysporum*. In a

study by Di Pietro *et al*. (2001), a targeted inactivation of *fmk1*, encoding a *F. oxysporum* f.sp. *lycopersici* MAPK orthologous to yeast Fus3/Kss1 MAPKs, produced mutants that were unable to penetrate the roots of tomato plants and did not produce any disease symptoms. The results from this study indicate the following roles of the MAPK pathway:

1. It plays a crucial role in root attachment during infection by soil-borne pathogens. The reason for this is not clear, although reduced expression of surface hydrophobins, a class of extracellular proteins implicated in fungal surface adhesion (Wosten *et al*. 1994), has been observed.
2. △*fmk1* mutants showed decreased production of PGs and PLs, two classes of pectinolytic enzymes that participate in the degradation of the plant cell wall. It, therefore, appears that *fmk1* controls the production of extracellular proteins involved in early stages of infection.

Among the components of signal transduction pathways, G proteins are heterotrimeric GTP-binding proteins involved in transducing signals from activated membrane receptors to a variety of intracellular targets (Recorbet *et al*. 2003). G protein α subunits have been shown to be implicated in the pathogenicity of *M. grisea* (Liu & Dean 1997), *Chryphonectria parasitica* (Murrill) Barr (*Endothia parasitica* (Murrill) P.J. Anderson *et* H.W. Anderson) (Choi *et al*. 1995), *Colletotrichum trifolii* Bain *et* Essary (Truesdell *et al*. 2000) and *Botrytis cinerea* Pers.: Fr (Gronover *et al*. 2001). Jain *et al*. (2002) isolated *fga1* encoding a Gα subunit that showed high similarity to those of G protein α family proteins from *F. oxysporum* f.sp. *cucumerinum*. △*fga1* mutants showed altered growth morphology, decreased conidiation on solid medium, and considerable, but not completely reduced, virulence. The mutants also showed a two-fold reduction in cAMP levels. In another study, *fgb1* encoding a Gβ subunit of *F. oxysporum*, was cloned (Jain *et al.* 2003). Virulence in △*fbg1* mutants of *F. oxysporum* f.sp. *cucumerinum* was significantly reduced when inoculated on cucumber plants. Reduction in conidiation as well as decreased intracellular cAMP levels was observed. The results from these studies suggest that the GαI and Gβ subunits control hyphal growth, conidiation and virulence in *F. oxysporum* via a cAMP-PKA pathway (Di Pietro *et al*. 2003). The MAPK and cAMP-PKA cascades both regulate virulence in *F. oxysporum*, as was

found in other fungal pathogens such as *M. grisea*, *Ustilago maydis* (de Candolle) Corda and *Candida albicans* (Robin) Berkhout (Lengeler *et al*. 2000).

Genes related to virulence in Fusarium oxysporum

Pathogenicity genes in fungi have been identified at an exponential rate in recent years. Indurm & Howlett (2001) tabulated 79 genes described at that time, and divided them into several categories, depending on their involvement in the formation of infection structures, cell wall degradation, response to the host environment, toxin biosynthesis, signal cascades, and novel functions. However, studies on genes related to pathogenicity in *F. oxysporum* have been limited. Two of the most important virulence genes associated with *F. oxysporum* are *FOW1* and *ARG1*.

FOW1

The pathogenicity mutant B60 of the melon wilt pathogen *F. oxysporum* f.sp. *melonis* was previously isolated by restriction enzyme-mediated DNA integrated mutagenesis (REMI) (Inoue *et al*. 2001). Molecular analysis of B60 identified the affected gene, designated *FOW1*, which encodes a protein with strong similarity to mitochondrial carrier proteins (MCPs) of yeast. Although the *FOW1* insertional mutant and gene-targeted mutants of *F. oxysporum* f.sp. *melonis* showed normal growth and conidiation in culture, they showed markedly reduced virulence as a result of a defect in the ability to colonise the plant tissue (Inoue *et al*. 2001). The *FOW1*-targeted mutants of the tomato wilt pathogen *F. oxysporum* f.sp. *lycopersici* also showed reduced virulence. The study done by Inoue *et al*. (2001) strongly indicates that *FOW1* encodes a mitochondrial carrier that is required specifically for colonisation of plant tissue by *F. oxysporum* (Inoue *et al*. 2002).

ARG1

REMI mutagenesis was used to tag genes required for pathogenicity in *F. oxysporum* f.sp. *melonis* (Namiki *et al.* 2001). Of the 1129 REMI transformants tested, 13 showed reduced pathogenicity on susceptible melon cultivars. One of the mutants, FMMP95-1, was an arginine auxotroph. Structural analysis of the tagged site in FMMP95-1

identified a gene, designated *ARG1*, which possibly encodes argininosuccinate lyase, an enzyme catalysing the last step for arginine biosynthesis. Complementation of FMM95-1 with the *ARG1* gene caused a recovery in pathogenicity, indicating that arginine auxotrophic mutation causes reduced pathogenicity in this pathogen (Namiki *et al*. 2001).

FACTORS CONTRIBUTING TO PATHOGENESIS

Temperature

The optimal growth of *F. oxysporum* was found to be between 25 and 28ºC. Cook & Baker (1983), in their review of the biological control of plant diseases, noted that the growth of Fusarium wilt pathogens is generally maximal at 28ºC, inhibited above 33ºC, and not favoured below 17ºC. The occurrence of Fusarium wilt diseases is also affected by soil temperature (Ben-Yephet & Shtienberg 1997). Ben-Yephet & Shtienberg (1994) described a parabolic relationship between substrate temperature and disease intensity, indicating that there were low and high temperature extremes at which wilt symptoms did not develop in carnation, and an optimum temperature at which the most severe disease occurred. In carnation, no wilt symptoms and very little colonisation were observed at 14°C, almost all stems were colonised at 18-20°C but remained symptomless, and at temperatures ranging between 23-26°C, wilt symptoms were severe (Fletcher & Martin 1972, Harling *et al*. 1988). The optimal temperature was 25-26°C. More severe symptoms of wilt in chickpea were observed at 25 and 30°C than at 10, 15 or 20°C (Bhatti & Kraft 1992). Temperature extremes at which Fusarium wilt of carnation developed, were influenced by solar radiation intensity and the inoculum concentration. For example, at 85% shade ($200\mu Em^{-2}s^{-1}$), the lower and upper temperatures at which symptoms did not develop were, respectively, 21.5°C and 30.7°C for 10^3 spores/ml, but 18.6°C and 33.0°C for 10^6 spores/ml (Ben-Yephet & Shtienberg 1994).

Harling *et al*. (1988) suggested that temperatures alter the balance between the plant host and the Fusarium wilt pathogen. Temperatures that favour the host's metabolism to increase relative to that of the pathogen would induce a resistant reaction, whereas temperatures that favour the pathogen would induce a susceptible reaction, resulting in wilt symptoms. The effect of temperature on wilt occurrence, however, may vary in different pathosystems (Ben-Yephet & Shtienberg 1997). Wilting of crucifers, caused by *F. oxysporum* f.sp. *conglutinans*, increased as temperatures increased for all pathosystems (highest temperature:) (Bosland *et al*. 1988). In banana, Brake *et al*. (1995) found that temperature was primarily affecting plant growth rather than influencing the aggressiveness of the pathogen. Ploetz *et al*. (1990) observed that, although *F. oxysporum* f.sp. *cubense* VCG 0120 is present in some tropical regions, it severely affects Cavendish banana only in the subtropics, indicating that temperature may have an important influence on disease development.

pH

Mycelia of *F. culmorum* (W.G. Smith) Saccardo, *F. graminearum* Schwabe (teleomorph = *Gibberella zeae* (Schwein.) Petch) and *F. oxysporum* grew within the range of pH 2-12. *Fusarium avenaceum* (Fr.) Sacc. started to grow at pH 3 and *F. graminearum* at pH 1. A pH of 6 was the most suitable for the growth of all species, while a highly acidic medium was unsuitable for sporulation of all species (Srobar 1978).

Acid soil (pH 4.2) supported growth of *Fusarium* through the soil, whereas a pH near neutrality prevented this growth (Wilson 1946). *In vitro* studies by Marshall & Alexander (1960) indicated that this probably represents microbial competition and antibiosis. Competitive effects of bacteria and actinomycetes dependent on higher soil pH have been demonstrated to lie in the competition for nutrients and to a lesser degree in antibiotic production. There is, however, a gap between *in vitro* demonstrations and *in situ* action. It, therefore, appears that the reason for "fertile" non-acid soils not

supporting *Fusarium* wilt disease production is the competitive action of a healthy population of soil microflora. Raising soil pH toward or slightly above neutrality appears to be a foundation in cultural control of Fusarium wilt, which commonly is a disease associated with acidic, sandy soils, rather than heavier soils with higher pH values (Woltz & Jones 1981). pH has been shown to influence germination of chlamydospores of *F. oxysporum*, although germination does seem to occur over a wide range of pH (Chuang 1991, Peng *et al*. 1999).

Nutrition

The stages of growth, decline or quiescence of a *Fusarium* population in soil depends on the ecological balance and nutrient availability. *Fusarium oxysporum* is very capable as an autotroph, requiring only a carbon source for structure and energy, and inorganic compounds to synthesize organic compounds such as sugars, lipids and amino acids (Woltz & Jones 1981). The list of essential nutrient elements for the growth, sporulation and virulence of *F. oxysporum* currently includes carbon, hydrogen, oxygen, nitrogen, phophorus, potassium, magnesium, sulphur, iron, manganese, molybdenum and zink (Steinberg 1950). Copper has not been shown to be indispensable as a nutrient for *F. oxysporum* (Steinberg 1950, Woltz & Jones 1971), while chloride is not essential to *F. oxysporum*, but may benefit the disease-producing functions of the fungus. Chloride activates a number of pectolytic and amylolytic enzymes, but a significant amount of this element is needed (Woltz & Jones 1981).

High levels of nitrogen fertilisation in agricultural soils generally lead to an increase in Fusarium wilt development (Woltz & Engelhard 1973, Woltz & Jones 1973). The nitrate form of nitrogen becomes increasingly unfavourable with increasing rates of application, while the ammonium form becomes more favourable for disease as the nitrogen application rate is increased. Woltz & Jones (1973) reported that *F. oxysporum* cultured on ammonium nitrogen was more virulent than the same fresh weight of the organism cultured on nitrate nitrogen. Effects of nitrate and ammonium sources on disease were apparently related to soil pH effects. Nitrate caused an elevation in soil pH while ammonium caused a reduction (Woltz & Jones 1973). In a

study by Walker (1971) it has been indicated that high nitrogen and low potassium favoured disease, while low nitrogen and high potassium retarded disease development. Relatively low levels of calcium appear more conducive to disease than normal levels (Edgington & Walker 1958, Corden 1965). Boron deficiency of host plants leads to an increase in disease severity (Keane & Sackston 1970).

CONCLUSION

For centuries *F. oxysporum* has caused vascular wilt diseases of many economically important agricultural crops (MacHardy & Beckman 1981, Pennypacker 1981). To distinguish among the many pathogenic forms in this morphologically homogenous fungus, the pathogen has been divided into *formae speciales*, based on its host range, and into races, based on its ability to cause diseases to specific crop cultivars (Gordon & Martyn 1997). Many strains of *F. oxysporum* are capable of heterokaryosis and can be divided into VCGs. VCG is useful for *formae speciales* and race identification when these characters aren't variable (Katan 1999). However in the case of diverse *ff. sp.* such as *cubense* which constitutes several VCGs it is not possible. The determination of VCG can, therefore, not be applied as a universal tool to identify *formae speciales* (Fravel *et al*. 2003). Neither can it be used to identify races within *formae speciales*. Race identification still relies strongly on pathogenicity testing, a phenotypic character that is often subjected to existing environmental conditions.

One of the concerns regarding the taxonomy of *F. oxysporum* is that the current nomenclature is not always supported by genetic and molecular analysis. In *F. oxysporum* f.sp. *lycopersici*, race designation is based on a single dominant resistance (R) gene function in the plant, and is not reflected in the molecular analysis of the fungal genome (Ori *et al*. 1997, Mes *et al*. 1999). In *F. oxysporum* f.sp. *cubense*, however, the same race includes several DNA fingerprinting groups, representing a number of VCGs, and the same fingerprint will include different races (Ploetz 1990, Bentley *et al*. 1995, Bentley *et al*. 1998). This confusion in the current nomenclature should be urgently addressed by utilizing appropriate molecular techniques and by

developing accurate molecular markers to assist plant breeders and biotechnologists with the improvement of germplasm against Fusarium wilt diseases.

Despite the substantial contribution that molecular techniques, such as sequencing, RFLP, RAPD, DNA Amplification Fingerprinting (DAF) and Amplified Fragment Lenth Polymorphism (AFLP) have made to diversity studies in *F. oxysporum*, it has not been able to sufficiently address or propose a new nomenclature that could replace the current system that is based on subjective phenotypic characters. There is still uncertainty about the polyphylytic nature of *formae speciales* and the phylogenetic relationships in some forms, such as the banana pathogen *F. oxysporum* f.sp. *cubense*. It is, therefore, necessary that the exact relationship between different varieties and potential patho-forms of *F. oxysporum* be investigated for identification purposes and the understanding of evolutionary relationships among species. We hope that tools such as AFLP analysis and short sequence repeats (SSRs) could eventually provide sufficient resolution in the analysis of this clonal fungus to address the current taxonomic limitations.

Virulence in *F. oxysporum*, and the specificity of its forms to a variety of agricultural crops, is still poorly understood. Apart from the pathogenic forms, *F. oxysporum* includes many non-pathogenic forms, often with a greater genetic diversity than that existing in the pathogenic members of the species (Appel & Gordon 1996). The reason why there is host specialization in *F. oxysporum,* and whether it is possible for non-pathogens to evolve into pathogens, has not been elucidated. Studies have been conducted to determine the role of cell wall degrading enzymes (CWDEs) in the pathogenesis of *F. oxysporum*. Roles have been proposed by which these enzymes contribute to symptom induction, but have not been substantiated (Cooper & Wood 1973). An observation made by Walton (1994) is that multiple genes could encode similar and functionally redundant enzyme activities in most plant pathogenic fungi. This could be the reason for the frequent observation in recent studies that transformation-mediated inactivation of individual CWDE-encoding genes had no detectable effect on virulence (Di Pietro & Roncero 1998, Ruiz-Roldán *et al*. 1999, Rogers *et al*. 2000, Gómez-Gómez *et al*. 2001, 2002). In contrast, the cAMP-PKA and

MAPK signalling transduction cascades have been implicated in pathogenesis of *F. oxysporum* through the generation of such pathogenicity mutants. Whether such genes involved in pathogenicity are present in both pathogenic and non-pathogenic isolates of *F. oxysporum*, or whether they are expressed and responsible for specificity only in certain *formae speciales* and races of *F. oxysporum*, needs to be further investigated.

Despite the information available on the general physiology of fungi, relatively little is known about its role in disease caused by *F. oxysporum*. Is there a correlation between growth rate or sporulation and virulence? Is infection by some forms and races of *F. oxysporum* related to their pH and temperature optima and nutritional status? Is the development of Fusarium wilt of banana in the subtropics related to pathogen behaviour or plant predisposition to cold temperatures? Better knowledge about the biology and physiology of *F. oxysporum* might contribute to a more complete understanding of Fusarium wilt diseases and their development.

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CHAPTER 2

Biological, physiological and pathogenic variation in a genetically homogenous population of *Fusarium oxysporum* f.sp. *cubense*

ABSTRACT

Fusarium oxysporum f.sp. *cubense* (*Foc*) is the causal agent of Fusarium wilt of banana. The pathogen is divided into three races and 21 vegetative compatibility groups (VCGs). Within a VCG, *Foc* proved to be relatively homogenous genetically. Previous studies showed that isolates of *Foc* differ in phenotypic characteristics, such as volatile production and cultural appearance, and in its virulence to a differential set of banana cultivars. These studies were performed with a set of isolates that represented different races and VCGs from different banana-producing countries. The aim of this study was to determine the phenotypic variation in the South African population of *Foc*, in which all isolates belong to VCG 0120. Twenty-six representative isolates of *Foc* 'subtropical' race 4 (VCG 0120) were selected for this study. Differences in growth rate were determined on potato dextrose agar, and the number of spores determined on carnation leaf agar. The number of microconidia, macroconidia and sporodochia was determined. Virulence was determined by inoculating 10-cm tissue culture banana plantlets of two Cavendish cultivars, Grand Nain and Williams with a spore suspension of 10^5 spores.ml^{-1}. Virulence was evaluated based on the development of symptoms in the rhizomes using a standardized disease rating scale. Cultural characteristics and virulence varied among isolates. The South African isolates can be divided into sporodochial, cottony and slimy pionnotal types. All isolates produced microconidia in abundance, but production of macroconidia and sporodochia varied. Disease severity varied from 0 (no vascular discoloration) to 5 (total discoloration of vascular tissue). This study showed that the South African population of *Foc* varies considerably in terms of phenotypic characteristics.

INTRODUCTION

Fusarium oxysporum Schlecth f.sp. *cubense* (EF Smith) Snyd. and Hans (*Foc*) is the soil-inhabiting fungus that causes Fusarium wilt of banana (Brandes 1919). The pathogen was responsible for one of the greatest epidemics in agricultural history when it devastated the Gros Michel-based banana export industry in tropical America and Africa (Stover 1962). Today, Fusarium wilt still results in severe losses in several banana-growing countries of the world (Viljoen 2002). The disease is a classic wilt disease of an agricultural crop, and is primarily caused by the colonization and blockage of the xylem vessels by the pathogen. Internal symptoms include the discoloration of the vascular system, which turns to a reddish-brown colour. External symptoms are the initial yellowing of the leaf margins of older leaves, before the yellowing progresses from the oldest to the youngest leaves. Leaves gradually collapse to form a "skirt" of dead leaves around the pseudostem and the plant eventually dies (Moore *et al*. 1995).

Fusarium oxysporum produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Booth 1971). According to Messiaen & Cassini (1981) there is a good deal of variation in spore morphology within the species, even within a specialised form or race, with respect to shape and size of macroconidia and the proportion of microconidia to macroconidia. Certain isolates do not produce any macroconidia (Messiaen & Cassini 1981). Smith *et al*. (1988) reported that *F. oxysporum* exhibits varying cultural morphology on Potato Dextrose Agar (PDA). The aerial mycelium first appears white and may then change to a variety of colours, ranging from violet to dark purple, according to the strain of *F. oxysporum.* If sporodochia are abundant, the culture may appear cream or orange in colour. The features used to classify *Foc* isolates into races prior to the 1980's included volatile production, cultural morphology and pathogenicity (Brandes 1919, Stover 1959, Stover & Malo 1972, Sun & Su 1978). The production of volatiles, or the lack thereof, led to the designation of the two types, 'Odoratum' and 'Inodoratum', respectively (Brandes 1919, Stover 1959). Vegetative compatibility group (VCG) and gas chromatographic work has verified that volatile production separates two distinct populations of *Foc* (Moore *et al*. 1993, Pegg *et al*. 1993). Waite & Stover (1960)

proposed a classification of *Foc* in various morphological types, namely sporodochial, sclerotial, cottony, ropy and slimy pionnotal types.

Pathogenic variability within *Foc* has led to its subdivision into races, differentiated by their ability to cause disease to specific banana cultivars. The term race is applied to describe the pathogenicity of different strains of *Foc* to a narrow range of banana cultivars. Three races (1, 2 and 4) of the pathogen affect banana plants, while race 3 is a pathogen of *Heliconia* (Waite 1963). Stover & Waite (1960) reported that race 1 affects Gros Michel and race 2 is pathogenic to Bluggoe and other closely related cooking bananas. Race 4 is the most destructive, and attacks Cavendish cultivars as well as race 1 and race 2 suscepts (Su *et al*. 1986). 'Tropical' and 'subtropical' strains of race 4 have been recognized, depending on their ability to attack Cavendish bananas under tropical and subtropical climatic conditions, respectively (Su *et al.* 1986, Brake *et al*. 1990, Ploetz *et al*. 1990, Pegg *et al*. 1993, 1994, Ploetz 1994, Bentley *et al*. 1998, Gerlach *et al*. 2000). Once individuals in *Foc* fuse asexually to form a stable heterokaryon (Lodwig *et al.* 1999), such individuals belong to the same VCG (Ploetz & Correl 1988). In the worldwide population of *Foc*, at least 21 VCGs have been identified (Ploetz 1990).

The confusion related to the nomenclature of *Foc* often complicates disease management efforts by plant pathologists and breeders. For instance, race designation currently relies entirely on symptom development under field conditions. Consequently, VCG 0120 will cause disease to Gros Michel in the tropics, but not to Cavendish bananas, thereby allocating this VCG to race 1. The same VCG will result in disease to Cavendish bananas in the subtropics, assigning it to race 4. Because disease development in VCG 0120 is governed by environmental conditions, management of this genetically uniform VCG in the tropics can be achieved by planting of Cavendish bananas, but not in the subtropics. It is hypothesised that the cold winter temperatures in the subtropics predisposes Cavendish bananas to infection by *Foc* VCG 0120 (Ploetz *et al*. 1990, Viljoen 2002). However, the possibility that some individuals in *Foc* are more competitive under the lower winter temperatures has not been investigated.

It has been found that a high soil pH reduces the incidence of Fusarium wilt diseases, while the source of nitrogen fertilizer can affect disease development. NO_3-N generally suppresses, while NH_4^+-N enhances Fusarium wilt incidence (Woltz & Engelhard 1973, Jones & Woltz 1975). The addition of $CaCO_3$ and NO_3^--N raises the soil pH and, thereby, results in a lower disease incidence (Jones & Woltz 1967, Jones & Woltz 1969, Jones & Woltz 1970). One explanation is that the lower disease incidence is due to the domination of advantageous and disease-suppressive bacteria in the root rhizosphere at a higher pH. Another explanation is that a higher pH results in the unavailability of micronutrients that are essential for the growth, sporulation and virulence of Fusarium wilt pathogens (Jones *et al*. 1989). The possibility that some isolates of *Foc* have a lower optimum pH has, as yet, not been considered.

Much effort has been made in the past to understand pathogenesis in *F. oxysporum* (Beckman & Halmos 1962, Beckman *et al*. 1962). It did, however, not lead to a better understanding of the underlying factors governing Fusarium wilt diseases. Stover's (1962) findings that isolates of *Foc* differ in phenotypic characteristics and in their virulence to a differential set of banana cultivars were performed with a set of isolates that represented different races and VCG's from different countries. In the current study, the biology, physiology and pathogenicity in a genetically uniform population of *Foc* from South Africa, which consists entirely of VCG 0120 'subtropical' race 4 (Viljoen 2002), was investigated. The aims were to obtain information on the variability of the phenotypic qualities of this pathogen, and to determine whether these could be correlated with virulence in order to understand pathogenicity in *Foc*. In addition, it was important to determine the diversity in temperature and pH regimes, and in nitrogen source utilization in isolates of *Foc*, in an attempt to gain a better understanding of Fusarium wilt of banana in the subtropics.

MATERIALS AND METHODS

Isolates used

Twenty-six isolates of *Foc* 'subtropical' race 4 (VCG 0120) were collected from diseased Cavendish bananas in three banana-growing areas in South Africa (Table 1). All isolates are maintained in the culture collection of the Forestry and Agricultural

Biotechnology Institute (FABI) in Pretoria, South Africa. Starter cultures were prepared by transfering isolates from water agar (WA) slants onto PDA (Biolab Diagnostics, Wadeville, South Africa) containing 0.02 $g.l^{-1}$ Novobiocin (Sigma-Aldrich, Steinheim, Germany).

Growth studies

Growth rate

Starter cultures were incubated at 25°C under a mixture of cool-white and near-ultraviolet fluorescent lights for 5 days. Mycelial disks (5 mm in diameter) were punched from culture edges and aseptically transferred to the centre of 90-mm-diameter Petri dishes containing PDA. The new plates were incubated at 25 and 30°C under a cool-white and near-ultraviolet fluorescent light with a 12-hr photoperiod. After 5 days, the colony diameter was measured using the digimatic electronic callipers (Mitutoyo, Andover, Hampshire, UK). The 5-mm disk was subtracted from this value. Five plates were incubated for each isolate, and the experiment was repeated.

Cultural morphology

Morphological types in *Foc* 'subtropical' race 4 VCG 0120 were determined using the classification system described by Waite & Stover (1960). In this system, the presence/absence of sporodochia, pionnotes and sclerotia were considered for classification into the type of culture morphology (Table 2). The abundance of aerial mycelia and the colony colour were also recorded.

Sporulation

Foc was transferred to carnation leaf agar (CLA) and PDA to determine the amount and form of sporulation by the individual isolates. After 5 days, the number of macroconidia, microconidia and sporodochia produced on CLA was counted in the vicinity of the carnation leaf in a 2 cm^2 field, using a stereomicroscope (Nikon, SMZ 645). Five replicate plates were counted per isolate, and an average was determined. The isolates were grouped according to the number of conidia and sporodochia

counted by using a scale from 0-3, where 0 = absent (0 spores), 1 = few (1-20 spores), 2 = regular (21-50 spores) and 3 = abundant (>50 spores).

Pathogenicity studies

Inoculum preparation

A liquid medium, to enhance spore formation, called Armstrong *Fusarium* medium (Booth 1977), was used to prepare the primary inoculum for the pathogenicity studies. Armstrong medium was made up in Erlenmeyer flasks and autoclaved. After the medium cooled down, separate flasks were inoculated with 21 different *Foc* isolates (Table 1) by transferring a small block of agar, cut from single-spored WA slants, to the sporulation medium. Thereafter it was placed on a rotary shake incubator with a rotation speed of 165 rpm under white light at 25°C for 5 days. After incubation, the sporulation medium was poured through cheesecloth to separate spores from mycelia. Spore suspension concentration was determined with a Haemacytometer, and adjusted with sterile distilled water to a final concentration of 5×10^6 spores.ml^{-1}.

Plant material

Disease-free Cavendish banana plantlets produced in tissue culture were obtained from Du Roi Laboratories in Letsitele, South Africa. Two susceptible Cavendish cultivar-types, Williams and Grand Nain, were used in two different pathogenicity assays. Banana plantlets were planted in a hydroponic system as follows: The soil was first removed from the roots, strips of sponge were then wrapped around the stems, secured by a lid, and the plants were subsequently placed in 250-ml plastic cups containing water (Fig. 1). After a week, the plants were supplemented with a hydroponic mixture consisting of 0.6 g.l^{-1} calcium nitrate monohydrate, 0.9 g.l^{-1} Agrasol® 'O 3:2:8 (Fleuron, P.O. Box 31245, Braamfontein, 2017) and 3 g.l^{-1} Micromax® (Fleuron). After another 7 days the plants were inoculated with the *Foc* spore suspensions.

Inoculation

Five tissue culture plantlets in a hydroponic system were inoculated with each isolate of *Foc*. The plants were first removed from their plastic cups and the roots slightly damaged by hand. The *Foc* spore suspension was then added to the cups to obtain a

final concentration of 10^5 spores.ml^{-1} before the plantlets were placed back in the plastic cups containing the spore suspension. No *Foc* spores were added to the control plants. The plants were maintained in a greenhouse at 25°C with a 12-hr photoperiod. After 6 weeks, disease severity was calculated using a standardized disease rating scale designed for Fusarium wilt of banana (Carlier *et al*. 2002) (Table 3). The plants were cut just above the roots, and disease severity caused by *Foc* isolates was based on the development of internal symptoms in the rhizomes of inoculated plants. The pathogen was re-isolated from representative diseased plants to prove Koch's Postulates.

Physiological studies

Temperature

To determine the effect of temperature on fungal growth, 5-mm-diameter disks were transferred from *Foc* culture edges and placed onto 90-mm-diameter Petri dishes containing PDA. Five repeats of each isolate were incubated at different temperatures in the dark. The temperatures selected were 5°C, 10°C, 15°C, 20°C, 25°C, 30°C and 35°C. After a period of 5 days, the colony diameter was measured as previously described.

pH

Citrate-phosphate buffer was prepared by titrating 0.1 M citric acid (N.T. laboratory supplies, Johannesburg, South Africa) with 0.2 M $Na_2HPO_4.12H_2O$ (Merck, Darmstadt, Germany) to a pH of 4 and 5. Phosphate buffer was prepared by titrating 0.2 M $Na_2HPO_4.12H_2O$ (Merck) with 0.2 M $NaH_2PO_4.2H_2O$ (Merck) to a pH of 6 and 7. Boric acid-borax buffer was prepared by titrating 0.2 M Boric acid (Sigma-Aldrich) with 0.05 M disodium tetraborate ($Na_2B_4O_7.10H_2O$) (Merck) to a pH of 8. Once the buffers were prepared, a basal medium was added to each litre of buffer. The basal medium consisted of 45 g sucrose (Saarchem, Unilab, Midrand, South Africa), 3 g $NaNO_3$ (Merck), 1.5 g K_2HPO_4 (Merck), 0.75 g $MgSO_4.7H_2O$ (Merck), 0.75 g KCl (N.T. laboratory supplies) and 0.015 g $FeSO_4$ (Riedel-de Haën AG, Seelze, Germany). Noble agar (15 g) (Difco, Becton, Dickinson and company, Franklin Lakes, USA) was added to each litre of buffer, with the exception of the pH 4 buffer, which received 18 g agar. The effect of pH on *Foc* growth was determined by placing

5-mm-diameter disks punched from the WA culture edges on five replicate plates containing the medium with different pH levels (pH 4, 5, 6, 7 and 8). The pH differential plates with *Foc* isolates were incubated at 25°C in darkness, and the colony diameters were measured after a period of 5 days.

Nitrogen utilisation

For comparison of isolates based on their nitrogen source assimilation, 2 $g.l^{-1}$ of each of four nitrogen sources was added to the basal media described by Patterson & Bridge (1994). The following nitrogen sources were evaluated: ammonium dihydrogen phosphate ($NH_4H_2PO_4$) (Sigma-Aldrich), sodium nitrate ($NaNO_3$) (Merck), sodium nitrite ($NaNO_2$) (Saarchem) and urea (Sigma-Aldrich). These sources were all added to the nitrogen assimilation medium prior to autoclaving. The final pH of all media was adjusted to 5.5, except for sodium nitrite, where the pH was set at 7 (to reduce potential nitrite toxicity). Noble agar without any supplements served as control medium. All media were autoclaved and poured into 90-mm-diameter Petri dishes. Disks (5 mm in diameter) from starter WA cultures were placed onto five replicate plates of nitrogen-amended medium. Plates were incubated at 25°C in darkness for 5 days and colony diameter was measured.

Statistical analysis

Data from the *in vitro* assays (growth study and physiological studies) were analysed based on the General Linear Model Procedure (GLM) as given in SAS (SAS institute Inc., SAS/STAT Users Guide, Version 8, Cary, NC: SAS Institute Inc. 1999). Differences between isolates and physiological treatments were significant at $P \leq 0.05$. Disease severity was calculated using the formula of Sherwood & Hagedorn (1958): Disease severity (%) = $\sum$ (number of plants in disease scale category) x (specific disease scale category) / (total number of plants) x (max. disease scale category) X 100.

RESULTS

Growth studies

Growth rate

Differences in growth rate among isolates of *Foc* subtropical race 4 (VCG 0120) were substantial ($F_{25;\ 390}$ = 357.86; P < 0.0001; R^2 = 0.958) (Fig. 2). An average colony diameter of more than 50 mm was achieved by five isolates (CAV 001, CAV 015, CAV 041, CAV 086, CAV 129), and these isolates had a significantly faster growth rate than the other isolates, supported by 95% confidence intervals. All other isolates had colony diameters between 30 and 50 mm. CAV 145 had the slowest growth rate of all isolates

Cultural morphology

According to their colony morphology, the different *Foc* isolates were divided into three morphological types, namely sporodochial, cottony and slimy pionnotal (Table 4). The sporodochial type was the most dominant morphological type containing 15 isolates, while six isolates could be described as cottony and five as slimy pionnotal. Isolates that produced a pink colony colour and abundant aerial mycelia were placed into category A (CAV 002, 015, 016, 041, 092) (Fig. 3). One isolate produced a dark pink colony colour and scant aerial mycelia and fell into category B (CAV 001), and most isolates produced a near purple colony colour, with aerial mycelia scant but produced around the colony edges, and fell into category C (CAV 009, 021, 022, 037, 045, 050, 069, 072, 094, 099, 105, 106, 108, 111, 129, 130, 137, 145, 147).

Sporulation

All isolates of *Foc* produced microconidia in variable amounts, but not all isolates produced macroconidia and sporodochia (Table 5). Microconidia were produced abundantly by nine isolates, regularly by 14 isolates and sparsely by only three isolates (CAV 099, 129, 145). Isolates CAV 069, 106 and 145 produced both macroconidia and sporodochia abundantly (Table 5). A little more than half of the isolates produced sporodochia, with only three isolates producing them abundantly.

Pathogenicity studies

Pathogenicity tests revealed significant variation in virulence among the different *Foc* isolates evaluated (Table 6). All isolates caused disease symptoms on banana plantlets, except for isolate CAV 099. A few strains proved to be highly virulent, such as CAV 045, CAV 092 and CAV 105. In the first trial on the Williams cultivar, CAV 045 and CAV 105 both resulted in a disease severity of 80.4%, while CAV 092 showed a disease severity of 84%. In the second trial on Grand Nain plantlets, CAV 092 and CAV 105 both had the second highest disease severity of 92.4%, and CAV 045 had the highest disease severity of 96%. Although there were differences in disease severity between the two trials, the same isolates that were considered highly virulent in the first trial were most virulent in the second trial, and those that were less virulent were so in both trials.

Physiological studies

Temperature

Differences in growth rate of *Foc* isolates were found at the different incubation temperatures ($F_{125;872}$ = 2197; $P < 0.0001$; R^2 = 0.997). The temperature assay revealed that the optimum temperature was 25ºC for all isolates (Fig. 4). At temperatures of 5 and 40ºC, no growth was observed for any of the isolates evaluated, while very little growth was observed at 10ºC and 35ºC. These results were supported by 95% confidence intervals.

pH

Differences in growth rate among isolates of *Foc* were detected at different pH values ($F_{82;578}$ = 404.84; $P < 0.0001$; R^2 = 0.983) (Fig. 5). All isolates grew most rapidly at pH 6, except for isolate CAV 037, which grew significantly faster at pH 7. No growth was detected at pH 8 for most *Foc* isolates, with the exception of isolates CAV 001, CAV 037 and CAV 111. All isolates grew at pH 4, but the growth rate was significantly slower than at pH 5, 6 and 7.

Nitrogen assimilation

Growth rate of isolates differed when grown on different nitrogen sources ($F_{104;735}$ = 237.50; $P < 0.0001$; R^2 = 0.971). Results revealed that sodium nitrite ($NaNO_2$) and

sodium nitrate ($NaNO_3$) had a stimulating effect on the growth of the isolates when compared to the control medium (Fig. 6). Urea did not have any significant effect on the growth of the isolates when compared to the control medium. Ammonium dihidrogen phosphate ($NH_4H_2PO_4$) was the only nitrogen source that had an inhibitory effect on growth of the isolates.

DISCUSSION

Despite being genotypically uniform, the population structure of *Foc* 'subtropical' race 4 (VCG 0120) proved to be phenotypically diverse. The consequences of this finding are important, as it implicates that variation in an asexually reproducing fungal pathogen populations does occur, at least in terms of virulence and culture morphology. The ability of some isolates to grow at different temperature and pH regimes makes *Foc* a flexible pathogen, and might explain its ability to survive and infect Cavendish bananas under different environmental and production conditions.

It has been well documented that genetically distinct isolates of *Foc* vary in their ability to attack different banana varieties, which resulted in their subdivision into biological races (Stover & Waite 1960, Su *et al*. 1986, Moore *et al*. 1995). Even genetically uniform populations of *Foc*, such as VCG 0120, might differ in their means to cause disease to bananas, for instance when this pathogen challenges Cavendish bananas under tropical and subtropical environmental conditions (Ploetz *et al*. 1990, Viljoen 2002). Under the same environmental conditions, however, isolates belonging to VCG 0120 have generally been found consistent in causing, or not causing, disease to Cavendish bananas. Variation in the level of virulence among isolates under the same environmental conditions has not previously been documented. This study, therefore, provides the first evidence that certain isolates of *Foc* consistently caused more severe disease to Cavendish bananas than others under controlled environmental conditions. This might aid us in understanding the underlying mechanisms of resistance by comparative studies on, for example, the transcriptional genomics of such isolates. The single isolate that proved to be avirulent to banana in this study (CAV 099) is a good candidate for such an investigation, as this isolate belonged to the same VCG as the more pathogenic ones

(Visser 2003). As most isolates have been obtained using the same procedures, we believe that it is unlikely that the loss in virulence is due to mutation on rich cultural medium, an event that has commonly been reported for *Fusarium* spp. in the past (Nelson *et al*. 1983).

Isolates of *Foc* 'subtropical' race 4 (VCG 0120) that grew more rapidly on artificial medium, such as CAV 001, 111 and 137, did not necessarily cause the highest disease severity in Cavendish banana plantlets in this study. Others that grew slower, such as CAV 147, resulted in a much higher disease incidence. Similarly, isolates that produced an abundance of microconidia in culture, such as CAV 001 and 002, did not result in more disease than the less abundant microconidium producers, such as CAV 129 and 145. Variation in fungal virulence to Cavendish bananas, therefore, appears not to be a function of growth rate and/or sporulation, as one would generally expect from a wilt pathogen. The reason for this is difficult to explain, other than that *Foc* performs differently in culture and within the plant. A possibility to consider is that factors other than the obstruction of sieve cells in the xylem are responsible for disease development in plants. These factors might include the production of toxins and the suppression of plant defence responses.

Studies by Beckman *et al*. (1960, 1962) have shown that temperature is important in the progress of *Foc* invasion and symptom development in banana. Maximum distribution of the pathogen in the vascular system and subsequent chlorosis of Gros Michel bananas occurred at a soil temperature of 26ºC, with a decrease at 22 and 30ºC. This, however, was not the case when Cavendish bananas were infected by *Foc* race 4 in the subtropics (Ploetz *et al*. 1990). In previous field studies it was observed that symptoms became most obvious in spring and early summer (Viljoen 2002). This suggests that infection, or at least establishment of the pathogen in the plant, takes place during winter when temperatures drop to below 15ºC in the subtropics. Symptom development, however becomes apparent only after winter, probably because of the greater distribution of the pathogen in the xylem vessels as a result of the higher transpiration tempo. The optimum cultural growth temperature for *Foc* in this study was 25ºC, similar to that reported for the root rot pathogen of bean *F. oxysporum* f.sp. *fabae* (Ivanovic *et al*. 1987) and the wilt pathogen of spinach *F. oxysporum* f.sp. *spinaciae* (Naiki & Morita 1983), with significantly inhibited growth

taking place below 15ºC and above 30ºC. These results, therefore, support Brake *et al*.'s (1995) views that the increased disease incidence in the subtropics is the result of the banana plant becoming more vulnerable to infection under lower winter temperatures, and not because the pathogen is more aggressive. Beckman *et al*.'s (1960, 1962) work, showing that continuity of occlusion was tenuous at 21ºC and absent at 27º, might explain why infection of Cavendish bananas by *Foc* 'subtropical' race 4 can progress in the roots during winter. However, infection is stopped during summer when temperatures rise to 34ºC, and when rapid and continuous occlusion might provide a high degree of resistance to fungal passage, similar as that reported in Gros Michel roots (Beckman *et al*. 1960, 1961).

Fusarium wilt is considered to be a disease associated with acidic sandy soils rather than heavier soils with higher pH values (Woltz & Jones 1981). In studies on *F. oxysporum* f.sp. *ciceris* (Sugha *et al*. 1994) and *F. oxysporum* f.sp. *dianthi* (Duskova & Prokinova 1989) it was found that a higher soil pH lead to a reduction of the disease. It was suggested that the lower disease incidence associated with a higher pH was due to its effect on the availability of micronutrients that are essential for the growth, sporulation and virulence of Fusarium wilt pathogens (Jones *et al*. 1989). Woltz & Jones (1981) suggested raising soil pH toward or slightly above neutrality to be a common denominator in cultural control of Fusarium wilt diseases. When hydrated lime [$Ca(OH)_2$] and ground limestone ($CaCO_3$) was added to the soil, the soil pH increased to levels that inhibited Fusarium wilt of tomato (Jones & Woltz 1967, Jones & Woltz 1969, Jones & Woltz 1970). This inhibition was most likely due to the decreased availability of micronutrients created by the increased pH of the soil solution (Jones & Woltz 1967, Jones & Woltz 1969).

The gap between *in vitro* demonstration and *in sito* action was brought to our attention in the pH assay during this study. Our results showed that optimal growth of *Foc in vitro* is at pH 6, similar to that reported for *F. culmorum* (W.G. Smith) Saccardo, *F. graminearum* Schwabe and *F. oxysporum* by Srobar (1978), and sometimes at pH 7. Growth was significantly slower at a pH of 4. Most studies on Fusarium wilt diseases, however, indicated that acid soil (pH 4.2) supported growth of *Fusarium* through the soil, whereas a pH near neutrality prevented this growth (Wilson 1946). Surprisingly, a study by Peng *et al*. (1999) reported that the greatest disease severity of Fusarium

wilt of banana occurred at pH 8 when chlamydospore germination was greatest. No growth of *Foc* took place in culture at pH 8 in this study. Peng and his co-workers (1999) further argued that acidic conditions reduced chlamydospore germination and disease severity, and that disease severity remained high under very alkaline conditions (pH 10). This was contradicted by Chuang (1991) who reported that germination of chlamydospores of *Foc* in soil was inversely correlated with soil pH from 4 to 8, but that the pathogen survived longer in very alkaline soil (pH 8, 9 and 10) than in very acidic soil (pH 2, 3 and 4). Stover (1962) also listed several investigations in Central America that stated that acidic soils increase Fusarium wilt of banana, and that alkaline soils decreased the disease incidence.

Previous studies demonstrated that the form of nitrogen in the soil is important in the development of Fusarium wilt diseases. When fields are fertilised with NO_3^-N, disease incidence is generally reduced when compared to fields fertilised with NH_4^+-N (Byther 1965). Results from this study, however, indicated that *Foc* grew significantly better on the NO_3^-N medium *in vitro* than on a NH_4^+-N medium. Comparison of *in vitro* with field data is apparently of little value in this instance, as our results on growth rate and disease incidence already indicated. Also, the complex ecology involving soil pH, fertiliser regimes and microbial composition can all contribute to disease progression. Nitrate and ammonium fertiliser apparently influence pH effects, with nitrate causing an increase and ammonium a reduction in soil pH (Woltz & Engelhard 1973). This might explain why disease incidence in the field is reduced when using a nitrate-based fertilizer instead of an ammonium-based fertiliser. The pH of the nitrite medium in this study was adjusted to 7 to prevent any toxicity effect (Patterson & Bridge 1994) when comparing growth of *Foc* on NO_2-N and NO_3-N –amended media. The effect of potential nitrite toxicity to *Foc* at other pH regimes has not been investigated, as such an exploration would fall outside the objectives of this study.

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Table1. Isolates of *Fusarium oxysporum* f.sp. *cubense* used for comparison of selective phenotypic characteristics.

Isolate number	Location	Cultivar	Phenotypic character studied				
			Growth study	Pathogenicity	Temperature	Nitrogen	pH
CAV 001	Port Edward	Williams	x	x	x	x	x
CAV 002	Port Edward	Williams	x	x	x	x	x
CAV 009	Ramsgate	Williams	x	x	x	x	x
CAV 015	Port Edward	Williams	x				
CAV 016	Marina Beach	Williams	x	x	x	x	x
CAV 021	Port Edward	Williams	x	x	x	x	x
CAV 022	Port Edward	Williams	x	x	x	x	x
CAV 037	Port Edward	Williams	x	x	x	x	x
CAV 041	Port Edward	Williams	x				
CAV 045	Port Edward	Williams	x	x	x	x	x
CAV 050	Burgershall	Chinese cavendish	x	x	x	x	x
CAV 069	Kiepersol	Williams	x				
CAV 072	Emmett	IGN	x	x	x	x	x
CAV 086	Kiepersol	IGN	x				
CAV 092	Kiepersol	IGN	x	x	x	x	x
CAV 094	Kiepersol	Williams	x				
CAV 099	Burgershall	Grand Nain	x	x	x	x	x
CAV 105	Kiepersol	Duran/ Cavendish	x	x	x	x	x
CAV 106	Kiepersol	Williams	x	x			
CAV 108	Burgershall	Israeli Grand Nain	x	x	x	x	x
CAV 111	Kiepersol	Chinese cavendish	x	x	x	x	x
CAV 129	Port Edward	Williams	x	x	x	x	x
CAV 130	Ramsgate	Williams	x	x	x	x	x
CAV 137	Munster	Williams	x	x	x	x	x
CAV 145	Tzaneen	IGN	x	x	x	x	x
CAV 147	Tzaneen	IGN	x	x	x	x	x

Table 2. Cultural growth types as defined for *Fusarium oxysporum* f.sp. *cubense* by Waite & Stover (1960).

Morphological type	***Description***
Sporodochial	Sporodochia are present, erumpent or submerged. Pionnotes present, aerial mycelium abundant and floccose. Colony colour variable from near white to pink or purple. Sclerotia present in variable amounts, some clones stable, others unstable, mutating to ropy and slimy pionnotal.
Cottony	Sporodochia absent. Pionnote present or absent. Aerial mycelium very abundant, fine and cottony. Colony colour white or pale pink sclerotia absent. Mutability stable.
Ropy	Sporodochia absent. Pionnotes present. Aerial mycelium very abundant; coarse, ropy, and often raised in tuffs. Often highly pigmented. Sclerotia absent. Usually highly unstable, mutating to slimy pionnotal.
Slimy pionnotal	Sporodochia absent. Pionnotes present and abundant. Aerial mycelium scant and appressed occur around the edges. Often highly pigmented. Sclerotia absent. Mutability stable.

Table 3. Disease severity rating scale used to record internal symptoms caused by *Fusarium oxysporum* f.sp. *cubense* in banana plants (Carlier *et al*. 2002).

Disease severity	**Disease symptoms**
0	Corm completely clean, no vascular discoloration.
1	Isolated points of discoloration in vascular tissue.
2	Discoloration of up to 1/3 of vascular tissue.
3	Discoloration of between 1/3 and 2/3 of vascular tissue.
4	Discoloration greater than 2/3 of vascular tissue.
5	Total discoloration of vascular tissue.

Table 4. Morphological types present in a South African population of *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120).

Isolate	**Sporodochial**	**Cottony**	**Slimy pionnotal**
CAV 001			X
CAV 002		X	
CAV 009	X		
CAV 015		X	
CAV 016		X	
CAV 021	X		
CAV 022	X		
CAV 037		X	
CAV 041		X	
CAV 045	X		
CAV 050	X		
CAV 069	X		
CAV 072			X
CAV 086		X	
CAV 092	X		
CAV 094			X
CAV 099	X		
CAV 105	X		
CAV 106	X		
CAV 108	X		
CAV 111			X
CAV 129			X
CAV 130	X		
CAV 137	X		
CAV 145	X		
CAV 147	X		

Table 5. The abundance of microconidia, macroconidia and sporodochia in 26 South African isolates of *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120). 0 indicates the absence of spores, 1 indicates the sparse (1-20 spores), 2 the regular (21-50 spores), and 3 the abundant (>50 spores) formation of spores.

Isolate	Microconidia	Macroconidia	Sporodochia
CAV 001	3	0	0
CAV 002	3	0	0
CAV 009	2	2	2
CAV 015	2	0	0
CAV 016	3	2	2
CAV 021	2	2	2
CAV 022	2	1	1
CAV 037	3	0	0
CAV 041	2	0	0
CAV 045	2	1	1
CAV 050	2	2	2
CAV 069	3	3	3
CAV 072	2	0	0
CAV 086	3	0	0
CAV 092	2	2	2
CAV 094	2	0	0
CAV 099	1	1	1
CAV 105	2	2	2
CAV 106	2	3	3
CAV 108	3	2	2
CAV 111	3	0	0
CAV 129	1	0	0
CAV 130	2	1	1
CAV 137	3	3	3
CAV 145	1	0	0
CAV 147	2	1	1

Table 6. Disease severity* in banana plants inoculated with different isolates of *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120).

	Disease severity (%)*	
Isolate	**Williams cv.**	**Grand Nain cv.**
CAV 001	8.4	3.6
CAV 002	8.4	12.0
CAV 009	16.8	3.6
CAV 016	24.0	12.0
CAV 021	32.4	20.4
CAV 022	24.0	39.6
CAV 037	32.4	43.2
CAV 045	80.4	96.0
CAV 050	24.0	32.4
CAV 072	8.4	8.4
CAV 092	84.0	92.4
CAV 099	0.0	0.0
CAV 105	80.4	92.4
CAV 106	20.4	16.8
CAV 108	20.4	20.4
CAV 111	15.6	3.6
CAV 129	31.2	44.4
CAV 130	16.8	8.4
CAV 137	15.6	20.4
CAV 145	48.0	31.2
CAV 147	56.4	39.6
Control	0.0	0.0

*Disease severity (%) = $\sum$ (number of plants in disease scale category) x (specific disease scale category) / (total number of plants) x (max. disease scale category) x 100 (Sherwood & Hagedorn 1958).

Figure 1. Disease-free banana plantlet, planted in a 250 ml cup, containing water and hydroponic mix.

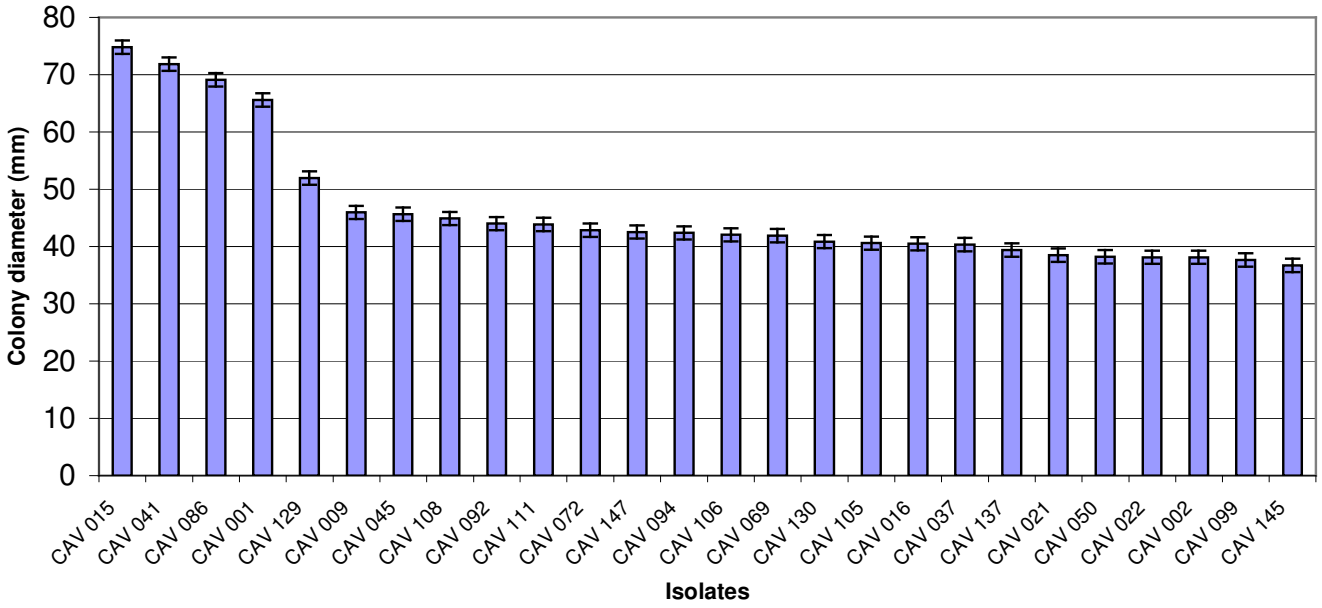

Figure 2. Growth rate of *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120) isolates from South Africa measured after incubation on potato dextrose agar at 25ºC for 5 days. Least Squares means (LS mean) was calculated taking into account the colony diameter in all isolates. Range bars indicate the 95% confidence interval for the different isolates.

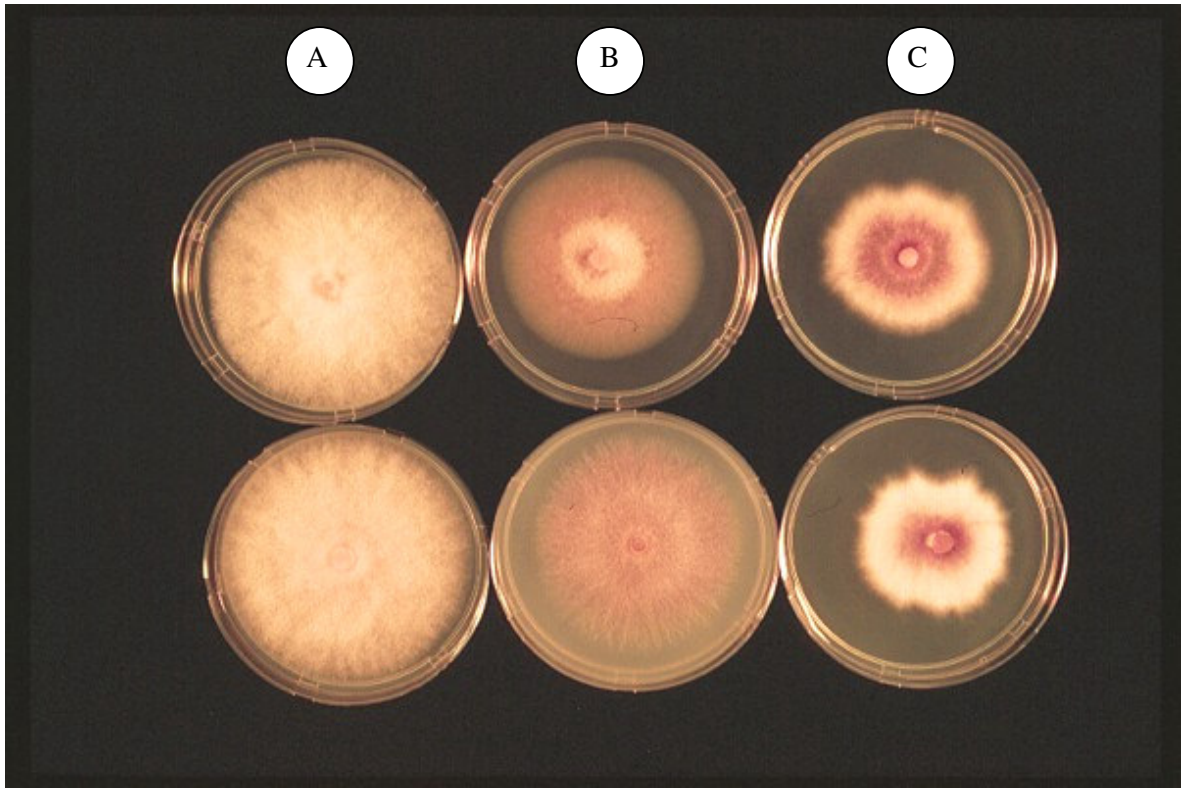

Figure 3. Cultural appearance of three isolates of *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120) on Potato Dextrose Agar. Plates A illustrate cultures that produced pink colonies with abundant aerial mycelia, plates B dark pink colonies with scant aerial mycelia, and plates C cultures with a near purple colony colour.

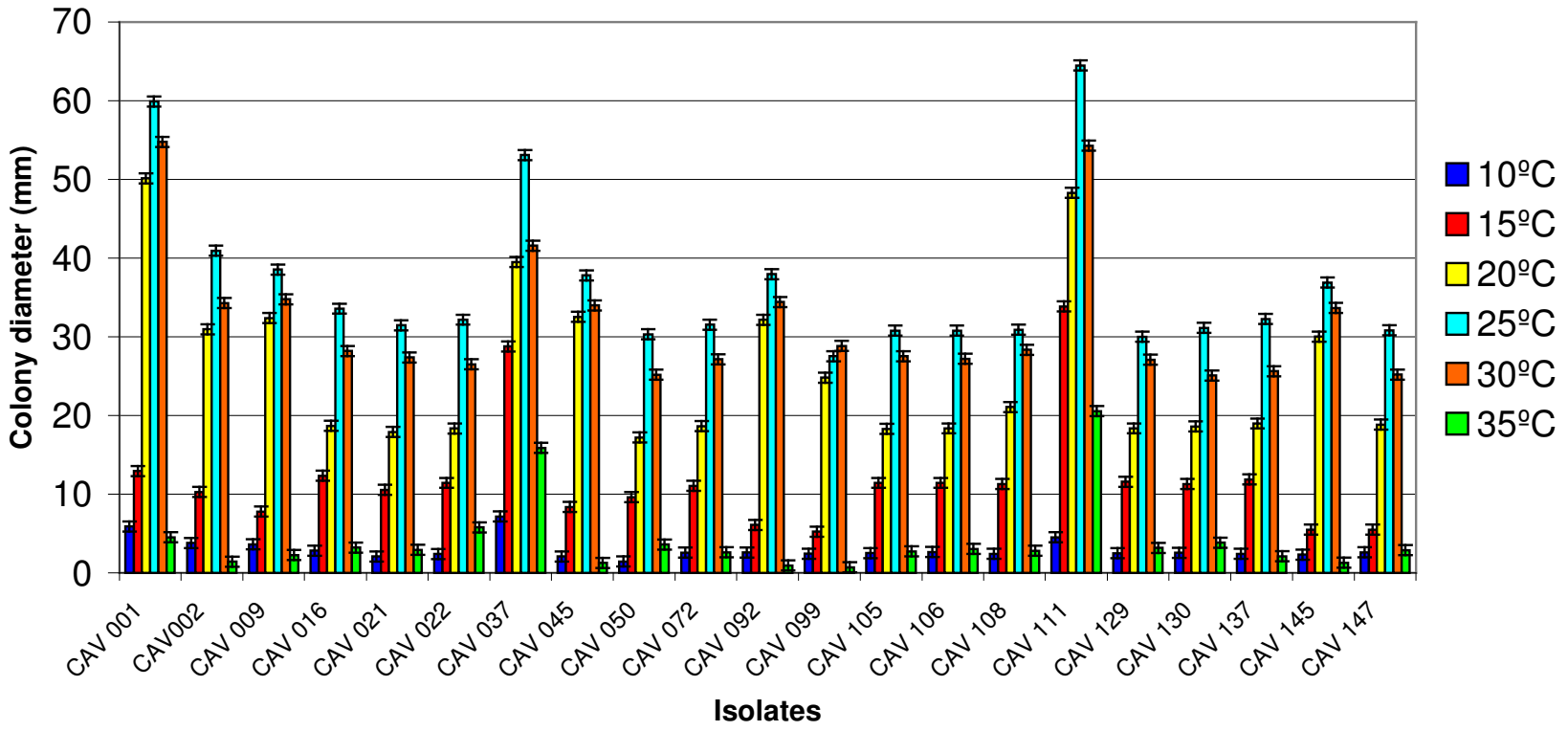

Figure 4. Growth rate of the different *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120) isolates at six different temperatures measured after 5 days. Least Square means was calculated, taking into account the colony diameter of all isolates per temperature. Range bars indicate a 95% confidence interval for each isolate at different temperatures.

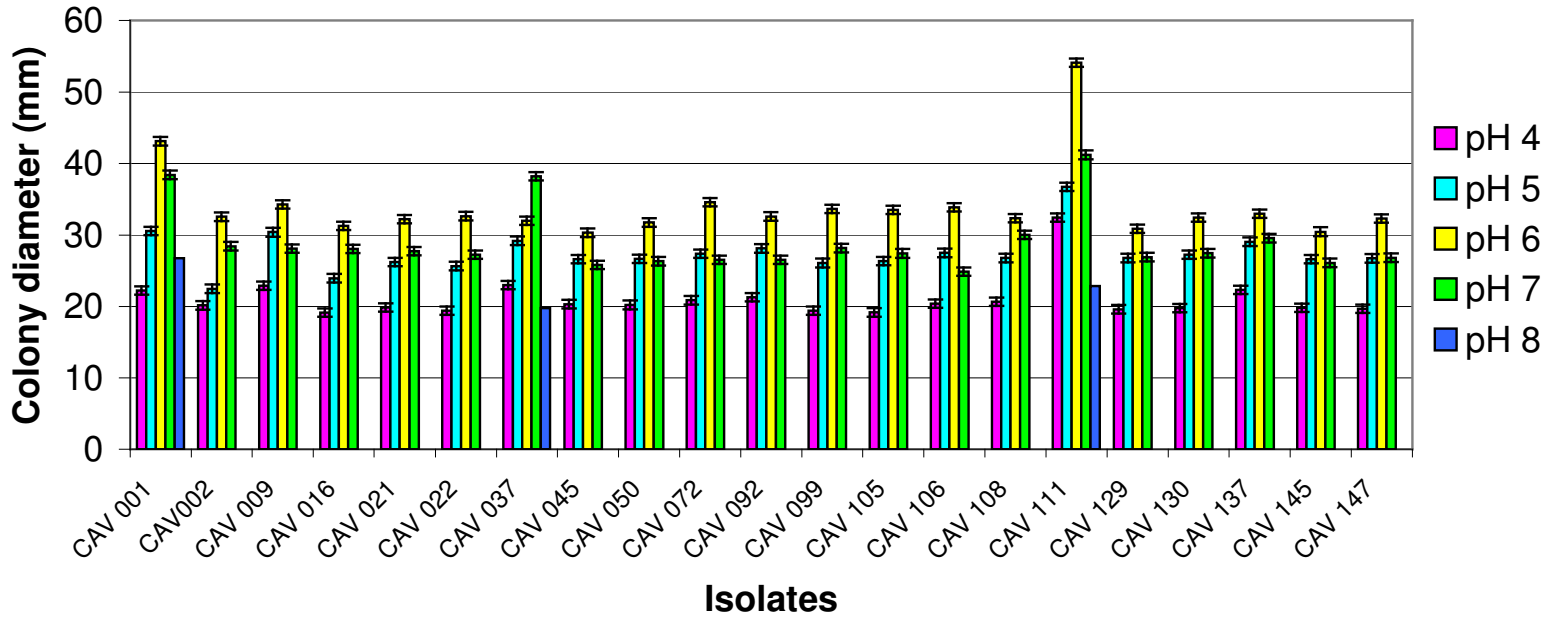

Figure 5. Growth rate of different *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120) isolates on media with different pH measured after 5 days. Least Square means was calculated taking in account the colony diameter for all isolates on different pH media. Range bars indicate the 95% confidence interval for isolates at different pH.

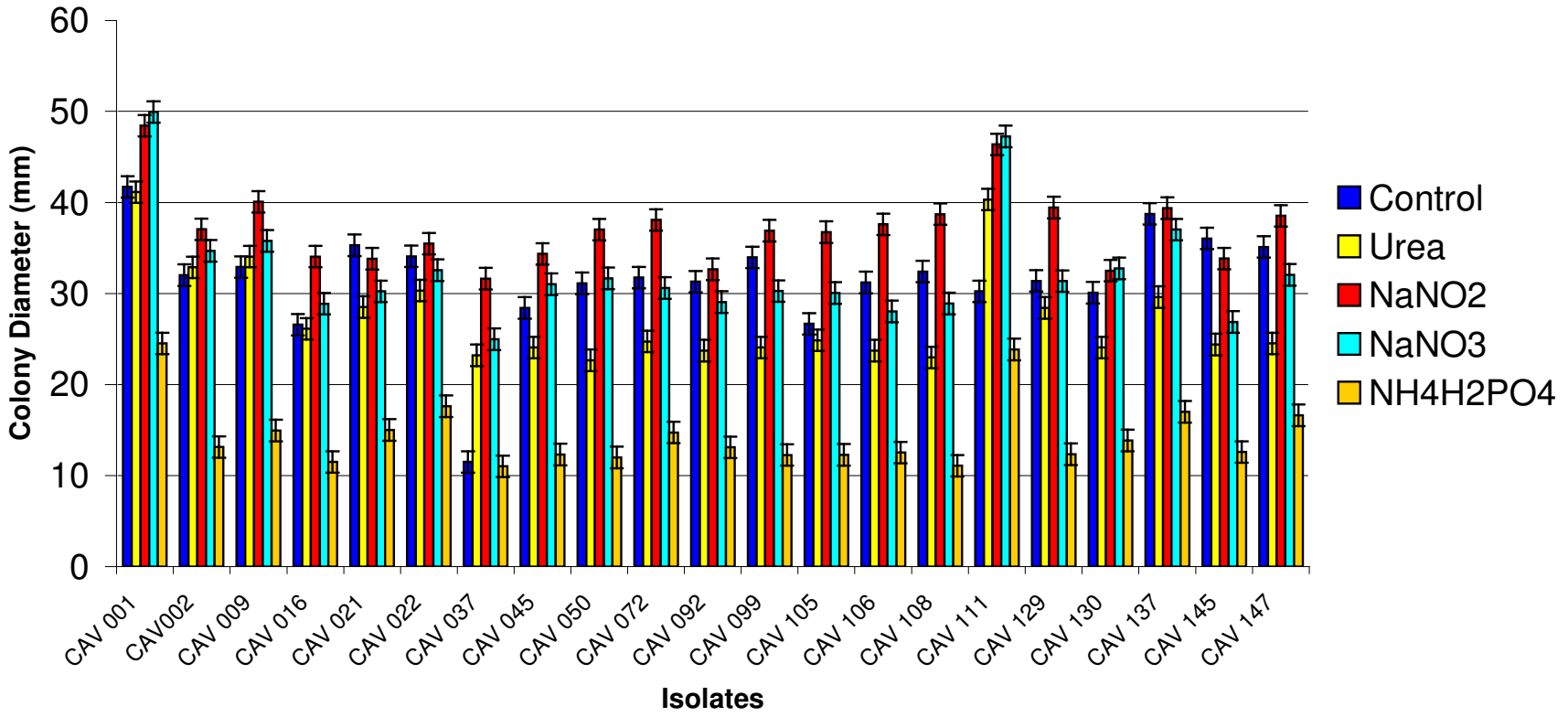

Figure 6. Growth rate of *Fusarium oxysporum* f.sp. *cubense* 'subtropical' race 4 (VCG 0120) isolates on different nitrogen source media, measured after 5 days. Least Square means was calculated taking in account the colony diameter for all isolates per nitrogen source. Range bars indicate the 95% confidence interval for each isolate supplemented with the different nitrogen source media.

CHAPTER 3

The application of high-throughput Amplified Fragment Length Polymorphisms in assessing genetic diversity in *Fusarium oxysporum* f.sp. *cubense*

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ABSTRACT

Fusarium oxysporum f.sp. *cubense* (*Foc*) is responsible for Fusarium wilt of bananas. The pathogen consists of several variants that are divided into three races and 21 VCGs. Several DNA-based techniques have previously been used to analyse the worldwide population of *Foc*, sometimes yielding results that were not always consistent. In this study, the high-resolution genotyping method of amplified fragment length polymorphism (AFLP) is introduced as a potentially effective molecular tool to investigate diversity in *Foc* at a genome-wide level. The population selected for this study included *Foc* isolates representing different vegetative compatibility groups and races, isolates of *F. oxysporum* f.sp. *dianthi*, a putatively non-pathogenic biological control strain *F. oxysporum* (Fo47), and *F. circinatum*. High-throughput AFLP analysis was attained using five different infrared dye-labelled primer combinations using a two-dye model 4200s LI-COR automated DNA analyser. An average of approximately 100 polymorphic loci were scored for each primer-pair using the SAGA^{MX} automated AFLP analysis software. Data generated from five primer-pair combinations were combined and subjected to Distance Analysis, which included the use of Neighbour-joining and a bootstrap of 1000 replicates. A tree inferred from AFLP distance analysis revealed the polyphyletic nature of the *Foc* isolates, and seven genotypic groups could be identified. The results indicate that AFLP is a powerful tool to perform detailed analysis of genetic diversity in the banana pathogen, *Foc*.

INTRODUCTION

Fusarium oxysporum Schlecth f.sp. *cubense* (EF Smith) Snyd. and Hans (*Foc*) is the soil-borne fungus responsible for a lethal disease in banana (*Musa* spp.) known as Fusarium wilt, also referred to as Panama disease (Brandes 1919, Stover 1962). Pathogenic variability within *Foc* has led to its subdivision into races based on ability to cause disease to certain cultivars in the field. Three races (1, 2 and 4) of the pathogen affect banana. Race 1 is pathogenic to Gros Michel, while race 2 affects Bluggoe and other closely related cooking bananas (Waite & Stover 1960, Moore *et al*. 1995). Race 4 causes disease in Cavendish cultivars as well as in those that are susceptible to race 1 and race 2 (Su *et al*. 1986). Both 'tropical' and 'subtropical' strains of race 4 have been recognised. *Foc* 'subtropical' race 4 attacks Cavendish bananas in countries such as South Africa, Australia, Taiwan and the Canary Islands (Su *et al.* 1986, Brake *et al*. 1990, Ploetz *et al*. 1990, Gerlach *et al*. 2000). 'Tropical' race 4, on the other hand, affects Cavendish bananas in the tropical regions of Southeast Asia and Australia (Pegg *et al*. 1993, 1994, Ploetz 1994, Bentley *et al*. 1998). Races of *Foc* have not been well defined (Ploetz 1994) and should not be confused with races in pathosystems for which host genes for resistance and susceptibility are known (Stover & Buddenhagen 1986).

In Fusarium wilt of banana, disease development relies heavily on the interaction between pathogen and plant genotypes, and appears to be strongly influenced by environmental conditions (Moore *et al*. 1993). This has previously been demonstrated by research inconsistencies which showed that *Foc* VCG 0120 caused disease to Cavendish bananas in the subtropics, but not in tropical regions (Su *et al*. 1986, Stover & Simmonds 1987). Since VCG 0120 causes disease to Cavendish bananas in the subtropics it would be identified as race 4, but because the same fungal phenotype does not cause disease to Cavendish bananas in the tropics, it might be considered as race 1. Another problem arose because 'tropical' and 'subtropical' isolates of race 4 proved to be genotypically different (Bentley *et al*. 1995, Bentley *et al*. 1998). Another reason for such inconsistencies is that *Foc* isolates were grouped into races that were determined by their pathogenicity to a limited number of banana differentials. In other *formae speciales* of *F. oxysporum* there is a defined genetic basis for race designation, meaning the cultivars contain certain resistance genes that

match specific avirulence genes in the pathogen (Bosland & Williams 1986, Ori *et al*. 1997).

Individual isolates of *Foc* within races can fuse asexually to form a stable heterokaryon (Puhalla 1985). According to Ploetz & Correll (1988) this trait is genetically controlled, and such individuals are said to belong to the same vegetative compatibility group (VCG). At least 21 VCGs have been identified worldwide within *Foc* (Ploetz 1990). Fifteen VCGs have been found in Asia, the centre of origin of bananas, where the pathogen is thought to have evolved (Pegg *et al*. 1994, Pegg *et al*. 1996). Only a few VCGs are found in Africa and the Americas (Ploetz 1993). After extensive sampling and comparison to Australian and Asian populations, it has been determined that only VCG 0120 is present in South Africa (Visser 2003). While VCGs provide a useful means of subdividing *Foc* into genetically isolated groups, they can be misleading in terms of true genetic relatedness among groups of isolates (Bentley *et al*. 1995).

A thorough understanding of the population diversity in *F. oxysporum* and the molecular events underlying the diversification process is essential for the development of a disease management strategy (Kistler 2001). Measuring diversity in a clonally reproducing fungus is complicated and requires a combination of phenotypic and genotypic tools. Several studies on the phenotypic diversity of the pathogen have been conducted. Cultural characteristics and volatile production can give some indication of physiological characteristics of *Foc* isolates, but these give no indication of genetic relatedness or diversity within or between groups of isolates (Brandes 1919, Stover 1959, Waite & Stover 1960).

Many types of molecular markers have been used to characterize genetic diversity in fungi (Anderson & Kohn 1995, Milgroom 1996). Previous studies conducted on worldwide populations of *Foc* included PCR methods such as randomly amplified polymorphic DNA analysis (RAPDs) (Bentley *et al*. 1995), DNA amplification fingerprinting (DAF) (Bentley *et al*. 1998, Gerlach *et al*. 2000), restriction fragment length polymorphisms (RFLPs) (Koenig *et al*. 1997) and DNA sequence analysis (O' Donnell *et al*. 1998). While these techniques were able to provide us with knowledge

related to the genetic diversity in *Foc*, they did not always agree in terms of genetic relationships among clonal lineages of this asexually reproducing pathogen.

Amplified fragment length polymorphism (AFLP) is a PCR-based DNA analysis technique that can detect variations in RFLPs on a genome-wide basis (Vos *et al*. 1995). Like RFLP analysis, AFLPs can detect size differences in restriction fragments caused by DNA insertions, deletions or changes in target restriction site sequences, but with less labour required. AFLPs have been increasingly used in analysis of fungal population structure (Majer *et al*. 1996, Gonzalez *et al.* 1998, DeScenzo *et al*. 1999, Purwantara *et al*. 2000, Zeller *et al*. 2000). The complex DNA fingerprinting patterns produced by the AFLP technique are reproducible and subsets of these data appear to show higher correlations to one another than is observed among many sets of RFLP or RAPD data (Spooner *et al*. 1996, Gonzalez *et al*. 1998). The utility, repeatability and efficiency of the AFLP technique lead to the broader application of this technique to analyse *Fusarium* populations (Baayen *et al*. 2000, Abd-Elsalam *et al*. 2002 a,b, Kiprop *et al*. 2002, Sivaramakrisnan *et al*. 2002, Abdel-Satar *et al*. 2003, Leslie *et al*. 2005). Therefore, the AFLP technique was selected to study relationships within and between natural populations of *Foc* isolates at the genome-wide level. A second objective was to investigate whether AFLPs could discriminate between VCGs of *Foc*. This would ensure that molecular techniques could be employed for the rapid identification of the fungus and determination of the VCG of this important banana pathogen.

MATERIALS AND METHODS

Fungal isolates

Isolates of *Foc* (35), *F. oxysporum* f.sp. *lycopersici* (*Fol*) (2), *F. oxysporum* f.sp. *melonis* (*Fom*) (2), one of *F. oxysporum* f.sp. *dianthi* (*Fod*), one non-pathogenic isolate *F. oxysporum* (Fo47) and one *F. circinatum* Nirenberg & O'Donnell were included in this study (Table 1). The isolates of *Foc* represent races 1 and 4, as well as 16 different VCG groups. These included six of the nine clonal lineages from DAF data described by Bentley *et al*. (1998). All fungal isolates used in this study are

maintained in the culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA isolation from fungal mycelia

Fresh fungal mycelia grown on half strength Potato Dextrose Agar (PDA) (Biolab Diagnostics, Wadeville, South Africa) containing Novobiocin (0.02 $g.l^{-1}$) (Sigma-Aldrich, Steinheim, Germany) were placed in 1.5 ml Eppendorf tubes. Three hundred µl DNA extraction buffer (200 mM Tris-HCl, pH 8; 150 mM NaCl; 25 mM EDTA, pH 8; 0.5% SDS) (Reader & Broda 1985) was added to each sample. The mycelium was homogenized in the buffer using a pestle. Tubes containing the samples were then frozen in liquid nitrogen, followed by 5 min incubation in boiling water. Phenol-chloroform (1:1) (Saarchem, Unilab, Midrand, South Africa) extractions were performed and the phases were separated by centrifugation at 14 000 rpm (r_{av} 16 cm) for 7 min at 4 ºC. The DNA was precipitated by adding 0.1 volume of 3 M NaAc (pH 5.5) (Saarchem) and two volumes of ice-cold absolute ethanol (Merck chemicals, Wadeville, South Africa) and inverting the tube five times, followed by centrifugation for 10 min at maximum speed (14 000 rpm) at 4 ºC. The precipitated DNA pellet was washed with 70% ethanol and centrifuged for 5 min at 5000 rpm at 4 ºC, after which the ethanol was discarded (Sambrook *et al*. 1989). The pellet was then dried in a vacuum centrifuge and resuspended in 200 µl sterile Sabax water (Adcock Ingram, Bryanston, South Africa). DNA was visualised on a 1% agarose gel (Roche Molecular Biochemicals, Manheim, Germany) after staining with 1.6 $\mu g.ml^{-1}$ ethidium bromide (EtBr) (Sigma). The DNA concentrations were determined using a spectrophotometer (Eppendorf bioPhotometer, Hamburg, Germany), and diluted to yield a concentration of 20 $ng.\mu l^{-1}$.

AFLP procedure

The AFLP procedure was carried out as reported by Vos *et al*. (1995) with a few modifications. Genomic DNA from all isolates was digested using 2 U *EcoR*I and 2 U *Mse*I endonucleases (Table 2) at 37°C for 2 hrs. It was subsequently ligated with corresponding site-specific adapters, namely 10 ρmol *Mse*I adapter and 1 ρmol *EcoR*I

adapter (Table 2) at 37°C for 3 hrs, using 1 U T4 DNA ligase (Roche Molecular Biochemicals). Pre-selective amplification of restriction fragments followed, using standard *EcoR*I and *Mse*I adapter primers (Table 2) complementary to the restriction sites and the adapter sequences containing no selective nucleotides. Pre-selective amplification and selective amplification conditions were as described by Vos *et al*. (1995). Amplification products were electrophoresed on a 1.2% agarose gel. The pre-amplification products were diluted (1:20) with low TE buffer (10 mM Tris-HCL, pH 8.0; 0.1 mM EDTA).

Selective amplification was achieved using standard *EcoR*I and *Mse*I adapter primers containing two additional nucleotides (Table 2). The *EcoR*I primers were 5' end labelled with infrared dye IRDye™ 700 or 800 (LI-COR, Lincoln, NE, USA). The primer combinations (Table 2) were selected from previous primer screening that were used for linkage mapping in *Gibberella moniliformis* Wineland (*F. verticillioides* (Sacc.) Nirenberg) (Jurgenson, Zeller & Leslie, 2002). All amplification steps were performed in a mastercycler (Eppendorf).

Electrophoresis and detection of AFLP fragments

Electrophoresis and detection of AFLP fragments were performed as described by Myburg *et al*. (2001), except for making use of 64 well combs (LI-COR) (0.25 mm spacer thickness) for gel loading.

Scoring of AFLP images

Digital AFLP images were scored using the SAGA-MX™ AFLP® analysis software program (Keygene, Wageningen, The Netherlands). AFLP fragments in the range of 50-750 bp were considered for analysis. Polymorphic regions were scored in order to determine the diversity among the isolates. An average of approximately 100 polymorphic loci were assayed simultaneously with each primer pair. If a band was present it was indicated by a "+" and if absent by means of a "-", while missing data was indicated with "F". In order to make grayscale values comparable from lane to lane, SAGA software begins by normalizing lanes using their grayscale value

distribution. The band scoring and detection parameters in SAGA were set on default (SAGA-MX™ AFLP® analysis software user manual).

AFLP Analysis

Five primer combinations were selected based on a high number of polymorphisms. Data files were generated by the SAGA-MX AFLP analysis software, based on presence or absence of loci. For analysis, data of the five primer combinations was combined. Data files were subjected to Distance analysis using the Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10 (Swofford 1999), which included Neighbour-joining as a tree building algorithm and a bootstrap of a 1000 replicates (Felsenstein 1985) for confidence support.

RESULTS

Genomic DNA of a high quality and with concentrations ranging from 100-500 ng/µl was obtained for all *Fusarium* isolates. After pre-selective amplification, all the products produced a smear of between 300 and 1200 bp. Through selective amplification, AFLP fingerprints of high standard were achieved for each of the five primer combinations (Fig. 1). While the isolates representing VCG 0120 proved to be similar, an obvious dissimilarity among the different VCGs of *Foc* was found (Fig. 1). *Fom*, *Fol* and *Fod* had different banding patterns when compared to *Foc*. The fingerprint of *F. circinatum* could easily be distinguished from that of *F. oxysporum*, with several unique bands.

AFLP fingerprinting of the five different primer combinations yielded a total of 499 polymorphic bands, ranging from 50-750 bp. This gave an average of 100 (99.8) polymorphic bands per primer combination. Polymorphic bands were found to exist between AFLP fingerprints produced by the various VCGs of *Foc* and other *formae speciales* of *F. oxysporum* (Fig. 1). These polymorphisms proved to be phylogenetically informative (Fig. 2). Seventy-five unique bands were observed for all the isolates examined (Table 3), but not all isolates had unique bands. *Fusarium oxysporum* of other *formae speciales* (*Fom*, *Fol* and *Fod*) gave unique bands in at least two of the primer combinations. *Fusarium circinatum* produced the most unique

bands for each primer combination, and primer combination E24 and M22c yielded the largest number of unique bands (Table 3).

Distance analysis of AFLP data clearly divided isolates of *Foc* into two major clades. The distinctiveness of these two clades was strongly supported by a 100% bootstrap (Fig. 2). Clade 1 contained VCGs 0120, 0120/15, 0121, 0122, 0126, 0129, 01213, 01213/16, 01216 and 01219. Clade 2 contained VCGs 0123, 0124, 0125, 01217 and 01218. The first clade included isolates that were collected from around the world including the South African isolates, while the second clade only contained isolates from Australasia. The *Foc* isolates from the second clade grouped closer to *F. oxysporum* of other *formae speciales* (*Fom*, *Fol* and *Fod*) than to *Foc* isolates of the first clade.

According to AFLP analysis, *Foc* isolates were divided into seven genotypic groups (Fig. 2). The first genotypic group contained VCG 0120 and VCG complex 0120/15. Genotypic group 2 included VCGs 0126, 0129, 0122, and 01219, and the third genotypic group included the 'tropical' race 4 isolates VCGs 01213, 01216 and 01213/01216. VCG 0121 was included in genotypic group 4, and VCGs 0123 and 01217 in genotypic group 5. VCG 01218 represented the sixth genotypic group, while Group 7 included VCGs 0124 and 0125.

DISCUSSION

AFLP analysis proved valuable in studying genetic diversity in a worldwide collection of *Foc*. The separation of *Foc* into two major clades by AFLP analysis is consistent with findings obtained when using various other DNA fingerprinting techniques (Boehm *et al*. 1994, Bentley *et al*. 1995, Koenig *et al*. 1997, Bentley *et al*. 1998, O' Donnell *et al*. 1998). However, better resolution within clades was obtained with AFLP analysis. For instance, DAF analysis grouped VCG 0129 with isolates belonging to VCGs 0120 and 0120/15 in DNA fingerprinting group (DFG) (Bentley *et al*. 1998). AFLP analysis, however, has been able to separate VCG 0129 from the group containing VCG 0120, and grouped it in DFG 2 with VCGs 0122, 0126 and 01219. This separation is to be expected since it is unlikely that VCG 0129, limited to

banana varieties in Queensland in Australia only, would fit closely with the genotypically highly uniform DFG 1 containing VCG 0120.

Isolates within each VCG generally produced similar banding patterns and were, therefore, closely related, independent of geographical origin or host source. VCG 0120 isolates are homogenous despite their geographical origins (Asia, Australia, South Africa, Central and South America) and different host cultivars (Cavendish, Highgate and Lady finger banana cultivars). This is important in terms of future disease management programmes. Firstly, we know that *Foc* clones are very stable, and that isolates of *Foc* do not easily mutate sufficiently to overcome plant resistance. This was proven by the management of *Foc* race 1 in Central America through the planting of resistant Cavendish cultivars (Stover 1962). Secondly, plant material should not be transported to regions where the plant could be susceptible to other VCGs or races of the pathogen. For example when Cavendish bananas, apparently resistant to VCG 0120 in the tropics is transported to the subtropics, the plant will succumb to the same VCG (Ploetz *et al*. 1990).

Foc isolates in clade 2 grouped closer to *F. oxysporum* of other *formae speciales* than to each other. This was previously also demonstrated with DNA sequence analysis of the nuclear and mitochondrial gene regions of four *formae speciales* of *F. oxysporum*, including *Foc* (O' Donnell *et al*. 1998). This result strongly supports the hypothesis of at least two independent evolutionary origins for *Foc*. The great diversity among Asian isolates supports the hypothesis that the pathogen has co-evolved with edible bananas and their diploid progenitors in Asia (Stover 1962, Vakili 1965, Stover & Buddenhagen 1986). If host pathogenicity has evolved convergently, it might be expected that each clonal lineage within *Foc* has unique pathogenic properties (O' Donnell *et al*. 1998). This could support observations by Koenig *et al*. (1997), where isolates of different clonal lineages have been recovered in different frequencies from different genotypes of banana, even when those banana cultivars are planted in the same field.

DFG 3 contains *Foc* 'tropical' race 4 isolates belonging to VCG 01213 and 01216. These isolates are known to be limited to Malaysia, Indonesia and northern Australia where they cause Fusarium wilt to Cavendish bananas in the tropics. AFLP analysis

showed that VCG 0121, previously considered to belong to *Foc* 'sub-tropical' race 4, grouped closely with the *Foc* 'tropical' race 4 isolates. In fact, VCG 0121 has also been closely related to VCGs 01213 and 01216 when phylogenically compared using DAF and PCR-RFLP analysis (Bentley *et al*. 1998, Bentley *et al*. 1999). This finding has tremendous implications, as a Cavendish variety with good tolerance to Fusarium wilt has been identified in Taiwan where VCG 0121 is the dominant *Foc* genotype (Hwang & Ko 2004). This would suggest that the Cavendish banana variety, GCTCV 218, could indeed show good tolerance to VCGs 01213 and 01216 in Malaysia, Indonesia and northern Australia.

No correlation between AFLP analysis and the existing race structure in *Foc* could be detected. According to the current race structure, race 1 and 'sub-tropical' race 4 are different, but AFLP analysis grouped some isolates belonging to these races together. These isolates all belong to VCG 0120 and they have also been shown in previous studies to be genetically related (Bentley *et al*. 1995, Koenig *et al*. 1997). The race designation for *Foc* is still based on field evaluation of a limited number of banana cultivars. Since climatic conditions in the field determine disease development in Cavendish bananas caused by VCG 0120 in the tropics and sub-tropics (Ploetz *et al*. 1990), the current race system cannot be considered accurate. We, therefore, believe that all isolates of VCG 0120 belong to the same race, despite differences in disease development under different environmental conditions. All other population analysis procedures, whether phenotypical (Boehm *et al*. 1994) or genotypical (Bentley *et al*. 1995, Bentley *et al*. 1998), support this opinion.

It is further possible that VCGs 01213 and 01216 (currently called 'tropical' race 4, and causing disease to Cavendish bananas in tropical Australia), VCGs 0124 and 0125 (currently including both race 1 and 2 isolates), and VCGs 0123 and 01217 (current races mostly undetermined and limited to Southeast Asia) all represent different races. Since a universally acceptable greenhouse inoculation technique does not exist (Bentley *et al*. 1998), and field confirmation of races is unreliable (Ploetz 1990), it is not yet possible to test the above groupings as potentially new races. Once a reliable greenhouse inoculation technique under controlled environmental conditions and involving more differential banana varieties has been developed, we

believe that the molecular-based subdivision of *Foc* should serve as framework for selecting candidates for new races in *Foc*.

An important reason why a new race structure could not be defined on vegetative compatibility only, is that a VCG does not give any indication of genetic relatedness among isolates belonging to different VCGs, or even of isolates within the same VCG. Isolates that are vegetatively compatible are thought to have identical alleles at each of the *vic* loci (Correll 1991). A mutation at a single *vic* locus, however, could result in closely related isolates becoming vegetatively incompatible (Bentley *et al*. 1995, Bentley *et al*. 1998). Also, some VCGs appear to sometimes produce heterokaryons between otherwise separate VCGs, such as VCGs 0120 and 01215, and VCGs 01213 and 01216 (Bentley *et al*. 1998, Ploetz 1990), and form VCG complexes. By using AFLP banding patterns, it was possible to both determine the genetic relationships among VCGs and the similarity between isolates within the same VCG.

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Table 1. Isolates of *Fusarium oxysporum* used for genetic diversity analysis through the application of Amplified Fragment Length Polymorphisms.

ISOLATE NR	*ORIGINAL NUMBER*	*VCGs*	*RACE*[2]	*HOST/CULTIVAR*	*COUNTRY*
CAV 002[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 009[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 045[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 092[1]		0120	ST race 4	Banana cv. Grand Nain	South Africa
CAV 099[1]		0120	ST race 4	Banana Cavendish cv.	South Africa
CAV 105[1]		0120	ST race 4	Banana Cavendish cv.	South Africa
CAV 129[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 145[1]		0120	ST race 4	Banana cv. Israeli Grand Nain	South Africa
CAV 147[1]		0120	ST race 4	Banana cv. Israeli Grand Nain	South Africa
CAV 287[1]	22615	0120	ST race 4	Banana cv. Lady finger	Australia
CAV 288[1]	23486	0120	ST race 4	Banana Cavendish cv.	Australia
CAV 291[1]	C1	0120	ST race 4	Banana Cavendish cv.	Canary Island
CAV 293[1]	IC-1	0120	ST race 4	Banana cv. Dwarf Cavendish	Canary Island
CAV 294[1]	34661	0120	Race 1	Banana cv. Highgate	Honduras
CAV 296[1]	STH1	0120	Race 1	Banana cv. Highgate	Honduras
CAV 297[1]	BR 13	0120/01215	unknown	-	Brazil
CAV 298[1]	BR 18	0120/01215	unknown	-	Brazil
CAV 299[1]	PD14-1	0120/01215	unknown	Banana cv. Gros Michel	Nigeria
CAV 300[1]	CV-1	01213	T race 4	Banana cv. Valery	Indonesia
CAV 301[1]	CV-2	01213	T race 4	Banana cv. Valery	Indonesia
CAV 312[1]	RPML 25	01213/01216	T race 4	Banana cv. Pisang Udang	Malaysia
CAV 313[1]	RPML 47	01213/01216	T race 4	Banana cv Pisang Awak legor	Malaysia
-	PPRI 4946[2]			Melon, Klapmuts	South Africa
CAV 317[2]	PPRI 4923			Melon, Klapmuts	South Africa
CAV 315[3]	PPRI 5456			*Lycopersicon esculentum*	South Africa
CAV 316[3]	PPRI 5457			*Lycopersicon esculentum*	South Africa
-	FOD 2[4]			-	South Africa
CAV 325[5]	FCC 41			Pine	South Africa
CAV 179[1]	23486	0120	ST race 4	Banana Cavendish cv.	Australia
CAV 180[1]	Taiwan 14	0121	ST race 4	-	Taiwan
CAV 181[1]	PHIL 36	0122	unknown	-	Philippines
CAV 182[1]	THAI 1-2	0123	1	-	Thailand
CAV 183[1]	23532	0124	1	-	Australia
CAV 184[1]	23906	0125	1	-	Australia

CAV 185[1]	PHIL 6	0126	unknown	-	Philippines
CAV 186[1]	24234	0129	ST race 4	-	Australia
CAV 192[1]	MAL 11	01216	T race 4	-	Malaysia
CAV 193[1]	MAL 6	01217	unknown	-	Malaysia
CAV 194[1]	INDO 5	01218	unknown	-	Indonesia
CAV 195[1]	INDO 25	01219	unknown	-	Indonesia
CAV 191[1]	INDO 160	0120/01215	unknown	-	Indonesia
CAV 196[6]	FO 47				

[1]*Fusarium oxysporum* f.sp. *cubense* (*Foc*); [2]*F. oxysporum* f.sp. *melonis* (*Fom*); [3]*F. oxysporum* f.sp. *lycopersici* (*Fol*); [4] *F. oxysporum* f.sp. *dianthi*; [5]*F. circinatum*; [6]non-pathogenic *F. oxysporum*; [7]T = tropical; ST = subtropical.

Table 2. Enzymes, adapters and primers used during the Amplified Fragment Length Polymorphism analysis of *Fusarium oxysporum*.

Restriction enzyme	**Sequence**
*Eco*RI (Roche)[1]	G↓AATT C C TTAA↑G
*Mse*I (NEB)[2]	T ↓TA A A AT ↑ T
Adapter	**Sequence**
*Eco*RI adapter (Inqaba Biotec)[3]	CTCGTAGACTGCGTACC CTGACGCATGGTTAA
*Mse*I adapter (Inqaba Biotec)[3]	GACGATGAGTCCTGAG TACTCAGGACTCAT
Primer	**Sequence**
*Eco*RI primer + 0 (Inqaba Biotec)[3]	5'-GAC TGC GTA CCA AAT C-3'
*Mse*I primer + 0 (Inqaba Biotec)[3]	5'-GAT GAG TCC TGA GTA A-3'
Primer combinations	
Labelled *Eco*RI primer +2 (Biolegio BV)[4]	***Mse*I primer +2 (Inqaba Biotec)[3]**
E22t (TC)	M21 (AA)
E21 (AA)	M23 (AG)
E21 (AA)	M22 (AC)
E22 (AC)	M23 (AG)
E24 (AT)	M22c (CC)

[1] Roche Molecular Biochemicals, Manheim, Germany.

[2] New England Biolabs Inc., USA.

[3] Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa.

[4] Biolegio BV Nijmegen/ Malden, The Netherlands.

Table 3. The number of unique bands for all *Fusarium* isolates examined per primer combination, obtained from Amplified Fragment Length Polymorphism fingerprints.

		PRIMER COMBINATION[1]				
Isolate number	**Designation[2]**	**E22t & M21**	**E21 & M23**	**E21 & M22**	**E22 & M23**	**E24 & M22c**
CAV 002	*Foc* VCG 0120, st. race 4	1	1		1	
CAV 099	*Foc* VCG 0120, st. race 4					2
CAV 129	*Foc* VCG 0120, st. race 4	1				
CAV 145	*Foc* VCG 0120, st. race 4	1				
CAV 317	*Fom*	1				2
CAV 315	*Fol*		1			1
CAV 316	*Fol*		2	1	1	4
-	*Fod*	1			1	2
CAV 325	*Fusarium circinatum*	9	4	5	7	8
CAV 180	*Foc* VCG 0121, st. race 4				3	1
CAV 181	*Foc* VCG 0122 race ?					1
CAV 182	*Foc* VCG 0123, race 1		1			
CAV 184	*Foc* VCG 0125, race 1					1
CAV 194	*Foc* VCG 01218, race1		1		2	1
CAV 195	*Foc* VCG 01219, race ?				1	
CAV 196	Non-pathogenic *Fusarium oxysporum*	3	1			2
Total		**17**	**11**	**6**	**16**	**25**

[1]E – Labeled *EcoR*I primer E22t: + TC selective nucleotides, E21: + AA selective nucleotides, E22: + AC selective nucleotides and E24: + AT selective nucleotides. M – MseI primer, M21: + AA selective nucleotides, M23: + AG selective nucleotides, M22: + AC selective nucleotides and M22c: + CC selective nucleotides.

[2]*Foc* = *Fusarium oxysporum* f.sp. *cubense*, *Fom* = *F. oxysporum* f.sp. *melonis*, *Fol* = *F. oxysporum* f.sp. *lycopersici* and *Fod* = *F. oxysporum* f.sp *dianthi* .

Figure 1. Gel image representing Amplified Fragment Length Polymorphism fragments of *Fusarium oxysporum* f.sp. *cubense* and other *F. oxysporum* isolates amplified with *Eco*RI+AT and *Mse*I+CC selective primers. *Foc* = *Fusarium oxysporum* f.sp. *cubense*, *Fom* = *F. oxysporum* f.sp. *melonis*, *Fol* = *F. oxysporum* f.sp. *lycopersici*, *Fod* = *F. oxysporum* f.sp *dianthi*.

A	50-700 Sizing Standard (LI-COR)
B	*Foc* VCG: 0120
C	*Foc* VCGs: 0120/15
D	*Foc* VCGs: 01213, 01213/16
E	*Fom*, *Fol*, *Fod*
F	*F. circinatum*
G	*Foc* VCGs: 0121, 0122, 0123, 0125, 0126, 0129, 01216, 01217, 01219
H	Non-pathogenic *F. oxysporum*

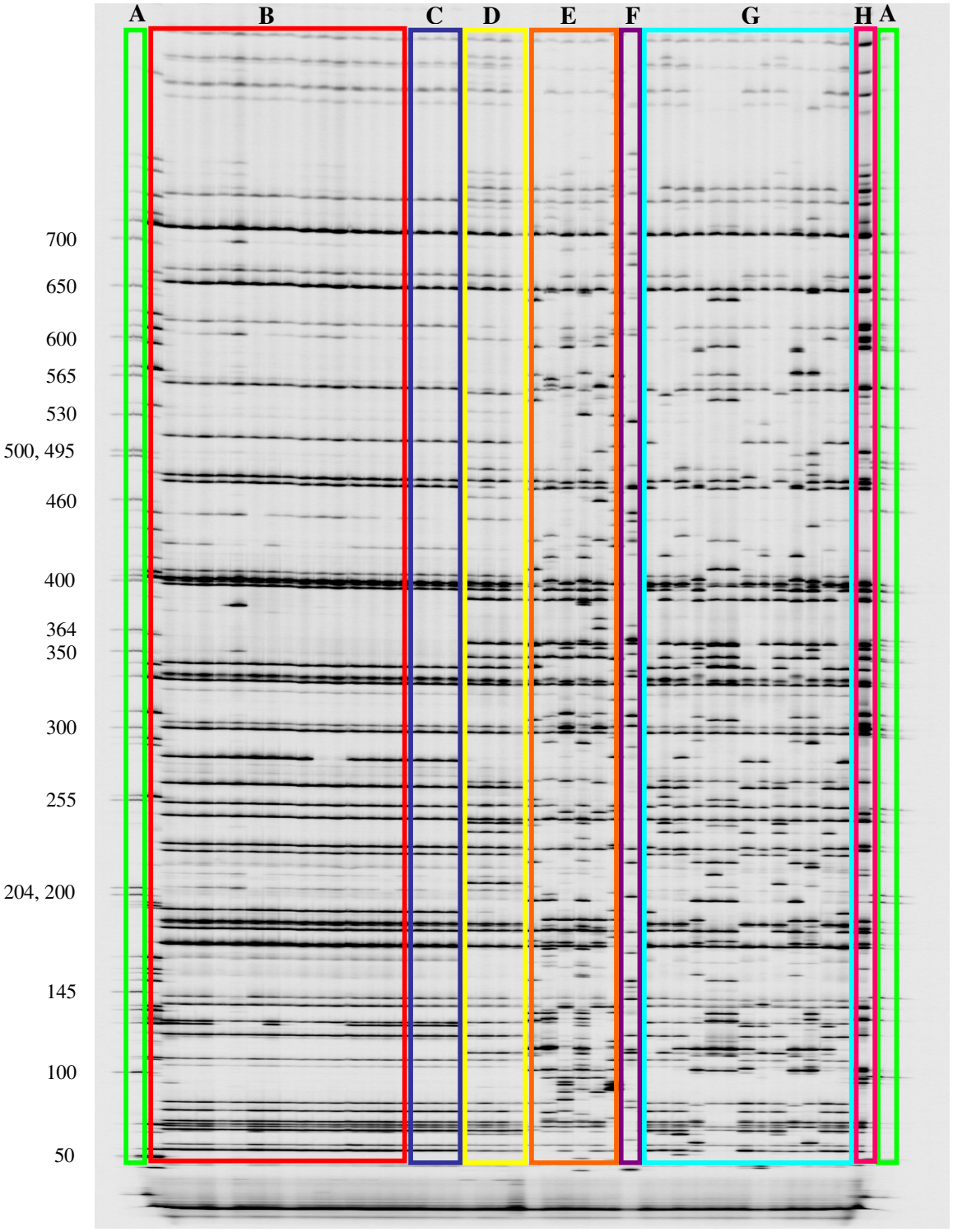
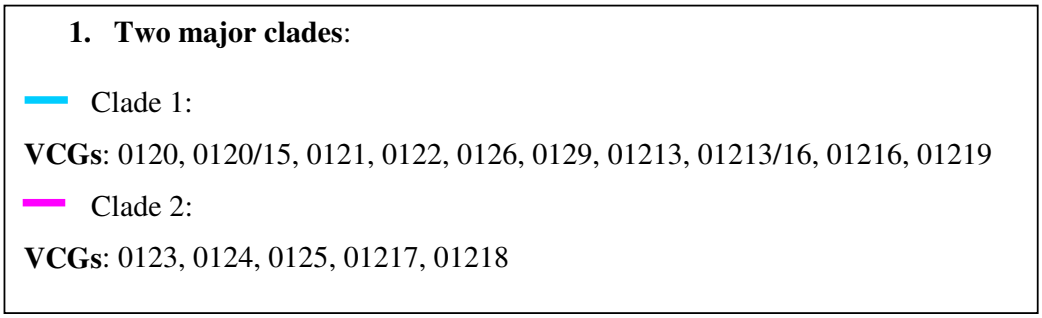
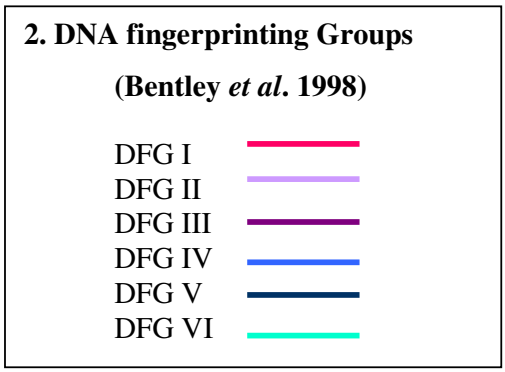
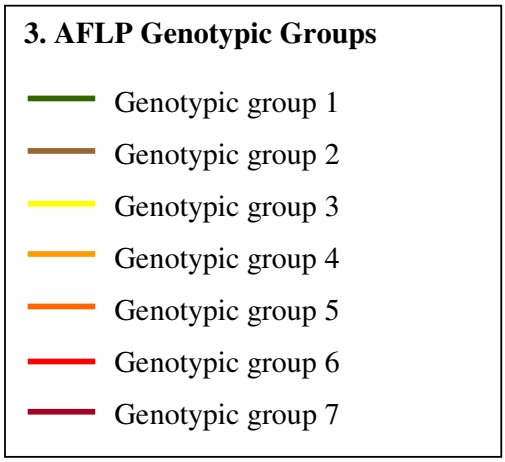
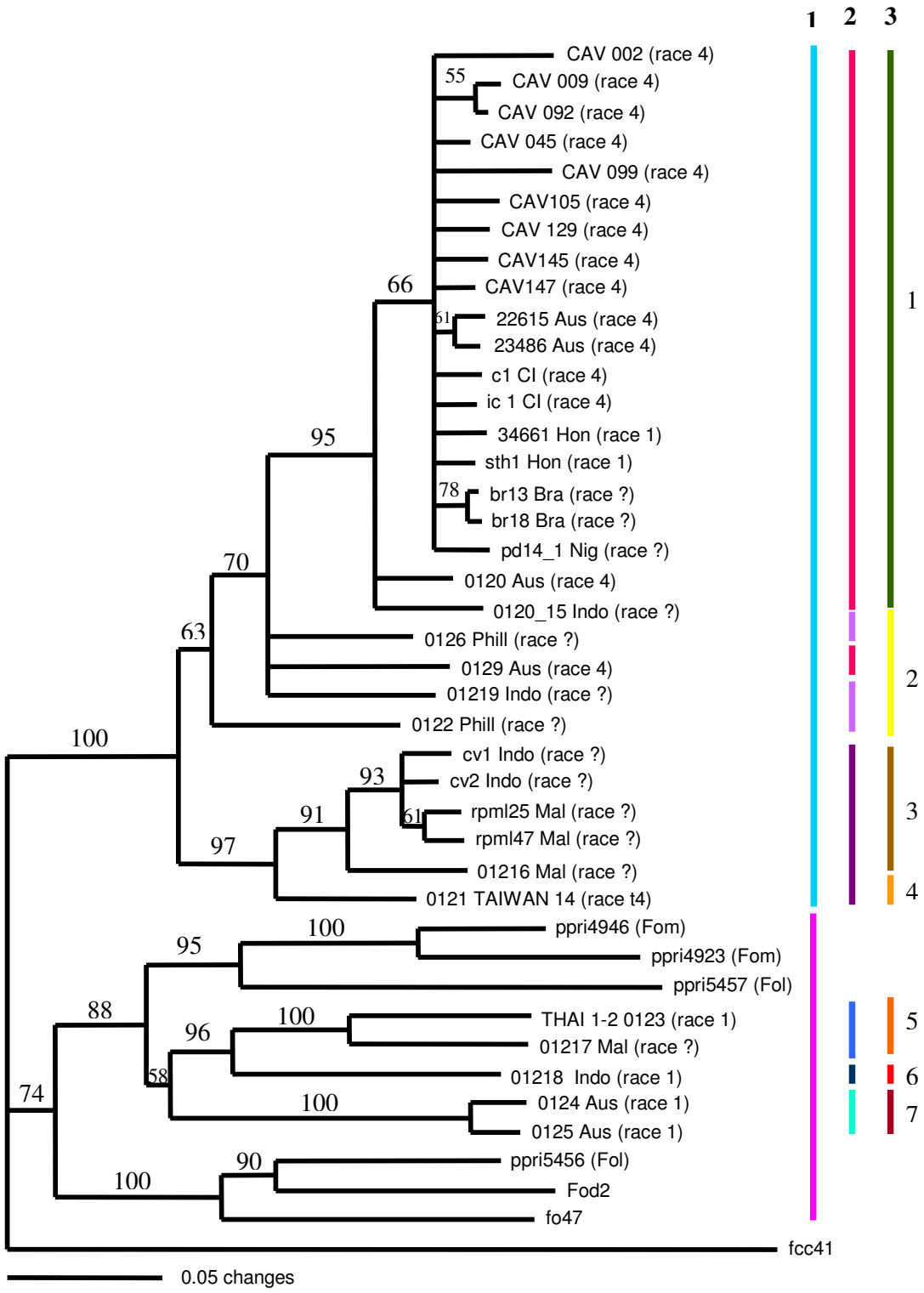

A
B
C
D
E
F
G
H
A
700
650
600
565
530
500, 495
460
400
364
350
300
255
204, 200
145
100
50

Figure 2. Phylogram inferred from Amplified Fragment Length Polymorphism. Distance analysis included Neighbour-joining as a tree building algorithm and a bootstrap of a 1000 replicates for confidence support. Bootstrap values are indicated above nodes.








1
2
3
CAV 002 (race 4)
55
CAV 009 (race 4)
CAV 092 (race 4)
CAV 045 (race 4)
CAV 099 (race 4)
CAV105 (race 4)
CAV 129 (race 4)
CAV145 (race 4)
66
CAV147 (race 4)
61
22615 Aus (race 4)
23486 Aus (race 4)
c1 CI (race 4)
ic 1 CI (race 4)
95
34661 Hon (race 1)
sth1 Hon (race 1)
78
br13 Bra (race ?)
br18 Bra (race ?)
pd14_1 Nig (race ?)
70
0120 Aus (race 4)
0120_15 Indo (race ?)
63
0126 Phill (race ?)
0129 Aus (race 4)
01219 Indo (race ?)
0122 Phill (race ?)
100
cv1 Indo (race ?)
93
cv2 Indo (race ?)
91
61
rpml25 Mal (race ?)
rpml47 Mal (race ?)
97
01216 Mal (race ?)
0121 TAIWAN 14 (race t4)
100
ppri4946 (Fom)
95
ppri4923 (Fom)
ppri5457 (Fol)
88
100
THAI 1-2 0123 (race 1)
96
01217 Mal (race ?)
58
01218 Indo (race 1)
74
100
0124 Aus (race 1)
0125 Aus (race 1)
90
ppri5456 (Fol)
100
Fod2
fo47
fcc41
0.05 changes
1
2
3
4
5
6
7

CHAPTER 4

Development of a VCG 0120-specific marker for *Fusarium oxysporum* f.sp. *cubense*

ABSTRACT

Fusarium oxysporum f.sp. *cubense* (*Foc*) causes Fusarium wilt of bananas. Only *Foc* 'subtropical' race VCG 0120 occurs in South Africa. The development of a marker, specific for *Foc* VCG 0120, would be very useful in terms of the rapid identification of the pathogen in culture, non-symptomatic planting material, water and soil. Genetic markers can efficiently be obtained by using Amplified Fragment Length Polymorphism (AFLP) fingerprinting, because no prior information on DNA sequence is required. The conversion of an AFLP marker to a simple single locus marker would allow less expensive and less laborious large-scale screenings. A fragment unique to *Foc* VCG 0120 was identified using AFLP fingerprinting. The VCG 0120-specific fragment (called Fragment-0120) was excised from an acrylamide gel and sequenced. The conversion of Fragment-0120 to a simple single locus marker, however, proved to be problematic. After single locus primers were designed to screen for internal polymorphic sites in *Foc* of different VCGs, it were determined that the internal sequence did not contain the original AFLP polymorphism. The flanking regions of the AFLP fragment was determined by inverse PCR, but the original AFLP polymorphism could still not be detected. The flanking regions did, however, produce single nucleotide polymorphisms (SNP's) that could prove useful in future. It is possible that the VCG 0120-specific marker was lost during the cloning procedure.

INTRODUCTION

Fusarium oxysporum Schlecth f.sp. *cubense* (EF Smith) Snyd. and Hans (*Foc*) is the causal agent of Fusarium wilt of banana (Brandes 1919). Three races of the pathogen have been identified based on their pathogenicity to certain cultivars. Race 1 is pathogenic to Gros Michel and AAB cultivars (Stover & Simmonds 1987) and race 2 attacks Bluggoe (Su *et al*. 1986, Stover & Simmonds 1987). Race 4 attacks Cavendish cultivars as well as those attacked by race 1 and race 2 (Su *et al*. 1986). Both 'tropical' and 'subtropical' strains of race 4 have been recognised. *Foc* 'subtropical' race 4 attacks Cavendish bananas in countries such as South Africa, Australia, Taiwan and the Canary Islands, where predisposition to cold stress plays an important role in disease developement (Su *et al.* 1986, Brake *et al*. 1990, Ploetz *et al*. 1990, Gerlach *et al*. 2000). 'Tropical' race 4, on the other hand, affects Cavendish bananas in the tropical regions of Southeast Asia and Australia (Pegg *et al*. 1993, 1994, Ploetz 1994, Bentley *et al*. 1998). No predisposition to cold stress is required for this variant of the pathogen to cause widespread destruction. Isolates of *Foc* can further be divided into vegetative compatibility groups (VCGs), which are genetically isolated groups within the fungus. Isolates that share identical alleles at the loci governing heterokaryon incompatibility, commonly referred to as *het* or *vic* loci, are vegetatively compatible (Leslie 1993). Conventionally, this is determined by the ability of nitrate non-utilising auxotrophic (*nit*) mutants to complement each other for nitrate utilisation (Correll *et al*. 1987). Twenty-one VCGs have been identified for *Foc* (Ploetz 1990). The only variant of the pathogen that occurs in South Africa, where Fusarium wilt causes severe damage to Cavendish bananas (Viljoen 2002), belongs to 'subtropical' race 4 and VCG 0120.

The use of molecular markers to detect plant pathogens can improve the accuracy of pathogen identification and reduce the time to process samples (Martin & Tooley 2004). Several molecular marker systems exist for phytopathogenic fungi, including Random Amplified Polymorphic DNA (RAPD) (Williams *et al*. 1990), DNA Amplified Fingerprinting (DAF) (Caetano-Anollés *et al*. 1991), Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al*. 1980), microsatellites or Simple Sequence Repeats (SSRs) (Tautz 1989) and Amplified Fragment Length

Polymorphisms (AFLP) (Vos *et al*. 1995). RAPDs and DAFs are PCR methods based on the amplification of anonymous DNA fragments. RFLP is used to identify a number of alleles at a designated locus (Botstein *et al*. 1980). Microsattellites are tandemly repeated motifs of one to six bases found in all prokaryotic and eukaryotic genomes analysed to date (Zane *et al*. 2002). In a study by Niepold & Schöber-Butin (1995), PCR primers were designed from the partial sequence of a tandem repeat satellite DNA, and were used to detect *Phytophthora infestans* (Mont.) de Bary in potato leaves of tuber slices 2 days after infection. By sequencing selected RAPD markers, Nicholson *et al*. (1998) developed PCR primers to detect *Fusarium culmorum* (W.G. Smith) Sacc. and *Fusarium graminearum* Schwabe in cereals.

AFLP markers have emerged as a genetic marker with broad applications in systematics, pathotyping, population genetics, DNA fingerprinting, local marker saturation and quantitative trait loci (QTL) mapping (Majer *et al*. 1996, Mueller & Wolfenbarger 1999). It is a PCR-based multi-locus fingerprinting technique which efficiently identifies DNA polymorphisms without prior information on the DNA sequence of the organism(s) (Vos *et al*. 1995). Although AFLP markers can be used for single locus assays, they can become too expensive and too laborious for large-scale single locus screenings. Specific AFLP markers, therefore, have to be converted into single locus PCR markers, such as cleaved amplified polymorphic site (CAPS) markers (Konienczny & Ausubel 1993) or sequence characterised amplified region (SCAR) markers (Paran & Michelmore 1993). These PCR-based methods are less laborious and expensive for simple locus assays (Brugmans *et al*. 2003). CAPS and SCARs are both conventional/standard PCR methods that amplify known segments of DNA sequences that lie between two inward-pointing primers. Other methods have been developed for the amplification of unknown DNA that flanks the region of known sequences. These include targeted gene walking PCR (TGW-PCR) (Parker *et al*. 1991), unpredictably primed PCR (UP-PCR) (Dominguez & Lopez-Larrea 1994) and inverse PCR (I-PCR) (Ochman *et al*. 1988, Triglia *et al*. 1988, Silver & Keerikatte 1989). I-PCR allows the amplification of sequences that lie outside the boundaries of known sequences by inverting the known sequence.

The development of a VCG 0120-specific marker for *Foc* is of great importance for the rapid identification and subsequent management of Fusarium wilt of banana in

South Africa. Such a marker would be able to accurately detect the pathogen in symptomless planting material or in pathogen-infested soil and water. The objective of the present study, therefore, was to convert an AFLP marker to sequence-specific or PCR-RFLP markers for *Foc* 'subtropical' race 4 VCG 0120.

MATERIALS AND METHODS

Fungal strains

Isolates of *Foc* (35), *F. oxysporum* f.sp. *lycopersici* (*Fol*) (2), *F. oxysporum* f.sp. *melonis* (*Fom*) (2), *F. oxysporum* f.sp. *dianthi* (*Fod*) (1), a non-pathogenic isolate of *F. oxysporum* (Fo47) and one isolate of *Fusarium circinatum* Nirenberg & O' Donnell were selected for AFLP fingerprinting (Table 1). The isolates of *Foc* represented races 1 and 4, as well as 16 different VCGs.

DNA isolation

Single-spore isolates of *Fusarium* were grown on half strength Potato Dextrose Agar (PDA) (Biolab Diagnostics, Wadeville, South Africa) containing 0.02 $g.l^{-1}$ Novobiocin (Sigma-Aldrich, Stanheim, Germany). After 10 days' growth at 25^{o}C, mycelia were harvested by scraping off fungal colonies from the agar surfaces using a sterile scalpel blade. Fresh fungal mycelia were then homogenized in DNA extraction buffer (200 mM Tris-HCl, pH 8; 150 mM NaCl; 25 mM EDTA, pH 8.0; 0.5% SDS), as described by Reader & Broda (1985). Genomic DNA was subsequently isolated using a phenol:chloroform (1:1) extraction method of Sambrook *et al*. (1989).

AFLP analysis

The standard AFLP procedure as described by Vos *et al*. (1995) was applied with a few modifications as indicated by Zeller *et al*. (2000). Pre-amplification was performed using standard *Eco*RI and *Mse*I adapter primers (Table 2) without any selective nucleotides. Selective amplification was achieved using standard *Eco*RI and *Mse*I adapter primers plus two selective nucleotides. The *Eco*RI primer was 5'-end labelled with infrared dye IRDye™ 800 (LI-COR, Lincoln NE, USA). The primer

combination used in this study was *Eco*RI primer E24 (AT) (Biolegio BV, Nijmegen/Malden, The Netherlands) and *Mse*I primer M22c (CC) (Inqaba Biotechnical Industries, Pretoria, South Africa) (Table 2). Electrophoresis and detection of AFLP fragments were performed on a model 4200S LI-COR® automated DNA analyser. The run parameters were set on 1500 V, 35 mA, 35 W, 48°C, signal filter 3, motor speed 3 and pixel size 16 bit. The collection time for scanning was set on 1 hr.

Excision and sequencing of AFLP fragment

The polyacrylamide gel was scanned on an Odyssey Infrared Imaging System (LI-COR) and fitted onto the grid pattern to allow careful positioning of bands. A DNA fragment unique to all *Foc* VCG 0120 isolates were then visually identified. Gel plugs containing the VCG 0120-specific fragments were excised using a scalpel, and successful fragment excision was verified by re-scanning of the gel. The gel plugs were placed in low TE-buffer (10 mM Tris-HCL pH 8; 0.1 mM EDTA and sterile distilled water) and frozen at -80°C for 30 min. This was followed by three freeze-thaw steps to extract DNA from the gel.

To determine the precision of the excision process, the putative VCG 0120-specific DNA fragment was re-amplified using the same primer combination and selective PCR conditions described above. The amplification product was then again separated on a LI-COR DNA analyser to determine whether the correct fragment was excised. It was also PCR amplified for separation on an agarose gel with un-labelled primers. The cycling profile before agarose gel electrophoresis included an initial denaturation for 2 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 50°C and elongation for 1 min at 72°C. A final elongation step was performed for 5 min at 72°C. After agarose gel electrophoresis, the DNA fragment was again excised and DNA extraction performed with a QIAquick® Gel Extraction Kit (QIAGEN, Valencia, USA) according to the manufacturers' instructions. The DNA fragment was then cloned using the pGEMT®-Easy vector system (Promega Corporation, Madison, USA) before being transformed into *Escherichia coli* JM 109

competent cells (Promega). Purification of plasmid DNA from the *E. coli* was done using the QIAprep® Spin Miniprep Kit (QIAGEN).

A PCR for the sequencing of the AFLP fragment was performed in a total reaction volume of 25 µl, consisting of 1 µl plasmid DNA, 0.3 µM of both the T7 forward and Sp6 reverse vector primers, 250 µM of each dNTP (Fermentas, Hanover, USA), 1 U *Taq* polymerase (Roche Molecular Diagnostics, Manheim, Germany) and 1 x PCR buffer including $MgCl_2$ (Roche Molecular Diagnostics). The following PCR conditions were used: An initial denaturation step of 2 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 50°C and elongation for 1 min at 72°C. A final elongation step was performed for 5 min at 72°C. The PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN) according to the manufacturers' instructions.

Sequencing of the VCG 0120-specific AFLP fragment was performed in a total volume of 10 µl containing 2 µl Big Dye, 1 µl 5 x Buffer, 4 µl DNA, 1 µM (1µl) of the forward or reverse primer and 2 µl Sabax H_2O. The T7 forward primer was used in the forward reaction and the Sp6 reverse primer in the reverse reaction, each to a final concentration of 1 µM. The sequencing product precipitation included transferring 5 µl to a 0.5 ml Eppendorf tube and adding 15 µl sterile Sabax water, 2 µl of 3 M sodium acetate (pH 5.5) and 50 µl 95% ethanol. The tubes were then centrifuged at 10 000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed with 200 µl 70% ethanol, and the tubes centrifuged at 10 000 rpm for 10 min. The ethanol was aspirated and the pellet dried under vacuum for approximately 15 min. DNA sequences were determined using the ABI PRISM™ Dye terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems Foster City, California), and visualised using Chromas software (Technelysium, Queensland, Australia). The sequence of the fragment was imported into GenBank (www.ncbi.nlm.nih.gov/entrez/) and the *F. graminearum* database (www.Broad.mit.edu.annotation/fungi/fusarium), and aligned to known sequences using the BLAST tool (Altschul *et al*. 1997).

Primer design and testing

The DNA sequence of the excised AFLP band was used to design primers specific to *Foc* VCG 0120 using Vector NTI® Suite V.6 (InforMax®, North Bethesda, USA). To make the PCR as stringent as possible, only primers with annealing temperatures higher than 55°C were designed. The two primers selected, tentatively called F120 and R120 (Inqaba Biotechnical Industries) (Table 2), were then tested for selective amplification of the genomic DNA of VCG 0120. A reaction mixture was prepared consisting of 70-120 ng genomic DNA from the respective *Fusarium* isolates, primers diluted to final concentration of 0.3 µM, 1 U *Taq* polymerase (Roche Molecular Diagnostics) and 250 µM of each dNTPs (Fermentas Life Sciences, Hanover, USA) The cycling profile consisted of an initial denaturation for 2 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 56°C and elongation for 1 min at 72°C. A final elongation step was performed for 5 min at 72°C. VCG 0120-specific amplification was then verified on an agarose gel.

DNA fragments that were produced following amplification of *Fusarium* isolates with the putative VCG 0120-specific primers were purified using the High Pure PCR Product Purification Kit (Roche Molecular Diagnostics). They were then sequenced in both directions using the primer set F120 and R120. The sequencing reaction was performed in a total volume of 10 µl containing 1 µM 3'/5' primer, 2 µl Big Dye, 1 µl 5 x Buffer, 4 µl DNA, and 2 µl Sabax H_2O. The cycle profile included 25 cycles at 96ºC for 10 sec, at 50ºC for 5 sec and at 60ºC for 4 min. The determined sequences were aligned using the software program DNAMan Demo Version 4.13 (Lynnon Biosoft, Quebec, Canada).

Inverse PCR

Genomic DNA (5 µg) of two isolates of *Foc*, representative of VCG 0120 (CAV 092) and 0126 (CAV 185), was selected for I-PCR. The DNA of each isolate was digested with each of five different restriction endonucleases (*Pst*I, *Bam*HI, *Rsa*I, *Hinf*I and *Scrf*I) (Fermentas) at 37ºC in a total volume of 100 µl for 16 hrs. The digested DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (CIAA)

(25:24:1) and the phases were separated by centrifugation for 7 min at 4ºC in a microcentrifuge. The aqueous phase was re-extracted with chloroform, and the DNA precipitated with a 0.1 volume 3 M sodium acetate (pH 5.5) and 2.5 volume absolute ethanol, followed by 30 min incubation at -70ºC. The DNA was then pelleted by centrifugation, vacuum dried and resuspended in 30 µl low TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The DNA was self-ligated for 16 hrs at 16ºC in 100 µl reaction volume, and the ligated DNA extracted once with phenol:chloroform, followed by CIAA extraction. The sample was ethanol precipitated as described above and re-suspended in 30 µl low TE buffer, pH 8.0.

PCR amplification steps were performed in 0.2-ml thin-walled tubes in a mastercycler (Eppendorf, Hamburg, Germany). Oligonucleotide inverse primers, InF-120 and InR-120 were designed with DNAMan Demo Version 4.13 and synthesized at Inqaba Biotechnical Industries (Table 2). One hundred ng of the self-ligated DNA was amplified using InF-120 and InR-120 primers at a final concentration of 0.2 µM, 1 U *Taq* polymerase (Roche Molecular Diagnostics), 1 x PCR buffer (Roche Molecular Diagnostics) and 250 µM of each dNTP (Fermentas Life Sciences). Gradient PCRs were performed in order to determine the optimum annealing temperature of the primers. The thermocycling profile included an initial denaturation step at 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 45 sec with a gradient of 5ºC and extenstion at 72ºC for 90 sec. A final extension step was performed at 72ºC for 10 min. The optimum annealing temperature was 54ºC.

The restriction endonuclease that gave a product closest to 1 Kb was *RsaI*. The inverse PCR products of this restriction enzyme were, therefore, cloned into the pGEMT®-Easy vector system (Promega) and transformed with *E. coli* JM109 competent cells (Promega). Colony PCRs were performed with the same conditions and thermocycling profile as described for sequencing PCR. The colony PCR products were purified using the High Pure PCR Product Purification Kit (Roche Molecular Diagnostics), and sequenced using the T7 forward and Sp6 reverse universal primers. Sequencing reaction conditions and precipitation were the same as described before. The determined sequences were verified using Chromas software and aligned using the software program BioEdit v 7.0.4, Biological Sequence

Alignment Editor for Windows 95/98/NT/2K/XP (Ibis Therapeutics, Carlsbad, CA, USA).

RESULTS

Excision and sequencing of AFLP fragment

A DNA fragment unique to *Foc* VCG 0120 was identified on the AFLP LI-COR gel image (Fig. 1) The fragment (called Fragment 0120) was then successfully excised after scanning on the Odyssey Infrared Imaging System (Fig. 2). Re-amplification of the excised fragment and subsequent running on a LI-COR gel confirmed that Fragment 0120 was indeed amplified, but a lot of background bands were also detected (Fig. 3A). The re-amplification products, therefore, were loaded onto an agarose gel to excise the fragment again (Fig. 3B), after which most of the background bands were lost (Fig. 3C). After the sequence of the AFLP fragment was determined and verified, BLAST results of this sequence on GenBank and the *F. graminearum* database showed no homology with any known sequence.

Primer design and testing

Primers designed for Fragment 0120 of *Foc* yielded PCR products of 160 bp for all *Fusarium* isolates included in this study (Fig. 4). When direct sequencing of the PCR products was performed, a single nucleotide polymorphism (SNP) was detected at base position 121 in some, but not all the VCGs. Isolates containing the SNP included VCGs 0123, 0124, 0125 and *F. oxysporum* f.sp. *melonis*, while VCGs 0120, 0121 and 0126 did not (Fig. 5).

Inverse PCR

Inverse PCR of the *Rsa*1-digested DNA of *Foc* VCG 0120 and 0126 yielded amplification products of approximately 1.4 Kb (Fig. 6). The I-PCR products contained Fragment 0120 and its flanking regions, as well as the *Eco*RI and *Mse*I restriction sites. SNPs were detected in the flanking regions at base positions: 136, 262, 367, 702, 744, 874 and 878 (Fig. 7).

DISCUSSION

A fragment unique to *Foc* VCG 0120 was produced by AFLP fingerprint analysis when using the primer combination of E24(AT) and M22c(CC). It was, however, not possible to convert this fragment into a single locus PCR marker. The reconstruction of single locus PCR markers from AFLP markers is difficult. In a previous study, six out of a potential 26 AFLP markers were successfully converted to sequence-specific PCR markers in barley and wheat (Shan *et al*. 1999). One of the reasons for this is that polyacrylamide gels often contain multiple fragments which are the result of co-isolation of background fragments (Meksem *et al*. 2001). In an attempt to recover a specific fragment, the wrong fragment might unintentionally be selected, and the fragment that provided the original polymorphism might be lost. The losing of Fragment 0120 could possibly be explained by the indiscriminate selection of a fragment from the original polymorphism that has separated into more than one band during the re-run on the polyacrylamide gel. In future studies, more polymorphic fragments should be selected for excision.

There are reasons, other than the loss of specific fragments, why polymorphic bands in multi-locus markers cannot be converted to single locus markers. AFLP fragments are often short, making it difficult to find SNPs and design internal primers that could be used to differentiate between alleles (Bradeen & Simon 1998). The SNP found within Fragment 0120 of some of the *Foc* isolates was not specific to VCG 0120 but, interestingly, distinguished between the two clades that constitute the worldwide population of *Foc* (Koenig *et al*. 1997, Bentley *et al*. 1998, O' Donnell *et al*. 1998, Chapter 3). The cloning procedure required for AFLP conversion to simple markers has also been reported to contribute to the loss of the original polymorphism (Wei *et al*. 1999). Considering this possibility, the original *Eco*RI/*Mse*I polymorphism could have been lost when the PCR-amplified fragments were cloned and internal sequences of fragments in *Foc* isolates were identical. A new approach to find the polymorphisms responsible for the VCG 0120-specific AFLP marker in selected fragments could entail direct sequencing of excised and re-amplified AFLP fragment, using the corresponding core primers without selective nucleotides as sequencing primers, to avoid cloning of the AFLP fragment (Brugmans *et al*. 2003).

From the I-PCR sequencing of Fragment 0120 in *Foc* VCG 0120 and VCG 0126, several new SNPs were identified. These SNPs might prove to be useful for designing primers that could, potentially, distinguish VCG 0120 from other VCGs of *Foc*. Before such primers are designed, it is suggested that the regions containing these SNPs are compared to determine whether they are also present in other VCGs. If they are, SCAR or CAPS markers could be developed to distinguish VCG 0120 from other VCGs in *Foc*.

Techniques other than AFLP analysis could be considered to develop simple markers for the rapid identification of phytopathogenic fungi. These include microsatellites or SSR (Tautz 1989), RFLPs (Botstein *et al*. 1980), representational difference analysis (RDA) (Lisitsyn *et al*. 1993) and RAPDs (Williams *et al.* 1990). Microsatellites and RFLP are single locus markers and, therefore, much simpler to work with, as no excising from gels is necessary. Some of these techniques, such as RFLPs and RDAs, might not provide sufficient resolution among VCGs of *Foc* to be considered for simple marker development. AFLP and RAPDs are multi-locus markers that need to be converted to single locus markers. Hoffman *et al*. (2003) converted a RAPD marker to a sequence tagged site (STS) marker for the identification of barley varieties. For this conversion, a mixed RAPD amplified fragment, which included all the fragments of the RAPD fingerprint, was cloned and size-selected, rather than being manually isolated and cloned from a single fragment on the gel. Despite successes like this, RAPDs have not gained wide acceptance for the development of genetic markers because of the lack of reproducibility among laboratories (Penner *et al*. 1993). In all these techniques, SNPs can be identified and SCAR or CAPS markers developed.

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Table 1. Isolates of *Fusarium oxysporum* used for Amplified Fragment Length Polymorphism analysis.

ISOLATE NR	*ORIGINAL NUMBER*	*VCGs*	*RACE*[7]	*HOST/CULTIVAR*	*COUNTRY*
CAV 002[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 009[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 045[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 092[1]		0120	ST race 4	Banana cv. Grand Nain	South Africa
CAV 099[1]		0120	ST race 4	Banana Cavendish cv.	South Africa
CAV 105[1]		0120	ST race 4	Banana Cavendish cv.	South Africa
CAV 129[1]		0120	ST race 4	Banana cv. Williams	South Africa
CAV 145[1]		0120	ST race 4	Banana cv. Israeli Grand Nain	South Africa
CAV 147[1]		0120	ST race 4	Banana cv. Israeli Grand Nain	South Africa
CAV 287[1]	22615	0120	ST race 4	Banana cv. Lady finger	Australia
CAV 288[1]	23486	0120	ST race 4	Banana Cavendish cv.	Australia
CAV 291[1]	C1	0120	ST race 4	Banana Cavendish cv.	Canary Island
CAV 293[1]	IC-1	0120	ST race 4	Banana cv. Dwarf Cavendish	Canary Island
CAV 294[1]	34661	0120	Race 1	Banana cv. Highgate	Honduras
CAV 296[1]	STH1	0120	Race 1	Banana cv. Highgate	Honduras
CAV 297[1]	BR 13	0120/01215	?		Brazil
CAV 298[1]	BR 18	0120/01215	?		Brazil
CAV 299[1]	PD14-1	0120/01215	?	Banana cv. Gros Michel	Nigeria
CAV 300[1]	CV-1	01213	T race 4	Banana cv. Valery	Indonesia
CAV 301[1]	CV-2	01213	T race 4	Banana cv. Valery	Indonesia
CAV 312[1]	RPML 25	01213/01216	T race 4	Banana cv. Pisang Udang	Malaysia
CAV 313[1]	RPML 47	01213/01216	T race 4	Banana cv Pisang Awak legor	Malaysia
CAV ?[2]	PPRI 4946			Melon, Klapmuts	South Africa
CAV 317[2]	PPRI 4923			Melon, Klapmuts	South Africa
CAV 315[3]	PPRI 5456			*Lycopersicon esculentum*	South Africa
CAV 316[3]	PPRI 5457			*Lycopersicon esculentum*	South Africa
	FOD 2[4]				South Africa
CAV 325[5]	FCC 41			Pine	South Africa
CAV 179[1]	23486	0120	ST race 4	Banana Cavendish cv.	Australia
CAV 180[1]	Taiwan 14	0121	ST race 4		Taiwan
CAV 181[1]	PHIL 36	0122	?		Phillipines
CAV 182[1]	THAI 1-2	0123	1		Thailand
CAV 183[1]	23532	0124	1		Australia
CAV 184[1]	23906	0125	1		Australia

CAV 185[1]	PHIL 6	0126	?	Phillipines
CAV 186[1]	24234	0129	ST race 4	Australia
CAV 192[1]	MAL 11	01216	T race 4	Malaysia
CAV 193[1]	MAL 6	01217	?	Malaysia
CAV 194[1]	INDO 5	01218	1	Indonesia
CAV 195[1]	INDO 25	01219	?	Indonesia
CAV 191[1]	INDO 160	0120/01215	?	Indonesia
CAV 196[6]	FO 47			

[1]*Fusarium oxysporum* f.sp. *cubense* (*Foc*); [2]*F. oxysporum* f.sp. *melonis* (*Fom*); [3]*F. oxysporum* f.sp. *lycopersici* (*Fol*); [4]*F. oxysporum* f.sp. *dianthi* (*Fod*); [5]*Fusarium circinatum*: [6]non-pathogenic *F. oxysporum*; [7]T = tropical; ST = subtropical; ? = race unknown.

Table 2. Oligonucleotide primers used for amplified fragment length polymorphism analysis of *Fusarium oxysporum* f.sp. *cubense*, and selective amplification of the inner and outer flanking regions of Fragment 0120.

Name	**Sequence (5'-3')**	**Design program**
E24 (AT)[1]	GACTGCGTACCAAATCAT	-
M22c (CC)[2]	GATGAGTCCTGAGTAACC	-
F-120[2]	GTCCTGAGTAACCCCGTCTT	Vector NTI
R-120[2]	TGAACTGTGGCCCTGTG	Vector NTI
InF-120[2]	GCCAATAGCCCGCATTAGACT	DNAman
InR-120[2]	CATTGCGGGAGTTTCATCG	DNAman

[1] Supplied by Biolegio Nijmegen/Malden, The Netherlands

[2] Supplied by Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa

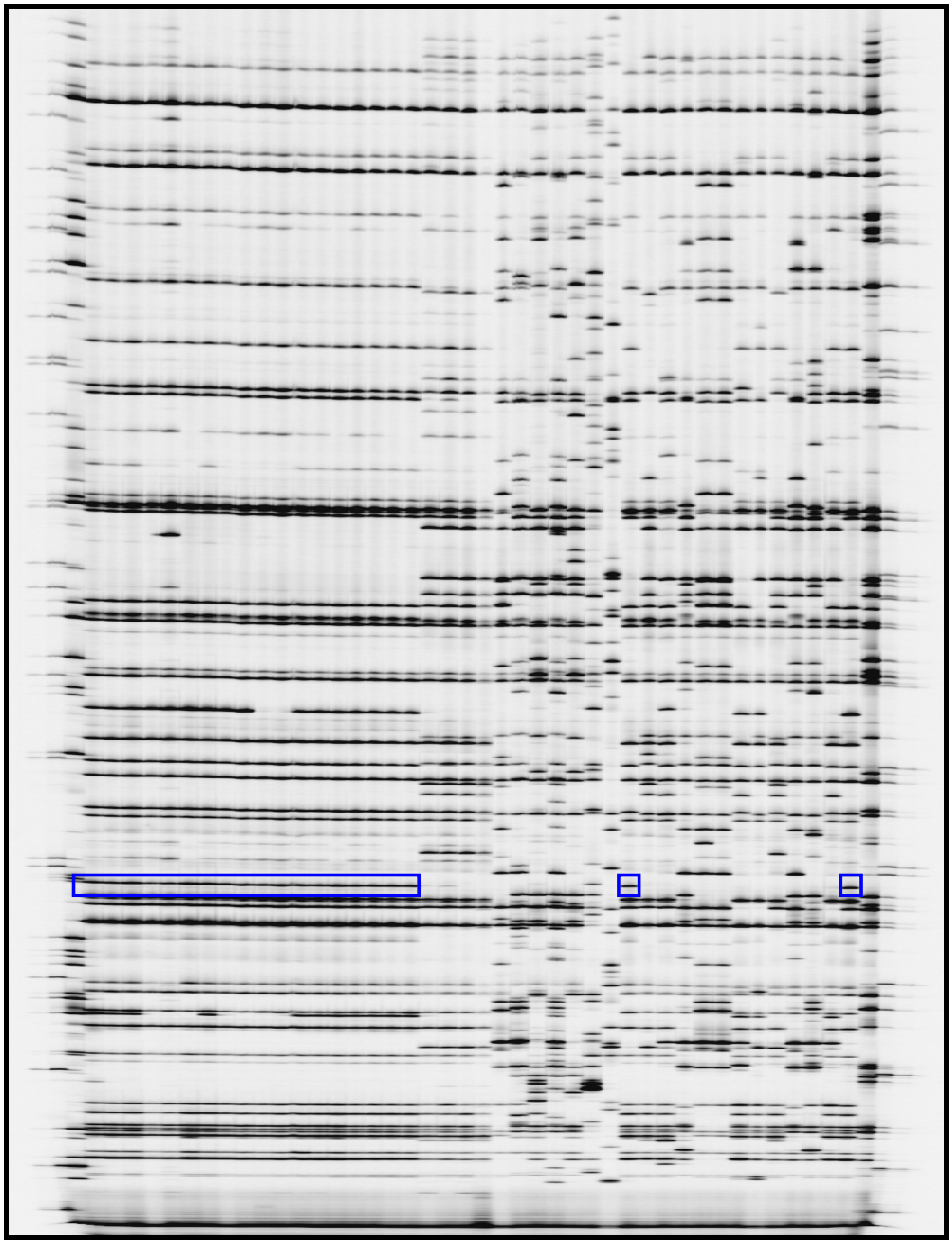

Figure 1. A LI-COR gel image representing Amplified Fragment Length Polymorphism fragments of *Fusarium oxysporum* f.sp. *cubense* and other *F. oxysporum* isolates following amplification with E24 (AT) and M22c (CC) primers. The fragment indicated in blue was specific to *Foc* VCG 0120 isolates.

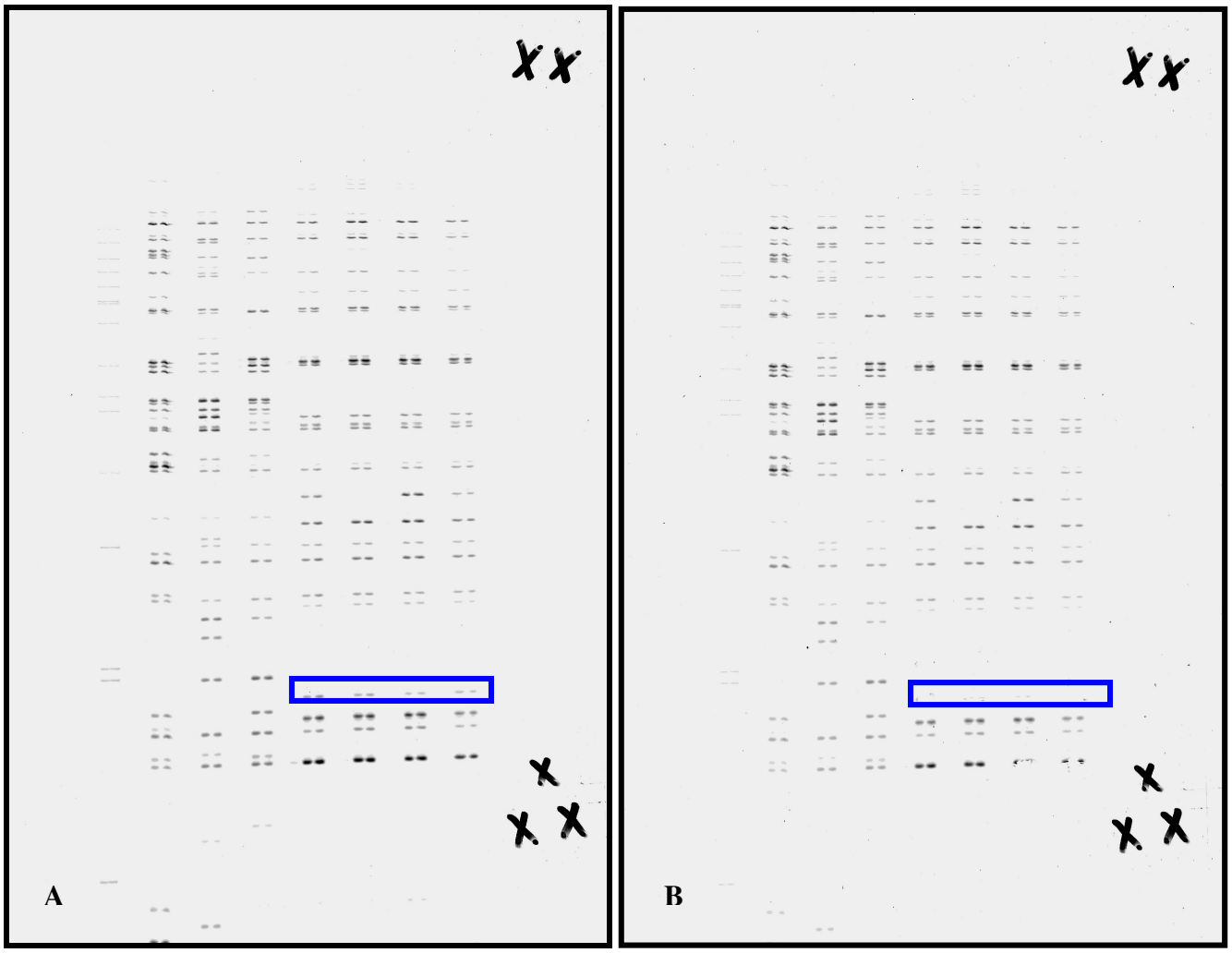

Figure 2. Positioning of an acrylamide gel with Amplified Fragment Length Polymorphism fingerprints of *Fusarium oxysporum* f.sp. *cubense* on a Odyssey Infrared Imaging system. A fragment unique to VCG 0120, indicated with blue frame (A) was excised, and successful fragment excision verified by rescanning of the gel (B).

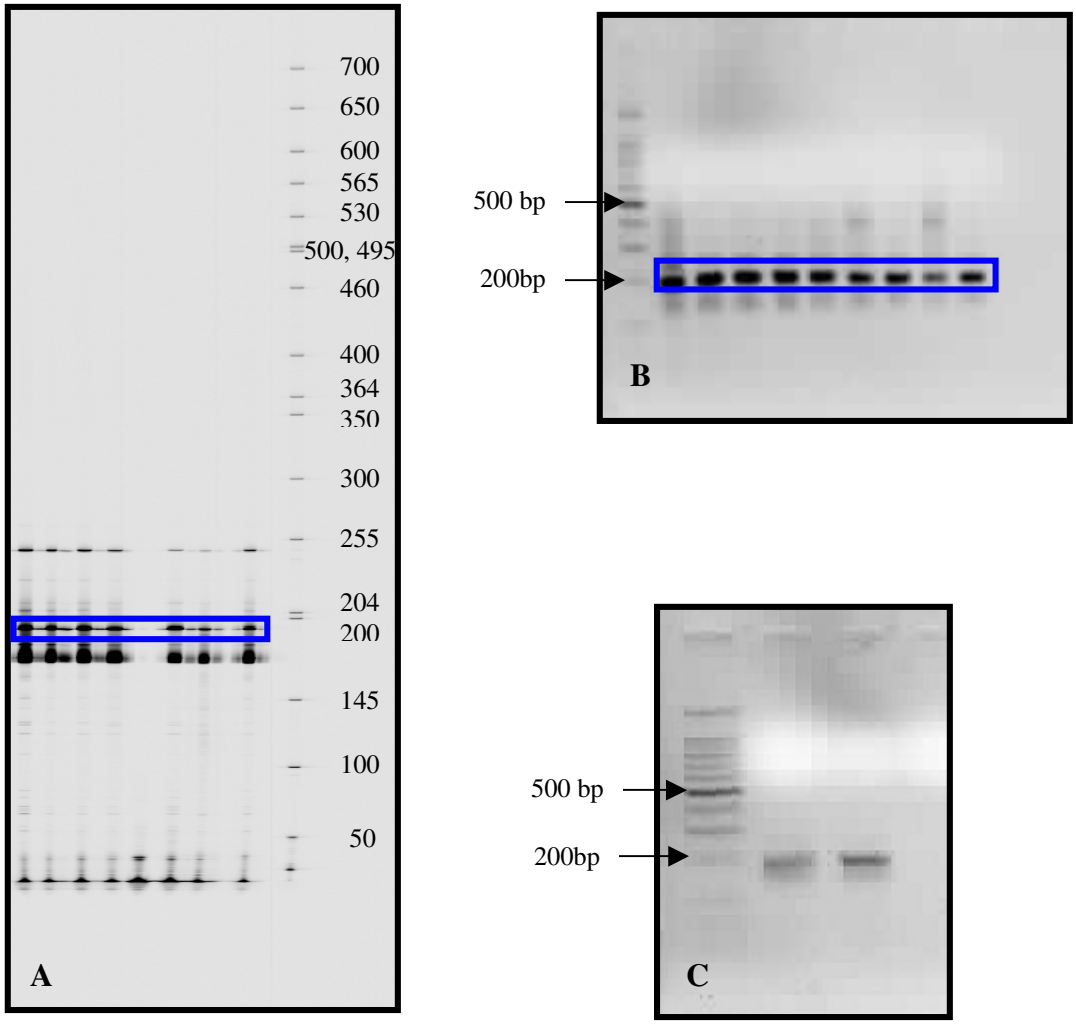

Figure 3. Amplification products of a fragment specific to isolates of *Fusarium oxysporum* f.sp. *cubense* VCG 0120 on LI-COR (A) and agarose (B) gels. The PCR products from agarose gel B was re-amplified and verified on a 2% agarose gel (C).

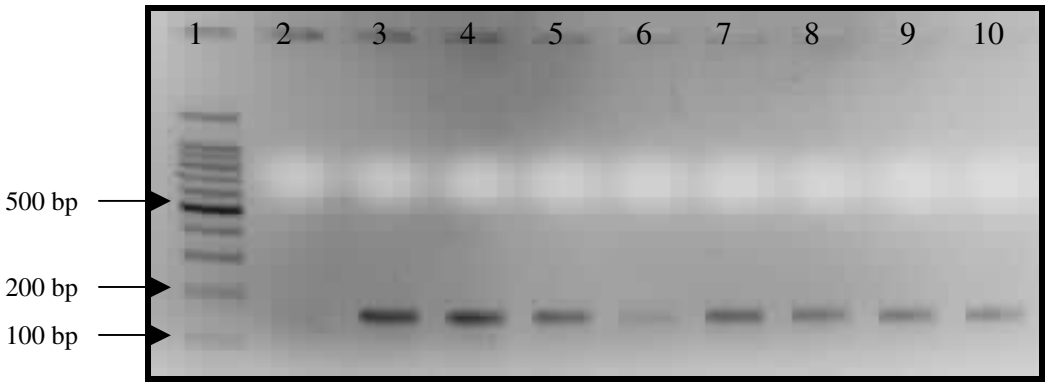

Figure 4. Fragment 0120-specific products of isolates of *Fusarium oxysporum* f.sp. *cubense* representing different VCGs when PCR-amplified with F-120 and R-120 primers. Lane 1: 100 bp marker (Promega), 2: negative control, 3: CAV 002 (VCG 0120), 4: CAV 009 (VCG 0120), 5: CAV 092 (VCG 0120), 6: CAV 300 (VCG 01213), 7: CAV 180 (0121), 8: CAV 182 (0123), 9: CAV 183 (VCG 0124), 10: CAV 317. CAV = Culture collection at FABI, University of Pretoria; VCG = Vegetative Compatibility Group.

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CAV002(0120)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV009(0120)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV092(0120)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV129(0120)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV145(0120)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV180(0121)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV182(0123)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV183(0124)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV184(0125)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
CAV185(0126)TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
fom         TGTCCTGAGTAACCCCGTCTTGTTTGAGGTTCGACTAGGG      40
Consensus   tgtcctgagtaaccccgtcttgtttgaggttcgactaggg
CAV002(0120)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV009(0120)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV092(0120)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV129(0120)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV145(0120)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV180(0121)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV182(0123)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV183(0124)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV184(0125)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
CAV185(0126)TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
fom         TCAAGTCTAATGCGGGCTATTGGCTGGTTGTGAGGCCAGA      80
Consensus   tcaagtctaatgcgggctattggctggttgtgaggccaga
CAV002(0120)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV009(0120)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV092(0120)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV129(0120)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV145(0120)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV180(0121)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV182(0123)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV183(0124)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV184(0125)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
CAV185(0126)GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
fom         GAAGCGGCCTTGACTCCATTGCGGGAGTTTCATCGATACT     120
Consensus   gaagcggccttgactccattgcgggagtttcatcgatact
CAV002(0120)CTTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV009(0120)CTTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV092(0120)CTTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV129(0120)CTTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV145(0120)CTTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV180(0121)CTTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV182(0123)ATTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV183(0124)ATTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV184(0125)ATTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
CAV185(0126)CTTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
fom         ATTCCGGCTGGTCAAGCACAGGGCCACAGTTCAA           154
Consensus    ttccggctggtcaagcacagggccacagttcaa
```

Figure 5. Sequence alignment of *Fusarium oxysporum* f.sp. *cubense* and *F. oxysporum* f.sp. *melonis* isolates representing Fragment 0120. Single nucleotide polymorphisms are highlighted.

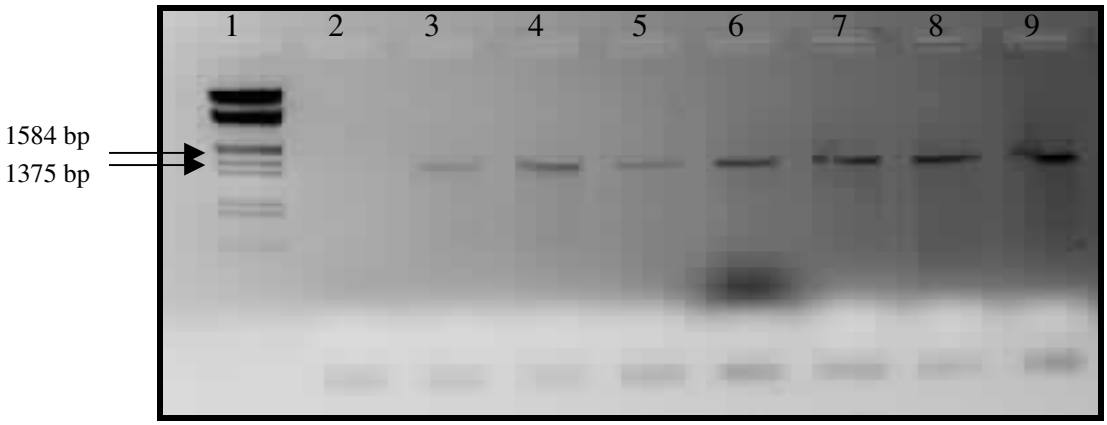

Figure 6. Amplification products of *Fusarium oxysporum* f.sp. *cubense* isolates with inverse PCR primers, InF-120 and InR-120. Lane 1: λ DNA marker, 2: negative control, 3 and 4: CAV 092 (VCG 0120), 5-9: CAV 185 (VCG 0126). CAV = Culture collection at FABI, University of Pretoria; VCG = Vegetative Compatibility Group.

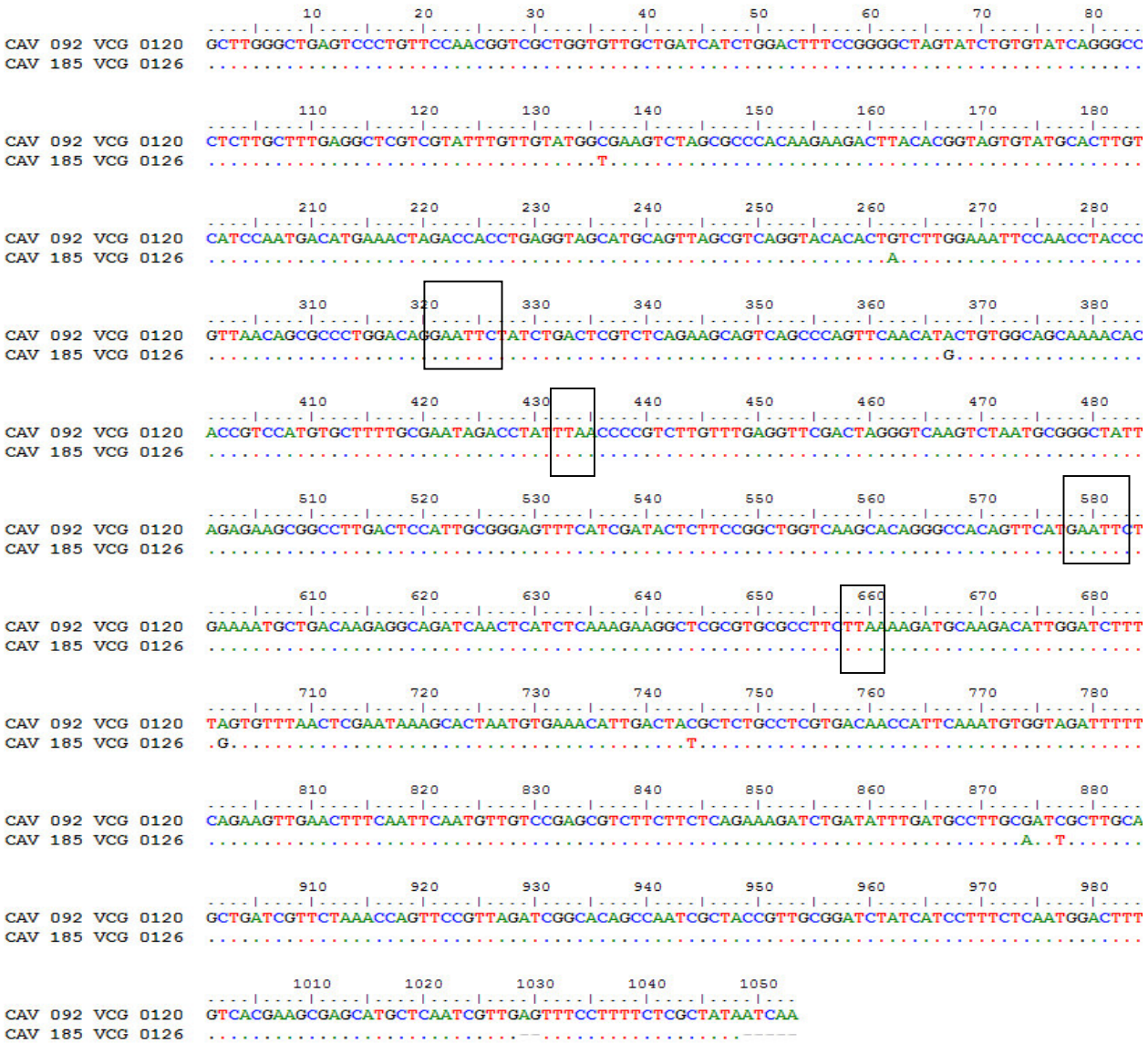

Figure 7. Alignment of two *Fusarium oxysporum* f.sp. *cubense* isolates representing VCG 0120 and VCG 0126. The sequences contain Fragment 0120 (between base positions 430 and 580) as well as its flanking regions as generated by inverse PCR. *Eco*RI and *Mse*I restriction sites are indicated by the black blocks. Single nucleotide polymorphisms were identified at base positions: 136, 262, 367, 702, 744, 874, 877.

CHAPTER 5

Comparative analysis of virulence-associated genes in *Fusarium oxysporum* isolates pathogenic and non-pathogenic to banana

ABSTRACT

Mitogen-activated protein kinases (MAPK) are responsible for signal transduction in eukaryotic systems. These MAPKs play noteworthy roles in pathogenesis, growth regulation, differentiation and survival. Polygalacturonases (PGs) are pectic enzymes responsible for the degradation of cell walls during infection and colonization of hosts. Xylanases act on xylan, which makes up a significant part of the hemicellulose fraction of plant cell walls. Recent studies have shown that MAPKs, PGs and xylanases are highly conserved in fungal pathogens, with a number of homologs to be found. The *fmk1* gene in *Fusarium oxysporum* encodes MAPK, the gene encoding the major endo-PG secreted by *F. oxysporum* is *pg1* and the *xyl3* gene is part of the family F xylanase genes. The objective of this study was to confirm the presence of *fmk1*, *pg1* and *xyl3* and to do a comparative analysis of the *fmk1* and *pg1* genes in isolates of *F. oxysporum* pathogenic and non-pathogenic to banana. These isolates included representatives of different races of *F. oxysporum* f.sp. *cubense* (*Foc*), other *formae speciales* of *F. oxysporum*, and non-pathogenic isolates of the fungus. *Fusarium circinatum* was included as outgroup. The complete *fmk1* gene of 1244 bp, including the four coding regions, the 740 bp fragments of the *pg1* including two introns and exon 4, and *xyl3* were sequenced and compared to known sequences using the BLAST tool on GenBank. The *fmk1* and *pg1* gene regions were analysed and phylogenetically compared. Amino acid sequences were highly similar among the different forms of *F. oxysporum*. The *fmk1* and *pg1* genes separate the forms of *F. oxysporum* into distinct groups, and proved to be valuable in phylogenetic differentiation. The high similarity in the two pathogenicity genes of different isolates indicated that, although these genes are present, expression and regulation patterns may vary.

INTRODUCTION

Fusarium oxysporum Schlecht causes vascular wilt diseases in a wide variety of crops (Beckman 1987). Based on host specificity, more than 120 different *formae speciales* of *F. oxysporum* have been recognized (Armstrong & Armstrong 1981). The soil-borne pathogen survives as chlamydospores, and germinates upon stimulus by the crop host to infect plants through their root systems. Infection most commonly takes place through wounds, but direct penetration is known to occur in some Fusarium wilt diseases (Brandes 1919, Pennypacker & Nelson 1972). To enter plant cells directly, *F. oxysporum* has to produce a wide variety of extracellular cell wall degrading enzymes (CWDEs), including endo- and exopolygalacturonases (PGs), xylanases, cellulases, proteases and pectate lyases. These enzymes may contribute to the degradation of the structural barriers constituted by plant cell walls (Christakopoulus *et al*. 1995, Di Pietro & Roncero 1996a, Di Pietro & Roncero 1996b, Huertas-Gonzales *et al*. 1999, Ruiz Roldán *et al*. 1999, García-Maceira *et al*. 2000).

One of the most important CWDEs in *F. oxysporum* is EndoPG. These enzymes result in the depolymerization of homogalacturan, a major component of the plant cell wall (Collmer & Keen 1986). When inoculated onto tomato cell walls, endoPG is the first enzyme activity detected in *F. oxysporum* cultures (Jones *et al*. 1972). A specific endoPG (PG1) was secreted during growth on pectin by isolates belonging to seven different *formae speciales* of *F. oxysporum*, which included *lycopersici, radicis-lycopersici, conglutinans, tuberosi, ciceris, melonis* and *niveum* (Di Pietro *et al*. 1998). It was concluded that PG1 is widely distributed in *F. oxysporum*, and that the PG1 locus was structurally conserved in most isolates (Di Pietro *et al*. 1998). Comparative analysis of an endoPG gene, *pg1*, in isolates of seven *Fusarium* species indicated that this region would be very useful for phylogenetic analysis in the genus *Fusarium* (Posada *et al*. 2000). Targeted inactivation of the genes encoding for the endoPGs secreted by *F. oxysporum*, *pg1* and *pg5*, has shown that these mutants retained their full virulence (Di Pietro & Roncero, 1998), and that they are not essential for pathogenesis on tomato plants (García-Maceira *et al*. 2001).

Endo-β-1,4-xylanases are produced by a number of plant pathogenic fungi and it has been suggested that they may play a role during infection (Walton 1994). Xylanases act on xylan, which makes up a significant part of the hemicellulose fraction of the plant cell wall

(Wong *et al*. 1988, Ruiz-Roldán *et al*. 1999). The presence of xylanases in *F. oxysporum* was previously reported (Jones *et al*. 1972, Alconada and Martínez 1994, Christakopoulos *et al*. 1996, Ruiz *et al*. 1997). In *F. oxysporum* f.sp. *lycopersici*, four xylanase genes (*xyl2*, *xyl3*, *xyl4* and *xyl5*) have recently been identified (Ruiz-Roldán *et al*. 1999, Gómez- Gómez *et al*. 2001, 2002). To degrade cellulose, cellulase enzymes C1 and C2 act upon native, insoluble cellulose to produce linear chains that are subsequently attacked by the cellulase enzyme Cx to produce cellobiose and glucose (MacHardy & Beckman 1981). *Fusarium* produces both Cx and C1 enzymes (Husain & Dimond 1960). Pectate lyases (PLs) catalyse the trans-elimination of pectate (Linhardt *et al*. 1986). No effect on the virulence of *F. oxysporum* f.sp. *lycopersici* was found when knocking out an individual PL gene (Di Pietro *et al*. 2003). The simultaneous disruptions of two PL genes, however, drastically reduced virulence in *F. oxysporum* f.sp. *pisi* (Rogers *et al*. 2000).

CWDEs and other virulence factors in *F. oxysporum* are transcribed by virulence genes. Seventeen virulence genes have already been characterized in *F. oxysporum*. One of these genes produces proteins that proved to be of great importance in disease development. These proteins are the mitogen-activated protein kinase (MAPK) (Di Pietro *et al*. 2001). MAPKs are involved in transducing a variety of extracellular signals and for regulating growth and differentiation processes in eukaryotic cells (Nishida & Gotoh 1993, Dickman & Yarden 1999, Schaeffer & Webber 1999). Di Pietro *et al*. (2001) identified the gene *fmk1* that encodes a MAPK in *F. oxysporum*. This gene is related to formation of infection hyphae, root attachment and invasive growth by *F. oxysporum* f.sp. *lycopersici* on tomato plants (Di Pietro *et al*. 2001).

The objective of this study was to verify the presence of three virulence genes, *fmk*1, *pg1 and xyl3*, in isolates of *F. oxysporum* pathogenic and non-pathogenic to banana. The phylogenetic relationship of two of these genes, *fmk*1 and *pg1*, in *F. oxysporum* isolates pathogenic and non-pathogenic to banana was also investigated.

MATERIALS AND METHODS

Fungal isolates

Twenty-seven isolates representing the different pathogenic races and selected VCGs in *Foc*, and one isolate each of *F. oxysporum* f.sp. *melonis*, *F. oxysporum* f.sp. *lycopersici* and the non-pathogenic *F. oxysporum* Fo-47, were included in this study (Table 1). An isolate of *F. circinatum* Nirenberg & O'Donnell, the causal agent of pitch canker of pines, was included as an outgroup.

DNA isolation

Single spore isolates were grown on half strength Potato Dextrose Agar (PDA) (Biolab Diagnostics, Wadeville, South Africa) containing 0.02 $g.l^{-1}$ Novobiocin (Sigma-Aldrich, Stanheim, Germany). Genomic DNA was isolated from fungal mycelia according to the method described by Sambrook *et al*. (1989). Fungal mycelia were homogenized in DNA extraction buffer (200 mM Tris-HCl, pH 8; 150 mM NaCl; 25 mM EDTA, pH 8.0; 0.5% SDS) (Reader & Broda 1985), and phenol:chloroform (1:1) extractions performed as described by Sambrook *et al*. (1989). The pellet was then dried in a speedy vac for 10 min and resuspended in 200 µl sterile Sabax water (Adcock Ingram, Bryanston, South Africa). DNA of some *Foc* isolates was obtained from Dr. S. Bentley, Indoroopily, Queensland, Australia (Table 1). DNA concentrations were determined using a spectrophotometer (Eppendorf bioPhotometer, Hamburg, Germany).

PCR amplification

Genomic DNA (40-100 ηg) was used for the amplification of the *fmk1* gene, the endoPG region (*pg1*), and the *xyl3* gene, using primers developed by Di Pietro *et al*. (2001), Posada *et al.* (2000) and Ruiz-Roldán *et al*. (1999), respectively (Table 2). Since the *fmk1* fragments were very long, Primer3 (Rozen & Skaletsky, 2000) and DNAMan Demo Version 4.13 (Lynnon Biosoft, Quebec, Canada) were used to design a set of primers for the amplification of the beginning (5') and end (3') sequences of the gene (Fig. 1, Table 2). The new reverse primer oliFmkA was used with the forward primer oliFmk1 to amplify the

5' end, and the new forward primer oliFmkB used with reverse primer oliFmk2 to amplify the 3' end of the gene. Both had a GC% of 50 and Tm of 60.40ºC.

The PCR was conducted in a Mastercycler (Eppendorf, Hamburg, Germany). The 25-µl reactions all contained 1 x PCR buffer (Roche Molecular Diagnostics, Mannheim, Germany) and 250 µM dNTPs (Fermentas Life Sciences, Hanover, USA). For amplification of the *fmk1* gene, 0.3 µM of both 3' (oliFmk2 and oliFmkA) and 5' primers (oliFmk1 and oliFmkB) (Inqaba Biotechnical Industries, Pretoria, South Africa), and 1 U *Taq* polymerase (Roche Molecular Diagnostics) were used. The thermocycling profile was set at an initial denaturation step of 94ºC for 5 min, followed by 35 cycles denaturation at 94ºC for 35 sec, annealing at 58ºC for 35 sec for oliFmk1/oliFmk2 and oliFmkB/oliFmk2, but at 61ºC for oliFmk1/oliFmkA (Fig. 1, Table 2), elongation at 72ºC for 90 sec, and final elongation at 72ºC for 7 min. For the endoPG gene region, 1.5 U of *Taq* polymerase (Roche Molecular Diagnostics) and oligonucleotide primers, UpperPG and LowerPG, at a final concentration of 1 µM were used. The thermocycling profile, described by Posada *et al.* (2000), included an initial step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, an annealing step at 52°C for 1 min, and an elongation step at 72 °C for 2 min. The final elongation step was performed at 72°C for 5 min. Finally, the reaction mixture for *xyl3* contained 1.5 U of Expand *Taq* polymerase (Roche Molecular Diagnostics), 1 x Expand buffer (Roche Molecular Diagnostics), 250 µM of each dNTP. The *xyl 3* oligonucleotide primers were used at a final concentration of 1 µM. The thermocycling profile used for amplification included an initial denaturation for 2 min at 96°C, followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 45 sec, elongation at 72°C for 45 sec and 30 cycles of denaturation for 30 sec at 94°C, annealing at 59°C for 45 sec, elongation at 72°C for 45 sec with a 0.5 sec/cycle increase in the extension time. A final elongation step at 72°C for 7 min was performed at the end of the cycling program.

Aliquots (5 µl) of all the PCR products were analysed on a 2% agarose gel (Sigma-Aldrich) in 1 x TAE buffer (Tris Acetic acid EDTA; pH 8.0), stained with 1.6 µg/ml ethidium bromide (EtBr) (Sigma-Aldrich) and the DNA visualised under UV illumination (Sambrook *et al*. 1989). Size estimates were done using molecular weight standards, a

100-bp ladder (Promega, Madison, Wisconsin) for smaller fragments, and λDNA/ *Eco*RI+*Hind*III marker 3 (Fermentas Life Sciences) for fragments larger than 1 kb.

Sequencing of fungal DNA

PCR products of genes were purified using the High Pure PCR Product Purification Kit (Roche Molecular Diagnostics) and sequenced, using the same set of primers as for PCR reactions, in both directions. A sequencing reaction was performed in a total volume of 10 µl containing 1 µM 3'/5' primer, 2 µl Big Dye, 1 µl 5 x Buffer, 4 µl DNA, and 2 µl Sabax H_2O. The cycle profile included 25 cycles at 96ºC for 10 sec, at 50ºC for 5 sec and at 60ºC for 4 min.

The sequencing product was precipitated by transferring 5 µl of sequencing product to a 0.5 ml Eppendorf tube, adding 15 µl sterile Sabax water, 2 µl of 3 M sodium acetate and 50 µl 95% ice-cold ethanol. The tubes were then centrifuged at 10 000 rpm for 30 min at 4ºC. The ethanol solution was removed, the pellet rinsed with 200 µl 70% ethanol, and the tubes centrifuged at 10 000 rpm for 10 min at 4ºC. The ethanol was aspirated and the pellet dried under vacuum for approximately 10 min. DNA sequences were determined using the BigDye® terminator Cycle version 3.1 cycle Sequencing (Applied Biosystems, Foster City, California).

DNA sequence analysis

DNA sequences were visualised and verified using Chromas software (Technelysium, Queensland, Australia). The software program BioEdit v 7.0.4, Biological Sequence Alignment Editor for Windows 95/98/NT/2K/XP (Ibis Therapeutics, Carlsbad, CA, USA) was used for DNA sequence alignment. The sequences were imported into GenBank (www.ncbi.nlm.nih.gov) and aligned with known sequences using the BLAST tool (Altschul *et al*. 1997). A model test was first performed using Modeltest 3.0 (Posada & Crandall 1998) to determine the substitution model for the correction of evolutionary distances. Data files were then subjected to Distance Analysis using the Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10 (Swofford 1999), which included the Neighbour-Joining (NJ) tree building algorithm and a bootstrap of 1000 replicates

(Felsenstein 1985) for confidence support. Translation to amino acid sequences was done in BioEdit.

RESULTS

Amplification of virulence genes

The complete *fmk1* (Fig. 2A), and partial *pg1* (Fig. 3) and *xyl3* (Fig. 4) gene regions were amplified with PCR fragments sizes of 1244, 740 and 260 bp, respectively. For the *fmk1* gene, the 5' end primer pair (oliFmk1 and oliFmkA) yielded PCR fragments of 220 bp (Fig. 2B) and the 3' end primer pair (oliFmk2 and oliFmkB) yielded PCR fragments of 364 bp (Fig. 2C). The amplification product of *pg1* included the full sequence of exon 4 and introns 3 and 4, and part of exons 3 and 5. BLAST searches on GenBank indicated a good homology with *fmk1* (AF286533; E-value: 0.0), *pg1* (U96456; E-value: 0.0) and *xyl3* (AF052582; E value: e^{-134}) genes of *F. oxysporum* f.sp. *lycopersici*.

Sequencing data analysis

Models that were obtained by modeltest 3.0 for *fmk1* and *pg1* data include HKY+G and TrNef+G, respectively. The HKY+G model allows the base frequencies to vary, and transitions and transversions have different substitutions rates. The TrNef+G model uses equal base frequencies. Both models allow for among-site variation, with no invariable sites and equal rates for all sites. Distance analysis based on *fmk1* and *pg1* sequences grouped *Foc* isolates into two major clades (Fig. 5 & 6). Clade 1 contained VCGs belonging to *Foc* race 4, and clade 2 consisted of isolates belonging to *Foc* races 1 and 2 (Jones 1999). The only discrepancy was VCG 01219 that was placed in clade 1 for the *fmk1* gene, and in clade 2 for the *pg1* gene.

Amino acid analysis

The amino acid sequences of the *fmk1*, *pg1* and the *xyl3* gene regions were deduced and compared among the different isolates. *Fusarium oxysporum* f.sp. *lycopersici* from GenBank (AF286533 for *fmk1*, U96456 for *pg1* and AF052582 for *xyl3*) was also included in the alignment. The results indicated high similarities for all gene regions. A similarity

of 99.9% was found for *fmk1* (Fig. 7), 97.5% for *pg1* (Fig. 8), and identical amino acid sequences for *xyl3* among the different isolates of *F. oxysporum*. Only two isolates' amino acid compositions were different in the *fmk1* region (23509 VCG 0129 and CAV 009 VCG 0120). All the changes were point mutations (substitutions), with transversions being more frequent than transitions.

DISCUSSION

The virulence-associated genes *fmk1*, *pg1* and *xyl3* were present in all isolates of *F. oxysporum*, pathogenic and non-pathogenic to banana. This demonstrates they are not unique to certain pathogenic forms of this vascular wilt pathogen. It does, however, not imply that they do not play a role in pathogenesis, since differences in the regulation of the gene expression patterns can exist (Balhadère & Talbot 2000). Differential expression of virulence genes upon infection of plant hosts, therefore, should in future be investigated. Their role in pathogenesis of *Foc* should also be substantiated by means of targeted inactivation. For instance, targeted inactivation of *fmk1* resulted in the loss of pathogenicity in *F. oxysporum* f.sp. *lycopersici* when inoculated onto tomato plants (Di Pietro *et al*. 2001). The disruption of particular genes, for instance those coding for single enzymes, however, do not always reduce the capacity of a pathogen to cause disease. Targeted inactivation of *pg1* and *pg5* encoding an endoPG and *pgx4* encoding an exoPG, individually, had no effect on virulence in *F. oxysporum* f.sp. *lycopersici* (Di Pietro & Roncero 1996b, 1998, Di Pietro *et al*. 1998, García-Maceira *et al*. 2000). Inactivation of *xyl3*, *xyl4* and *xyl5,* individually, also did not suppress the ability of *F. oxysporum* f.sp. *lycopersici* to cause disease in tomato plants (Ruiz-Roldán *et al*. 1999, Gómez-Gómez *et al*. 2001, 2002). This, however, could probably be attributed to the presence of additional CWDE genes in the pathogen (Apel-Birkhold & Walton 1996, Wu *et al*. 1997, Gómez-Gómez *et al*. 2001, 2002).

Sequence analyses of *fmk1* and *pg1* yielded regions with different levels of variability, and proved useful for comparing *F. oxysporum* isolates. Both genes divided *Foc* into two major clades, similar to the ones previously reported by Boehm *et al*. (1994), Bentley *et al*. (1995), Bentley *et al*. (1998), Koenig *et al*. (1997), and O' Donnell *et al*. (1998). This study represents the first record where sequences of virulence genes were applied for comparative

analysis of *formae speciales* within *F. oxysporum*. Previously, the comparative analysis of a *pg1* gene in seven *Fusarium* species proved to be valuable (Posada *et al*. 2000). In this study, both coding and two intron sequences contained in the *endopg* fragment were compared to evaluate their potential use in phylogenetic studies. It was concluded that this *pg1* region would be very useful for phylogenetic analysis in the genus *Fusarium*.

The high similarity in amino acid sequences among *fmk1*, *pg1* and *xyl3* genes in *F. oxysporum* pathogenic and non-pathogenic to banana suggests that these genes play a limited role in host specificity. This information indicates structural conservation of the genes. This was also found to be true in previous studies on PGs (Di Pietro & Roncero 1998, Posada *et al*. 2000). *Fmk1* is a homologue of *pmk1*, a MAP kinase gene identified in *Magneporthe grisea* (T.T. Hebert) M.E. Barr. It has been found that *pmk1* and its homologues are essential for appressorium formation in foliar fungal pathogens (Lev *et al*. 1999, Takano *et al*. 2000, Xu & Hamer 1996). The *pmk1* pathway is also conserved for regulation of hyphal growth, mating, conidiation and conidial germination in different fungal pathogens (Xu 2000). The conserved structure among MAPK members suggests a high degree of function relatedness (Di Pietro *et al*. 2001).

Isolation and characterization of virulence genes in phytopathogenic fungi is not only important for a better insight into disease development, but these genes and their corresponding proteins are also key targets for approaches to improve disease control strategies. Depending on the nature of virulence genes, plants can be treated with microbial elicitors or genetically modified to recognise and resist pathogens. Fumonisin B1, a known phytotoxin produced by the necrotrophic pathogen *Fusarium verticilliodes* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon) in maize might act as an elicitor switching on active plant defence and cell death programmes in *Arabidopsis* (Stone *et al*. 2000). Transgenic plants can also be developed to bind specific virulence proteins produced by pathogens. For example, plant polygalacturonase inhibitor proteins (PGIPs) can bind fungal PGs. Inhibition specificities and kinetics, however, might vary within and among species. Bean PGIP, for instance, inhibited fungal PGs from *Aspergillus niger* van Tieghen, *Fusarium moniliforme* and *Botrytis cinerea* Pers. ex. Fr., while pear PGIP inhibited only *B. cinerea*, and tomato PGIP inhibited both *A. niger* and *B. cinerea* (Stotz *et al*. 2000).

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Table 1. Isolates of *Fusarium oxysporum* selected for PCR amplification of virulence genes and genetic diversity analysis.

ISOLATE NR.	**ORIGINAL NR.**	***FORMAE SPECIALIS***	**VCGs**	**HOST/CULTIVAR**	COUNTRY
CAV 002		*cubense*	0120	Cavendish Williams	South Africa
CAV 009		*cubense*	0120	Cavendish Williams	South Africa
CAV 092		*cubense*	0120	Cavendish Grand Nain	South Africa
CAV 291	C1	*cubense*	0120	Cavendish	Canary Island
CAV 298	BR 13	*cubense*	0120/15		Brazil
	23631[1]	*cubense*	01211	SH 3142 (AA)	Wamuran, Australia
CAV 612[1]	RP CR1-1	*cubense*	01215	Gros Michel (AAA)	Costa Rica
CAV 618[1]	PHIL 10	*cubense*	0122	Cavendish Grand Nain	Philippines
CAV 599[1]	PHIL 6	*cubense*	0126	Latundan	Philippines
	RP 51 (A1-1)[1]	*cubense*	01210	Apple	Florida, United States
CAV 603[1]	INDO 25	*cubense*	01219	Pisang Ambon Putih	Indonesia
	F9 130[1]	*cubense*	0121	Cavendish	Taiwan
CAV 610[1]	MAL 32	*cubense*	01213	Pisang Rastali (AAB)	Malaysia
	RPML 25[1]	*cubense*	01213/16	Pisang Udwang	Malaysia
	INDO 47[1]	*cubense*	01216	Cavendish Grand Nain	Indonesia
CAV 630[1]	23532	*cubense*	0124	Lady finger	Ormeau, Australia
CAV 606[1]	THAI 13	*cubense*	0124/5	Kluai Namwa	Thailand
	8605[1]	*cubense*	0125	Lady finger	Tallebudera, Australia
	22993[1]	*cubense*	0128	Blue Java (ABB)	South Johstone, Australia
CAV 623[1]	24218	*cubense*	01220	Cavendish Williams	Carnavon Western, Australia
	RP 58 (STMP1)[1]	*cubense*	01212	Ney poovan	Tanzania
	MAL 5[1]	*cubense*	0123	Pisang awak (ABB)	Malaysia
	23509[1]	*cubense*	0129	Lady finger	Gunulda, Australia
	MAL 7[1]	*cubense*	01217	Pisang Rastali (AAB)	Malaysia

CAV 194[1]	INDO 5	*cubense*	01218	Pisang siem (ABB)	Indonesia
CAV 189[1]	RP MW 40	*cubense*	01214	Harare	Malawi
CAV 617[1]	23 707	*cubense*	0129/11	Lady finger	Kadanga, QLD, Australia
CAV 315	PPRI 5456	*lycopersici*		*Lycopersicon esculentum*	South Africa
CAV 317	PPRI 4946	*melonis*		melon	South Africa
CAV 196	FO 47	Non-pathogenic			
CAV 325	FCC 41	*F. circinatum*		pine	South Africa

[1] DNA received from Dr. S. Bentley, QDPI, Indoroopily, Queensland, Australia.

Table 2. Oligonucleotide primers used for the amplification of *fmk1*, *pg1* and *xyl3* regions.

Gene	Primer name	Reference	Sequence (5'→3')	Tm (ºC)
fmk1	oliFmk1 (forward)	Di Pietro *et al*. (2001)	TGTCCCGATCGAACCCCCC	66.55
	OliFmkA (forward)	Designed in this study	CAACAGCTTCATCTCTCGCA	60.40
	oliFmk2 (reverse)	Di Pietro *et al*. (2001)	CTGTTACCTCATAATCTCCTGG	60.81
	OliFmkB (reverse)	Designed in this study	ACCATTTGACCGCTAGACCA	60.40
Pg1	Upper PG (forward)	Posada *et al*. (2000)	ATCTGGCCATGTCATTGA	62.18
	Lower PG (reverse)	Posada *et al*. (2000)	GGTCGGCTTTCCAGTAGG	55.34
xyl3	*Xyl3*- sense (forward)	Ruiz-Roldán *et al*. (1999)	TTTTTCGGTTCTCCTCGCTCTCGC	66.28
	Xyl3-antisense (reverse)	Ruiz-Roldán *et al*. (1999)	ACTCTCGTGGGACCTAAACC	67.98

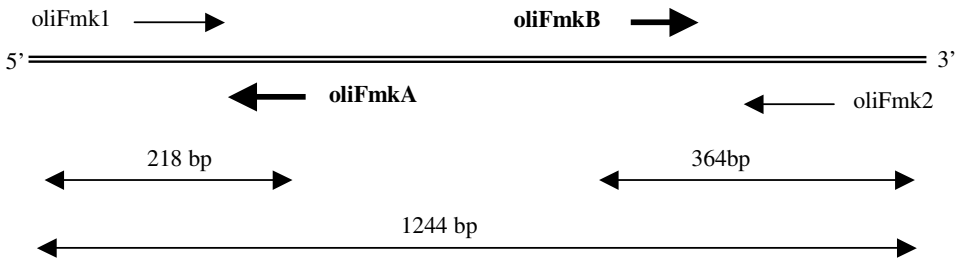


Figure 1. Amplification of the *fmk1* region in *Fusarium oxysporum*. oliFmk1 / oliFmk2 produced the full length gene of 1244 bp. oliFmk1 / oliFmkA produced a 218 bp fragment containing the 5' end of the *fmk1* gene. oliFmk2 / oliFmkB produced a 364 bp fragment containing the 3' end of the *fmk1* gene.

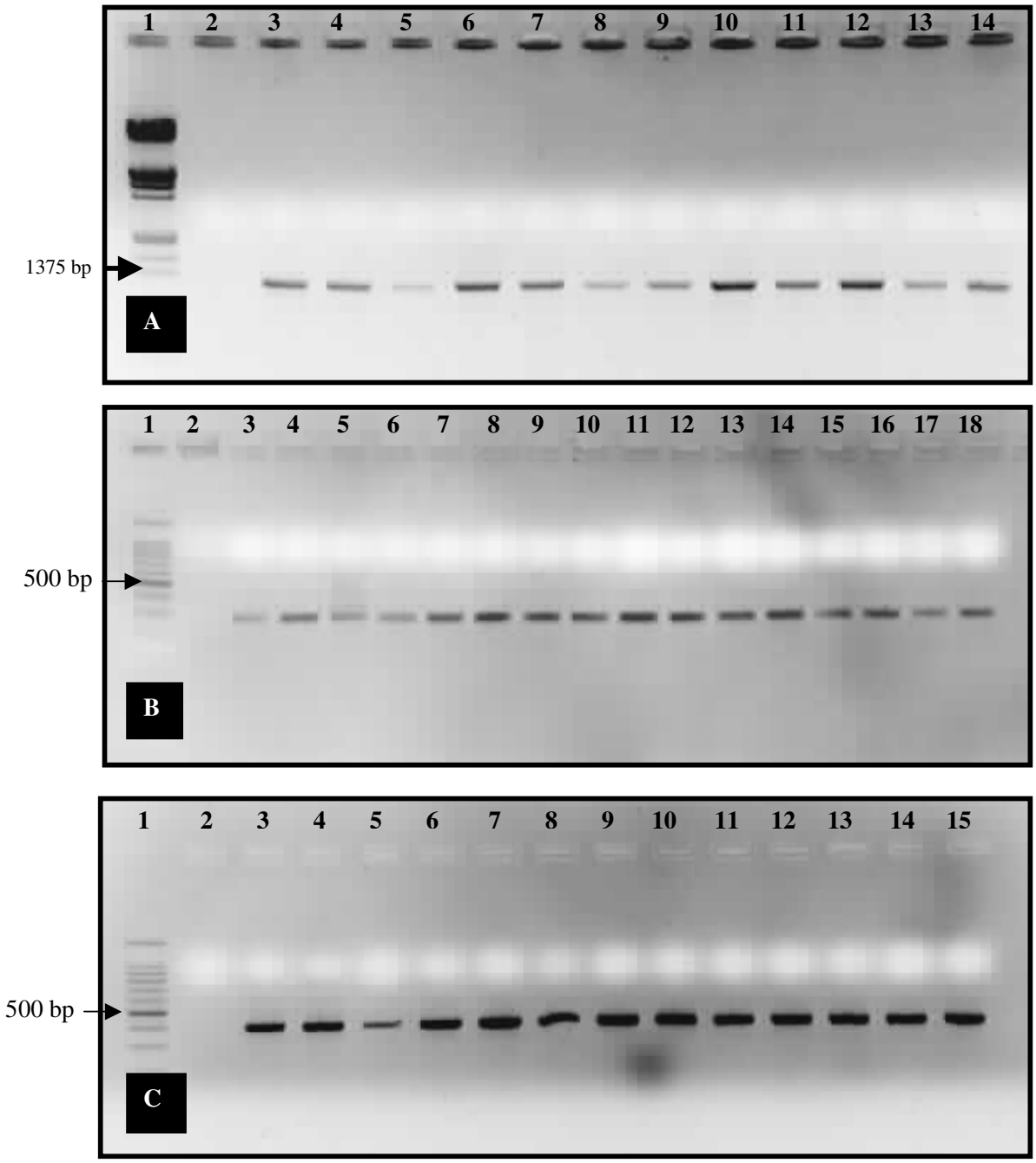


Figure 2. PCR amplification products of *Fusarium oxysporum* f.sp. *cubense* isolates representing A: full length of *fmk1* gene (1244 bp), B: 5' end of *fmk1* gene (220 bp), C: 3' end of *fmk1* gene (364 bp). A: Lane 1: λ DNA molecular weight marker 3, 2: negative control, 3: CAV 291, 4: CAV 298, 5: 23631, 6: RP CR1-1, 7: PHIL 10, 8: PHIL 6, 9: RP 51, 10: INDO 25, 11: F9 130, 12: MAL 32, 13: RPML 25, 14: INDO 47 and 15: 23532. B: Lane 1: 100 bp molecular weight marker, 2: negative control, 3: 23631, 4: PHIL 10, 7:

PHIL 6, 6: RP 51, 7: INDO 25, 8: F9 130, 9: 23532, 10: THAI 13, 11: 8605, 12: 24218, 13: RP 58, 14: MAL 5, 15: MAL 7, 16: INDO 5, 17: RP MW 40 and 18: 23707. C: Lane 1: 100 bp molecular marker, 2: negative control, 3: F9 130, 4: MAL 32, 5: RPML 25, 6: INDO 47, 7: 23532, 8: THAI 13, 9: 8605, 10: 22993; 11: 24218, 12: RP 58, 13: MAL 5, 14: 23509 and 15: MAL 7.

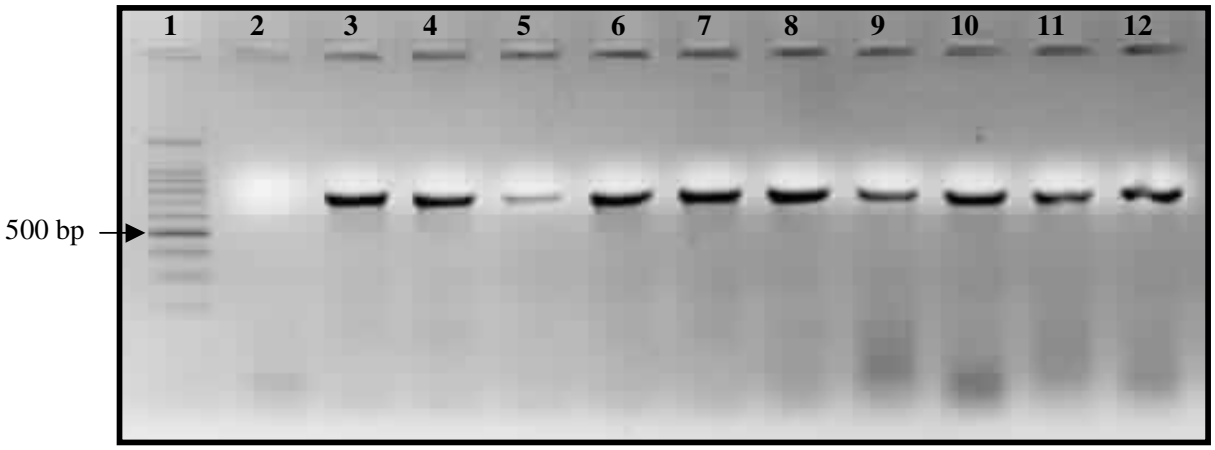


Figure 3. PCR amplification products of *Fusarium oxysporum* f.sp. *cubense* representing the *pg1* gene (740 bp fragment). Lane 1: 100 bp molecular weight marker, 2: negative control, 3: CAV 002, 4: CAV 009, 5: CAV 092, 6: CAV 291, 7: CAV 298, 8: 23631, 9: RP CR1-1, 10: PHIL 10, 11: PHIL 6 and 12: RP 51.

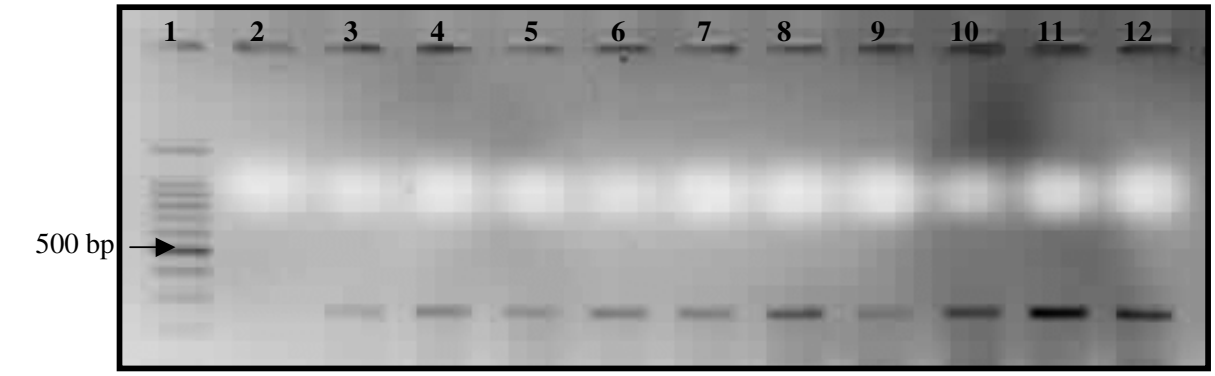


Figure 4. PCR amplification products of *Fusarium oxysporum* f.sp. *cubense* representing the *xyl3* gene region (260 bp fragment). Lane 1: 100 bp molecular weight marker, 2: negative control, 3: CAV 291, 4: CAV 298, 5: 23631, 6: RP CR1-1, 7: PHIL 10, 8: PHIL 6, 9: RP 51, 10: INDO 25, 11: F9 130 and 12: MAL 32.

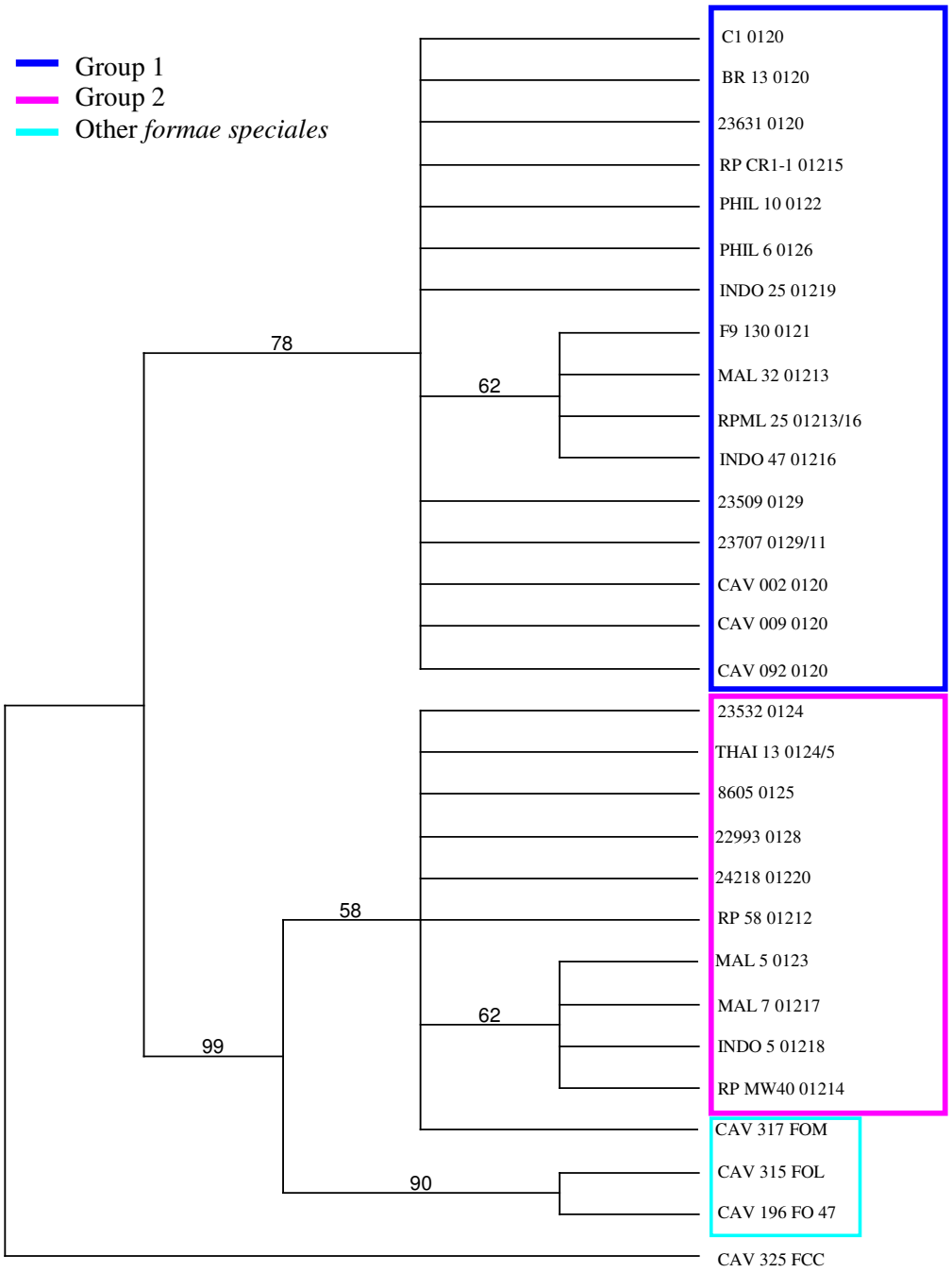


Figure 5. Bootstrap tree of the *Fusarium oxysporum* isolates based on *fmk1* sequence analysis. Distance analysis included Neighbour-joining as a tree building algorithm and a bootstrap of a 1000 replicates for confidence support. Bootstrap values are indicated above the nodes.

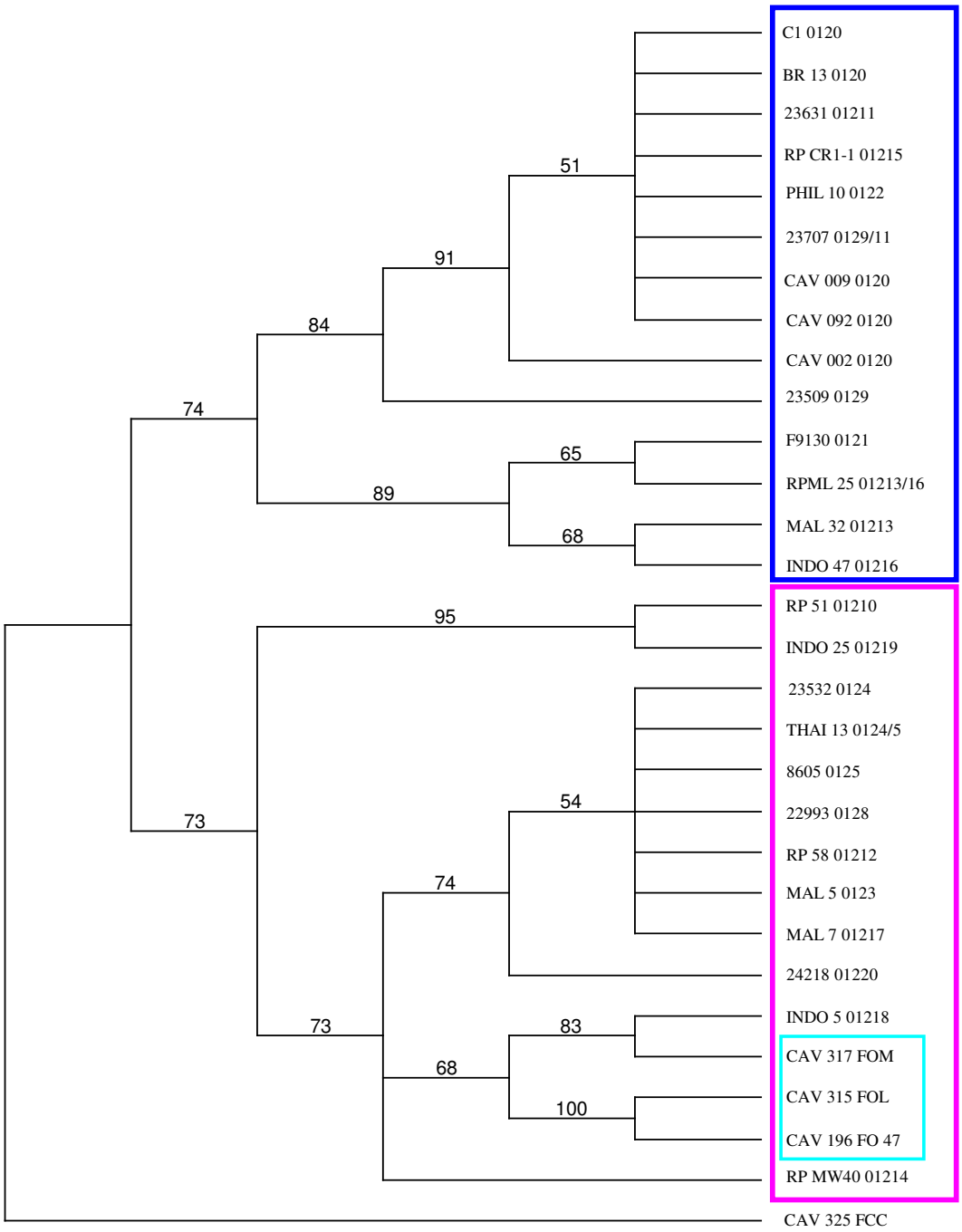


Figure 6. Bootstrap tree of *Fusarium oxysporum* isolates based on *pg1* gene sequence analysis. Distance analysis included Neighbour-joining as a tree building algorithm and a bootstrap of a 1000 replicates for confidence support. Bootstrap values are indicated above nodes.

```
                    10         20         30         40         50

23509_0129    MSRSNPPNAA GSRKISFNVS EQYDIQDVVG EGAYGVVSSA IHKPSGQKVA
CAV009_0120   MSRSNPPNAA GSRKISFNVS EQYDIQDVVG EGAYGVVSSA IHKPSGQKVA
AF286533Fol   MSRSNPPNAA GSRKISFNVS EQYDIQDVVG EGAYGVVCSA IHKPSGQKVA

              ....|....| ....|....| ....|....| ....|....| ....|....|
                       60         70         80         90        100

23509_0129    IKKITPFDHS MFCLRTLREM KLLRYFNHEN IISILDIQKP RNYESFNEVY
CAV009_0120   IKKITPFDHS MFCLRTLREM KLLRYFNHEN IISILDIQKP RNYESFNEVY
AF286533Fol   IKKITPFDHS MFCLRTLREM KLLRYFNHEN IISILDIQKP RNYESFNEVY

              ....|....| ....|....| ....|....| ....|....| ....|....|
                      110        120        130        140        150

23509_0129    LIQELMETDM HRVIRTQDLS DDHCQYFFYQ TLRDLKALHS ANVLHRDLKP
CAV009_0120   LIQELMETDM HRVIRTQDLS DDHCQYFIYQ TLRALKAMHS ANVLHRDLKP
AF286533Fol   LIQELMETDM HRVIRTQDLS DDHCQYFIYQ TLRALKAMHS ANVLHRDLKP

              ....|....| ....|....| ....|....| ....|....| ....|....|
                      160        170        180        190        200

23509_0129    SNLLLNANCD LKVCDFGLAR SAASQEDNSG FMTEYVATRW YRAPEIMLTF
CAV009_0120   SNLLLNANCD LKVCDFGLAR SAASQEDNSG FMTEYVATRW YRAPEIMLTF
AF286533Fol   SNLLLNANCD LKVCDFGLAR SAASQEDNSG FMTEYVATRW YRAPEIMLTF

              ....|....| ....|....| ....|....| ....|....| ....|....|
                      210        220        230        240        250

23509_0129    KEYTKAIDVW SVGCILAEML SGKPLFPGKD DHHQLTLILD VLGTPTMEDY
CAV009_0120   KEYTKAIDVW SVGCILAEML SGKPLFPGKD DHHQLTLILD VLGTPTMEDY
AF286533Fol   KEYTKAIDVW SVGCILAEML SGKPLFPGKD YHHQLTLILD VLGTPTMEDY

              ....|....| ....|....| ....|....| ....|....| ....|....|
                      260        270        280        290        300
23509_0129    YGIKSRRARE YIRSLPFKKK VPFRTLFPKT SDLALDLLEK LLAFNPVKRI
CAV009_0120   YGIKSRRARE YIRSLPFKKK VPFRTLFPKT SDLALDLLEK LLAFNPVKRI
AF286533Fol   YGIKSRRARE YIRSLPFKKK VPFRTLFPKT SDLALDLLEK LLAFNPVKRI

              ....|....| ....|....| ....|....| ....|....| ....|....|
                      310        320        330        340        350

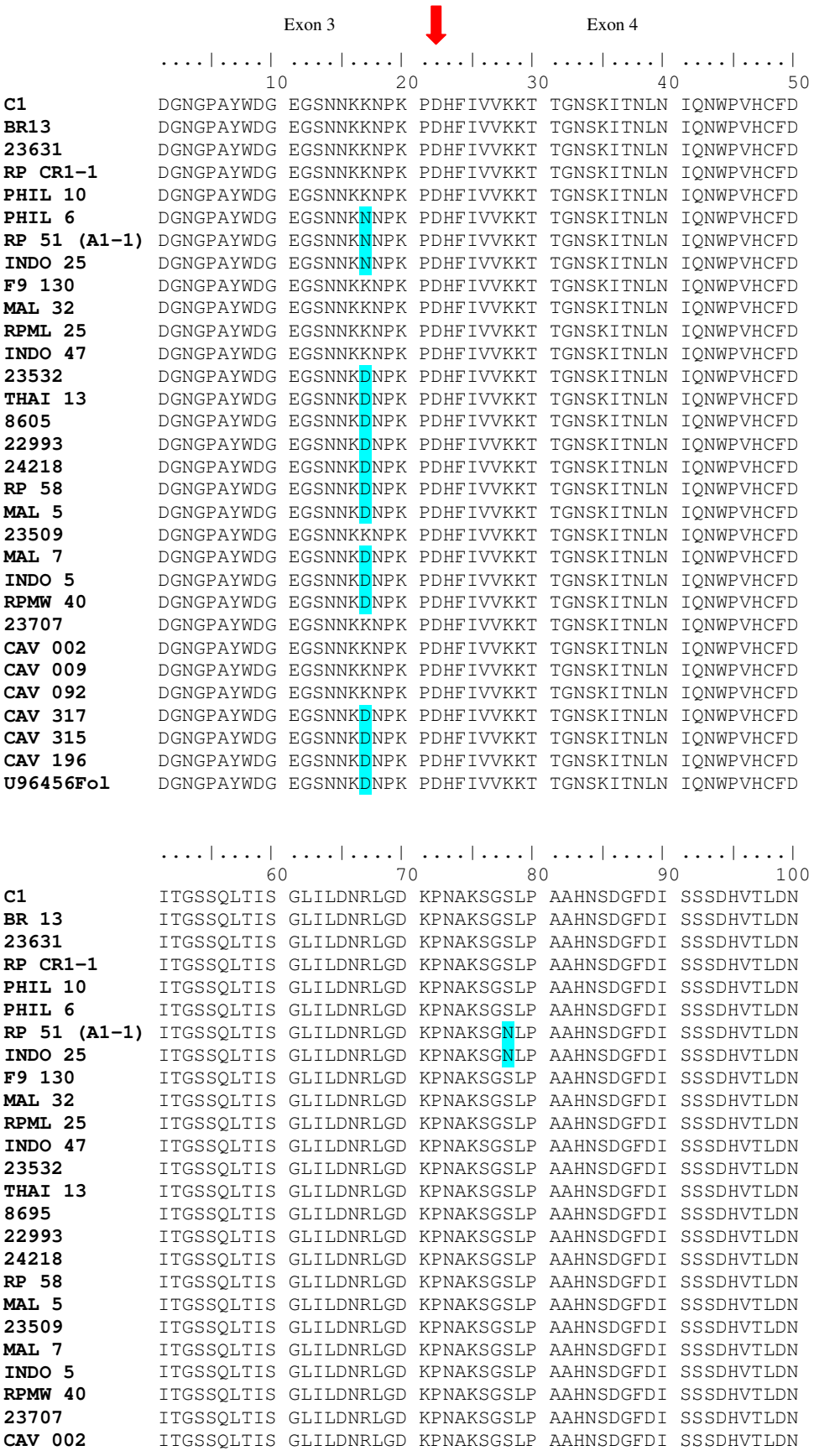
23509_0129    TVEEALKHPY LEPYHDPEDE PTAPPIPEEF FDFDKHKDNL SKEQLKQLIY
CAV009_0120   TVEEALKHPY LEPYHDPEDE PTAPPIPEEF FDFDKHKDNL SKEQLKQLVY
AF286533Fol   TVEEALKHPY LEPYHDPEDE PTAPPIPEEF FDFDKHKDNL SKEQLKQLIY

              ....|

23509_0129    QEI
CAV009_0120   QEI
AF286533Fol   QEI
```

■ Amino acids at certain positions different from the rest of the *F. oxysporum* isolates

Figure 7. Amino acid (aa) sequence alignment of two *Fusarium oxysporum* f.sp. *cubense* isolates that had different aa at certain positions compared with *F. oxysporum* f.sp. *lycopersici* from GenBank (AF 286533).


Exon 3
Exon 4
10 20 30 40 50
C1 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
BR13 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
23631 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
RP CR1-1 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
PHIL 10 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
PHIL 6 DGNGPAYWDG EGSNNKNNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
RP 51 (A1-1) DGNGPAYWDG EGSNNKNNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
INDO 25 DGNGPAYWDG EGSNNKNNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
F9 130 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
MAL 32 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
RPML 25 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
INDO 47 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
23532 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
THAI 13 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
8605 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
22993 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
24218 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
RP 58 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
MAL 5 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
23509 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
MAL 7 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
INDO 5 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
RPMW 40 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
23707 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
CAV 002 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
CAV 009 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
CAV 092 DGNGPAYWDG EGSNNKKNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
CAV 317 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
CAV 315 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
CAV 196 DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
U96456Fol DGNGPAYWDG EGSNNKDNPK PDHFIVVKKT TGNSKITNLN IQNWPVHCFD
60 70 80 90 100
C1 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
BR 13 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
23631 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
RP CR1-1 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
PHIL 10 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
PHIL 6 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
RP 51 (A1-1) ITGSSQLTIS GLILDNRLGD KPNAKSGNLP AAHNSDGFDI SSSDHVTLDN
INDO 25 ITGSSQLTIS GLILDNRLGD KPNAKSGNLP AAHNSDGFDI SSSDHVTLDN
F9 130 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
MAL 32 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
RPML 25 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
INDO 47 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
23532 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
THAI 13 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
8695 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
22993 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
24218 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
RP 58 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
MAL 5 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
23509 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
MAL 7 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
INDO 5 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
RPMW 40 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
23707 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
CAV 002 ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN

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Cav 009       ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
CAV 092       ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
CAV 317       ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
CAV 315       ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
CAV 196       ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN
U96456Fol     ITGSSQLTIS GLILDNRLGD KPNAKSGSLP AAHNSDGFDI SSSDHVTLDN

                     ....|....| ....|....| ....|....| ....|....| ....|....|
                             110        120        130        140        150
C1            IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
BR 13         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
23631         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
RP CR1-1      IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
PHIL 10       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
PHIL 6        IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
RP 51 (A1-1)  IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
INDO 25       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
F9 130        IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
MAL 32        IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
RPML 25       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
INDO47        IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
23532         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
THAI13        IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
8605          IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
22993         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
24218         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
RP 58         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
MAL 5         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
23509         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
MAL 7         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
INDO 5        IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
RPMW 40       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
23707         IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
CAV 002       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSMGSVGGKS NNVVNGVQFL
CAV 009       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
CAV 092       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
CAV 317       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
CAV 315       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
CAV 196       IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL
U96456Fol     IHVYNQDDCV AVTSGTNIIV SNMYCSGGHG LSIGSVGGKS NNVVNGVQFL

                                                Exon 5
                     ....|....| ....|....| ....|....| ....|....| ....|....|
                             160        170        180        190        200
C1            DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
BR 13         DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
23631         DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
RP CR1-1      DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
PHIL 10       DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
PHIL 6        DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
RP 51 (A1-1)  DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
INDO 25       DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
F9 130        DSQIVNSENG CRIKSNSGTT GTIENVTYQN IALTNISKYG VDVQQDYLNG
MAL 32        DSQIVNSENG CRIKSNSGTT GTIENVTYQN IALTNISKYG VDVQQDYLNG
RPML 25       DSQIVNSENG CRIKSNSGTT GTIENVTYQN IALTNISKYG VDVQQDYLNG
INDO 47       DSQIVNSENG CRIKSNSGTT GTIENVTYQN IALTNISKYG VDVQQDYLNG
23532         DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
THAI 13       DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
8605          DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
22993         DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
24218         DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
RP 58         DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
MAL 5         DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
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23509          DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
MAL 7          DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
INDO 5         DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
RPMW 40        DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
23707          DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
CAV 002        DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
CAV 009        DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
CAV 092        DSQIVNSENG CRIKSNSGTT GTIANVTYQN IALTNISKYG VDVQQDYLNG
CAV 317        DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
CAV 315        DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
CAV 196        DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG
U96456Fol      DSQIVNSENG CRIKSNSGTT GTIANVTYQN ISLTNISKYG VDVQQDYLNG

               ...

C1             GPT
BR 13          GPT
23631          GPT
RP CR1-1       GPT
PHIL 10        GPT
PHIL 6         GPT
RP 51 (A1-1)   GPT
INDO 25        GPT
F9 130         GPT
MAL 32         GPT
RPML 25        GPT
INDO 47        GPT
23532          GPT
THAI 13        GPT
8605           GPT
22993          GPT
24218          GPT
RP 58          GPT
MAL 5          GPT
23509          GPT
MAL 7          GPT
INDO 5         GPT
RPMW 40        GPT
23707          GPT
CAV 002        GPT
CAV 009        GPT
CAV 092        GPT
CAV 317        GPT
CAV 315        GPT
CAV 196        GPT
U96456Fol      GPT
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Amino acids at certain positions different from the rest of the *F. oxysporum* isolates

Figure 8. Amino acid (aa) sequence alignment of *pg1* gene region of all *Fusarium oxysporum* isolates compared with *F. oxysporum* f.sp. *lycopersici* from GenBank (U 96456).

SUMMARY

Fusarium oxysporum Schlecht. causes vascular wilt diseases to many economically important agricultural crops. The fungus is taxonomically complex, and consists of many pathogenic and non-pathogenic forms that are morphologically similar. One of the most important pathogenic forms is *F. oxysporum* f.sp. *cubense* (EF Smith) Snyd. and Hans (*Foc*), the causal agent of Fusarium wilt of banana. *Foc* has a worldwide distribution and consists of three races (1, 2 and 4) and 21 vegetative compatibility groups (VCGs). Race 4 is divided into 'tropical' and 'subtropical' isolates, dependant on their ability to cause disease to Cavendish bananas in the tropics and subtropics, respectively. In *Foc*, the same race includes several DNA fingerprinting groups and represents a number of VCGs. In this thesis, the diversity in *Foc* has been investigated by means of phenotypic and genotypic analysis. An attempt was made to develop a molecular marker for VCG 0120, the most widely distributed VCG of the pathogen in the world, and to understand virulence in the fungus.

An important finding in this study was that the genotypically uniform population of *Foc* 'subtropical' race 4 (VCG 0120) is phenotypically diverse. This study provided the first evidence that certain genetically uniform isolates of *Foc* caused more severe disease to Cavendish bananas than others under controlled environmental conditions. Variation in fungal virulence to Cavendish bananas does not appear to be a function of growth tempo and/or sporulation, but could rather be due to the influence of other factors such as the production of toxins and suppression of plant defence responses. The finding that *Foc* 'subtropical' race 4 isolates showed optimal growth at 25ºC, supports previous views that the increased disease incidence on Cavendish bananas in the subtropics is primarily a function of the banana plant being more vulnerable to infection under winter temperatures, rather than the pathogen becoming more competitive. *Foc* grew better on nitrate medium than on ammonium medium *in vitro*, which does not reflected Fusarium wilt development in the field. The more pronounced disease development in soils fertilized with NH_4-nitrogen, compared to NO_2-nitrogen, is because nitrate causes an increase in pH and ammonium a decrease. Fusarium wilt is associated with acidic soils rather than with alkaline soil.

Previous studies gave a good indication of the diversity of the worldwide population of *Foc*, but did not always agree in terms of genetic relationships among clonal lineages of this pathogen. Amplified fragment length polymorphism (AFLP) analysis of *Foc* isolates supported previous findings that divided a worldwide population of *Foc* into two major clades, but gave higher resolution within clades. It further suggests that the current race designation cannot be considered accurate, since race 1 and race 4 isolates grouped together. These isolates are currently assigned to different races based on their pathogenicity to Cavendish bananas under field conditions. Another important finding was that VCG 0121, previously considered to belong to 'subtropical' race 4, grouped closer to isolates of VCGs 01213 and 01216 that belong to 'tropical' race 4 than other 'subtropical' race 4 isolates. This suggests that the Cavendish banana variety 'GCTCV 218' that proofed to be tolerant to *Foc* VCG 0121 in Taiwan, could also be tolerant to VCGs 01213 and 01216, the 'tropical' race 4 currently causing devastating losses to Cavendish bananas in Malaysia, Indonesia and northern Australia.

AFLP analysis provided sufficient polymophisms among VCGs of *Foc* for conversion to simple single locus markers. In an effort to develop a VCG 0120-specific marker, a DNA fragment was isolated and analysed for single nucleotide polymorphisms (SNPs) that could potentially be developed into sequence characterised amplified region (SCAR) or cleaved amplified polymorphic site (CAPS) markers. Due to problems arising during the cloning of the excised AFLP fragment, the original polymorphism was lost. The conversion from AFLP marker to a sequence-specific PCR or PCR-RFLP marker could, therefore, not be achieved in this study. SNPs were found in the flanking regions of the AFLP fragment that could potentially distinguish between *Foc* VCG 0120 and other VCGs of *Foc*.

Three virulence-associated genes, *fmk1*, *pg1* and *xyl3* were found in *Foc*, but were not unique to the banana pathogen. Sequence analysis of *fmk1* and *pg1* from a diverse range of *F. oxysporum* isolates proved that these genes were useful in phylogenetic analysis of a worldwide *Foc* population. They can also be applied for comparative analysis of *formae speciale*s within *F. oxysporum*. From the high similarity in amino acid sequences among *fmk1*, *pg1* and *xyl3* genes in *F. oxysporum* pathogenic and non-

pathogenic to banana, it can be concluded that these genes play a limited role in host specificity.