EVOLUTION AND DETECTION OF *FUSARIUM OXYSPORUM* F.SP. *CEPAE* IN ONION
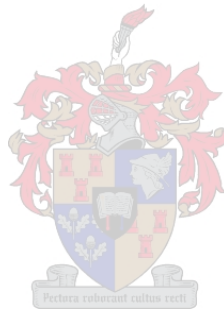
MICHAEL J. SOUTHWOOD

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Supervisor: Dr. A. McLeod

Co-supervisor: Prof. A. Viljoen

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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Michael John Southwood   Date

SUMMARY

In the Western Cape onion industry in South Africa, *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *cepae* (H.N. Hans.) W.C. Snyder & H.N. Hans. (*Focep*) has been identified as the leading cause of harvest and storage losses. This pathogen is of world-wide importance and causes Fusarium basal rot of onions (*Allium cepa*), affecting all onion growth stages. No information is available on the evolution, genetic diversity, molecular detection and inoculum sources of the South African *Focep* population.

Similar to what is the case for South Africa, limited information is available on *Focep* in other regions of the world. World-wide, four vegetative compatibility groups (VCGs) and two single-member VCGs (SMVs) have been identified among two Japanese and 19 Colorado (USA) isolates. This polyphyletic origin of *Focep* suggested by VCG analyses was confirmed through molecular analyses of isolates from a few countries. Only the mating type (*MAT*)*1-1* idiomorph has been reported for *Focep* isolates from Welsh onion (*Allium fistulosum*).

The development of sustainable management strategies of *Focep* is dependent on knowledge of (i) the genetic diversity and evolution of *Focep,* (ii) whether high throughput molecular methods can be developed for identifying the most virulent and widespread *Focep* genotypes and (iii) the role of seedlings and seeds as primary inoculum sources, and the *Focep* genotypes associated with these growth stages. Therefore, the three main aims of the current study were to investigate the aforementioned three aspects.

In the first aim of the study, the genetic diversity and evolution of *Focep* was investigated using a collection of 79 *F. oxysporum* isolates from South Africa (27 *Focep* and 33 non-pathogenic isolates) and Colorado (19 *Focep* isolates). VCG analyses revealed the presence of six VCGs, four among the Colorado *Focep* isolates (VCGs 0421, 0422, 0423 and 0424) and two among the South African bulb-associated isolates (VCGs 0425 and 0426). VCG 0421 and VCG 0425 were the two main VCGs in Colorado and South Africa, respectively. Four SMVs and one heterokaryon self-incompatible (HSI) isolate were also identified. The polyphyletic nature of *Focep* in

South Africa and Colorado was shown through a combined translation elongation factor 1α (EF-1α) and mitochondrial small-subunit (mtSSU) phylogeny. The phylogeny divided the *Focep* isolates into two main clades, of which one contained the two main VCGs (0421 and 0425), SMVs and non-pathogenic isolates. The second, ancestral clade contained the HSI isolate, VCGs 0422, 0423 and 0424, and non-pathogenic isolates. Unlike the clade containing the two main VCGs, which were highly virulent toward onion bulbs, the ancestral clade contained isolates that were mostly moderately virulent. The incongruence of the EF-1α and mtSSU datasets with an intergenic spacer (IGS) region data set, and the presence of both *MAT* idiomorphs within the same isolate for some isolates, suggested possible exchange of genetic material between isolates.

The second aim of the study was to develop molecular methods for identifying the two main *Focep* VCGs (0425 and 0421), using DNA fingerprinting methods and sequence-characterized amplified region (SCAR) markers. These techniques were first developed using the *F. oxysporum* isolates from the first aim, and were then used to investigate the prevalence of VCG 0425 among 88 uncharacterized *F. oxysporum* isolates from onion bulbs in South Africa. Two random amplified polymorphic DNA primers provided two diagnostic amplicons for VCG 0425, but attempts to develop SCAR markers from these amplicons were unsuccessful. In contrast, an inter-retrotransposon amplified polymorphism (IRAP) fingerprinting method enabled the developed of a multiplex IR-SCAR polymerase chain reaction method that detected the VCG 0421, 0425 and SMV 4 isolates as a group. Fingerprinting and SCAR marker testing of the 88 uncharacterized *F. oxysporum* isolates from South Africa (65 *Focep* and 23 non-pathogenic) confirmed that VCG 0425 is the main VCG in South Africa associated with mature onion bulbs, since 63 of the *Focep* isolates had the molecular characteristics of VCG 0425.

The third aim of the study was to determine whether seed and seedling transplants are inoculum sources of *Focep,* and whether the same genotype (VCG 0425) that dominated on mature bulbs could be detected from these sources. *Focep* isolates were obtained from seven of the 13 investigated onion seed lots, as well as from onion seedling transplants that were collected from all five onion nurseries in the Western Cape. *Focep* seedling infection more than doubled from the 6-week growth

stage to the 14-week growth stage. Seed infections by *Focep* were low, but the seedborne nature of *Focep* was confirmed by showing that a green fluorescent protein labelled *Focep* transformant could be transmitted from infected soil to onion seed via the onion bulbs and seedstalks. It is thus clear that commercial seed and seedlings are inoculum sources of *Focep*. However, the *Focep* genotypes on seed and seedlings are different from those in mature bulbs and were not dominated by VCG 0425. Furthermore, most ($\leq$ 60%) of the seed and seedling isolates were moderately virulent, as compared to the mostly highly virulent isolates from mature bulbs.

OPSOMMING

In die Wes-Kaapse uiebedryf in Suid-Afrika is *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *cepae* (H.N. Hans.) W.C. Snyder & H.N. Hans. (*Focep*) geïdentifiseer as die vernaamste oorsaak van oes- en opbergingsverliese. Hierdie patogeen is van wêreldwye belang; dit veroorsaak Fusarium-bolvrot van uie (*Allium cepa*) en affekteer alle plantgroeistadia. In Suid-Afrika is daar geen inligting beskikbaar oor die evolusie, genetiese diversiteit, molekulêre opsporing en inokulumbronne van die *Focep*-populasie nie.

Soortgelyk aan wat die geval in Suid-Afrika is, is daar beperkte inligting beskikbaar oor *Focep* in ander wêrelddele. Wêreldwyd is daar vier vegetatiewe versoenbaarheidsgroepe (VVGe) en twee enkellid VVGe (ELVe) geïdentifiseer onder twee Japannese en 19 Colorado (VSA) isolate. Hierdie veelvuldige oorsprong van *Focep* wat deur VVG-analise voorgestel was, is deur die molekulêre analises van isolate uit 'n paar ander lande bevestig. Slegs die paringstipe (*PT*)*1-1* idiomorf is vir *Focep*-isolate uit Walliese-tipe uie (ook bekend as 'lenteuie' in Suid Africa) (*Allium fistulosum*) berig.

Die ontwikkeling van volhoubare bestuurstrategieë vir *Focep* steun op kennis van (i) die genetiese diversiteit en evolusie van *Focep,* (ii) of hoë-deurset molekulêre metodes ontwikkel kan word vir die identifisering van die mees virulente en wydverspreide *Focep*-genotipes en (iii) die rol van saailinge en saad as primêre inokulumbronne, en die *Focep*-genotipes wat met hierdie groeistadia geassosieer word. Daarom was die hoof doelstellings van hierdie studie om die bogenoemde drie aspekte te bestudeer.

Om die eerste doel van die studie te bereik is die genetiese diversiteit en evolusie van *Focep* bestudeer deur gebruik te maak van 'n versameling van 79 *F. oxysporum*-isolate uit Suid-Afrika (27 *Focep* en 33 nie-patogeniese isolate) en uit Colorado (19 *Focep*-isolate). VVG-analises het die teenwoordigheid van ses VVGe aangetoon – vier onder die Colorado *Focep*-isolate (VVGe 0421, 0422, 0423 en 0424) en twee onder die Suid-Afrikaanse bol-geassosieerde isolate (VVGe 0425 en 0426). VVG 0421 en VVG 0425 was die twee hoof VVGe in onderskeidelik Colorado en

Suid-Afrika. Vier ELVe en een meerkernige self-onversoenbare (MSO) isolaat is ook geïdentifiseer. Die veelvuldige oorsprong van *Focep* in Suid-Afrika en Colorado is ook aangetoon deur 'n gekombineerde translasie verlengings faktor 1α (VF-1α) en mitokondriale klein-subeenheid (mtKSE) filogenie. Dié filogenie het die *Focep*-isolate in twee groepe verdeel, waarvan die een groep die twee hoof VVGe (0421 en 0425), ELVe en nie-patogeniese isolate bevat het. Die tweede, basal groepering het die MSO-isolaat, VVGe 0422, 0423 en 0424, en nie-patogeniese isolate bevat. In teenstelling met die eersgenoemde groepering wat hoogs virulente isolate van uiebolle bevat het, het die basale groepering isolate bevat wat meestal matig virulent was. Die inkongruensie van die VF-1α en mtKSE-datastelle met 'n intergeen-gespasieerde (IGS) area datastel – asook die teenwoordigheid van beide PT-idiomorwe binne dieselfde isolaat by sommige isolate – het op 'n moontlike uitruiling van genetiese materiaal tussen isolate gedui.

Die tweede doel van die studie was om molekulêre metodes te ontwikkel vir die identifisering van die twee hoof *Focep* VVGe (0425 en 0421) deur gebruik te maak van DNA-vingerafdrukke en nukleotied-gekarakteriseerde geamplifiseerde area (NKAA) merkers. Hierdie tegnieke is ontwikkel deur van die *F. oxysporum*-isolate van die eerste doelstelling gebruik te maak en is daarna gebruik om die frekwensie van VVG 0425 onder 88 ongekarakteriseerde *F. oxysporum*-isolate van uiebolle in Suid-Afrika te ondersoek. Twee gerandomiseerde geamplifiseerde polimorfiese DNS (RAPD) merkers het twee diagnostiese nukleotiedbasis-areas vir VVG 0425 gelewer, maar pogings om NKAA-merkers uit hierdie geamplifiseerde nukleotiedbasis-areas te onwikkel was onsuksesvol. In teenstelling hiermee het 'n inter-retrotransposon geamplifiseerde polimorfisme (IRAP) vingerafdrukmetode die ontwikkeling van 'n multipleks IR-NKAA polimerase kettingreaksiemetode moontlik gemaak wat die VVG 0421-, VVG 0425- en ELV 4-isolate as 'n groep aangedui het. Vingerafdruktoetsing en NKAA-merkertoetsing van die 88 ongekaraktariseerde *F. oxysporum* isolate van Suid-Afrika (65 *Focep* en 23 nie-patogenies) het bevestig dat VVG 0425 die hoof VVG in Suid-Afrika is wat met volwasse bolle geassosieer word, aangesien 63 van die *Focep*-isolate die molekulêre eienskappe van VVG 0425 gehad het.

Die derde doel van die studie was om vas te stel of saad en saailinge inokulumbronne van *Focep* is, en of dieselfde genotipe (VVG 0425) wat op volwasse bolle dominant is, waargeneem kon word op hierdie bronne. *Focep*-isolate is verkry van sewe van die 13 uiesaadlotte asook van uiesaailinge wat in al vyf uiesaailingkwekerye in die Wes-Kaap versamel is. *Focep*-saailinginfeksie was meer as dubbel in die 14-week groeistadium as wat dit in die 6-week stadium was. Saadinfeksies deur *Focep* was laag, maar die saadgedraagde aard van *Focep* is bevestig deur aan te toon dat 'n *Focep*-transformant wat met 'n groen fluoreserende proteïen geëtiketeer is, van geïnfekteerde grond na uiesaad oorgedra kon word via die uiebolle en -saadstele. Dit is dus duidelik dat kommersiële saad en saailinge as inokulumbronne van *Focep* dien. Die *Focep*-genotipes op saad en saailinge verskil egter van dié in volwasse bolle en is nie deur VVG 0425 gedomineer nie. Verder was die meeste ($\leq 60\%$) saad- en saailingisolate matig virulent, in teenstelling met die meestal hoogs virulente isolate uit volwasse bolle.

ACKNOWLEDGEMENTS

CONTENTS

1. THE EVOLUTION, DETECTION AND EPIDEMIOLOGY OF *FUSARIUM OXYSPORUM* F.SP. *CEPAE* IN ONION

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important fresh vegetables in the world, covering 21.9% of all land planted to vegetables. In 2007, green and dried onions were grown on almost 3.8 million hectares worldwide, yielding an estimated 70.2 million tons at an average of 18.8 tons/ha (Food and Agricultural Organization of the United Nations, 2009). In South Africa, onion is also an important vegetable crop, with an estimated 7000 hectares cultivated on an annual basis (personal communication, J. van Zijl, Hygrotech Seed, South Africa). The first onions were cultivated in South Africa by the survivors of the Haarlem shipwreck in 1647, and favorable reports of its production contributed to the decision made by the Dutch to start a colony in the Cape (Van Rooyen and Comrie, 1995).

In South Africa, a large number of onion varieties are grown as seed crops (used for the production of seed) and bulb crops (for fresh market consumption). Onion bulb crops are established using either direct sowing of seed or onion transplants produced by nurseries. The method of crop establishment differs for production regions. For example, in the Western Cape Province, most bulb crops are established using transplants, whereas growers in the rest of the country make use of mainly direct sowing. The amount of onion seed used in South Africa per planting season is estimated at 33.4 tons. This comprises 15.4 tons of seed for direct sowing purposes and 18 tons for transplants. A wide range of open-pollinated and hybrid onion varieties is available for direct sowing and transplant production on the local market, with 36 open-pollinated and 45 hybrid varieties currently registered in South Africa. Of these, 50 are short-day varieties and 31 intermediate-day varieties.

World-wide, one of the constraints in onion production is Fusarium basal rot of onion, caused by *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *cepae* (H.N. Hans.) W.C. Snyder & H.N. Hans (*Focep*) (Schwartz and Mohan, 2008). This pathogen has been reported from several regions in the world, including the Netherlands, Uruguay,

Japan, Turkey, the United Kingdom and the USA. *Focep* causes both field and storage losses. Significant losses, up to 50%, have been reported from several areas in the world where onions are cultivated extensively (Lacy and Roberts, 1982; Everts *et al.*, 1985; Cramer, 2000; Özer *et al.*, 2002; Swift *et al.*, 2002; Koike *et al.*, 2007; Galván *et al.*, 2008, Dissanayake *et al.*, 2009a, b).

In South Africa, Doidge *et al.* (1953) was the first to report *Focep* as the causal agent of onion basal rot. Subsequently, the majority of Fusarium basal rot losses have been reported from the Western Cape Province, since this is the only area in South Africa where onions are grown and harvested in the summer months. Harvest and storage losses are particularly severe when hot and dry conditions prevail during spring. The first report of serious *Focep* onion losses occurring on certain farms in the Caledon-Riviersonderend district in this province appeared in 1973 (Holz, 1973; Holz and Knox-Davies, 1976). Today, the main Western Cape onion production area is the Koue Bokkeveld, where the first noticeable losses due to Fusarium basal rot were reported in the early 1990s. The disease continues to be a problem in the Koue Bokkeveld where field and storage losses of 15% and 25%, respectively, have been reported from 2004 to 2006 (M. Smit, Du Toit Vegetables, South Africa, personal communication).

Focep is one of more than 120 formae speciales of *F. oxysporum* that are identified according to their host range (Baayen *et al.*, 2000). In the past two decades, our understanding of the evolution and genetic diversity of some formae speciales and non-pathogenic *F. oxysporum* isolates has increased substantially (Gordon and Martyn, 1997; O'Donnell *et al.*, 1998; Baayen *et al.*, 2000; O'Donnell *et al.*, 2009). Increased knowledge on the evolution and genetic diversity of pathogenic *F. oxysporum* and non-pathogenic *F. oxysporum* isolates is important, since this can give an indication of the ability of the pathogen to adapt to changing management strategies. Furthermore, hypotheses could be formed on how pathogenicity is acquired, and what the potential risks may be of using non-pathogenic *F. oxysporum* isolates as biological control agents (Assigbetse *et al.*, 1994; Baayen *et al.*, 1998; Bruton and Damicone, 1999; Baayen *et al.*, 2000; Skovgaard *et al.*, 2001; Fravel *et al.*, 2003; Lievens *et al.*, 2008; O'Donnell *et al.*, 2009). Although *Focep* is an economically important pathogen of onions, very few studies have been conducted on

the evolution and genetic diversity of this pathogen. The available studies are limited to a vegetative compatibility (VCG) study by Swift *et al.* (2002) and multi-gene sequence analyses studies by Galván *et al.* (2008) and Dissanyake *et al.* (2009a, b), none of which included South African *Focep* isolates.

A small number of scientific studies have been conducted on *Focep* epidemiology, especially primary inoculum sources of *Focep* (Abawi and Lorbeer, 1971, 1972). Knowledge on primary inoculum sources is important, since pathogen-free plant material forms an integral part of integrated disease management strategies (Agrios, 2005). One factor contributing to the limited information on the epidemiology and inoculum sources of *Focep* is the difficulty in differentiating *Focep* from other non-pathogenic *F. oxysporum* isolates that are ubiquitous in soil and plant material. As such, molecular discrimination and detection methods that can rapidly differentiate *Focep* from non-pathogens will be helpful. These methods have only been developed for a few formae speciales and races, which does not include *Focep,* due to the time consuming and labour intensive nature of the development of such methods (Lievens *et al.*, 2008). Reporter gene-labeled *F. oxysporum* forma specialis transformants can also facilitate differentiation of pathogenic and non-pathogenic isolates within plant tissue under controlled environmental conditions (Nahalkova and Fatehi, 2003; Michielse and Rep, 2009).

The following literature review will firstly discuss evolutionary biological aspects and genetic diversity in *F. oxysporum,* with an emphasis on *Focep* and other bulb and corm rot formae speciales. Secondly, the development and use of DNA-based detection systems for formae speciales of *F. oxysporum* will be elaborated on, followed by a brief discussion on the potential use of reporter-labeled fungi for studying the ecology and infection of fungi. Thirdly, for *Focep*, epidemiological aspects such as inoculum sources, survival and spread will be discussed. Lastly, the different commercial control options available for Fusarium basal rot management will be highlighted.

THE GENUS *FUSARIUM*

Fusarium is an anamorphic genus within the phylum Ascomycota (Agrios, 2005). Because *Fusarium* is an important and mycotoxigenic fungal genus, it has received much attention with regard to its identification systems (Geiser *et al.*, 2004). The need for accurate but simplistic identification of *Fusarium* species was identified as far back as 1935. The genus *Fusarium* was first properly classified when Wollenweber and Reinking (1935) divided approximately 1000 described *Fusarium* species into 16 sections, reducing the number of species to 65. Snyder and Hansen (1941, 1945) developed a nine-species system for *Fusarium*, grouping them into sections that did not reflect the now known phylogenetic diversity. In 2004, over 150 phylogenetically and/or morphologically distinct species were accepted by *Fusarium* taxonomists (Seifert and Lévesque, 2004). The use of phylogenetic studies is important for *Fusarium* species identification efforts, as it provides an excellent framework for objective species recognition (Geiser *et al.*, 2004). More recently, Leslie and Summerell (2006) produced a laboratory manual in which 70 *Fusarium* species are described.

Due to limitations in morphological species recognition, and incorrect and confusing application of *Fusarium* species names, Geiser *et al*. (2004) generated a database containing phylogenetically diverse translation elongation factor 1-alpha gene (EF-1α) sequences from the genus *Fusarium*. This database, called FUSARIUM-ID v. 1.0, can be accessed at http://fusarium.cbio.psu.edu, and is useful for identifying *Fusarium* species.

FUSARIUM OXYSPORUM

In the early years of *Fusarium* species identification, Wollenweber and Reinking (1935) placed *F. oxysporum* into the section (*Gruppe*) Elegans along with nine other species in three subsections (*Untergruppen*). Subsequently, Snyder and Hanson (1940) collapsed section Elegans into the single species, *F. oxysporum,* due to the morphological divisions within section Elegans being small and highly variable (Nelson, 1991). Although this consolidation has received wide acceptance, it is well

acknowledged that *F. oxysporum* is comprised of a wide diversity of strains. However, the extent to which differences are sufficient to justify different species within *F. oxysporum* is an ongoing debate (Gordon and Martyn, 1997).

Molecular phylogenetic analyses have emphasized the diversity within *F. oxysporum.* Multi-gene sequence phylogenies have previously identified three to four distinct clades that span the known breadth of diversity in *F. oxysporum* (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000; Mbofung *et al.,* 2007). More recently, O'Donnell *et al.* (2009) identified 254 unique two-locus haplotypes among 850 *F. oxysporum* isolates that were grouped into seven haplotype groups. Due to the well acknowledged diversity in *F. oxysporum,* it is often referred to as a species complex, i.e. as the *F. oxysporum* species complex or FOSC (O'Donnell *et al.*, 2009). *Fusarium oxysporum* is nested within the *Gibberella* clade, even though it has no known sexual stage (O'Donnell *et al.*, 1998; Leslie and Summerell, 2006).

Formae speciales and non-pathogenic isolates. *Fusarium oxysporum* isolates are ubiquitous soil-borne fungi that include pathogenic and non-pathogenic strains. In *F. oxysporum*, the forma specialis designation is used to distinguish host-specific pathogenic isolates from non-pathogenic isolates that are morphologically similar and indistinguishable from each other. Most *F. oxysporum* isolates commonly isolated from soil are non-pathogenic, and some are even biocontrol agents of pathogenic formae speciales strains (Fravel *et al.*, 2003). There are more than 120 formae speciales in *F. oxysporum* that attack different host plants (Baayen *et al.*, 2000; Fravel *et al.,* 2003). These pathogens, depending on their specific forma specialis, can cause vascular wilts, damping-off and several rots (root, stem, bulb, tuber and corm) in a large number of plant species (Nelson *et al.*, 1981; Baayen *et al.*, 2000; Vakalounakis *et al.*, 2005; Michielse and Rep, 2009). In addition to being plant pathogens, some *F. oxysporum* members are also clinically important, and can cause localized or deeply invasive life-threatening infections in humans and animals (Ortoneda *et al.,* 2004; O'Donnell *et al.*, 2007).

In agriculture, most of the *F. oxysporum* formae speciales cause economically important vascular wilts, but only eight formae speciales have been reported to cause rots of bulbs and corms. The latter formae speciales are common on plants such as

onion, lily and gladiolus. Some of the symptoms caused by bulb and corm rot formae speciales include brown to black discoloration of the basal plate, roots and scales, with bulbs and corms not showing outward symptoms at first. This might later change into a firm, dry rot. Above-ground parts often turn yellow and die prematurely (Agrios, 2005; Lievens *et al.,* 2008). The formae speciales that cause bulb and corm rots include *F. oxysporum* f.sp. *cepae* (onions), f.sp. *tulipae* Apt (tulip), f.sp. *gladioli* [Massey] Snyder & Hansen (gladiolus), f.sp. *croci* Boerema & Hamers (crocus), f.sp. *hyacinthi* Muller (hyacinthus), f.sp. *narcissi* Snyder & Hansen (narcissus), f.sp. *lilii* Imle (lily) and f.sp. *cyclaminis* Gerlach (cyclamen) (Boerema and Hamers, 1988; Roebroeck and Mes, 1992; Baayen *et al.,* 1998). The degree of specificity among members of this family is, however, questionable. For example, *F. oxysporum* f.sp. *tulipae* isolates have cross-pathogenicity to both gladiolus and lily in the *Iris* family (Baayen *et al.,* 1998).

Vegetative compatibility groups. Formae speciales of *F. oxysporum* are often classified into VCGs, where isolates that belong to the same VCG can form a heterokaryon through hyphal anastomosis. Buxton (1962) was the first to demonstrate heterokaryon formation between two mutants of *Fusarium* after ultra-violet irradiation of isolates. Subsequently, Puhalla (1985) demonstrated heterokaryon formation of nitrate non-utilizing (*nit*) mutants on minimal medium (Kistler, 1997). Today, this method is widely used for determining VCGs in *F. oxysporum.* VCG analyses in different formae speciales have shown that many formae speciales have more than 10 VCGs, of which only a few are common and widespread. However, there are a few formae speciales (*asparagi* and *opuntiarum*) that contain many VCGs of which none is dominant (Baayen *et al.,* 2000). The number of VCGs in bulb and corm rot formae speciales can vary from one (f.spp. *lilii* and *tulipae*) to five (f.sp. *gladioli*) (Baayen *et al.*, 2000).

Races. Some of the formae speciales of *F. oxysporum* have been subdivided into races, which are defined by the virulence of an isolate to a range of varieties (Correll, 1991; Abo *et al.*, 2005). For some formae speciales (e.g. f.sp. *dianthi*) up to 11 races have been identified, whereas for others, for example *Focep, F. oxysporum* f.sp. *lilii* and f.sp. *tulipae* (Jacobson and Gordon, 1991; Aloi and Baayen, 1993; Havey, 1995; Schreuder *et al*., 2000), no races are known.

GENETIC DIVERSITY AND EVOLUTION WITHIN *FUSARIUM OXYSPORUM* FORMAE SPECIALES

Knowledge on the genetic diversity and evolution of *F. oxysporum* formae speciales is important, since it can give an indication of the pathogen's potential to overcome management strategies, such as fungicide control and host resistance (Assigbetse *et al*., 1994; Bruton and Damicone, 1999). Genetic diversity in a pathogen population is also important for screening germplasm collections for potential resistance to all forms of the specific pathogen (Gordon and Martyn, 1997). Furthermore, evolutionary relationships can provide clues as to how pathogenicity traits are obtained and transferred in populations (Baayen *et al.*, 1998; Lievens *et al*., 2008). In *F. oxysporum,* genetic and evolutionary studies have mainly been aimed at developing hypotheses for understanding the evolution of different formae speciales, races and VCGs (Gordon and Martyn, 1997; Skovgaard *et al.*, 2001; Fourie *et al.*, 2009).

Investigations into the evolution and genetic diversity in *F. oxysporum* have shown that the evolution of formae speciales, races and VCGs can differ for each specific forma specialis. Multi-gene sequence phylogenies revealed that only a few formae speciales in *F. oxysporum* are monophyletic, i.e. derived from a common ancestor, whereas many are polyphyletic, thus having multiple independent origins (Baayen *et al.,* 2000). The latter calls into question the value of the 'forma specialis' nomenclature (O'Donnell *et al.*, 2009). The race and VCG relation of a forma specialis is complex and differs from one forma specialis to the next. Consequently, the processes by which these races and VCGs evolve within a specific forma specialis are also likely to differ (Correll, 1991; Baayen *et al*., 2000). For some formae speciales, the race corresponds to the VCG; for others, one VCG may contain more than one race; and for yet others, multiple VCGs exist for a single race (Correll, 1991). For those formae speciales where a close genetic relatedness is often found among races, it has been hypothesized that races develop in a stepwise process rather than evolving independently (Gordon and Martyn, 1997). This stepwise pattern of race evolution has only been shown for *F. oxysporum* f.sp. *ciceris* that causes Fusarium wilt of chickpeas (Jiménez-Gasco *et al.,* 2004).

Techniques that have been used for investigating diversity and evolution in *F. oxysporum* include VCG typing, DNA gene sequence data, restriction fragment length polymorphism (RFLP) analyses of amplified polymerase chain reaction (PCR) products, DNA fingerprinting techniques and mating type idiomorphs. Due to the extensive nature of the available literature on the diversity and evolution of formae speciales, VCGs and races, examples will mainly be provided for bulb and corm rot formae speciales in the following section. Information on *Focep* is discussed in the following section.

Vegetative compatibility grouping. VCG determination is a powerful phenotypic method to define genetic relationships within *F. oxysporum* formae speciales isolates (Puhalla, 1985; Lievens *et al*., 2008). Since VCGs involve the genetics of the fungus, they are useful for characterizing the genetic diversity within a forma specialis (Puhalla, 1985; Correll, 1991; Leslie and Summerell, 2006). However, it is important to note that VCGs may reflect genetic similarities but not the degree of genetic differences among isolates (Kistler, 1997).

VCGs have proved to be an excellent predictor of evolutionary origin in *F. oxysporum* (Elias *et al.*, 1993). Molecular data have confirmed these early observations, since most (80%) of the formae speciales that have more than one VCG have a polyphyletic origin, whereas most monophyletic formae speciales consist of only one VCG (Baayen *et al*., 2000; Skovgaard *et al*., 2001). Therefore, a forma specialis that has more than one VCG is likely to be polyphyletic. It is, however, important to note that VCG should only be used as a predictor of evolutionary origin, since for some formae speciales, such as *F. oxysporum* f.sp. *asparagi*, different VCGs may have the same evolutionary origin (Baayen *et al.*, 2000). This may be due to the fact that vegetative compatibility in *F. oxysporum* is controlled by at least seven heterokaryon incompatibility loci (Ploetz, 1999), and if a mutation occurs in one of these genes, isolates are no longer vegetatively compatible (Correll, 1991). Therefore, molecular techniques are required to confirm the evolutionary origin suggested by VCG data (Baayen *et al.*, 2000).

VCGs have also been used successfully to differentiate pathogens from non-pathogens. Since the VCGs of non-pathogens are in general different from the VCGs

of pathogenic isolates, it has been suggested that pathogens and non-pathogens have independent evolutionary origins (Correll, 1991; Gunn and Summerell, 2002; Leslie and Summerell, 2006; Lievens *et al*., 2008). However, for some formae speciales, non-pathogens have been shown to belong to the same VCG as pathogenic isolates. For example, in *F. oxysporum* f.sp. *melonis*, four non-pathogenic isolates were included in two VCGs (0131 and 0134) associated with pathogenic isolates (Appel and Gordon, 1994). Intergenic spacer (IGS) region sequence phylogenies showed that the IGS sequences of these non-pathogenic isolates did not cluster with *F. oxysporum* f.sp. *melonis* VCG 0131 and 0134 isolates (Appel and Gordon, 1996). Thus, the non-pathogens were not as genetically similar to the pathogens as was initially suggested by VCG analyses.

Gene sequence and PCR-restriction fragment length polymorphism (RFLP) analyses. Genetic diversity and evolution of *F. oxysporum* formae speciales can be studied using robust multi-gene sequence phylogenies, viz. mitochondrial small subunit (mtSSU), EF-1α and the IGS region of ribosomal DNA (rDNA) (Appel and Gordon 1996; O'Donnell *et al.*, 1998; Baayen *et al.*, 2000; Geiser *et al*., 2004). Of these three commonly targeted regions, the mtSSU region is the least informative (Cunnington, 2006), whereas the EF-1α and IGS rDNA seem to have significant phylogenetic signals (O'Donnell *et al.*, 2009). Other genes that have occasionally been investigated include polygalacturonases, phosphate permease, β-tubulin, nitrate reductase and a mitochondrial repeat region (Di Pietro and Roncero, 1996; Skovgaard *et al*., 2001; Fourie *et al.*, 2009).

In bulb and corm rot formae speciales, Baayen *et al.* (2000) used combined mtSSU and EF-1α phylogenies to show that *F. oxysporum* f.sp. *gladioli* is polyphyletic and consists of distinct lineages, whereas *F. oxysporum* f. sp. *lilii* and f.sp. *tulipae* are monophyletic. The phylogenies could not resolve VCGs within *F. oxysporum* f.sp. *gladioli*, since more than one VCG was present within a distinct lineage (Baayen *et al*., 2000). The analyses further showed that non-pathogenic *F. oxysporum* isolates from other hosts formed clonal lineages with some of the aforementioned formae speciales, suggesting the same evolutionary origin for pathogens and non-pathogens, although the pathogenicity of the non-pathogens was not tested specifically on the hosts of interest (Baayen *et al.*, 2000). Similarly,

O'Donnell *et al.* (2009), using EF-1α and IGS datasets, found that of 27 investigated non-pathogens; 13 had two-locus haplotypes that matched those of known formae speciales, but these were also isolated from other hosts.

Several studies in other formae speciales of *F. oxysporum*, but not in bulb and corm rot formae speciales, have used the IGS region to resolve VCGs and lineages (Appel and Gordon, 1996; Alves-Santos *et al*., 1999; Lori *et al*., 2004; Abo *et al.*, 2005; Enya *et al*., 2008; Fourie *et al.*, 2009). The IGS region is the area between the large and small rDNA subunit repeats and is known to have evolved more rapidly than any other region in rDNA repeats (Edel *et al*., 1995; Appel and Gordon, 1996; Zambounis *et al*., 2007). In *F. oxysporum*, studies using the IGS region consist of either restriction fragment length polymorphism (RFLP) analyses of the IGS region (Appel and Gordon, 1995; Alves-Santos *et al*., 1999; Lori *et al*., 2004; Abo *et al.*, 2005) or sequence and phylogenetic analyses of a partial region of the IGS (Appel and Gordon, 1996; Mbofung *et al.*, 2007; Enya *et al*., 2008; Fourie *et al.*, 2009). Strains that are highly similar in sequence within the IGS region are assumed to have a close relationship (Abo *et al*., 2005).

Although being highly polymorphic, the utility of the IGS region for phylogenetic studies will be determined by the evolution of this region (Appel and Gordon, 1996). Limitations in the usefulness of the IGS region for evolutionary inference was first suggested by Mbofung *et al.* (2007), since their IGS data were incongruent with EF-1α and mtSSU data sets. A similar finding was made by Fourie *et al*. (2009) for *F. oxysporum* f.sp. *cubense*, and O'Donnell *et al.* (2009) who investigated a large number of *F. oxysporum* formae speciales isolates of clinical and agricultural importance. It can therefore be concluded that, although widely used, the homoplastic evolutionary history provided by IGS data sets obscures accurate phylogenetic relationships within *F. oxysporum* (O'Donnell *et al.*, 2009). Mbofung *et al.* (2007) suggested the following as potential reasons for the homoplasy in IGS data sets: (i) the presence of divergent copies whose presence is due to an ancient hybridization event that were distributed unequally among lineages, (ii) unequal rates of evolution between gene sequence regions and (iii) incomplete concerted evolution of the IGS region. The data of Apple and Gordon (1996) suggested that the incongruency of the IGS data set may be due to sexual reproduction. Using IGS

sequence data, they suggested the possibility of past somatic or sexual interactions between *F. oxysporum* f.sp. *melonis* VCG 0131 and 0134, since one of their race 1 isolates contained two IGS sequence types, one suggesting an affiliation with VCG 0131 and the other with VCG 0134. O'Donnell *et al.* (2009) hypothesized that horizontal gene transfer, possibly mediated by parasexuality, may be the cause of incongruency in IGS datasets.

Random Amplified Polymorphic DNA (RAPD) analyses. RAPD analysis is a technique in which short oligonucleotide sequences, usually 10-mer with a minimum G+C content of 50%, are used in a PCR to amplify random, often repetitive fragments in the target genome (Williams *et al.*, 1990; Kelly *et al.*, 1994; Edel *et al.*, 1995). The size of the amplified amplicons can then be compared to those of other isolates after band separation on agarose gels. RAPD analyses are useful for higher level resolution identification within *F. oxysporum* at the forma specialis, race and VCG levels. Numerous studies have demonstrated the use of RAPD molecular markers for the identification of formae speciales (Manulis *et al.*, 1994; García-Pedrajas *et al.*, 1999; Chiocchetti *et al.*, 2001; Cramer *et al.*, 2003; Wang *et al.*, 2008), races (Grajal-Martin *et al.*, 1993; Assigbetse *et al.*, 1994; Migheli *et al.*, 1998; Jiménez-Gasco *et al.*, 2001; Lin *et al.*, 2008) and VCGs (Bentley *et al.*, 1994; Kalc Wright *et al.*, 1996) within *F. oxysporum*. A disadvantage of RAPD analysis is that this technique suffers from interlaboratory reproducibility, and sometimes also from within-laboratory variability (Jones *et al.*, 1997). Furthermore, the usefulness of RAPD data for evolutionary inference is limited, since the fingerprints obtained cannot be scored as loci and alleles, because the sequence of the amplicons is unknown and fragments of the same size may differ in sequence (McDonald, 1997).

A limited number of RAPD analyses have been conducted on bulb and corm rot associated formae speciales. RAPDs have been used to separate VCGs of *F. oxysporum* f.sp. *gladioli* into two groups (Mes *et al.*, 1999) and to distinguish *F. oxysporum* f.sp. *gladioli* race 1 isolates from race 2 isolates (de Haan *et al.*, 2000).

Amplified fragment length polymorphism (AFLP) analyses. AFLP analyses consist of the restriction digestion of high quality genomic DNA with two restriction enzymes, followed by the ligation of adapters containing ends that are

complementary to the digested genomic DNA. Subsequently, two rounds of PCR amplifications are conducted, i.e. a pre-amplification PCR with non-selective primers (primer sequence similar to the adapter sequences), followed by a second round of amplification (selective amplification) with primers that can have one to three selective nucleotide base pairs. The amplified products can be separated on polyacrylamide gels or, if fluorescence primers were used, a sequencer can be employed for this purpose (Vos *et al.*, 1995; Habera *et al.*, 2004). The advantage of AFLP analysis is that this is a highly reproducible technique that can be automated for high throughput analyses. Similar to RAPDs, the observed amplicons in fingerprints cannot be assumed to be loci and alleles (McDonald, 1997).

AFLPs have been used in *F. oxysporum* to investigate the clonality and evolutionary origin in plant-associated isolates, including bulb and corm rot formae speciales (Baayen *et al.*, 2000; Groenewald *et al.*, 2006). This method has also been used to identify clonal lineages among *F. oxysporum* isolates that are pathogenic to humans (O'Donnell *et al.*, 2004). In corm and bulb rot formae speciales, Baayen *et al.* (2000) found that VCGs of *F. oxysporum* f.sp. *gladioli* had independent origins, but that there were also two VCGs that had the same origin and could not be differentiated from each other based on AFLP analysis.

Mating type idiomorphs. Mating type (*MAT*) genes are commonly viewed as the main regulatory genes required for successful crosses between strains of filamentous fungi, with these systems ranging from simple to complex. As such, identification of functional *MAT* genes suggests that fungi may still have the potential for sexual reproduction. Information on the presence of *MAT* idiomorphs across phylogenetic clades can further be used to formulate hypotheses on the potential occurrence of sexual reproduction (O'Donnell *et al.*, 2004; Fourie *et al.*, 2009). Therefore, the presence of *MAT* genes has been investigated in several formae speciales in *F. oxysporum* (Arie *et al.*, 2000; Yun *et al.*, 2000; O'Donnell *et al.*, 2004; Enya *et al.*, 2008; Fourie *et al.,* 2009; Lievens *et al.*, 2009a). It is important to note that, although both Arie *et al.* (2000) and Yun *et al.* (2000) found that *F. oxysporum* carries functional mating type genes, *F. oxysporum* is strictly known as an asexual fungus (Jiménez-Gasco and Jiménez-Díaz, 2003; Lievens *et al.*, 2008).

In *F. oxysporum* and the *Gibberella fujikuroi* complex, the mating type (*MAT*) locus is a single regulatory locus (Arie *et al.,* 2000; Yun *et al.*, 2000). In these ascomycetes, the *MAT* locus has two *MAT* alleles, which are named idiomorphs (*MAT1-1* and *MAT1*-2) as the two alleles share no significant sequence similarity, even though they map to the same position on homologous chromosomes (Coppin *et al.*, 1997; Arie *et al.*, 2000). The *MAT1-1* and *MAT1-2* nomenclature is used, as this nomenclature was suggested by Turgeon and Yoder (2000) for fungi that have a single mating type locus that is designated *MAT1* (Yoder *et al.,* 1986). The two idiomorphs at the *MAT1* locus are distinguished from each other by the presence of an alpha box motif in *MAT1-1* and a high mobility group (HMG) motif in *MAT1-2.* In heterothallic fungi, two individuals of opposite idiomorphs (*MAT1-1* and *MAT1-2*) are required for sexual reproduction, whereas in homothallic fungi both idiomorphs are present within the same individual (Arie *et al.*, 2000). Arie *et al.* (2000) and Yun *et al.* (2000) successfully cloned both *MAT* idiomorphs from *F. oxysporum* f.sp. *lycopersici* isolates.

Several PCR primers have been published for the amplification of mating type idiomorphs from *F. oxysporum* or the *G. fujikuroi* complex. Prior to their publication it was difficult to determine whether asexual fungi had *MAT* genes, because they could not be crossed with each other using media known to allow sexual reproduction in other *Fusarium* spp. (Arie *et al.*, 2000). Arie *et al.* (1999) and Kerényi *et al.* (1999) were the first to develop PCR primers for the amplification of only the *MAT1-2* idiomorph in the *G. fujikuroi* complex, using the conserved HMG region of the *MAT1* locus. Subsequently, Arie *et al.* (2000) and Yun *et al.* (2000) were the first to publish primer pairs for the amplification of both the *MAT1-1* and *MAT1-2* idiomorphs from *F. oxysporum* isolates. They were also the first to show conclusively that *F. oxysporum* is heterothallic, since isolates contained either one of the *MAT* idiomorphs, but not both idiomorphs in the same individual. The primers of Arie *et al.* (2000) and Yun *et al.* (2000) have been used for the amplification of *MAT* idiomorphs from *F. oxysporum* by several investigators (O'Donnell *et al.*, 2004; Kawabe *et al.*, 2007; Enya *et al.*, 2008; Dissanayake *et al.*, 2009a; Fourie *et al.*, 2009). Other primers that have also been published for the amplification of *MAT* idiomorphs in *F. oxysporum* or the *G. fujikuroi* complex are MAT1L, MAT1R, MAT2L and MAT2R (Abo *et al.*, 2005), Gfmat2c (Steenkamp *et al.*, 2000), FOM211, FOM212, FOM112 and FOM111

(O'Donnell *et al.*, 2004), FF1 (Visser, 2003; Fourie *et al.*, 2009) and GFMH2 and GFMH1 (Kerényi *et al*., 1999). These PCR-based investigations into the presence of mating type idiomorphs in *F. oxysporum* formae speciales (*apii*, *cubense*, *lycopersici*, *radicis-lycopersici*, *spinaciae*, *vasinfectum*) have shown that isolates either contain one or the other mating type, confirming that *F. oxysporum* formae speciales are heterothallic (Arie *et al.*, 2000; Yun *et al.,* 2000, O'Donnell *et al.*, 2004). Only one study, by Kawabe *et al*. (2007), has thus far investigated the *MAT* idiomorphs in non-pathogenic *F. oxysporum* isolates from spinach plants, which also showed that the isolates were heterothallic.

MECHANISMS INVOLVED IN THE EVOLUTION OF *FUSARIUM OXYSPORUM*

The main mechanisms that have been hypothesized to play a role in the evolution of *F. oxysporum* include parasexuality; which requires a heterokaryotic state; horizontal gene transfer, sexual reproduction (mating systems) and transposable elements (Buxton, 1962; Leslie, 1993; Kuhn *et al*., 1995; Teunissen *et al.*, 2002). There is more support for the involvement of some of these mechanisms than for others. Here, these mechanisms will be discussed on the basis of the available evidence, although some of this evidence is still only circumstantial. In addition to these mechanisms, evolutionary forces that are known to be important in other organisms, such as natural mutation, natural selection, genetic drift and gene flow (McDonald and Linde, 2002), most likely also play a role in the evolution of *F. oxysporum.* In presumably asexual fungi, such as *F. oxysporum*, the role of mutation is of particular importance (Taylor *et al.*, 1999; Jiménez-Gasco *et al.*, 2004).

Sexual reproduction. Evolution theory predicts that sexual reproduction plays an important role in pathogen population structure. Sexual reproduction can result in new fungal strains resistant to fungicides, or new pathogenic races overcoming cultivar disease resistance (Gordon and Martyn, 1997; Arie *et al*., 2000). However, sexual reproduction needs to be frequent if it is to play an imperative role in the generation and preservation of genotype diversity in fungal field populations (Kerényi *et al*., 1999).

The role of sexual reproduction in *F. oxysporum* is uncertain. The *F. oxysporum* species complex is considered to be predominantly asexual, given that no sexual stage has been identified (Gordon and Martyn, 1997; Fourie *et al.*, 2009). Therefore, the identification of functional mating type idiomorph genes by Arie *et al.* (2000) in *F. oxysporum* f.sp. *lycopersici* was unexpected. Subsequent to this study, several others have also shown that in *F. oxysporum* formae speciales populations, both mating type idiomorph genes are present. However, studies attempting matings between isolates containing different idiomorphs have not yet been successful. For example, although Kawabe *et al.* (2005) identified three phylogenetic lineages within *F. oxysporum* f.sp. *lycopersici* that each had a different *MAT* idiomorph, successful matings between these different lineages containing opposite *MAT* idiomorphs could not be achieved (Kawabe *et al.*, 2005). A similar finding was made by Fourie *et al.* (2009) for *MAT* idiomorph genotyping and matings of *F. oxysporum* f.sp. *cubense* lineages containing opposite *MAT* idiomorphs. Even though the sexual stage has not been found in current populations, sexual reproduction may have occurred in the early evolution of this species complex in ancient lineages (Taylor *et al.*, 1999; O'Donnell *et al.*, 2004; Fourie *et al.*, 2009). Taylor *et al.* (1999) found some evidence for the involvement of sexual reproduction in *F. oxysporum* f.sp. *cubense* by re-analyzing published data.

Horizontal gene transfer. Horizontal gene transfer (HGT), also known as lateral gene transfer, is the incorporation of genetic material from any exogenous source into an organism (Andersson, 2009). Obtaining evidence for the occurrence of HGT in eukaryotes is difficult. Consequently, evidence for the role of HGT in fungi is limited, but literature on HGT suggests that this mechanism may have been more important in the evolution of fungi than in any other eukaryote (Rosewich and Kistler, 2000; Andersson, 2009).

HGT has been hypothesized to play a role in the evolution of *F. oxysporum*. This hypothesis comes from studies on virulence genes and transposable elements. Daboussi *et al.* (2002) hypothesized that horizontal gene transfer may have contributed to the discontinuous distribution of the *Fot1* transposon in *F. oxysporum*. Van der Does *et al.* (2008) hypothesized that virulence loci may have spread along clonal lines of *F. oxysporum* f.sp. *lycopersici* due to HGT, as the virulence genes they

studied were identical in all the investigated *F. oxysporum* f.sp. *lycopersici* isolates. Furthermore, most of these genes were also located on the same chromosome region, which was hypothesized to be a conditionally dispensable chromosome since (i) it was the smallest chromosome, (ii) the region was highly enriched with transposable element sequences and (iii) some deletions can be tolerated in the region without affecting vegetative growth (Rep *et al.*, 2004; Van der Does *et al.*, 2008).

Heterokaryosis. The definition of heterokaryosis is the coexistence of genetically different nuclei in the same cytoplasm (Webster, 1974). The evolutionary advantage of heterokaryosis in haploid fungi is that it provides the organism with many of the advantages of heterozygosity found in diploid organisms. In ascomycetes, heterokaryons exist briefly during the sexual stage of reproduction, but are uncommon in the vegetative phase of these fungi. In the vegetative phase, heterokaryons can be formed through mutation in a multinucleate thallus, anastamosis and nuclear exchange between strains of closely related fungi, or formation of multinucleate spores (Webster, 1974). In nature, heterokaryosis in general do not appear to be a significant source of natural variation in ascomycetes (Clutterbuck, 1996; Newton *et al.*, 1998). However, there is one interesting example of heterokaryosis in nature for the ascomycete *Cryphonectria parasitica* (Murrill) Barr. In this pathogen, natural populations can include heterokaryotic isolates that contain near isogenic nuclei that only differ at a few known loci and the *MAT* locus. How these heterokaryons are formed and maintained in natural populations is not yet clear (McGuire *et al.*, 2004, 2005). In *F. oxysporum,* heterokaryosis has not been postulated as having a role in evolution, but since this is a prerequisite for parasexuality, it is important to take note of the occurrence of heterokaryosis in other fungi.

Parasexuality. In fungi, parasexuality is the phenomenon where genetic recombination occurs without meiosis (Tinline and MacNeil, 1969; Kendrick, 2000). The main steps required for parasexuality are (i) fusion of cells and the formation of a heterokaryon, (ii) formation of heterozygous diploids through fusion of nuclei in vegetative hyphae, (iii) somatic recombination (mitotic recombination) and (iv) non-meiotic reduction of the changed nuclei through chromosome loss to the haploid state (Tinline and MacNeil, 1969; Kendrick, 2000). Proof of parasexuality would thus include the demonstration of heterokaryosis, formation of heterozygous diploids, and

recovery of diploid and haploid segregant genotypes in the progeny (Webster, 1974). Unlike meiosis, somatic recombination in the parasexual cycle usually involves the crossing-over of only one or a few chromosomes (Kendrick, 2000).

In *F. oxysporum*, similar to most ascomycete fungi, parasexuality has been shown under laboratory conditions only (Webster, 1974; Molnar *et al.*, 1990; Leslie, 1993; Teunissen *et al.*, 2002). One reason that may limit parasexuality in nature as a mechanism of generating genetic diversity, is the prerequisite for the formation of a heterokaryon. Due to the presence of vegetatively incompatible (*vic*) genes in *F. oxysporum*, parasexuality would only occur between isolates belonging to the same VCG (Elias and Schneider, 1992). Since VCGs are seen as clonal lineages (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000), this would not lead to the generation of much genetic variation. However, Molnar *et al.* (1990) were able to use *vic* strains to provide some evidence for the occurrence of parasexual recombination between incompatible strains using (i) auxotrophic mutants and (ii) protoplast fusion. The latter circumvents the vegetative incompatibility problem, but not the former. Using the auxotrophic mutants, Molnar *et al.* (1990) came to the conclusion that vegetative incompatibility does not mean that an absolute barrier exists against parasexual gene exchange, since the barrier can be broken by using strong selection pressure such as minimal media. In *F. oxysporum,* under laboratory conditions, parasexuality has been shown to lead to the massive exchange of parental DNA, involving chromosome rearrangements and recombination, even when isolates from the same VCG, but from different races, were investigated (Teunissen *et al.*, 2002).

Some circumstantial evidence for the occurrence of parasexuality in naturally occurring *F. oxysporum* populations is the occurrence of genetic duplication and aneuploidy (Kistler *et al.,* 1995; O'Donnell *et al.*, 1998), as well as the variable number of chromosomes between VCGs of the same forma specialis (Boehm *et al*., 1994; Migheli *et al*., 1995; O'Donnell *et al*., 1998). Teunissen *et al.* (2002) even found that two *F. oxysporum* f.sp. *lycopersici* isolates from the same VCG differed markedly in terms of their chromosome number. The aforementioned genome phenomena are analogous to signatures that are present in the parasexual cycle in *Candida albicans* (Forche *et al.,* 2008). Parasexuality has also been hypothesized to contribute to the horizontal transfer of pathogenicity genes (Baayen *et al*., 2000) and

may involve the transfer of conditionally dispensable chromosomes between strains (Covert, 1998).

In contrast to most fungi, for which only circumstantial evidence and laboratory induced parasexuality are available, the formation of heterokaryons by vegetatively incompatible individuals and parasexuality is thought to play a role in the evolution of the ascomycete *C. parasitica* under natural field conditions (McGuire *et al.*, 2005). It is unclear what mechanisms would allow one to overcome the obstacle of vegetative incompatibility in this system, since *vic* genes also determine vegetative compatibility of *C. parasitica*. McGuire *et al.* (2004) hypothesized that disruption of the normal mating process in *C. parasitica* could assist the formation of heterokaryons between vegetative incompatible strains, since vegetative incompatibility is suppressed during the sexual stage. For example, heterokaryons could perhaps form beneath the perithecia. Alternatively, or in addition to this, transient heterokaryons could be formed between incompatible strains, followed by the loss of one of the chromosomes containing the *vic* loci (McGuire *et al.,* 2005).

Transposable elements. Transposable elements are relative short DNA sequences (typically < 25 000 bp) that move around in the genome using different strategies to replicate and insert (Jurka, 2008). These elements can cause marked changes in host genomes, including partial or total gene inactivation, changes in gene transcription and the rearrangement of genomic information (Daboussi and Capy, 2003). Therefore, transposable elements are valuable factors in genome evolution and organization (Hua-Van *et al*., 2000; 2001).

Transposable elements are estimated to compose 5% of the *F. oxysporum* genome and are associated with spontaneous genetic change (Hua-Van *et al*., 2001; Roncero *et al*., 2002; Lievens *et al*., 2008). Several families of transposable elements are present in the genome of *F. oxysporum*. Some of these transposons include *Fot1* (Pasquali *et al*., 2004), impala (Hua-Van *et al*., 2001), Tfo1 (Okuda *et al*., 1998) and Folyt1 (Gomez-Gomez *et al*., 1999).

Transposons are also thought to play an important role in the evolution of *F. oxysporum*, based on several observations. Firstly, in several formae speciales and

races, pathogenicity or virulence have been associated with transposable element sequences. This enabled the development of markers specific for these groups based on the transposon sequences or flanking regions (Fernandez *et al*., 1998; Chiocchetti *et al*., 1999; Mes *et al*., 2000; Pasquali *et al*., 2004; Lievens *et al*., 2008). Secondly, a putative dispensable genomic region in *F. oxysporum* f.sp. *lycopersici* that contains genes that promote virulence towards tomato was shown to be rich in transposable elements (Van der Does *et al.*, 2008). Thirdly, the movement of transposable elements can result in mutations in genes that are important for pathogenicity. In this sense, Migheli *et al*. (2000) was able to show that the transposon *impala* transposes randomly, can generate mutants impaired in their pathogenicity and can even cause pathogenic isolates to become non-pathogenic.

MOLECULAR DISCRIMINATION OF *FUSARIUM OXYSPORUM* FORMA SPECIALIS, VCGs AND RACES

Accurate discrimination and detection techniques of plant pathogens are needed for the correct implementation of disease management strategies. The use of molecular detection methods can also save diagnosis time. Within *F. oxysporum*, rapid methods for differentiating non-pathogens from pathogens are especially important, since gene sequence data that are useful for identifying other fungal pathogens are often not reliable for distinguishing closely related pathogenic and non-pathogenic *F. oxysporum* isolates (Lievens *et al.*, 2008). One of the exceptions to the latter is the usefulness of the IGS region for distinguishing some formae speciales from non-pathogens (Zambounis *et al.*, 2007).

Two of the main DNA-based methods currently used for the rapid detection and identification of plant pathogens are (i) PCR amplification of the pathogen with PCR primer pairs that only amplify the targeted pathogen of interest and (ii) DNA micro- or macro-arrays that involve the hybridization of PCR products amplified from a putative positive sample, to a membrane (macro-array) or glass slide (micro-array) containing short oligonucleotides that are specific to the pathogen(s) of interest (Lievens and Thomma, 2005; Vincelli and Tisserat, 2008). The first approach has the advantage of being high throughput, although the number of pathogens that can be

tested is limited compared to the DNA array-based methods. The identification and detection of pathogens using PCR amplification with pathogen-specific primers can be conducted using either conventional PCR or real-time PCR. Conventional PCR identifications are based on the presence or absence of a specific sized amplicon on agarose gels, whereas real-time PCR employs the detection of a fluorescent signal that is indicative of a positive amplification. Real-time PCR has the advantage of being highly sensitive as well as quantitative (Schaad and Frederick, 2002; Vincelli and Tisserat, 2008).

The detection and identification of plant pathogens using pathogen-specific PCR primers should preferably use primers that target known virulence genes. The strong link between some of these genes and pathogenicity makes them excellent markers for host-specific pathogenicity (Lievens *et al.,* 2008). Alternatively, primers can be developed using cloned gene sequence data in a sequence characterized amplified region (SCAR) approach (Schaad and Frederick, 2002; Lievens *et al.*, 2008). The rapid detection and identification of *F. oxysporum* forma specialis, races and VCGs have been conducted using both of the aforementioned approaches.

Primers targeting the IGS region. The IGS region often contains a high number of single nucleotide polymorphisms (SNPs) that can be useful for developing pathogen-specific primers (Edel *et al*., 1995; Appel and Gordon, 1996; Lori *et al*., 2004; Abo *et al.,* 2005; Zambounis *et al*., 2007). Only one report has been published that used the IGS region for designing primers that were specific for *F. oxysporum* f.sp. *vasinfectum* (Zambounis *et al.*, 2007)*.* The IGS-based primers were capable of distinguishing *F. oxysporum* f.sp. *vasinfectum* isolates from other *F. oxysporum* formae speciales and non-pathogenic isolates, and could quantify the pathogen directly from soil and plant material.

Primers targeting virulence genes. Information on genes involved in the virulence and host specificity of *F. oxysporum* formae speciales has only recently become available, and only for *F. oxysporum* f.sp. *lycopersici*. Van der Does *et al.* (2008) and Rep *et al*. (2004) were the first to show that identification of *F. oxysporum* f.sp. *lycopersici* based on host-specific virulence genes can be very robust. In addition to this, Lievens *et al*. (2009b) convincingly showed that identification of *F.*

oxysporum f.sp. *lycopersici* and races can also be done by using host-specific effector genes. The aforementioned pathogen-specific and race-specific primers were all developed from a genomic region in *F. oxysporum* f.sp. *lycopersici* that contain the 'secreted in xylem' (*SIX*) genes.

The *SIX* proteins are secreted in the xylem vessels during colonization of tomato plants, and by themselves or as part of a larger group confer the ability to cause tomato wilt. In total, seven *SIX* genes (*SIX1* to *SIX*7) have been identified. Of these *SIX* genes, two (*SIX3* and *SIX4*) are avirulent (unpublished data in Lievens *et al.*, 2009b; Van der Does *et al.,* 2008). Lievens *et al*. (2009b) screened a large world-wide collection of *F. oxysporum* isolates for the presence of the seven *SIX* genes. They showed that *SIX4* can be used for the identification of *F. oxysporum* f.sp. *lycopersici* race 1 strains, whereas *SIX1*, *SIX*2, *SIX3* and *SIX5* can be used to identify isolates belonging to forma specialis *lycopersici*. Additionally, polymorphisms in *SIX3* can be used to differentiate race 2 from race 3 isolates (Lievens *et al.*, 2009b).

Polygalacturonases are cell-wall-degrading enzymes produced by several pathogens which may play a role in the host-pathogen interaction. Hirano and Arie (2006) developed primers from the genes encoding an endo polygalacturonase (*pg1*) and exo polygalacturonase (*pgx4*) to differentiate *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *radicis-lycopersici*, respectively, from each other. However, these primers showed cross-reaction with other formae speciales (Hirano and Arie, 2006).

SCAR primers developed from sequence-unbiased genotyping techniques. For discrimination of formae speciales and races of *F. oxysporum*, results obtained from sequence-unbiased genotyping techniques can be used to develop simple and more reliable SCAR primers for identification of the pathogen of interest (Lievens *et al*., 2008). Most SCAR markers have been developed using RAPD genotyping data. This approach was succesful for the identification of *F. oxysporum* f.sp. *ciceris* and each of its pathogenic races 0, 1A, 5 and 6 (Jiménez-Gasco and Jiménez-Diaz, 2003), f.sp. *cucumerinum* (Lievens *et al*., 2007), f.sp. *radicis-cucumerinum* (Lievens *et al*., 2007) and f.sp. *basilici* (Chiocchetti *et al*., 2001). RAPD-derived SCAR markers have also been developed for the identification of *F. oxysporum* f.sp. *ciceris* isolates of the wilt-inducing pathotype (Kelly *et al*., 1998), isolates from the four pathogenic races

(0, 1A, 5 and 6) of *F. oxysporum* f.sp. *ciceris* (Jiménez-Gasco and Jiménez-Diaz, 2003) and the highly virulent isolates of seven *F. oxysporum* f.sp. *phaseoli* races (Alves-Santos *et al*., 2002). Some of the RAPD fragments that were used for SCAR marker development had similarity to transposons (Jiménez-Gasco and Jiménez-Diaz, 2003).

SCAR markers developed from transposon sequences or insertional sites. Several studies succeeded in identifying formae speciales or races within *F. oxysporum* using SCAR markers that were designed from sequence regions with homology to transposons (Fernandez *et al.*, 1998; Chiocchetti *et al.*, 1999; Pasquali *et al.*, 2004, 2007; Lievens *et al.*, 2007). When using this approach, it is important that the sequences used should be stably inserted at specific sites (Pasquali *et al.*, 2004). The usable lifespan of transposable element markers will increase significantly if the transposable elements are linked to non-dispensable genes (like pathogenicity) (Alves-Santos *et al*., 2002).

PCR primers that were developed using transposable element sequences have been used as sensitive diagnostic tools for the detection of *F. oxysporum* f.sp. *albedinis* (Fernandez *et al*., 1998), f.sp. *dianthi* races 1, 2, 4 and 8 (Chiocchetti *et al*., 1999) and f.sp. *chrysanthemi* (Pasquali *et al*., 2004). The PCR primers for detection of *F. oxysporum* f.sp. *albedinis* were developed by screening a genomic library for DNA clones containing copies of the *Fot* 1 transposon, followed by cloning and SCAR marker development (Fernandez *et al*., 1998). The transposon-based markers for the detection of *F. oxysporum* f.sp. *dianthi* races and *F. oxysporum* f.sp. *chrysanthemi* were developed using an inverse PCR technique that targeted transposons (*Fot* 1 or *impala*). The inverse PCR technique is a method that allows the acquisition of a sequence region that flank the transposon. Therefore, primers could be developed that overlapped the 3' or the 5' end of the transposon and its flanking genomic region (Chiochetti *et al.*, 1999; Pasquali *et al.*, 2004).

Another approach for developing SCAR markers for *F. oxysporum* is the use of a method known as the inter-retrotransposon amplified polymorphism (IRAP) technique. The IRAP PCR technique was first applied to barley and is based on the amplification of flanking regions between long terminal repeat (LTR) sequence sites

where transposons insert (Kalendar *et al.*, 1999; Pasquali *et al.*, 2007). Inter-retrotransposon sequence-characterized amplified region (IR-SCAR) has been used to develop a specific set of primers to differentiate *F. oxysporum* f.sp. *lactucae* race 1 isolates from other *F. oxysporum* isolates (Pasquali *et al.*, 2007).

THE USE OF REPORTER GENES FOR STUDYING FUNGAL ECOLOGY AND INFECTION

Reporter genes convey a detectable phenotype to transformed microbes, and thus allow their visualization and discrimination from other fungi and microbes in plants and soil. The visualization of reporter gene-labelled fungi associated with plant tissue has increased our understanding of microbial behavior and ecology (Nahalkova and Fatehi, 2003). For example, reporter gene-labelled pathogens have been used successfully for studying host infection and colonization by various plant pathogens (Lagopodi *et al.*, 2002; Nahalkova and Fatehi, 2003; Si-Ammour *et al.*, 2003).

Some of the reporter genes used for transformation of fungi in ecological studies include the green fluorescent protein (GFP), GFP-enhanced color variants (egfp, ygfp, ecfp, sgfp) and reef coral proteins like *Discosoma* sp. red fluorescent protein (DsRed) (Kantakamalakul *et al.*, 2003; Shaner *et al.*, 2004). GFP is derived from the jellyfish *Aequorea Victoria*, and is a relatively small protein (Sheen *et al.*, 1995; Tsien, 1998; Bottin *et al.*, 1999; Lorang *et al.,* 2001). The green fluorescence is visible in whole organisms when excited by UV or blue light (Sheen *et al.*, 1995), generating a highly visible, efficiently emitting internal fluorophore (Tsien, 1998). DsRed occurs naturally in the colored body parts of the reef coral *Discosoma* species (Tsien, 1998; Nahalkova and Fatehi, 2003). This protein results in bright red cytoplasmic fluorescence of the fungus, and can be used in a similar way as the GFP gene (Mikkelsen *et al.*, 2003).

Reporter genes have been used successfully for investigating the colonization of host tissue by *F. oxysporum* formae speciales isolates without changing the virulence of the transformants (Aboul-Soud *et al.*, 2004). Lagopodi *et al.* (2002) used GFP to label *F*. *oxysporum* f. sp. *radicis-lycopersici*, showing the complete

colonization process as well as the first steps of contact between the fungus and the host at the root hair zone. The red fluorescent protein, DsRed2, has also been used successfully for monitoring the infection of *F. oxysporum* f. sp. *lycopersici* (Nahalkova and Fatehi, 2003).

The labeling of pathogenic and non-pathogenic *F. oxysporum* with different reporter genes of which the spectral properties do not overlap, has been used to study the interactions between pathogenic and biological control isolates. Bolwerk *et al.* (2005) labelled *F. oxysporum* f. sp. *radicis-lycopersici* and beneficial *Fusarium* strains with GFP and an enhanced cyan fluorescent protein (*ecfp*) gene, to show that the mechanism of biocontrol by the beneficial *Fusarium* strain entailed competition for niches and nutrients. This mechanism of biocontrol was also found in the study of Nahalkova and Fatehi (2003) for *F. oxysporum* f. sp. *lycopersici*. Paparu *et al*. (2009) stably transformed *F. oxysporum* isolates with either the GFP or DsRed fluorescent protein genes to study plant colonization by endophytic non-pathogenic *F. oxysporum* isolates. They concluded that active growth of the transformed endophyte was limited to the root epidermis (Paparu *et al*., 2009).

FUSARIUM SPECIES ON *ALLIACEAE*: PATHOGENIICTY AND SYMPTOMS

Various species within the genus *Fusarium* are pathogenic towards onions, including *Focep* and *F. proliferatum* (T. Matsushima) Nirenberg (Dugan *et al*., 2003; Du Toit *et al*., 2003; Stankovic *et al.*, 2007; Crowe, 2008). These two pathogens are of great concern to onion growers across the globe (Dugan *et al*., 2003; Du Toit *et al*., 2003; Stankovic *et al*., 2007; Crowe, 2008; Galván *et al.*, 2008). Other *Fusarium* species pathogenic within the *Alliaceae* include *F. redolens* Wollenw. on Welsh onion (Shinmura, 2002) and *F. culmorum* (W.G. Smith) Sacc. on garlic (*A. sativum*) (Schwartz and Mohan, 2008).

***Fusarium oxysporum* f.sp. *cepae*.** On onion, *Focep* symptoms are observed on numerous plant parts, including roots, the basal stem plate, bulb scales and leaves. The main symptoms include basal rot, yellowing of leaves and necrosis from the leaf

tips down (Gabor, 1996; Agrios, 2005; Koike *et al*., 2007). Fusarium rot within the stem plate is usually dry and firm (Agrios, 2005). Following rot development within the stem plate, roots can easily become detached and can even separate from the stem plate during harvest. Mycelial growth can also sometimes be observed on the basal portions of the outer bulb scales. Losses in storage are often greater than losses in the field, mainly due to latent infections and secondary infections caused by other microbes (Cramer, 2000). In addition to being pathogenic to onion, *Focep* has also been shown to cause the same symptoms on Welsh onion (*Allium fistulosum* L.).

As for almost all other *F. oxysporum* formae speciales, the identification of *Focep* involves the use of pathogenicity tests. In the literature, several different pathogenicity tests have been used for identifying *Focep.* For instance, Abawi and Lorbeer (1971) and Dissanayake *et al.* (2009a) evaluated seedling emergence after inoculation of onion seed. Swift *et al*. (2002) evaluated the incidence of *F. oxysporum* colonies developing on cross-sections of surface sterilized onion bulbs five days after inoculation. *Focep* inoculation of soil was used by Abawi and Lorbeer (1972), Lopez and Cramer (2004) and Galván *et al*. (2008). Abawi and Lorbeer (1972) and Lopez and Cramer (2004) evaluated damping-off and root plate necrosis of onion seedlings, while Gálvan *et al*. (2008) only assessed the percentage seedling emergence. No races have been reported for *Focep* (Havey, 1995). However, *Focep* isolates have been found to differ in virulence responses on resistant selections (Galván *et al*., 2008; Dissanayake *et al.*, 2009b), and are known to show various degrees of aggressiveness (Schwartz and Mohan, 2008).

Fusarium proliferatum. The pathogenic nature of *F. proliferatum* among members of the *Alliaceae* has been shown for garlic in addition to onion (Simey, 1990; Stankovic *et al.*, 2007). The pathogen causes severe bulb rot symptoms on these hosts. Unlike *Focep, F. proliferatum* mainly causes storage losses and not field losses (Cramer, 2000; Dugan *et al*., 2003; Du Toit *et al*., 2003; Crowe, 2008). Bulb rot of lily has also been shown to be caused by *F. proliferatum,* specifically *F. proliferatum* var. *minus* (Baayen *et al.,* 1998).

GENETIC DIVERSITY AND EVOLUTION IN *FOCEP*

Only two studies have investigated the VCG status of *Focep* isolates, and reported the presence of four VCGs and two single-member isolates. One VCG, numbered VCG 0420 (Yoo *et al.*, 1993; Katan, 1999), was identified for two Japanese isolates, and the other three VCGs (VCGs 0421, 0422 and 0423) were identified among 19 *Focep* isolates from Colorado, USA (Swift *et al.*, 2002). The Colorado isolates also included two *Focep* isolates that were single-member isolates, and these therefore did not receive a VCG number (Swift *et al.,* 2002).

The polyphyletic nature of *Focep* suggested by VCG analysis was confirmed using phylogenetic analyses of IGS sequence data (Dissanayake *et al.*, 2009a) and AFLP data (Galván *et al.*, 2008). These studies, however, did not include any information on the VCG status of isolates. The IGS and AFLP phylogenies not only showed the polyphyletic nature of *Focep,* but also showed that some *Focep* isolates are genetically more similar to isolates from other formae speciales isolates than to other *Focep* isolates (Galván *et al.*, 2008; Dissanyake *et al.*, 2009a). The study of Mbofung *et al*. (2007) which only included one *Focep* isolate also showed, using various gene regions (EF-1α, mtSSU and IGS), that *Focep* was genetically closely related to isolates from other formae speciales. The *Focep* isolates used in the aforementioned studies originated from various regions in the world including The Netherlands, Germany, France, Spain, Turkey, Australia, Argentina, Uruguay, the USA (California) (Galván *et al.*, 2008) and several regions within Japan (Dissanayake *et al.*, 2009a). The isolates from Japan were isolated from Welsh onion (*A. fistulosum*), but some were also shown to be pathogenic towards *A. cepa*, and could, thus, be designated as *Focep* (Dissanyake *et al.*, 2009b). The genetic diversity in *F. oxysporum* isolates from Welsh onion that varied in virulence (low to high) was further investigated using the IGS region. The analyses showed that there was a tendency for grouping of isolates according to their virulence, but that some isolates with low virulence were divergently placed in clades that contained highly virulent isolates (Dissanayake *et al.*, 2009a).

The mating type idiomorphs associated with *Focep* have only been investigated for 30 *F. oxysporum* isolates pathogenic to Welsh onion, using the *MAT*

primers of Arie *et al*. (2000) (Dissanayake *et al.,* 2009a). Among these isolates, of which 18 were confirmed to be *Focep,* only the *MAT1-1* idiomorph was present. The study did not include the analyses of non-pathogenic *F. oxysporum* isolates from onions.

INOCULUM SOURCES AND EPIDEMIOLOGY OF *FUSARIUM OXYSPORUM* F.SP. *CEPAE*

Inoculum sources. Once successfully established, *Focep* can affect numerous future *Alliaceae* crops, as the fungus can survive in infested fields as chlamydospores for many years. It is, therefore, important to identify the origins of Fusarium basal rot inoculum and the contribution of each of the inoculum sources to yield losses. This information is required for the implementation of preventative control strategies. The inoculum sources of *Focep* potentially include soilborne chlamydospores, seedling transplants and seed.

The primary source of *Focep* inoculum under field conditions is chlamydospores (Cramer, 2000). Soils with a long history of Fusarium basal rot usually have a high population of chlamydospores available for early season infection. Abawi and Lorbeer (1972) showed that up to 90% of *Focep* conidia can be transformed into chlamydospores within the first 14 days after being added to organic rich soil. Chlamydospore production is optimal at high temperatures (25 to 45°C) and pH (H$_2$O) levels of 6 to 7 (Sood, 1996). The formation of chlamydospores from macroconidia is also enhanced through damaged onion bulbs that leak their cell content (Sood, 1996). Under favorable conditions, *Focep* chlamydospores can survive in soil for considerable periods of time (Abawi and Lorbeer, 1971, 1972; Brayford, 1996; Gabor, 1996; Sood, 1996). For other *F. oxysporum* formae speciales, such as *F. oxysporum* f.sp. *narcissi,* chlamydospores have been shown to survive for up to 20 years in soil (Hiltunen *et al*., 1995). Chlamydospores are either free between-soil particles or embedded in organic tissue, as they are mostly formed in and on infected roots (Abawi and Lorbeer, 1971, 1972; Sherf and Macnab, 1986).

Onion transplants and seed are potential inoculum sources of *Focep*. Seedling transplants are well-known inoculum sources of *Focep,* which can result in the dissemination of the pathogen over very long distances, causing Fusarium basal rot losses in mature bulbs (Abawi and Lorbeer, 1972; Özer and Köycü, 2004; Schwartz and Mohan, 2008). The role of seed as *Focep* inoculum source is uncertain. Reports on the seedborne nature of *Focep* have only been published in non-peer reviewed journals that are not readily available (Boff *et al*., 1995; El-Zawahry *et al*., 2000). Since other *F. oxysporum* formae speciales have been shown to be seedborne, this possibility also exists for *Focep*. Other formae speciales that have been shown to be seedborne include *F. oxysporum* f.sp. *lactucae* infecting lettuce (Pasquali *et al*., 2005, 2007), *F. oxysporum* f.sp. *basilici* infecting sweet basil (Chiocchettti *et al.*, 2001; Pasquali *et al.*, 2006) and *F. oxysporum* f.sp. *radicis-lycopersici* infecting tomato (Menzies and Jarvis, 1994).

Infection and subsequent disease development. The primary method of *Focep* infection is through direct penetration of the basal plate, but infection can also occur through natural wounds, through the roots, and directly through basal portions of bulb scales when inoculum levels in the soil are high (Abawi and Lorbeer, 1971; Sherf and Macnab, 1986; Cramer, 2000). *Focep* infection can also take place through maggot, onion smut or pink root damaged areas (Everts *et al*., 1985; Sherf and Macnab, 1986; Schwartz and Mohan, 2008). Although not proven for *Focep*, a strong nematode-fungus complex has been shown to exist between Fusarium wilt and infection by root-knot, lesion and other nematodes (Agrios, 2005). The physiological state of the host can also influence *Focep* infection; for example, infection is more severe in slow growing seedlings (Krueger *et al*., 1989).

The inoculum density of *Focep* in soil influences disease development. Larger amounts of inoculum are usually needed in organic rich soil to cause a significant amount of Fusarium basal rot (Abawi and Lorbeer, 1972; Sherf and Macnab, 1986). The inoculum density of *Focep* has also been shown to be directly correlated with the amount of onion seedling damping-off (Abawi and Lorbeer, 1972). Inoculum densities in naturally infected soil can vary from 22 to 6500 *Focep* colony-forming units per gram dried soil (Abawi and Lorbeer, 1972; Everts *et al*., 1985).

Focep infection and subsequent disease development are influenced by temperature. Successful infection is most often favored by high temperatures (25 to 35°C) and moderate to low moisture conditions (Smith *et al*., 1988; Snowdon, 1991; Schwartz and Mohan, 2008). Similarly, a temperature increase from 10 to 32°C causes an escalation in seedling damping-off symptoms (Abawi and Lorbeer, 1972). As the season progresses, an increase in the percentage of *F. oxysporum* isolates pathogenic to onion take place, with populations increasing from 25-56% at mid-season to 62-90% later in the season (Everts *et al*., 1985).

CONTROL OPTIONS FOR FUSARIUM BASAL ROT

Control options in South Africa. Control options in South Africa focus on the prevention and management of Fusarium basal rot, as it is virtually impossible to cure this disease. Control options include a three to four year rotation schedule, thiram and benomyl seed coatings, biological control with *Trichoderma* and *Bacillus* species in onion seedling nurseries, transplanting only healthy seedlings, and good water scheduling to keep soils cool and to promote root development (K. Smit, Middeltuin Farm, South Africa, personal communication).

Chemical control. Successful chemical control options against *Focep* include metham-sodium fumigation (Jaworski *et al*., 1978) and the use of benzimidazole fungicides (Holz, 1973; Schwartz and Mohan, 2008). Good Fusarium basal rot control has also been achieved with fludioxanil and thiophanate methyl on garlic (Dugan *et al*., 2007), and with benomyl and carbendazim on *Iris* and *Narcissus* (Gould and Miller, 1971a, 1971b; Hanks, 1996). Benomyl was also shown to be the best *Gladiolus* corm dip treatment against *F. oxysporum* f.sp. *gladioli* (Ram *et al*., 2004). Chemical control studies against *F. oxysporum* f.sp. *cubense* on banana revealed successful inhibition of mycelial growth with prochloraz and propiconazole and a reduction in disease severity in the greenhouse using benomyl and demethylation-inhibiting fungicides (Nel *et al.*, 2007).

Biological control. *Trichoderma harzianum* Rifai seed treatment was shown to be successful in decreasing *Focep* incidence in onion sets (Coşkuntuna and Özer,

2008). Other successful biological control examples include control of Fusarium basal rot on *Narsiccus* by *Streptomyces* (Hiltunen *et al*., 1995), and the suppression of Fusarium wilt of banana by using non-pathogenic *F. oxysporum* isolates and *Pseudomonas fluorescens* strain WCS 417 (Nel *et al*., 2006). Significant reductions of Fusarium wilt of tomato has also been achieved by non-pathogenic *F. oxysporum* isolates and strains of *T. hamatum*, *P. fluorescens* and *Burkholderia cepacia* (Larkin and Fravel, 1998).

Cultural control. Although chemical seed treatment is standard practice in the vegetable industry, some of the most effective control strategies still involve the use of pathogen-free seed, transplant certification (Chiocchettti *et al.*, 2001; Pasquali *et al.*, 2006) and using healthy propagation material (Sherf and Macnab, 1986; Howard *et al*., 1994; Koike *et al*., 2007). Proper crop rotation with non-host crops, avoiding rotations with *Allium* spp. such as shallots, bunching onions and leeks, forms the basis of successful disease management (Abawi and Lorbeer, 1972; Everts *et al*., 1985; Sherf and Macnab, 1986; Howard *et al*., 1994; Havey, 1995; Cramer, 2000). Other cultural control measures include the removal of alternative hosts like *Oxalis corniculata* (Abawi, 1971; Holz, 1973; Elmer and Lacy, 1987), minimizing mechanical wounding to underground plant parts (Everts *et al*., 1985) and burning or deep ploughing infected crop residue (Schwartz and Mohan, 2008). In addition, commercial varieties with high levels of tolerance are available to onion growers (Cramer, 2000; Koike *et al*., 2007).

Integrated disease control. As for the control of *F. oxysporum* f.sp. *ciceris* wilt of chickpea (Landa *et al*., 2004), more than one control option should be integrated into a commercially acceptable program for successful management of Fusarium basal rot in onion. Although integrated programmes will differ from one another, they should as a rule centre around the most prominent risk factors contributing to Fusarium basal rot losses. These risk factors pertain to pathogen contaminated seedling transplants, soil temperature, levels of soil acidity, osmotic potential, nutrient make-up of the soil, inoculum density, water-holding capacity, nematode counts and cultivar susceptibility.

CONCLUSION

Although some limited information is available on the genetic diversity and evolution of *Focep* in some regions of the world, no information is available for South Africa. One and three VCGs, respectively, have been reported from two investigated isolates from Japan and 19 from Colorado (Yoo *et al.*, 1993; Katan, 1999; Swift *et al.*, 2002). No races have been identified for *Focep* (Havey, 1995). *Focep* has a polyphyletic evolutionary origin based on molecular analyses of isolates collected from Japan and nine other countries (The Netherlands, Germany, France, Spain, Turkey, Australia, Argentina, Uruguay and the USA) (Galván *et al*., 2008; Dissanayake *et al.*, 2009a). Information on the mating type idiomorphs in *Focep* is limited to a study from Japan, which showed that only the *MAT*1-1 idiomorph was present among *Focep* isolates obtained from Welsh onion (Dissanayake *et al.,* 2009a). None of the studies mentioned investigated the genetic diversity and evolutionary origin of non-pathogenic *F. oxysporum* isolates from onion. Therefore, it is currently unknown whether non-pathogenic and pathogenic *F. oxysporum* isolates have the same evolutionary origin. It is also unknown whether pathogenic and non-pathogenic isolates can be distinguished from each other based on housekeeping gene sequence data such as the IGS region, or whether other molecular approaches, such as the development of SCAR markers, will be required to differentiate between these groups.

The contribution of seed and seedling infection to the epidemiology of *Focep* in South Africa is unknown. Although onion transplants are known to act as inoculum sources of *Focep* (Abawi and Lorbeer, 1972; Özer and Köycü, 2004; Schwartz and Mohan, 2008), it is not clear whether the *Focep* genotypes obtained from symptomless onion transplants are the same as those found on diseased onion bulbs in storage. The contribution of seed as an inoculum source of *Focep* is uncertain world-wide. Therefore, more information is required on the seedborne nature of *Focep*. It is, for instance, not known whether *Focep* can spread systemically from onion bulb basal plates through the seed stalk to seed in the umbel. Investigations into the latter can be aided by the use of reporter gene-labelled *Focep* isolates, since non-pathogenic *F. oxysporum* isolates endogenous to onion tissue and latent *Focep* bulb infections can interfere with conclusions made based on isolation studies for *F. oxysporum*.

Considering the importance of Fusarium basal rot in South Africa, and the limited knowledge available on the epidemiology and genetic diversity of *Focep* in the country, it is clear that research on these aspects is urgently required. The research should address the genetic diversity and evolution of pathogenic and non-pathogenic *F. oxysporum* isolates from onion by using multi-gene sequence and phylogenetic analyses, *MAT* idiomorph genotyping and VCG status determination. It will also be useful to compare the genetic diversity of South African *Focep* isolates to that of isolates from other regions of the world, especially where specific VCGs have been identified, such as Colorado. Results from the characterization studies will not only shed light on the genetic diversity and evolution of *Focep* in South Africa, but will also determine whether the development of SCAR markers will be required for discriminating between pathogenic and non-pathogenic isolates.

To better understand the epidemiology of the disease in South Africa, it will be important to determine whether seedling transplants and seed are inoculum sources of *Focep*. The seedborne nature of *Focep* should not only be investigated through isolation studies from seed, but should also be confirmed through the use of reporter gene-labelled isolates. Altogether, this research will help the industry to make informed disease management decisions and also to optimize current integrated management strategies. Furthermore, genetic diversity and evolutionary studies will allow predictions on the potential of *Focep* to adapt to new management strategies, and in future will allow us to determine whether current *Focep* populations have been replaced by new genotypes that may have increased ecological fitness and virulence.

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2. GENETIC DIVERSITY AND EVOLUTION OF *FUSARIUM OXYSPORUM* F. SP. *CEPAE* IN SOUTH AFRICA AND COLORADO (USA)

ABSTRACT

Fusarium oxysporum f.sp. *cepae* (*Focep*) is the causal agent of Fusarium basal rot of onions, a disease of world-wide importance. In South Africa and Colorado, limited information is available on the evolution and genetic diversity of this forma specialis. Therefore, 79 *F. oxysporum* isolates from onions were characterized with regard to their pathogenicity, vegetative compatibility group (VCG) status, three gene sequence regions and mating type idiomorphs. The isolates included 19 Colorado and 27 South African *Focep* isolates, and 33 non-pathogenic *F. oxysporum* isolates from onion bulbs in South Africa. The Colorado *Focep* isolates consisted of four vegetative compatibility groups (VCG 0421 to 0424) and one single-member VCG (SMV 4). Only one of the South African *Focep* isolates was vegetatively compatible with Colorado VCG 0423, with the remaining isolates grouping into two new VCGs (VCG 0425 and VCG 0426), three single-member VCGs (SMV 1, 2 and 3), and one heterokaryon self-incompatible (HSI) isolate. The dominant VCGs in Colorado and South Africa were VCG 0421 (47% of isolates) and VCG 0425 (74% of isolates), respectively. A combined translation elongation factor 1α (EF-1α) and mitochondrial small-subunit (mtSSU) phylogeny confirmed the polyphyletic nature of *Focep,* and showed that most of the isolates clustered into two large well supported clades ($\geq$ 79% bootstrap, 0.96 probability). The one clade contained the two dominant VCGs (0421 and 0425), both highly virulent to onion, as a putative clonal group along with SMVs and non-pathogenic isolates. The other clade was ancestral and included the HSI isolate, VCG 0422, 0423 and 0424, and non-pathogenic isolates. Most of the *Focep* isolates in this clade was only moderately virulent. The data set of the intergenic spacer (IGS) region of the rDNA was incongruent with that of the EF-1α and mtSSU, and suggested the exchange of genetic material between isolates. This was also suggested by mating type (*MAT*) idiomorph genotyping, since *(i) MAT1-1* and *MAT1-2* idiomorph containing isolates were distributed across phylogenetic clades and (ii) some isolates, including all VCG 0421 isolates and some non-pathogenic *F. oxysporum* onion isolates, contained both *MAT* idiomorphs within the same isolate.

INTRODUCTION

Onion bulb diseases caused by *Fusarium* can be subdivided into Fusarium basal rot and Fusarium bulb rot caused by *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *cepae* (H.N. Hans.) W.C. Snyder & H.N. Hans. and *Fusarium proliferatum* (T. Matsushima) Nirenberg, respectively (Dugan *et al*., 2003; Du Toit *et al*., 2003; Crowe, 2008). Both of these diseases have been reported from several regions in the world, including the Netherlands, Uruguay, Serbia and the USA, although bulb rot was reported for the first time in the USA only in 1997 (Mohan *et al.,* 1997; Dugan *et al*., 2003; Du Toit *et al*., 2003; Stankovic *et al*., 2007; Crowe, 2008; Galván *et al.*, 2008). *Fusarium oxysporum* f.sp. *cepae* (*Focep*) is economically the most important *Fusarium* disease, as it causes field and storage losses. In contrast, *F. proliferatum* has only been reported as causing storage losses, even though infection takes place in the field (Dugan *et al*., 2003; Du Toit *et al*., 2003; Crowe, 2008).

Doidge *et al*. (1953) first reported *Focep* as the causal agent of onion basal rot in the Western Cape Province of South Africa. In 1973, Holz also reported *Focep* as the causal agent of serious onion harvest losses on certain farms in the Caledon-Riviersonderend district of the Western Cape (Holz and Knox-Davies, 1976). Since then, the Koue Bokkeveld region in the Western Cape has increasingly become the location for the production of export quality Pukekohe-type onions, with the first noticeable losses due to Fusarium basal rot being reported in the early 1990s (M. Smit, Du Toit Vegetables, South Africa, personal communication). The disease continues to be problematic in the Koue Bokkeveld where in recent years (2004, 2005 and 2006) field and storage losses of 15% and 25%, respectively, have been reported (unpublished data). Until now, Fusarium bulb rot has not been reported in South Africa.

Focep is one of more than 120 different formae speciales within the *F. oxysporum* species complex that contains pathogenic and non-pathogenic isolates (Kistler, 1997; Fravel *et al*., 2002; Lori *et al*., 2004). The forma specialis designation is used to distinguish host-specific pathogenic isolates from non-pathogenic isolates, since many isolates are non-pathogenic and can promote plant growth and/or have potential as biocontrol agents (Fravel *et al.,* 2002; Michielse and Rep, 2009). Isolates

within a specific forma specialis can further be subdivided into races, which are identified based on virulence reactions on a set of differential host cultivars (Correll, 1991; Aloi and Baayen, 1993). Another phenotypic method used to define genetic relationships within formae speciales of *F. oxysporum* isolates is vegetative compatibility groups (VCGs) (Puhalla, 1985; Lievens *et al*., 2008). VCG analysis involves the use of different chlorate resistant nitrate non-utilizing (*nit*) mutants. Two isolates belong to the same VCG when hyphae of their different *nit* mutants can fuse and form a heterokaryon when grown on nitrate deficient medium. VCGs are based on the genetics of the fungus, rather than on the host-pathogen interaction itself (Puhalla, 1985), and are therefore useful for characterizing the genetic diversity within a forma specialis (Correll, 1991; Leslie and Summerell, 2006; Lievens *et al*., 2008), and for distinguishing pathogens from non-pathogens (Gunn and Summerell, 2002).

Knowledge of the genetic diversity and evolution of *F. oxysporum* formae speciales is important for developing sustainable control strategies and breeding programs. It may also provide (i) an indication of the evolutionary potential of pathogens to overcome management strategies such as host resistance (Assigbetse *et al*., 1994; Bruton and Damicone, 1999; Skovgaard *et al.*, 2001) and (ii) clues as to how pathogenicity traits are acquired and transferred in populations (Baayen *et al.*, 1998; Lievens *et al*., 2008). Genetic diversity and evolution of *F. oxysporum* formae speciales can be studied using robust multi-gene sequence phylogenies, viz. mitochondrial small subunit (mtSSU), translation elongation factor 1-alpha gene (EF-1α), and the intergenic spacer (IGS) region of rDNA (Appel and Gordon 1996; O'Donnell *et al.*, 1998; Baayen *et al.*, 2000; Geiser *et al*., 2004; O'Donnell *et al.*, 2009). Additionally, information on the presence of mating type (*MAT*) idiomorphs, *MAT1-1* and *MAT1-2*, in isolates and across phylogenetic clades can be used to formulate hypotheses on the potential of sexual reproduction or other mechanisms that may contribute to the generation of genetic diversity (O'Donnell *et al.*, 2004). However, it is important to note that, although isolates of *F. oxysporum* contain functional *MAT* genes (Yun *et al*., 2000), it is predominantly known as an asexual fungus (Jiménez-Gasco and Jiménez-Díaz, 2003; Lievens *et al*., 2008; Michielse and Rep, 2009). Yet, based on a re-analyses of *F. oxysporum* f.sp. *cubense* DNA data,

Taylor *et al.* (1999) suggested that there is evidence of sexual reproduction in this species complex.

Limited information is available on the genetic diversity and evolution of *Focep* populations world-wide, whereas no information is available for South African isolates. The first genetic studies conducted on *Focep* used VCG analyses, which showed that two Japanese isolates belonged to the same VCG (Yoo *et al.*, 1993), namely VCG 0420 (Katan, 1999; Swift *et al.*, 2002). Subsequently, 19 *Focep* isolates from Colorado were tentatively classified into three VCGs (VCG 0421, 0422 and 0423) and two single-member VCGs (SMVs) (Swift *et al*., 2002). The polyphyletic origin of *Focep* in several countries has recently been shown by Galván *et al*. (2008) and Dissanayake *et al.* (2009a) using an AFLP- and IGS phylogeny, respectively. Dissanayake *et al.* (2009a) also showed that only the *MAT1-1* idiomorph was present among 18 *Focep* as well as 13 *F. oxysporum* isolates that are pathogenic to Welsh onion in Japan. No races have been identified within *Focep* (Havey, 1995), although isolates have been found to differ in virulence responses on resistant selections (Özer *et al.*, 2004; Galván *et al*., 2008; Dissanayake *et al.*, 2009a).

The correct identification of *F. oxysporum* formae speciales is dependent on the use of an appropriate pathogenicity testing method, except for a few formae speciales where pathogen-specific molecular markers have been developed (Lievens *et al.*, 2008). Unfortunately, for many host systems, including onions, the best pathogenicity testing method is controversial due to the inevitable variation involved in biological systems where both the host and pathogen are influenced by environmental and other factors (Aloi and Baayen, 1993; Clark *et al*., 1998; Teunissen *et al*., 2003). Several pathogenicity tests and criteria have been published for *Focep* identification. Some discrepancies in these methods result from the use of either onion seedlings or bulbs for pathogenicity testing, since *Focep* can cause both seedling and post-harvest bulb damage. Published pathogenicity tests and criteria for *Focep* identification include the evaluation of (i) seedling emergence, damping-off and stunted growth after inoculation of onion seed (Abawi and Lorbeer, 1971; Dissanayake *et al.*, 2009a, 2009b), (ii) damping-off and root plate necrosis of onion seedlings planted in inoculated soil (Abawi and Lorbeer, 1972; Lopez and Cramer, 2004), (iii) the incidence of *F. oxysporum* colonies developing on cross sections of

surface-sterilized onion bulbs 5 days after inoculation (Swift *et al*., 2002) and (vi) percentage seedling emergence after pathogen inoculation of soil (Galván *et al*., 2008).

The aim of this study was to first determine the pathogenicity of 60 *F. oxysporum* isolates collected from onion bulbs in South Africa using a robust pathogenicity test. This pathogenicity test was also used to validate the pathogenicity results of the 19 *Focep* Colorado isolates from the work of Swift *et al.* (2002). Secondly, the VCGs of South African *Focep* isolates were determined and compared with VCGs previously identified in Colorado (Swift *et al.*, 2002). Lastly, the genetic diversity and evolution of Colorado and South African *Focep* isolates and non-pathogenic onion *F. oxysporum* isolates from South Africa were investigated using multi-gene sequence and phylogenetic analyses of the EF-1α, mtSSU and IGS regions and *MAT* idiomorph genotyping.

MATERIALS AND METHODS

Fungal isolates. Nineteen *Focep* isolates previously characterized by Swift *et al.* (2002) were kindly provided by H.F. Schwartz (Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins). The isolates originated from three regions in Colorado, USA. These were the north eastern (Weld County), south eastern (Otero County), and western (Delta, Mesa and Montrose Counties) regions. The VCG status of each of these isolates was previously reported (Swift *et al.*, 2002).

A few *Fusarium* isolates were used as negative controls in pathogenicity studies. These isolates included two *F. graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch) isolates (SP 249 and C 381 AU) from wheat plants in Moorreesburg, South Africa, and isolates from other formae speciales of *F. oxysporum*. The formae speciales isolates included *F. oxysporum* f.sp. *lini* (CBS 259.52), *F. oxysporum* f.sp. *gladioli* (CBS 137.97), *F. oxysporum* f.sp. *lycopersici* (CBS 412.90) from the CBS Fungal Biodiversity Centre (Utrecht, The Netherlands), *F. oxysporum* f.sp. *lycopersici* (PPRI 5457) from the South African National

Collection of Fungi (PPRI, Pretoria, South Africa) and *F. oxysporum* f.sp. *cubense* (CAV 005 and CAV 013) isolates from KwaZulu-Natal, South Africa.

***Fusarium* isolations from onion bulbs in South Africa**. Onion bulbs, symptomatic and asymptomatic, were collected from five regions, within three provinces, in South Africa over a 3-year period, from 2005 to 2007. The three provinces were Limpopo Province, the Northern Cape Province and the Western Cape Province, with the three regions in the latter being the Koue Bokkeveld, Boland and Southern Cape (Fig. 1). The collected bulbs represented 27 different onion varieties. After removal of the outer two fleshy bulb scales and 3 to 5 mm of the outer basal plate tissue, small tissue pieces (1 to 3 mm in diameter) were taken from inside the basal plate and placed onto potato dextrose agar (PDA; Biolab, Gauteng, South Africa) supplemented with 0.04 g Streptomycin sulphate per litre agar (PDA$^+$). Plates were incubated at 25ºC ± 4ºC on the laboratory bench and were inspected regularly for fungal growth. All isolates were hyphal tipped, followed by single-spore isolation. All *Fusarium* isolates were tentatively identified to the species level using conidial morphology as described by Leslie and Summerell (2006). Isolates were stored in 15% glycerol at -80°C. All single-spored isolates were submitted to the fungal culture collection of Stellenbosch University, Department of Plant Pathology.

Genomic DNA extraction. DNA was extracted from *Fusarium* cultures by first growing isolates on PDA for 2 weeks at room temperature. Mycelia were harvested from the plates and DNA was extracted with the Wizard® SV Genomic DNA Purification system (Promega Corporation, Madison, WI, USA) according to the manufacturer's recommendations.

Identification of *Fusarium* species using PCR amplification and sequencing of the EF-1α region. *Fusarium* isolates that were isolated from onion bulbs in South Africa were identified to the species level using sequence data of the partial region of the EF-1α gene, and Blast analyses in the FUSARIUM-ID v. 1.0 database (http://fusarium.cbio.psu.edu) (Geiser *et al*., 2004). The EF-1α gene was also sequenced from all the Colorado *Focep* isolates.

PCR amplification of the EF-1α gene was conducted using primers EF-1 (5'-ATGGGTAAGGAAGACAAGAC) and EF-2 (5'-GGAGGTACCAGTCATCATGG-T) (O'Donnell *et al.*, 1998). The PCR reaction consisted of 1 unit BIOTAQ^{TM} DNA polymerase (Bioline, London, United Kingdom), 1 x PCR buffer, 0.2 μM of each primer, 4 mM MgCl$_2$, 1 mg/ml bovine serum albumine (BSA) Fraction V (Roche Diagnostics South Africa, Randburg, South Africa), 200 μM of each dNTP and 5 μl genomic DNA in a final reaction volume of 40 μl. Amplification was carried out in a 2700 GeneAmp thermocycler (Applied Biosystems, Foster City, CA) using an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 1 min, with a final extension step at 72°C for 7 min. Amplicons were separated on 1% agarose gels, stained with ethidium bromide and visualized under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK).

PCR amplification products were purified using the Wizard SV gel and PCR clean-up system (Promega Corporation) following the manufacturer's protocol. The PCR products were sequenced in both directions using the ABI PRISM BigDye Terminator version 3.0 Cycle Sequencing Ready reaction kit (Applied Biosystems) according to the manufacturer's specifications. The sequencing amplification conditions consisted of the following steps: 94°C for 5 min, followed by 30 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min, with a final extension step at 60°C for 30 s. Analyses of the resulting fragments were done on an ABI 3130XL Genetic Analyzer (Perkin-Elmer, Norwalk, CN). Geneious Pro (Biomatters Ltd., Auckland, New Zealand) was used to view ABI trace files and to obtain consensus double strand sequences for each isolate.

Pathogenicity tests. A preliminary pathogenicity trial evaluated the reliability of various inoculation assays using onion bulbs. In these assays, the inoculation on lower, middle and upper bulb cross-sections, small basal plate pieces and whole bulbs were compared to one another. The most reliable assay was the whole bulb inoculation method (unpublished data). This assay was chosen for pathogenicity testing.

All the *Fusarium* isolates from onion bulbs in South Africa (indicated with STE-U numbers; n = 64) and Colorado (n = 19) were included in pathogenicity tests (Table 1). Coastal Cream onion bulbs, a locally produced Pukekohe-type onion, were used in pathogenicity testing, as this cultivar is economically important and also susceptible to Fusarium basal rot. Onion bulbs used in the pathogenicity studies were purchased from a local onion grower after a minimum storage period of 13 weeks. Single-spored *Fusarium* isolates were grown for 3 weeks on PDA$^+$. Spores were harvested from plates using sterile distilled water, and adjusted to a final concentration of 5×10^4 conidia/ml. Prior to inoculation of onion bulbs, the dry bulb scales, one of the fleshy inner scales, as well as the roots were removed. The basal plate was wounded using a sterile scalpel (approximately 20 shallow wounds per plate), and inoculated with a 1-ml spore suspension using small droplets. The bulbs were left to dry in a laminar flow cabinet before being transferred to brown paper bags. These bags were placed in moisture chambers for a 6-week period. The bags were kept closed for the first 48 hours in order to create a saturated environment. The incubation conditions were 24 to 26$^\text{o}$C, a minimum of 60% relative humidity, and natural light conditions.

After 6 weeks incubation, 5 mm of the outer basal plate was cut off, and the percentage necrosis was evaluated on the cut basal plate section. The pathogen was re-isolated from the basal plates onto PDA$^+$, thus fulfilling Koch's postulates. Pathogenicity testing of each isolate was conducted using four onion bulbs and was repeated at least once. The pathogenicity assays included negative controls consisting of two *F. graminearum* isolates, four formae speciales of *F. oxysporum* (two *cubense* isolates, two *lycopersici* isolates, and one isolate each of *gladioli* and *lini*), a non-pathogenic *F. oxysporum* isolate from onion (identified in the first pathogenicity experiments) and a water control. The positive control consisted of a highly pathogenic *Focep* isolate, also identified in the first pathogenicity experiment.

Vegetative compatibility tests. The VCG status of 27 *Focep* isolates from South Africa that were identified in the pathogenicity tests was determined. Nine isolates from Colorado (Foc 1, 4, 21, 22, 23, 31, 32, 41 and 42) that represented the previously identified three VCGs (VCG 0421, 0422 and 0423) and two single-member VCGs (SMV 4 and SMV 5) from this region (Swift *et al.*, 2002) were used to

determine whether any of the South African isolates belonged to these VCGs. The VCGs of five non-pathogenic *F. oxysporum* isolates (STE-U 6675, 6682, 6683, 6684 and 6688) were also determined.

VCG determination was done using standard techniques as previously described (Leslie and Summerell, 2006). *Nit* mutants were first generated as spontaneous chlorate-resistant sectors on media containing 4% chlorate (ClO_3^-). The chlorate concentration was altered for all of the isolates for which *nit* mutants failed to generate. The *nit* mutants that were generated were differentiated into *nit1*, *nit3* and Nit M classes based on their growth on phenotypic media that consisted of minimal medium containing different nitrogen sources (NH_4^+, NO_3^-, NO_2^- and hypoxantine). Mutants in the *nit1*, *nit3* and Nit M classes were then used to force heterokaryons. A line of robust mycelial growth between complementary *nit* mutants from the different isolates indicated that the two isolates belonged to the same VCG.

For all the isolates used in the vegetative compatibility tests, heterokaryon self compatibility (HSC) was first determined by pairing different *nit1* and Nit M mutants of the same isolate with each other. Subsequently, all 27 *Focep* isolates from South Africa were compared amongst each other to determine their VCG status. Fourteen isolates that represented all the different South African *Focep* VCGs and SMVs were then tested against the nine Colorado isolates and the five non-pathogenic South African *F. oxysporum* isolates. The VCG test was repeated for the 36 *Focep* isolates from South Africa and Colorado to confirm their VCG status.

PCR amplification and sequencing of the mtSSU and IGS regions. Isolates of which the multi-locus gene sequences were determined included all *F. oxysporum* isolates from onion bulbs in South Africa (pathogenic and putative non-pathogenic isolates) as well as the *Focep* isolates from Swift *et al.* (2002).

The mtSSU rDNA region was amplified using primers MS1-1 (5'-GTGTTCCAGTATGGGGAGC) and MS2-1 (5'-CTTCACTACTGGTGTCAGAAA-CG), which were slightly modified from published primers and allowed the amplification of all isolates. The PCR reaction mixture contained 1 unit BIOTAQ™ (Bioline),, 1 x PCR buffer, 0.2 μM of each primer, 3 mM MgCl₂, 1 mg/ml BSA

Fraction V, 200 μM of each dNTP and 5 μl genomic DNA in a total volume of 40 μl. The amplification condition was similar to that of the EF-1α region. PCR product purification, sequencing and the compilation of consensus sequence data were conducted as described for the EF-1α region.

A partial region of the IGS (approximately 1.6 kb) was amplified using primers PNFo (5'-CCCGCCTGGCTGCGTCCGACTC) and PN22 (5'-CAAGCATA-TGACTACTGGC) (Edel *et al*., 1995). The PCR reaction mixture contained 1.5 units BIOTAQ^{TM} (Bioline), 1 x PCR buffer, 0.2 μM of each primer, 2.8 mM MgCl$_2$, 0.8 mg/ml BSA Fraction V, 250 μM of each dNTP and 10 μl genomic DNA in a total volume of 25 μl. The amplification conditions were similar to that of the mtSSU and EF-1α regions, except that an extension time of 2 min. was used. Sequencing reactions were conducted using primers PNFo, PN22 and IGS2 (internal reverse primer: 5'-GCCGGATTTGCTCCCTTCT) (Fourie *et al.*, 2009), as described for the EF-1α region.

Consensus sequences for EF-1α, mtSSU and IGS were deposited in GenBank (http://www.ncbi.nml.nih.gov/) (Table 1).

Phylogenetic analyses. Automated sequence alignments were done using MAFFT version 5.85 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) for the EF-1α, MtSSU and IGS regions, followed by manual adjustments of the alignments in Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002). In addition to the Colorado and South African *F. oxysporum* isolates, reference sequences of other *F. oxysporum* formae speciales (O'Donnell *et al.*, 1998; Baayen *et al*., 2000; Mbofung *et al.*, 2007) were also included in the alignments. One *Focep* isolate from Germany (NRRL 22538) used in the study of Mbofung *et al.* (2007) was the only *Focep* isolate in GenBank for which EF-1α, mtSSU and IGS sequences were available. NRRL 25184 and NRRL 28387 that were used as outgroups in the EF-1α and mtSSU trees, were also used as outgroups in the studies of Baayen *et al.* (2000) and O'Donnell *et al*. (1998). Sequences used as outgroups in the IGS phylogeny were *F. subglutinans* (AY 249387.1) and *F. avenaceum* (FJ 154746) (Schweigkofler *et al*., 2004).

The relatedness of *Focep* isolates from Colorado and South Africa to *Focep* isolates from other regions of the world was investigated by constructing an expanded EF-1α phylogeny. This phylogeny included the *Focep* EF-1α sequences from the study of Galván *et al.* (2008) that were the only GenBank *Focep* sequences, in addition to the sequences of isolate NRRL 22538 from Mbofung *et al.* (2007), which could be compared to sequence data from the current study. Although Dissanayake *et al.* (2009a) submitted IGS sequences of 18 *Focep* isolates in Genbank, these sequences could not be included in the IGS phylogeny of the current study, since the sequences had a length of 660 bp and did not overlap with the 1.6 kb fragment amplified from Colorado and South African isolates.

Maximum parsimony analysis was conducted on the alignments using PAUP v.4.0b10 (Swofford, 2003). The heuristic search option with 10 random taxon additions was used, and tree bisection and reconstruction (TBR) was used as the branch swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. In all cases, statistical support for the most parsimonious tree was derived by running 1 000 bootstrap replicates. A partition homogeneity test was conducted in PAUP to test the pairwise congruence between the three gene area datasets (EF-1α, mtSSU and IGS).

Bayesian analysis was conducted using MrBayes v. 3.1.2. (Ronquist and Huelsenbeck, 2003). The program MrModeltest (J.J.A. Nylander, available from the internet: www.ebc.uu.se/systzoo/staff/nylander.html was used for selecting the optimal model of sequence evolution for each clade alignment. The likelihood and prior settings were changed in MrBayes according to the models found with MrModeltest for each partition. Markov chains were initiated from a random tree and run for two million generations, keeping one out of every 100th generation. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. The average deviation of split frequencies was 0.0101 for the combined EF-1α and mtSSU dataset, 0.0123 for the EF-1α and Galván *et al.* (2008) dataset, and 0.0201 for the IGS dataset. The potential scale reduction factors (PSRF) were one for each of the parameters. The first 140 000 generations (burn-in) were discarded for the combined EF-1α and mtSSU sequences, 142 000 generations for the EF-1α and Galván *et al.*

(2008) sequences, and 330 000 generations for the IGS sequences. The remaining samples were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches.

Amplification of *MAT* idiomorphs. The presence of *MAT* idiomorphs (*MAT1-1* and *MAT1-2*) in each of the *F. oxysporum* isolates from onion was determined using several different primer pairs. The annealing positions of the primers are shown in Fig. 2. Amplification of the *MAT1-1* idiomorph was conducted using primer pairs (i) FOM111 and FOM112 (O'Donnell *et al.*, 2004), (ii) Falpha1 and Faplha2 (Arie *et al.*, 2000) and (iii) FOM111 and Falpha2. Amplification of the *MAT1-2* idiomorph was conducted using two primer pair combinations consisting of (i) FOM211 and FOM212 (O'Donnell *et al.*, 2004) and (ii) FOM211 (O'Donnell *et al.*, 2004) and GFMH2 (Kerényi *et al.*, 1999). Attempts to amplify the *MAT1-2* idiomorph using primer pairs FHMG11 and FHMG12 (Arie *et al.*, 1999, 2000) and GFMH1/GFMH2 (Kerényi *et al.*, 1999), with several different PCR reaction- and amplification conditions were unsuccessful.

Amplification with primer pairs FOM111/FOM112 and FOM211/212 was conducted in a total reaction volume of 25 μl containing 1 x PCR buffer (Immolase, Bioline), 0.25 mM dNTPs, 0.35 μM of each primer, 50 ng genomic DNA, 1 U Immolase Taq (Bioline) and 3 mM $MgCl_2$. The amplification reaction of primer pair Falpha1/Falpha2 consisted of 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.3 μM of each primer, 50 ng DNA and 1 U BIOTAQ^{TM} (Bioline) in a total volume of 25 μl. Amplification with primer pairs FOM111/Falpha2 and FOM211/GFMH2 consisted of 1 x PCR buffer (Supertherm Gold), 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.3 μM of each primer, 50 ng DNA and 1 U SuperTherm Gold DNA polymerase (JMR Holdings, Kent, UK) in a total volume of 25 μl. Each set of PCR amplifications included a negative control where water was added to the reaction instead of genomic DNA.

Amplification conditions for all five primer pairs were similar, except that the annealing temperature differed for the primer pairs. The amplification conditions consisted of 1 cycle denaturing at 95°C for 8 min, 36 cycles of 30 s at 94°C, 30 s at

50°C (Falpha1/Falpha2) or 54°C (FOM111/FOM112 and FOM211/212) or 57°C (FOM211/GFMH2) or 53°C (FOM111/Falpha2), 40 s at 72°C, and a final extension step of 6 min at 72°C. PCR amplicons were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light using a GeneGenius Gel Documentation and Analysis System.

The identity of the amplified PCR products with primer pairs FOM211/212, FOM211/GFMH2 and FOM111/Falhpa2 was investigated using sequence analyses of PCR products obtained from a subset of 31 isolates that represented different VCGs and phylogenetic clades. Sequence reactions and amplifications were done as described for the EF-1α region using primers that were used to amplify the genes.

RESULTS

***Fusarium* isolations from onion bulbs**. In total, 64 *Fusarium* isolates were obtained from onion bulbs. Most of the isolates (62.5%) were collected from the Koue Bokkeveld (Table 1). Tentative morphological identification of these *Fusarium* isolates revealed four species, namely *F. oxysporum* (60 isolates), *F. proliferatum* (two isolates), *F. solani* (Mart.) Sacc. (one isolate) and an unidentifiable species (one isolate).

***Fusarium* species identification using PCR amplification and sequencing of the EF-1α region**. Morphological species identifications were confirmed by Blast analyses. Four *Fusarium* species were identified among the 64 selected isolates, of which *F. oxysporum* was predominant (93.8%) (99.8% sequence similarity). The three other *Fusarium* species were all obtained from areas outside the Koue Bokkeveld and included one *F. solani* (99.9% sequence similarity), one *F. acutatum* Nirenberg & O'Donnell (99.8% sequence similarity) and two *F. proliferatum* (97% and 99% sequence similarity) isolates. All 40 *Fusarium* isolates collected from the Koue Bokkeveld were identified as *F. oxysporum* (Table 1).

Pathogenicity tests. In total, the pathogenicity of 83 *Fusarium* isolates was tested, which included all the South African *Fusarium* isolates from onions (64

isolates), and the 19 *Focep* Colorado isolates from the study of Swift *et al*. (2002). Pathogenic isolates, i.e. *Focep* isolates, were classified as either being highly virulent or moderately virulent. Highly virulent isolates caused 70 to 100% necrosis of the cut basal plate section in four inoculated bulbs in two repeat experiments, whereas moderately virulent isolates only caused 20 to 70% necrosis. The basal plate necrosis caused by *Focep* isolates had a characteristic Fusarium dry rot symptom (Fig. 3). Non-pathogenic *F. oxysporum* isolates all caused less than 10% basal plate necrosis (Table 1). The negative control bulbs, which were inoculated with two *F. graminearum* isolates, six other *F. oxysporum* formae speciales isolates, a non-pathogenic *F. oxysporum* isolate and a water control, remained asymptomatic until the end of each assay. On a few occasions, the negative control bulbs did show some internal necroses, but these were not observed in repeat experiments or in more than one of the inoculated bulbs. In all experiments, the positive control, consisting of a highly pathogenic *Focep* isolate, caused 80 to 100% necrosis in all four inoculated bulbs.

Among the South African and Colorado isolates there were highly virulent and moderately virulent isolates. Almost all of the South African *Focep* isolates were highly virulent and originated from the Koue Bokkeveld. Isolates from Limpopo, the Boland, and the Northern and Southern Cape were predominantly non-pathogenic (Table 1). Fourteen of the 19 *Focep* Colorado isolates were highly virulent. The remaining five isolates were moderately virulent causing 20 to 50% necrosis. These five isolates were from VCG 0423 (isolates Foc 42, Foc 47, Foc 48 and Foc 50 from Mesa) and VCG 0422 (isolate Foc 32 from Delta) (Table 1). Only one South African *Focep* isolate, which belonged to VCG 0423 (STE-U 6659), was moderately virulent.

The other *Fusarium* species (*F. solani, F. acutatum* and *F. proliferatum*) obtained from onion bulbs caused 0 to 16% basal plate necrosis, except for one *F. proliferatum* isolate that caused 44% necrosis of the cut basal plate section (Table 1). However, the symptoms caused by the latter *F. proliferatum* isolate, unlike that caused by *Focep* isolates, included a light brown bulb necrosis of the outer dry and fleshy scales progressing downward and inward from the top halve of the bulbs.

Vegetative compatibility test. The recovered *nit* mutants were divided into three phenotypic classes, namely *nit1* (47.3% of total), Nit M (39.5% of total) and *nit3* (13.2% of total). Most of the *nit* mutants were stable on minimal media slants in storage, with a few individuals reverting back to the wild type after a few months. Although a Nit M and *nit1* pairing gave a clearer result than a *nit3* and *nit1* pairing (Leslie and Summerell, 2006), Nit M mutants could not be obtained for two of the *Focep* isolates (STE-U 6650 and 6659) from South Africa, and were substituted by *nit3* mutants. Only one of the Koue Bokkeveld *Focep* isolates (STE-U 6655) was heterokaryon self-incompatible (HSI).

The identities of the three Colorado VCGs 0421, 0422 and 0423 (Swift *et al.*, 2002) in nine of the evaluated isolates were confirmed, as none of the representative isolates from each of the three VCGs anastomosed with each other. However, contrary to the findings of Swift *et al.* (2002), isolates Foc 22 (previously VCG 0421) and Foc 23 (previously SMV 5) anastomosed with each other, and thus now represent a new VCG, VCG 0424. Colorado isolate Foc 21 did not anastomose with any other isolate, thus retaining its status as a SMV, and was renamed as SMV 4 for the purpose of the current study. One of the pathogenic isolates from South Africa in the Koue Bokkeveld (STE-U 6659) was vegetatively compatible with the two VCG 0423 representative isolates Foc 41 and Foc 42 from Colorado, and could thus be classified into this VCG (Table 1).

Two new VCGs were identified among the remaining 26 South African *Focep* isolates. These VCGs, excluding the SMVs, were given numbers following on the VCG numbering system of Swift *et al.* (2002) and the new VCG (VCG 0424) identified in this study. VCG 0425 represented the bulk (74%) of the *Focep* isolates from South Africa, including 19 isolates from the Koue Bokkeveld and one isolate (STE-U 6662) from the Boland. VCG 0426 included 2 isolates, one each from Limpopo (STE-U 6637) and the Koue Bokkeveld (STE-U 6638). The assignment of VCG numbers 0424 to 0426 to *Focep* isolates was acknowledged by T. Gordon (Department of Plant Pathology, University of California). Three of the South African *Focep* VCGs were comprised of single-member isolates (STE-U 6636, 6645 and 6658), obtained from the Limpopo (STE-U 6636) and Koue Bokkeveld (STE-U 6645

and 6658), and will hereafter be referred to as SMV 1, SMV 2 and SMV 3, respectively (Table 1).

Only five non-pathogenic *F. oxysporum* isolates (STE-U 6675, 6682, 6683, 6684 and 6688) from onions in South Africa were subjected to VCG analysis. Each of these isolates represented SMV isolates (Table 1), and were not vegetatively compatible with any of the *Focep* VCGs or SMVs from South Africa or Colorado.

Phylogenetic analyses. The PCR amplification product sizes that were obtained for the mtSSU, EF-1α and IGS regions were 650 bp, 620 bp and 1.6 kb, respectively.

The first phylogeny that was constructed consisted of a combined EF-1α and mtSSU phylogeny, since partition homogeneity testing indicated that these data sets could be combined (*p*-value = 0.315). The phylogeny contained four main clades (clades 1 to 4) and one large sub-clade (4a), all with high bootstrap support ($\geq$ 79%) and probability ($\geq$ 0.96) values (Fig. 4). Clade 1 (100% bootstrap support, 1.00 probability) formed the basal clade and contained all the VCG 0422, 0423 and 0424 isolates (mostly Colorado *Focep* isolates), as well as two non-pathogenic isolates from South Africa (STE-U 6675 and 6685) and the one HSI isolate (STE-U 6655). Clade 2 (100% bootstrap support, 1.00 probability) contained only two isolates from Limpopo (RSA); one a *Focep* (SMV 1) isolate and the other a non-pathogenic isolate (STE-U 6674). Clade 3 (94% bootstrap support, 0.98 probability) did not contain any onion *F. oxysporum* isolates, but included the three *F. oxysporum* f.sp. *cubense* (NRRL 26029, 26024 and 25603) and two *F. oxysporum* f.sp. *lactucae* (F 9501 and FK 09701) isolates that were shown by O'Donnell *et al*. (1998) and Mbofung *et al*. (2007), to represent the most ancestral lineage within their *F. oxysporum* phylogenies. Clade 4 (79% bootstrap support and 0.96 probability value) contained a large well supported sub-clade 4a (98% bootstrap support, 1.00 probability). The majority of *F. oxysporum* f.sp. *cepae* isolates grouped in clade 4a. The EF-1α and mtSSU sequences of the isolates in clade 4a were highly similar indicating that they might be clonal. Within clade 4a STE-U 6639 showed variation that can be attributed to an indel of 15 bp in the EF-1α region. Clade 4a consisted of *Focep* isolates from three VCGs (including the two main VCGs 0421 and 0425), three *Focep* SMVs (SMV 2, 3 and 4),

two non-pathogenic *F. oxysporum* isolates (STEU 6687 and 6695) from the Koue Bokkeveld region in South Africa and one (STE-U 6638) of the two VCG 0426 isolates. The other isolate of VCG 0426 (STE-U 6637) from the Limpopo clustered outside of sub-clade 4a, but still within clade 4. The other *F. oxysporum* isolates that also clustered in clade 4, but outside of sub-clade 4a, mainly consisted of non-pathogenic *F. oxysporum* isolates from different regions in South Africa, other *F. oxysporum* formae speciales isolates and a *Focep* isolate from Germany (NRRL22538).

The relatedness of the South African and Colorado *Focep* isolates to the *Focep* isolates from the study of Galván *et al.* (2008) was investigated using an expanded EF-1α phylogeny, since mtSSU sequences were not available for the isolates of Galván *et al.* (2008). The expanded EF-1α phylogeny included nine *Focep* sequences from Galván *et al.* (2008) that represented six countries (Fig. 5). The topology and clade distribution of the EF-1α phylogeny was comparable to that of the combined EF-1α and mtSSU phylogeny, except that isolate NRRL28371 grouped into a distinct fifth clade. In the expanded EF-1α phylogeny, all nine Galván *et al.* (2008) sequences grouped within clade 4 (81% bootstrap support and 1.00 probability), with only two of the sequences, DSM 62306 from California and EZA from Australia, clustering within sub-clade 4a (99% bootstrap support, 1.00 probability). Two of the Galván *et al.* (2008) *Focep* sequences, one from Germany (CBS 148-25) and one from Uruguay (UR17-8), clustered in clade 4 with the South African VCG 0426 isolate that did not cluster in sub-clade 4a. None of the Galván *et al.* (2008) *Focep* sequences clustered within the basal clade 1 containing *Focep* isolates from Colorado and South Africa.

The IGS data set could not be combined with the EF-1α or mtSSU data set, since partition homogeneity tests with IGS-EF-1α and IGS-mtSSU showed the datasets to be significantly different (*p*-values < 0.05). Parsimony and Bayesian analysis of the IGS data yielded two clades with high bootstrap support and probability values (100% and 1.00 for clade 1; 79% and 1.00 for clade 2) (Fig. 6). When compared to the combined mtSSU and EF-1α phylogeny, the IGS phylogeny only supported the same clade 1 containing *Focep* VCGs 0422, 0423, 0424 and the HSI isolates. The one exception was the divergent placement of the EF-1α-mtSSU-clade 1 isolate, Foc 50, from Colorado (Mesa) that clustered into clade 2 in the IGS

phylogeny among the bulk of the non-pathogenic and *Focep* EF-1α-mtSSU-clade 4a isolates. This result was confirmed by repeating the sequencing of the three gene areas for Foc 50 from new DNA. The non-pathogenic isolate STE-U6675 also did not cluster in clade 1, but grouped basal in clade 2. Even though STE-U6675 had high support in clustering with the other clade 1 isolates, the long branch lengths in the EF-1α-mtSSU phylogeny indicate it to also be different. Within the IGS phylogeny, clade 2 contained all the *Focep* VCG 0421, 0425 and 0426 isolates, as well as most of the non-pathogenic and all of the Mbofung *et al*. (2007) reference isolates. Clade 2 also contained the four *Focep* SMVs 1, 2, 3 and 4 isolates. Within the combined EF-1α and mtSSU phylogeny, SMV 2, 3 and 4 grouped within clade 4, but SMV 1 clustered into a separate clade (clade 2). The two *F. oxysporum* f.sp. *lactucae* isolates, F 9501 and FK 09701, that represented the most basal isolate clade in the study of Mbofung *et al*. (2007), grouped basal within clade 2.

Amplification of *MAT* idiomorphs. The three *MAT1-1* primer pairs differed in their success rate in amplification of the *MAT1-1* idiomorph from various isolates, whereas the two *MAT1-2* primer pairs were equally effective in amplifying the *MAT1-2* idiomorph. For amplification of the *MAT1-1* idiomorph, primer pair FOM111/Falpha2 was most successful, and resulted in the amplification of an approximately 390-bp PCR product from all isolates that were designated as having this idiomorph (Table 1). However, for some isolates other amplicons, in addition to the 390-bp amplicon, were also amplified. Amplification of these isolates with the other *MAT1-1* primer pairs yielded only the one expected amplicon size. The primer pair FOM111/FOM112 only yielded the expected 1 kb PCR product in 41% of the isolates that were identified as *MAT1-1* by primer pair FOM111/Falpha2. Similarly, primer pair Falpha1/Falpha2 only yielded the expected 380-bp product in 60% of the known *MAT1-1* isolates. Attempts to improve the amplification efficiency of the latter two primer pairs by lowering the stringency of reactions (increased magnesium chloride concentration) and amplifications (lower annealing temperatures) conditions, did not result in the amplification of the *MAT1-1* idiomorph from more isolates. For amplification of the *MAT1-2* idiomorph, the FOM211/FOM212 and FOM211/GMH2 primer pairs amplified an approximate 700-bp and 600-bp *MAT1-2* PCR product, respectively, from the same set of isolates.

Almost all of the onion *F. oxysporum* isolates had the *MAT1-1* idiomorph, except for isolate STE-U 6669. Furthermore, for some isolates the *MAT1-1* and the *MAT1-2* idiomorphs were identified within the same isolate (Table 1, Fig. 6). In *Focep* EF-1α-mtSSU-clade 1 and IGS-clade 1, the Colorado *Focep* isolates (VCG 0422, VCG 0423 and VCG 0424 isolates) and the South African *Focep* HSI isolate all only contained the *MAT1-1* idiomorph. In *Focep* EF-1α-mtSSU-clade 4 and IGS-clade 2 the South African *Focep* isolates (VCG 0423, 0425, 0426, SMV 1, 2 and 3) all only contained the *MAT1-1* idiomorph. However, the Colorado *Focep* isolates (VCG 0421 and SMV 4) in these clades all contained the *MAT1-1* and *MAT1-2* idiomorphs within the same isolate. The non-pathogenic *F. oxysporum* isolates from onion contained the *MAT1-1*, *MAT1-2* or a combination of both *MAT1-1* and *MAT1-2* idiomorphs within the same isolate (Table 1, Fig. 6).

The identity of a subset of the amplified *MAT* PCR products was confirmed using sequencing analyses. The consensus sequences (387 bp) obtained from 31 randomly selected isolates with primer pair FOM111 and Falpha 2 had 98% to 100% similarity to *F. oxysporum MAT1-1* GenBank sequences (AY52742 and AY527425) from the study of O'Donnell *et al.* (2004). The *MAT1-1* consensus sequences (GenBank accession GU060619; isolate STE-U 6647) obtained for 27 of the isolates were identical, whereas the consensus sequences (GenBank accession GU060620; isolate STE-U 6640) of the other four isolates (STE-U 6676, STE-U 6640, STE-U 6668 and Foc 8) differed by the same five base pairs from these isolates. The consensus sequences (523 bp) amplified from 18 isolates with primer pair FOM211/GFMH2 or FOM211/FOM212, had 99% similarity to the *MAT1-2 F. oxysporum* f.sp. *lycopersici* GenBank accession AB011378 (Arie *et al.,* 2000). The *MAT1-2* consensus sequences (GenBank accession GU060621; isolate Foc 25) obtained from 10 of the isolates were identical, whereas the consensus sequences (GenBank accession GU060622; isolate STE-U 6667) from the remaining eight isolates (STE-U 6667, STE-U 6678, STE-U 6676, STE-U 6663, STE-U 6677, STE-U 6674, STE-U 6668, STE-U 6672) differed by only one base pair from these isolates.

DISCUSSION

Fusarium oxysporum was the main *Fusarium* species associated with asymptomatic and decayed onion bulbs in South Africa. Most of the *F. oxysporum* isolates were from the Koue Bokkeveld, since this region has a long history of concentrated onion production through the warm summer months when *Focep* is most active. Pathogenicity studies showed that most *Focep* isolates, as expected, were from this region, but non-pathogenic isolates were also obtained. Most *F. oxysporum* isolates from the Northern and Southern Cape, Limpopo and Boland were non-pathogenic. Only two *F. proliferatum* isolates were obtained. Since Koch's postulate was fulfilled for one of the isolates, this is the first report of *F. proliferatum* causing bulb rot in South Africa.

The pathogenicity test that was used in this study to identify *Focep* isolates was conducted under stringent conditions, i.e. high inoculum concentration and favorable environmental conditions. It is, therefore, unlikely that South African isolates would have been identified as non-pathogenic when they were indeed pathogenic. At most, the stringent inoculation conditions could have resulted in some non-pathogens being identified as *Focep.* However, this seems unlikely since none of the four other *F. oxysporum* formae speciales and the two *F. graminearum* isolates was able to cause disease. The pathogenicity tests showed that, of the 60 South African *F. oxysporum* isolates, 27 were *Focep* and 33 were non-pathogenic. The pathogenicity test further confirmed the pathogenicity of the Colorado *Focep* isolates (Swift *et al.,* 2002).

Since only one cultivar (Coastal Cream) was used for identifying *Focep* isolates, the presence of different races within the collection of *F. oxysporum* isolates cannot be excluded. The existence of races in *Focep* is a possibility since more than 20 onion varieties are grown on a commercial scale in Colorado and South Africa. Races can be selected by the widespread deployment of resistant cultivars, although in some formae speciales races seem to have developed even though there was no selection for resistance breaking races (Jiménez-Gasco *et al.*, 2004). Although no races have been identified in *Focep* thus far (Havey, 1995), races have been reported for another bulb rot pathogen, *F. oxysporum* f.sp. *gladioli,* that attacks *Gladiolus*,

Freesia, *Crocus* and *Iris* species (Mes *et al*., 1994; Baayen *et al*., 2000; de Haan *et al*., 2000).

VCG analyses of South African and Colorado *Focep* isolates resulted in the identification of three new VCGs (0424 to 0426), in addition to the previously reported four VCGs (0420 to 0423) (Yoo *et al.*, 1993; Swift *et al*., 2002). Furthermore, four single member VCGs, three from South Africa (SMV 1 to 3) and one from Colorado (SMV 4) are also now known. These single member VCGs cannot receive official VCG status until more isolates are identified that can anastomose with them. Although Swift *et al.* (2002) previously identified three Colorado *Focep* VCGs (VCG 0421, 0422 and 0423), the current study identified one new VCG, VCG 0424, among the collection of isolates used in their study. Only one of the South African *Focep* isolates belonged to a VCG, VCG 0423, previously identified in Colorado, with the other isolates fitting into two new VCGs (VCG 0425 and VCG 0426). In each region, one VCG dominated, namely VCG 0421 (47% of isolates) in Colorado and VCG 0425 (74% of isolates) in South Africa. This suggests that within each region one VCG has been selected that is most successful at infecting the wide range of varieties that are grown, and adapting to the local environmental conditions. The relative large number of *Focep* VCGs and SMVs is in contrast to the few VCGs identified in other bulb and corm rot formae speciales. *Fusarium oxysporum* f.sp. *tulipae* and *F. oxysporum* f.sp. *lilii* each only contains one VCG, whereas *F. oxysporum* f.sp. *gladioli* contains two VCGs (Baayen *et al*., 1998; Roebroeck and Mes, 1992).

A combined EF-1α and mtSSU phylogeny, which contained four well supported clades (clades 1 to 4), for the most part supported the putative clonal nature of the *Focep* VCGs. In other *F. oxysporum* formae speciales, mtSSU and/or EF-1α analyses have also shown the clonal nature of VCGs, but that more than one VCG can be included within a clonal lineage (Kistler, 1997; O'Donnell *et al.*, 1998; Baayen *et al*., 2000). The latter was true for *Focep* VCGs 0421, 0425, 0426 (but only one of the two isolates) and three single member VCGs (SMV 2, 3 and 4) that formed a clonal lineage within clade 4a. The remaining VCGs (VCGs 0422, 0423 and 0424) clustered into clade 1, with VCG 0422 and 0423 clustering together in a well supported clade (88% bootstrap support, 0.95 probability) and the two clonal isolates from VCG 0424

grouping basal to these two VCGs. An important aspect that was supported by the EF-1α and mtSSU phylogeny is the re-classification of the Colorado *Focep* isolate Foc 22 into the same VCG (VCG 0424) as isolate Foc 23, since these two isolates formed a clonal lineage. The separate grouping of the two VCG 0426 isolates (STE-U 6636 and 6638), one in sub-clade 4a and the other basal to this clade but still within clade 4, is unexpected. However, Lievens *et al*. (2007) also found that isolates within two of the VCGs of *F. oxysporum* f.sp. *cucumerinum* were not clonal but polyphyletic.

Several hypotheses can be formed to explain the observation that putative non-pathogenic *F. oxysporum* isolates were present in both of the *Focep* EF-1α-mtSSU-clades (4a and 1), and that some of these were genetically similar to *Focep* isolates. These putative non-pathogenic isolates can either be (ii) true non-pathogenic isolates, (ii) *Focep* isolates that are a different race that cannot infect the cultivar (Coastal Cream) used in the pathogenicity study, (iii) *Focep* isolates that lost their pathogenictiy or (iv) *Focep* isolates that emerged from local non-pathogenic strains. The evolution of *F. oxysporum* formae speciales isolates from soil or endophytic non-pathogenic *F. oxysporum* isolates is very difficult to prove conclusively, but has been hypothesized by Gordon and Okamoto (1992) and Skovgaard *et al*. (2002).

The combined EF-1α and mtSSU phylogeny, as well as an expanded EF-1α phylogeny which contained sequences from *Focep* isolates from other regions of the world, confirmed the polyphyletic origin of *Focep* as reported by Galván *et al.* (2008) and Dissanayake *et al.* (2009a). In other bulb and corm rot formae speciales, either a polyphyletic (f.sp. *gladioli*) or monophyletic (f.sp. *lilii* and *tulipae*) origin has been identified (Baayen *et al*., 1998, 2000). The expanded EF-1α and the combined EF-1α and mtSSU phylogenies each contained the same four main clades, although a fifth clade was present in the expanded EF-1α that only contained one *F. oxysporum* isolate from lily. Most of the *Focep* isolates clustered within clades 1 and 4a of these phylogenies. The remaining *Focep* isolates clustered in clade 2 (only 1 isolate) or basal to clade 4a, within clade 4. *Focep* clade 4a seems to be the clade that has a world-wide distribution since it included the major VCGs (0425 and 0421) from South Africa and Colorado, as well as two isolates from California and Australia. The *Focep* SMV 1 isolate in clade 2, may either be a newly emerging *Focep* clade that could increase in frequency in future, or it may be a clade that is less successful at

infecting all the cultivars and adapting to local environmental conditions in the Koue Bokkeveld, especially since it was only found in the Limpopo Province. *Focep* clade 1, which mainly contained isolates from Colorado but also one HSI isolate and non-pathogenic isolates from South Africa, grouped basal in all the phylogenies. The basal *Focep* clade 1 may be an ancient lineage from which other *Focep* clades may have evolved. This clade also grouped basal (EF-1α and mtSSU phylogeny) to sequences in *F. oxysporum* clades that were identified as the most basal clades in the studies of O'Donnell *et al.* (1998), Mbofung *et al.* (2007) and Baayen *et al.* (2000). Interestingly, *Focep* isolates within clade 1 also contained the largest variation in virulence (20% to 100% necrosis), with most isolates being moderately virulent. This variance in isolate virulence may be due to the virulence of the isolates being reduced during storage, or due to genetic differences in virulence of the isolates on cultivar Coastal Cream.

The *Focep* IGS data set was incongruent with the EF-1α and mtSSU data sets. Similar observations were made in other *F. oxysporum* studies, including studies on *F. oxysporum* f.sp. *cubense* (Fourie *et al*., 2009), *F. oxysporum* f.sp. *lactucae* (Mbofung *et al.*, 2007) and several plant and human pathogens within the *F. oxysporum* species complex (O'Donnel *et al.*, 2009). In some formae speciales, the IGS has been useful for resolving VCGs (Alves-Santos *et al*., 1999; Lori *et al*., 2004; Enya *et al*., 2008) and lineages (Fourie *et al.*, 2009), differentiating pathogenic isolates from non-pathogens (Mbofung *et al.*, 2007), and distinguishing pathogenic isolates with different virulence characteristics (Dissanayake *et al*., 2009a). However, this was not true for the collection of *Focep* isolates used within the current study. This was most evident in the divergent placement of one of the Colorado VCG 0423 isolate (Foc 50) in the IGS phylogeny. This isolate was placed in clade 1 in the EF-1α-mtSSU phylogeny, but grouped with EF-1α-mtSSU clade 4a isolates in the IGS phylogeny. One possible explanation could be genetic recombination or genetic exchange between isolate Foc 50 and an isolate within EF-1α-mtSSU-clade 4a. If this was through parasexual recombination, the vegetative incompatibility of isolates would have to be overcome. A similar observation was made for one of the non-pathogenic *F. oxysporum* isolates (STE-U6673) that also had a divergent placement in the IGS phylogeny.

Several reasons, aside from genetic recombination or exchange, can explain the incongruence of the *Focep* IGS data set with those of the EF-1α and mtSSU regions. Mbofung *et al*. (2007) suggested that (i) the IGS may have the same systematic problem as the ITS2 region in *Fusarium*, with divergent copies being present due to an ancient hybridization event that were distributed unequally among lineages, (ii) the gene sequence regions have unequal rates of evolution or (iii) incomplete concerted evolution occurred in the IGS region. The study of Apple and Gordon (1996) suggested that genetic recombination or exchange of genetic material in *F. oxysporum* f.sp. *melonis* may indeed be the reason for incongruency. By using a cloning and sequencing approach they found that one isolate contained two polymorphic IGS sequence copies that each belonged to a different VCG (Apple and Gordon, 1996). O'Donnell *et al.* (2009) hypothesized that horizontal gene transfer, possibly mediated by parasexuality, might be the cause of incongruency in IGS datasets.

This is the first study to report the presence of two different *MAT* idiomorphs within the same *F. oxysporum* isolate, although this species complex is generally considered to be heterothallic (Arie *et al*., 2000). It is important to note that the identification of the specific *MAT1-1* and *MAT1-2* idiomorphs in the current collection of isolates was dependent on the use of the correct primer pair combinations, which is important to consider when conducting *MAT* genotyping. Almost all the *Focep* isolates contained the *MAT1-1* idiomorph, except for the non-pathogenic isolate STE-U 6669 from Limpopo that only had a *MAT1-2* idiomorph. The distribution of isolates that contained both idiomorphs within the same isolate was VCG specific, since all the VCG 0421 isolates contained both idiomorphs within the same isolate. Additionally, several of the non-pathogenic isolates and SMV 4 also contained both idiomorphs in the same isolate. The distribution of *F. oxysporum* isolates that contained either the *MAT1-1* or both *MAT1-1* and *MAT1-2* idiomorphs was not specific to any of the phylogenetic clades in the EF-1α-mtSSU or IGS phylogenies.

There are several factors that suggest that the exchange of genetic material and recombination among *Focep* isolates from different VCGs, and non-pathogenic onion *F. oxysporum* isolates, may play an important role in the evolution of *Focep*. These

include (i) the incongruence of the IGS with the mtSSU and EF-1α data sets, (ii) the identification of both *MAT1-1* and *MAT1-2* idiomorphs within the same isolate, (iii) the polyphyletic nature of VCG 0426 and (iv) the genetic similarity of some *Focep* and non-pathogenic *F. oxysporum* isolates based on three gene sequence regions.

The mechanisms by which exchange of genetic material and recombination takes place between *F. oxysporum* isolates could involve sexual reproduction, parasexuality, horizontal gene transfer or mutation and transposition. The role of sexual reproduction in *F. oxysporum* is uncertain since no teleomorph has been described (Koenig *et al*., 1997; Baayen *et al*., 2000). Therefore, this species complex is considered to be predominantly asexual, although sexual reproduction may have occurred in the early evolution of this species complex in ancient lineages (Taylor *et al*., 1999; O'Donnell *et al*., 2004; Fourie *et al*., 2009). Since several studies have shown that the mtSSU (mitochondrial) and EF-1α (nuclear) phylogenies in *F. oxysporum* are congruent (Baayen *et al*., 2000; Skovgaard *et al*., 2001; Mbofung *et al*., 2007), sexual reproduction and/or parasexuality is most likely a rare event. The congruency of these gene areas would rather support predominantly asexual reproduction, since asexual reproduction would result in the mitochondrial and nuclear genomes being inherited as a unit yielding congruent phylogenies (Schurko *et al*., 2009).

Parasexuality has been proposed to play a role in exchange of genetic material in some *F. oxysporum* formae speciales (Molnar *et al.*, 1990; Leslie, 1993; Teunissen *et al.*, 2002). This mechanism may be limited to vegetative compatible strains since a pre-requisite for parasexuality is the fusion of hyphae that allows the formation of a heterokaryon. However, in *F. oxysporum,* evidence for the occurrence of parasexuality between vegetatively incompatible strains has been shown, but only under laboratory conditions (Molnar *et al.*, 1990). In contrast, in another ascomycete, *Cryphonectria parasitica* (Murrill) Barr, the formation of heterokaryons by vegetatively incompatible individuals and parasexuality is thought to play a role in the evolution of this pathogen under natural field conditions (McGuire *et al.*, 2005). Parasexuality could contribute to the horizontal transfer of pathogenicity genes or conditionally dispensable chromosomes between strains (Covert, 1998; Baayen *et al*., 2000). On this point it is interesting to note that in *F. oxysporum* f.sp. *dianthi* and *F.*

oxysporum f.sp. *cubense* a strong association has been found between chromosome number and VCG, races and lineages (Boehm *et al*., 1994; Migheli *et al*., 1995; O'Donnell *et al*., 1998). Furthermore, some evidence for the role of dispensable chromosomes in the virulence of *F. oxysporum* has also been found (Van der Does *et al*., 2008; Chakrabarti *et al.,* 2009; Ellis *et al*., 2009). The presence of both *MAT* idiomorphs in *Focep* VCG 0421 isolates and some non-pathogenic isolates could possibly be due to the presence of additional chromosomes, and therefore future investigations should investigate the chromosome number in different *Focep* VCGs.

This study confirmed *Focep* as the causal organism of Fusarium basal rot of onion in South Africa. This is also the first report of *F. proliferatum* causing bulb rot of onion in South Africa. Three new *Focep* VCGs and three new SMVs were identified among *Focep* isolates from South Africa and Colorado, increasing the total number of official *Focep* VCGs to seven. In each of these regions, one VCG dominated, namely VCG 0421 in Colorado and VCG 0425 in South Africa. Multi-locus phylogenies (i) supported the putative clonal nature of the main *Focep* VCGs (0421 and 0425), (ii) identified an ancient *Focep* lineage from which other *Focep* clades containing the major VCGs (0421 and 0425) may have evolved, and (iii) confirmed the polyphyletic origin of *Focep*. Possible genetic exchange between isolates via parasexuality and/or sexual reproduction was supported by incongruent nuclear datasets, the polyphyletic nature of one *Focep* VCG and the presence of both *MAT1-1* and *MAT1-2* idiomorphs within the same isolate.

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Table 1. Characteristics (pathogenicity, vegetative compatibility group and mating type idiomorph) of *Fusarium* isolates collected from different regions in South Africa, and their GenBank accession number for three sequenced regions

Isolate	Species name	Regional origin	Year[a]	Disease severity[b] (%)	VCG[c]	*MAT* idiomorph	GenBank Accession number mtSSU	TEF	IGS
STE-U6636[d]	*Focep* [e]	Limpopo, RSA	2005	100	SMV 1	*MAT1-1*	GU166046	GU165888	GU165967
STE-U6637	*Focep*	Limpopo, RSA	2005	83	0 426	*MAT1-1*	GU166047	GU165889	GU165968
STE-U6638	*Focep*	Koue Bokkeveld, RSA	2005	97	0 426	*MAT1-1*	GU166048	GU165890	GU165969
STE-U6639	*Focep*	Koue Bokkeveld, RSA	2005	83	0 425	*MAT1-1*	GU166049	GU165891	GU165970
STE-U6640	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166050	GU165892	GU165971
STE-U6641	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166051	GU165893	GU165972
STE-U6642	*Focep*	Koue Bokkeveld, RSA	2005	90	0 425	*MAT1-1*	GU166052	GU165894	GU165973
STE-U6643	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166053	GU165895	GU165974
STE-U6644	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166054	GU165896	GU165975
STE-U6645	*Focep*	Koue Bokkeveld, RSA	2005	100	SMV 2	*MAT1-1*	GU166055	GU165897	GU165976
STE-U6646	*Focep*	Koue Bokkeveld, RSA	2005	99	0 425	*MAT1-1*	GU166056	GU165898	GU165977
STE-U6647	*Focep*	Koue Bokkeveld, RSA	2005	83	0 425	*MAT1-1*	GU166057	GU165899	GU165978
STE-U6648	*Focep*	Koue Bokkeveld, RSA	2005	91	0 425	*MAT1-1*	GU166058	GU165900	GU165979
STE-U6649	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166059	GU165901	GU165980
STE-U6650	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166060	GU165902	GU165981
STE-U6651	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166061	GU165903	GU165982
STE-U6652	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166062	GU165904	GU165983
STE-U6653	*Focep*	Koue Bokkeveld, RSA	2005	92	0 425	*MAT1-1*	GU166063	GU165905	GU165984
STE-U6654	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166064	GU165906	GU165985
STE-U6655	*Focep*	Koue Bokkeveld, RSA	2005	77	HSI	*MAT1-1*	GU166065	GU165907	GU165986
STE-U6656	*Focep*	Koue Bokkeveld, RSA	2005	83	0 425	*MAT1-1*	GU166066	GU165908	GU165987
STE-U6657	*Focep*	Koue Bokkeveld, RSA	2005	100	0 425	*MAT1-1*	GU166067	GU165909	GU165988
STE-U6658	*Focep*	Koue Bokkeveld, RSA	2005	100	SMV 3	*MAT1-1*	GU166068	GU165910	GU165989

Isolate	Species name	Regional origin	Year[a]	Disease severity[b] (%)	VCG[c]	MAT idiomorph	GenBank Accession number mtSSU	TEF	IGS
STE-U6659	*Focep*	Koue Bokkeveld, RSA	2005	50	0 423	*MAT1-1*	GU166069	GU165911	GU165990
STE-U6660	*Focep*	Koue Bokkeveld, RSA	2006	100	0 425	*MAT1-1*	GU166070	GU165912	GU165991
STE-U6661	*Focep*	Koue Bokkeveld, RSA	2006	99	0 425	*MAT1-1*	GU166071	GU165913	GU165992
STE-U6662	*Focep*	Boland, RSA	2006	97	0 425	*MAT1-1*	GU166072	GU165914	GU165993
Foc 1[f]	*Focep*	Eastern Colorado, USA	1992	91	0 421	*MAT1-1&MAT1-2*	GU166073	GU165915	GU165994
Foc 4[f]	*Focep*	Southern Colorado, USA	1988	100	0 421	*MAT1-1&MAT1-2*	GU166074	GU165916	GU165995
Foc 8	*Focep*	Western Colorado, USA	1993	100	0 421	*MAT1-1&MAT1-2*	GU166075	GU165917	GU165996
Foc 25	*Focep*	Western Colorado, USA	1999	100	0 421	*MAT1-1&MAT1-2*	GU166076	GU165918	GU165997
Foc 27	*Focep*	Western Colorado, USA	1999	79	0 421	*MAT1-1&MAT1-2*	GU166077	GU165919	GU165998
Foc 28	*Focep*	Western Colorado, USA	1999	84	0 421	*MAT1-1&MAT1-2*	GU166078	GU165920	GU165999
Foc 22[f, g]	*Focep*	Western Colorado, USA	1999	100	0 424	*MAT1-1*	GU166079	GU165921	GU166000
Foc 201 A	*Focep*	Eastern Colorado, USA	1998	100	0 421	*MAT1-1&MAT1-2*	GU166080	GU165922	GU166001
Foc 201 D	*Focep*	Eastern Colorado, USA	1998	100	0 421	*MAT1-1&MAT1-2*	GU166081	GU165923	GU166002
Foc 204 A	*Focep*	Eastern Colorado, USA	1998	100	0 421	*MAT1-1&MAT1-2*	GU166082	GU165924	GU166003
Foc 31[f]	*Focep*	Western Colorado, USA	1999	80	0 422	*MAT1-1*	GU166083	GU165925	GU166004
Foc 32[f]	*Focep*	Western Colorado, USA	1999	20	0 422	*MAT1-1*	GU166084	GU165926	GU166005
Foc 41[f]	*Focep*	Western Colorado, USA	1999	100	0 423	*MAT1-1*	GU166085	GU165927	GU166006
Foc 42[f]	*Focep*	Western Colorado, USA	1999	50	0 423	*MAT1-1*	GU166086	GU165928	GU166007
Foc 48	*Focep*	Western Colorado, USA	1999	50	0 423	*MAT1-1*	GU166087	GU165929	GU166008
Foc 50	*Focep*	Western Colorado, USA	1999	30	0 423	*MAT1-1*	GU166088	GU165930	GU166009
Foc 47	*Focep*	Western Colorado, USA	1999	46	0 423	*MAT1-1*	GU166089	GU165931	GU166010
Foc 21[f]	*Focep*	Western Colorado, USA	1999	100	SMV 4	*MAT1-1&MAT1-2*	GU166090	GU165932	GU166011
Foc 23[f,h]	*Focep*	Western Colorado, USA	1999	75	0 424	*MAT1-1*	GU166091	GU165933	GU166012
STE-U6663	*F. oxysporum*	Southern Cape, RSA	2005	1	n.d.	*MAT1-1&MAT1-2*	GU166092	GU165934	GU166013
STE-U6664	*F. oxysporum*	Boland, RSA	2005	4	n.d.	*MAT1-1*	GU166093	GU165935	GU166014
STE-U6665	*F. oxysporum*	Boland, RSA	2005	1	n.d.	*MAT1-1*	GU166094	GU165936	GU166015

Isolate	Species name	Regional origin	Year[a]	Disease severity[b] (%)	VCG[c]	MAT idiomorph	GenBank Accession number mtSSU	TEF	IGS
STE-U6666	*F. oxysporum*	Boland, RSA	2005	0	n.d.	*MAT1-1*	GU166095	GU165937	GU166016
STE-U6667	*F. oxysporum*	Limpopo, RSA	2005	7	n.d.	*MAT1-1&MAT1-2*	GU166096	GU165938	GU166017
STE-U6668	*F. oxysporum*	Limpopo, RSA	2005	0	n.d.	*MAT1-1&MAT1-2*	GU166097	GU165939	GU166018
STE-U6669	*F. oxysporum*	Limpopo, RSA	2005	0	n.d.	*MAT1-2*	GU166098	GU165940	GU166019
STE-U6671	*F. oxysporum*	Limpopo, RSA	2005	3	n.d.	*MAT1-1*	GU166099	GU165941	GU166020
STE-U6672	*F. oxysporum*	Limpopo, RSA	2005	0	n.d.	*MAT1-1&MAT1-2*	GU166100	GU165942	GU166021
STE-U6673	*F. oxysporum*	Limpopo, RSA	2005	8	n.d.	*MAT1-1*	GU166101	GU165943	GU166022
STE-U6674	*F. oxysporum*	Limpopo, RSA	2005	0	n.d.	*MAT1-1&MAT1-2*	GU166102	GU165944	GU166023
STE-U6675	*F. oxysporum*	Limpopo, RSA	2005	1	SMV 5	*MAT1-1*	GU166103	GU165945	GU166024
STE-U6676	*F. oxysporum*	Limpopo, RSA	2005	3	n.d.	*MAT1-1&MAT1-2*	GU166104	GU165946	GU166025
STE-U6677	*F. oxysporum*	Northern Cape, RSA	2005	0	n.d.	*MAT1-1&MAT1-2*	GU166105	GU165947	GU166026
STE-U6678	*F. oxysporum*	Northern Cape, RSA	2005	0	n.d.	*MAT1-1&MAT1-2*	GU166106	GU165948	GU166027
STE-U6679	*F. oxysporum*	Northern Cape, RSA	2005	0	n.d.	*MAT1-1*	GU166107	GU165949	GU166028
STE-U6680	*F. oxysporum*	Northern Cape, RSA	2005	0	n.d.	*MAT1-1*	GU166108	GU165950	GU166029
STE-U6681	*F. oxysporum*	Koue Bokkeveld, RSA	2005	9	n.d.	*MAT1-1&MAT1-2*	GU166109	GU165951	GU166030
STE-U6682	*F. oxysporum*	Koue Bokkeveld, RSA	2005	0	SMV 6	*MAT1-1&MAT1-2*	GU166110	GU165952	GU166031
STE-U6683	*F. oxysporum*	Koue Bokkeveld, RSA	2005	1	SMV 7	*MAT1-1&MAT1-2*	GU166111	GU165953	GU166032
STE-U6684	*F. oxysporum*	Koue Bokkeveld, RSA	2005	8	SMV 8	*MAT1-1*	GU166112	GU165954	GU166033
STE-U6685	*F. oxysporum*	Koue Bokkeveld, RSA	2005	0	n.d.	*MAT1-1&MAT1-2*	GU166113	GU165955	GU166034
STE-U6686	*F. oxysporum*	Koue Bokkeveld, RSA	2005	8	n.d.	*MAT1-1*	GU166114	GU165956	GU166035
STE-U6687	*F. oxysporum*	Koue Bokkeveld, RSA	2006	1	n.d.	*MAT1-1*	GU166115	GU165957	GU166036
STE-U6688	*F. oxysporum*	Koue Bokkeveld, RSA	2006	0	SMV 9	*MAT1-1&MAT1-2*	GU166116	GU165958	GU166037
STE-U6689	*F. oxysporum*	Koue Bokkeveld, RSA	2007	0	n.d.	*MAT1-1&MAT1-2*	GU166117	GU165959	GU166038
STE-U6690	*F. oxysporum*	Koue Bokkeveld, RSA	2007	0	n.d.	*MAT1-1&MAT1-2*	GU166118	GU165960	GU166039
STE-U6691	*F. oxysporum*	Koue Bokkeveld, RSA	2007	2	n.d.	*MAT1-1&MAT1-2*	GU166119	GU165961	GU166040

Isolate	Species name	Regional origin	Year[a]	Disease severity[b] (%)	VCG[c]	*MAT* idiomorph	GenBank Accession number		
							mtSSU	TEF	IGS
STE-U6692	*F. oxysporum*	Koue Bokkeveld, RSA	2007	0	n.d.	*MAT1-1&MAT1-2*	GU166120	GU165962	GU166041
STE-U6693	*F. oxysporum*	Koue Bokkeveld, RSA	2007	0	n.d.	*MAT1-1&MAT1-2*	GU166121	GU165963	GU166042
STE-U6694	*F. oxysporum*	Koue Bokkeveld, RSA	2007	3	n.d.	*MAT1-1&MAT1-2*	GU166122	GU165964	GU166043
STE-U6695	*F. oxysporum*	Koue Bokkeveld, RSA	2007	0	n.d.	*MAT1-1*	GU166123	GU165965	GU166044
STE-U6696	*F. oxysporum*	Koue Bokkeveld, RSA	2007	0	n.d.	*MAT1-1&MAT1-2*	GU166124	GU165966	GU166045
STE-U6946	*F. acutatum*	Northern Cape, RSA	2005	15	n.d.	n.d.	n.d.	n.d.	n.d.
STE-U6947	*F. proliferatum*	Limpopo, RSA	2005	44	n.d.	n.d.	n.d.	n.d.	n.d.
STE-U6948	*F. solani*	Southern Cape, RSA	2005	0	n.d.	n.d.	n.d.	n.d.	n.d.
STE-U6949	*F. proliferatum*	Southern Cape, RSA	2005	16	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Year of collection.

[b] For disease severity determination, the basal plates of four onion bulbs were inoculated with *Fusarium* in two repeat experiments. An isolate was designated as being pathogenic towards onion and thus as *Focep,* if the isolate caused 20% or more necrosis of the basal plate as an average over the eight onion bulbs.

[c] The VCG code consists of a four digit number, with the first three digits corresponding to forma specialis, and the fourth digit to designate VCGs within the forma specialis. For *Fusarium oxysporum* isolates that did not anastomose with any other isolate, a single member VCG (SMV) number was assigned.
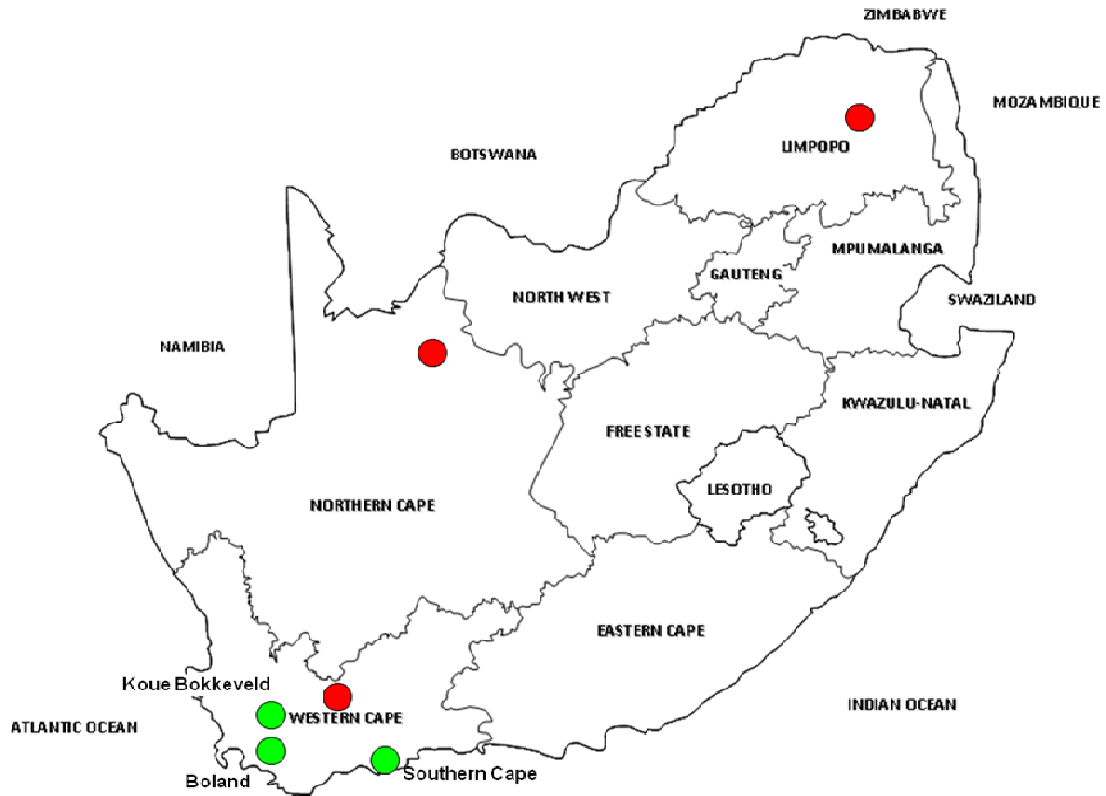
[d] The isolates collected from onion in South Africa were deposited in the fungal culture collection of Stellenbosch University, Department of Plant Pathology.

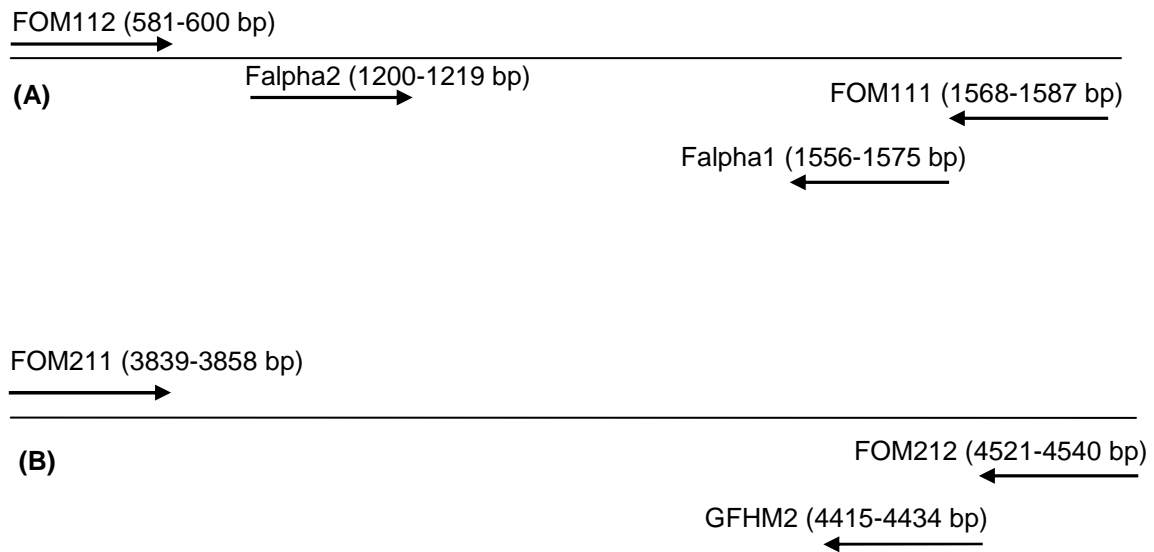[e] *Focep = Fusarium oxysporum* f.sp. *cepae.*

[f] The nine representative *Focep* isolates from Colorado used in VCG determination of the *Focep* isolates from South Africa.

[g] Isolate Foc 22 was formerly numbered as VCG 0421 (Swift *et al*., 2002). VCG and phylogenetic analysis in the current study placed this isolate into a new Colorado VCG, namely VCG 0424.

[h] Isolate Foc 23 was formerly identified as a SMV with no assigned VCG coding (Swift *et al*., 2002). VCG and phylogenetic analysis in the current study placed this isolate into a new Colorado VCG, namely VCG 0424.

Fig. 1. *Fusarium* isolates were obtained from onion bulbs collected in three provinces (Limpopo, Western Cape and Northern Cape) in South Africa. Within the Western Cape Province isolates were obtained from three regions, i.e. Koue Bokkeveld, Boland and the Southern Cape.

FOM112 (581-600 bp)

(A)

Falpha2 (1200-1219 bp)

FOM111 (1568-1587 bp)

Falpha1 (1556-1575 bp)

FOM211 (3839-3858 bp)

(B)

FOM212 (4521-4540 bp)

GFHM2 (4415-4434 bp)

Fig. 2. Schematic diagram (not drawn to scale) showing the annealing positions of primers that amplify *Fusarium oxysporum* mating type (*MAT*) idiomorphs (A) *MAT1-1* and (B) *MAT1-2*. The annealing positions of the *MAT1-1* primers are based on base pair assignments in Genbank accession AB011379, and that of the *MAT1-2* idiomorph on base pair assignments in accession AB011378.
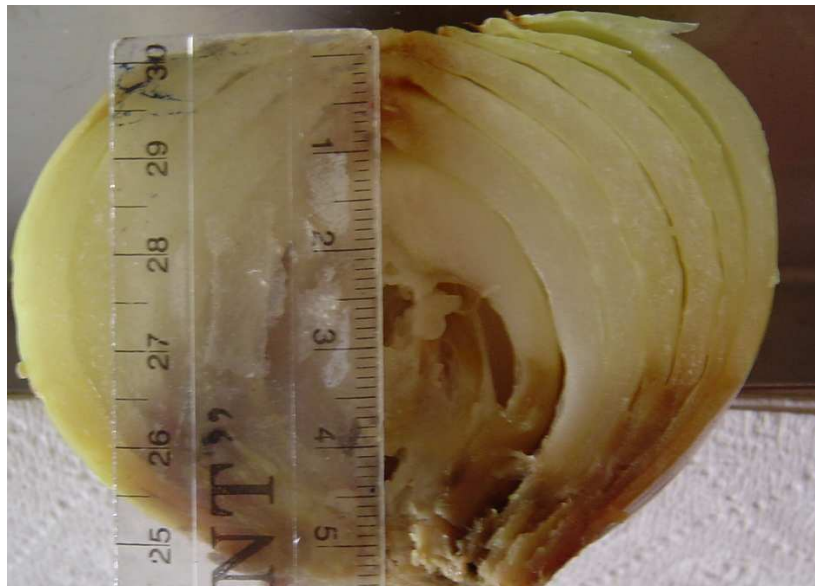

Fig. 3. Characteristic *Fusarium* dry rot symptom caused by *F. oxysporum* f.sp. *cepae* on Coastal Cream Pukekohe onions after inoculation with a highly virulent isolate.
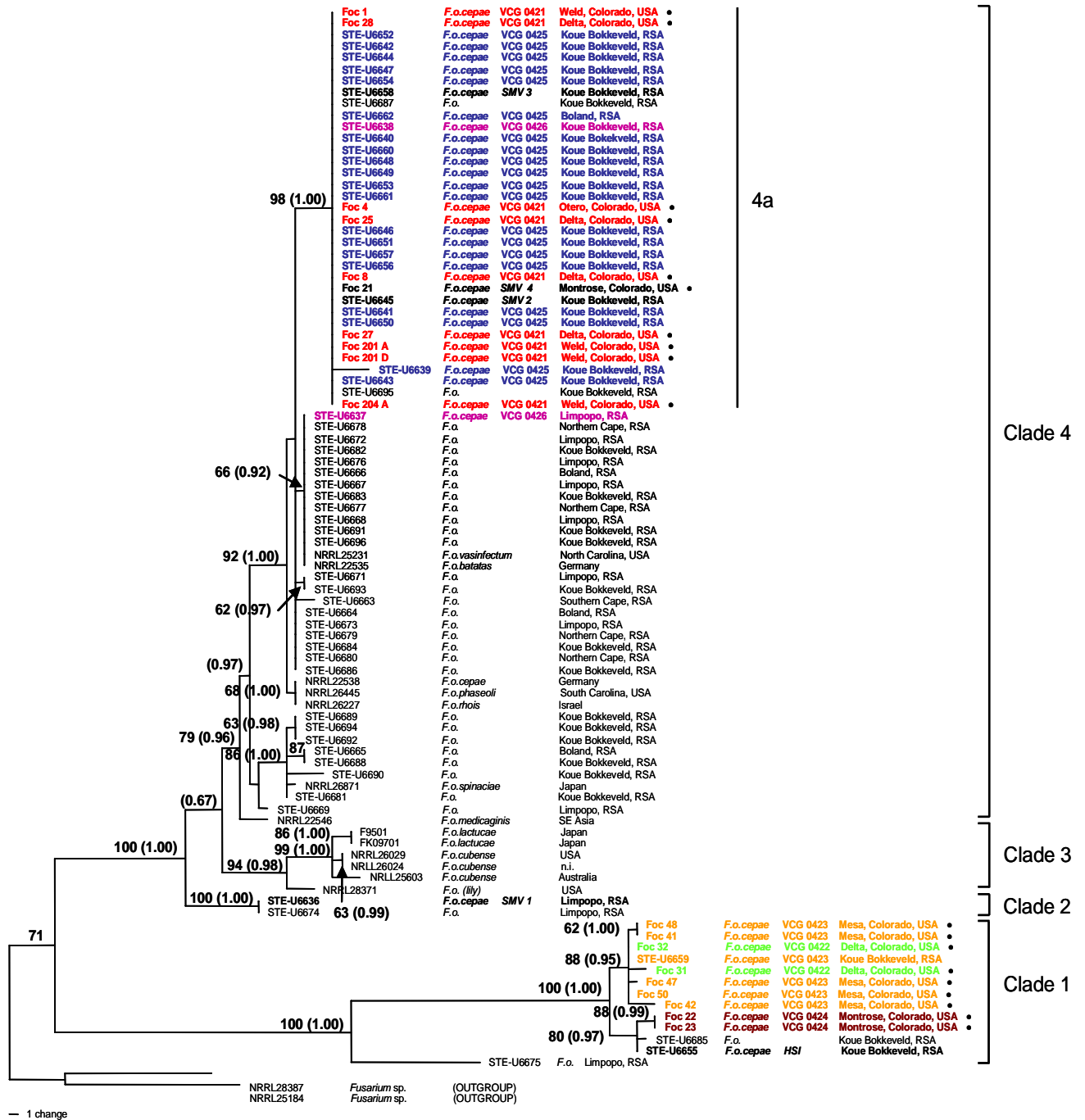
Fig. 4. Phylogeny of *Fusarium oxysporum* (*F.o.*) isolates based on the combined analysis of translation elongation factor and mitochondrial small subunit sequences. The tree presents one of 100 equally parsimonious trees of a heuristic search. Numbers within the tree represent the bootstrap values (1000 replicates) followed by probability values in brackets. Only bootstrap values above 60% are shown. Length = 219, CI = 0.863, RI = 0.977 and RC = 0.844. South African (RSA) *F. oxysporum* f.sp. *cepae* (*F.o.cepae*) and *F.o.* sequences have STE-U codes. United States (USA) Colorado *F.o.cepae* sequences are followed by "●". Each of the specific *F.o.cepae* vegetative compatibility groups (VCGs) are colour coded. Sequences of *F.o.cepae* single member VCGs (SMV) and heterokaryon self incompatible (HSI) isolates are shown in bold. The place of origin of isolates is shown to the right of the phylogram. NRRL 25184 and NRRL 28387 (Baayen *et al.*, 2000) were used as outgroups to root the phylogram.
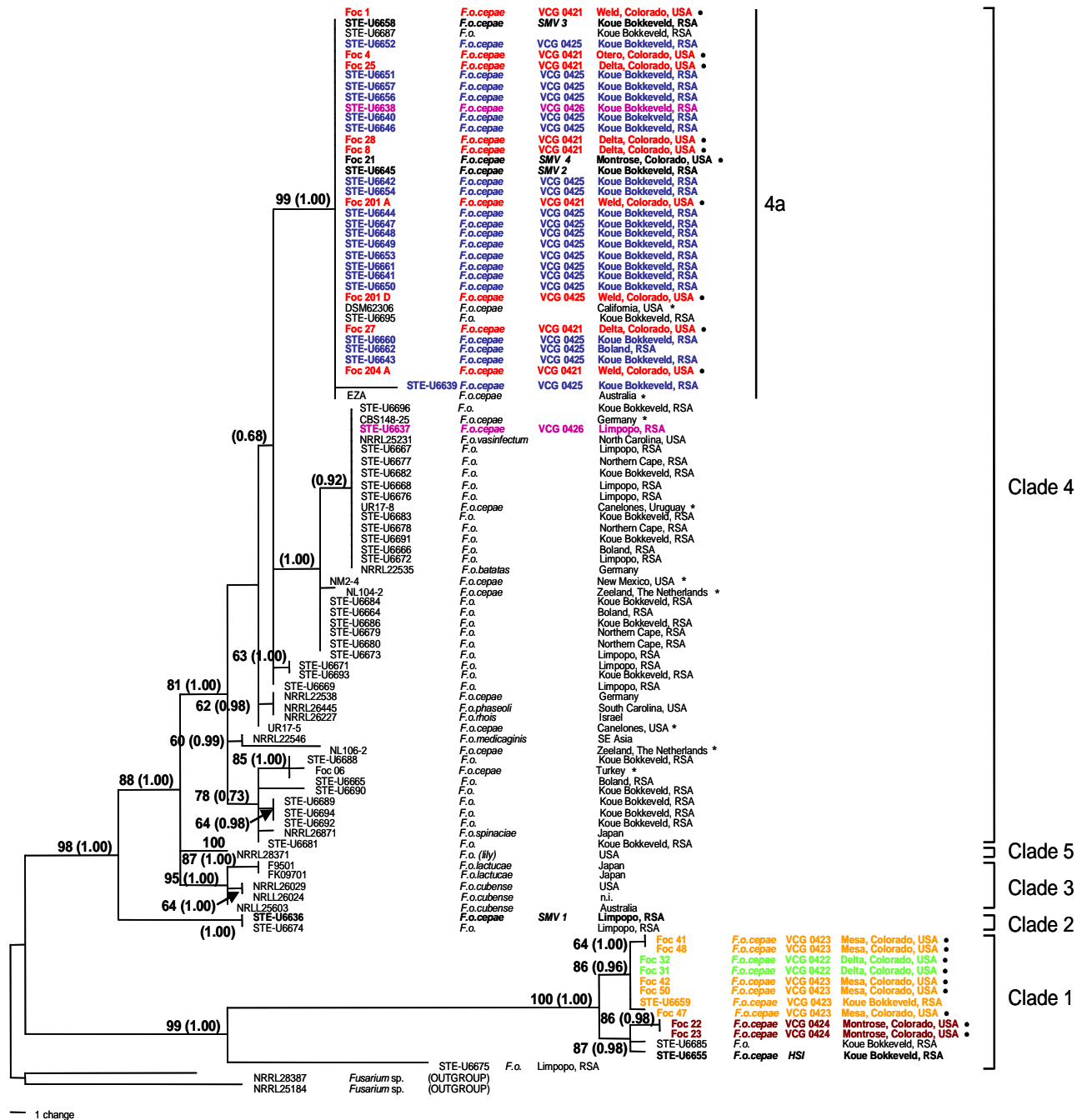
Fig. 5. Phylogeny of *Fusarium oxysporum* (*F.o.*) isolates based on translation elongation factor sequences. The tree presents one of 100 equally parsimonious trees of a heuristic search. Numbers within the tree represent the bootstrap values (1000 replicates) followed by probability values in brackets. Only bootstrap values above 60% are shown. Length = 152, CI = 0.868, RI = 0.978 and RC = 0.849. South African (RSA) *F. oxysporum* f.sp. *cepae* (*F.o.cepae*) and *F.o.* sequences have STE-U codes. United States (USA) Colorado *F.o.cepae* sequences are followed by "●" and those from the study of Gálvan *et al.* (2008) are followed by "*". Each of the specific *F.o.cepae* vegetative compatibility groups (VCGs) are colour coded. Sequences of *F.o.cepae* single member VCGs (SMV) and heterokaryon self incompatible (HSI) isolates are in bold. The place of origin of isolates is shown to the right of the phylogram. NRRL 25184 and NRRL 28387 (Baayen *et al.*, 2000) were used as outgroups to root the phylogram.
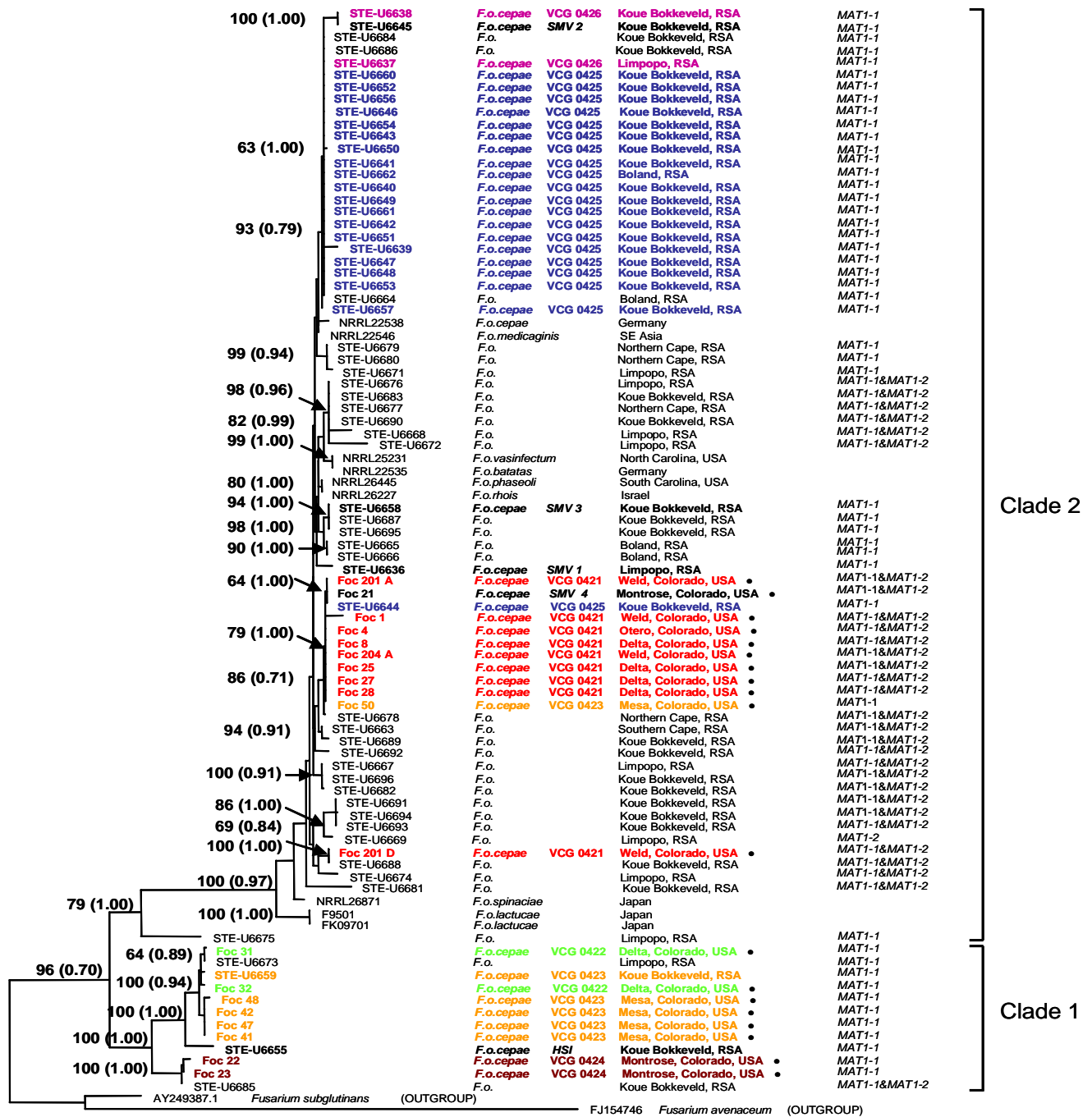
Fig. 6. Phylogeny of *Fusarium oxysporum* (*F.o.*) isolates based on intergenic spacer (IGS) region sequences. The tree presents one of 100 equally parsimonious trees of a heuristic search. Numbers within the tree represent the bootstrap values (1000 replicates) followed by probability values in brackets. Only bootstrap values above 60% are shown. Length = 1242, CI = 0.781, RI = 0.913 and RC = 0.713. South African (RSA) *F. oxysporum* f.sp. *cepae* (*F.o.cepae*) and *F.o.* sequences have STE-U codes. United States (USA) Colorado *F.o.cepae* sequences are followed by "●". Each of the specific *F.o.cepae* vegetative compatibility groups (VCGs) are colour coded. Sequences of *F.o.cepae* single member VCGs (SMV) and heterokaryon self incompatible (HSI) isolates are in bold. The place of origin of isolates is shown to the right of the phylogram, followed by the mating type *(MAT)* idiomorph identified within each isolate. Accession numbers AY249387.1 (*F. subglutinans*) (Schweigkofler *et al*., 2004) and FJ154746 (*F. avenaceum*) (Nalim *et al*., 2009) were used as outgroups to root the phylogram.

3. IDENTIFICATION OF THE TWO MAIN VEGETATIVE COMPATIBILITY GROUPS OF *FUSARIUM OXYSPORUM* F.SP. *CEPAE* USING DNA FINGERPRINTING AND SEQUENCE CHARACTERIZED AMPLIFIED MARKERS

ABSTRACT

Fusarium oxysporum f.sp. *cepae* (*Focep*), which causes basal rot of onions, consists of seven known vegetative compatibility groups (VCGs 0420 to 0426) and four single-member VCGs (SMV 1 to 4). *Focep* populations in South Africa and Colorado each comprise one main VCG, namely VCG 0425 and 0421, respectively. The aim of this study was to develop DNA fingerprinting methods for identifying VCGs 0425 and 0421, using a collection of 79 previously characterized *F. oxysporum* isolates. Inter-retrotransposon sequence-characterized amplified region (IR-SCAR) markers were also developed for identifying the two main VCGs and SMV 4 isolates. Finally, the prevalence of VCG 0425 among South African *Focep* isolates was investigated through fingerprinting and SCAR marker testing of a collection of 88 uncharacterized *F. oxysporum* isolates (65 *Focep* and 23 non-pathogenic *F. oxysporum* isolates). Evaluation of 20 random amplified polymorphic DNA (RAPD) primers showed that only two primers (OPA-05 and OPI-18) provided informative fingerprints for VCG 0425 isolates, which included two diagnostic amplicons. Attempts to develop SCAR markers from these amplicons were not successful. Analysis of the RAPD data using an unweighted paired group method with arithmetic averages revealed the clustering of the two main VCGs into two distinct sub-clades, which excluded all non-pathogenic isolates. Inter-retrotransposon amplified polymorphism fingerprinting using one primer pair (Hani and Gagi) revealed the same three diagnostic amplicons for VCGs 0421 and 0425, and only two of the three amplicons for SMV 4. This allowed the development of a multiplex IR-SCAR PCR method for the identification of the aforementioned *Focep* isolates. Fingerprinting and SCAR marker testing of the uncharacterized collection of *F. oxysporum* isolates confirmed that VCG 0425 is the main VCG in South Africa, with all but three of the 65 *Focep* isolates having the molecular characteristics of this VCG.

INTRODUCTION

Fusarium oxysporum Schlechtend.:Fr. f.sp. *cepae* (H.N. Hans.) W.C. Snyder & H.N. Hans (*Focep*), which causes basal rot of onions, consists of seven known vegetative compatibility groups (VCGs 0420 to 0426) and four single-member VCGs (SMV 1 to 4). To date, VCG analyses have only been conducted using *Focep* isolates collected from South Africa and Colorado, USA (Chapter 2; Swift *et al*., 2002), with one exception. This exception involves VCG 0420, represented by two isolates, which was reported from Japan (Yoo *et al*., 1993). Among the *Focep* VCGs from Colorado and South Africa, two predominate, namely VCG 0425 in South Africa (74% of isolates) and VCG 0421 in Colorado (47% of isolates). Only VCG 0423 is shared between the two regions (Chapter 2; Swift *et al*., 2002). Pathogenicity testing of isolates representative of the *Focep* VCGs and SMVs showed that those in VCGs 0421, 0424, 0425 and 0426, and SMVs 2 and 4, were highly virulent, whereas isolates from VCG 0422 and 0423 were moderately virulent (Chapter 2).

Multi-locus sequence analyses of South African and Colorado *Focep* isolates and non-pathogenic *F. oxysporum* isolates from onions in South Africa have shown that all of these isolates are closely related. Combined translation elongation factor 1α (EF-1α) and mitochondrial small-subunit (mtSSU) phylogenies showed that the two main VCGs, VCG 0421 and 0425, form a putative clonal lineage. This *Focep* clonal lineage also included two non-pathogenic *F. oxysporum* isolates, three SMVs (2, 3 and 4) and one VCG 0426 isolate (STE-U 6638). An intergenic spacer (IGS) phylogeny also grouped the main *Focep* VCGs into a single clade. Similar to the EF-1α and mtSSU regions, some non-pathogenic *F. oxysporum* isolates also had IGS sequences that were identical to those of VCG 0421 and 0425 isolates (Chapter 2). Therefore, these sequence regions are not useful for identifying *Focep* isolates. This is in contrast to studies on *F. oxysporum* f.sp. *dianthi* and f.sp. *vasinfectum*, where the IGS region was useful for discriminating pathogens from non-pathogens (Lori *et al*., 2004; Zambounis *et al*., 2007). Furthermore, in *F. oxysporum* f.sp. *cubense*, the IGS region was useful for developing a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for distinguishing lineages within this pathogen (Fourie *et al.*, 2009).

DNA fingerprinting methods have been used with success for high level resolution and identification of *F. oxysporum* at the forma specialis, race and VCG levels. Two of the most widely used fingerprinting methods include amplified fragment length polymorphism (AFLP) analysis (Baayen *et al.,* 2000; Vakalounakis *et al.*, 2005) and random amplified polymorphic DNA (RAPD) analysis (Kelly *et al*., 1994; Kistler, 1997; Clark *et al*., 1998; De Haan *et al.*, 2000; Jiménez-Gasco *et al*., 2001; Vakalounakis *et al*., 2004; Lievens *et al*., 2007). AFLP analyses have been used to distinguish between *F. oxysporum* f.sp. *melonis* and f.sp. *radicis-cucumerinum* (Vakalounakis *et al*., 2005), to differentiate some VCGs of *F. oxysporum* f.sp. *cubense* (Groenewald *et al.*, 2006) and to distinguish VCGs within a number of *F. oxysporum* formae speciales causing wilt and rot diseases (Baayen *et al*., 2000). RAPD analyses have been used to identify, for example, highly virulent strains of *F. oxysporum* f.sp. *ciceris* and f.sp. *phaseoli* (Kelly *et al*., 1994; Alves-Santos *et al*., 2002), distinguish between races of *F. oxysporum* f.sp. *pisi* and f.sp. *ciceris* (Grajal-Martin *et al*., 1993; Manulis *et al*., 1994; Jiménez-Gasco *et al*., 2001) and between VCGs of *F. oxysporum* f.sp. *cubense* and f.sp. *dianthi* (Bentley *et al*., 1994; Kalc Wright *et al*., 1996).

A new fingerprinting method that has recently been developed for differentiating *F. oxysporum* f.sp. *lactucae* race 1 isolates is a sequence-biased method that targets transposons. This method, known as inter-retrotransposon amplified polymorphism polymerase chain reaction (IRAP-PCR), targets the long terminal repeat (LTR) insertion sites of transposons and, therefore, amplifies genomic regions between LTRs (Pasquali *et al.*, 2007). It is better to target the LTR insertion sites than transposons, since these sites are more conserved, and transposition events do not influence their position in the genome, as can be the case with transposons (Hua-Van *et al*., 2001; Roncero *et al*., 2002; Lievens *et al*., 2008).

Several studies have used DNA fingerprinting data to develop sequence-characterized amplified region (SCAR) markers for the rapid and accurate identification of formae speciales, races or VCGs of *F. oxysporum*. SCAR makers provide a simple and cost effective way of identifying these groups, which can be used inter-laboratorily (Lievens *et al.*, 2008). The latter is important, since RAPD analysis, especially, suffers from inter-laboratory reproducibility, and sometimes also from intra-laboratory variability (Jones *et al.*, 1997). RAPD analysis remains an important approach for developing SCAR

markers for high resolution identification of *F. oxysporum* groups, since 64% (14/22) of all SCAR markers that have been developed have used this approach. The remaining SCAR markers (36%) were developed using either the IRAP-PCR approach (only one marker) or various molecular approaches that targeted transposable elements (Lievens *et al.*, 2008).

The aims of this study were, firstly, to develop DNA fingerprinting methods for identifying the two main *Focep* VCGs (0421 and 0425) using a collection of 79 previously characterized *Focep* isolates. These included 19 *Focep* isolates from Colorado and 27 from South Africa, as well as 33 non-pathogenic isolates of *F. oxysporum* from onion (Chapter 2). Secondly, the fingerprint data were used to develop SCAR markers for the detection of VCGs 0421 and 0425 and SMV 4 isolates among the South African and Colorado *Focep* isolates. Finally, the fingerprint methods and SCAR markers were used to further investigate the prevalence of VCG 0425 among 88 uncharacterized *F. oxysporum* isolates from onion bulbs in South Africa.

MATERIALS AND METHODS

Fungal isolates. Seventy nine previously characterized *F. oxysporum* isolates from onions (Chapter 2) were used for fingerprinting and SCAR marker development. The isolates consisted of 27 *Focep* isolates from South Africa, 19 *Focep* isolates from Colorado, and 33 non-pathogenic *F. oxysporum* isolates from onion bulbs in South Africa. The isolates represented six *Focep* VCGs and four SMVs. The collection of isolates included nine isolates of VCG 0421 and 20 isolates of VCG 0425 (Chapter 2). Six *F. oxysporum* isolates from other formae speciales were also used as controls in the development of the DNA fingerprinting methods. These isolates were CAV 005 and CAV 013 (*F. oxysporum* f.sp. *cubense*), PPRI 5457 and CBS 412.90 (*F. oxysporum* f.sp. *lycopersici*), CBS 137.97 (*F. oxysporum* f.sp. *gladioli*) and CBS 259.51 (*F. oxysporum* f.sp. *lini*). Three of these isolates as well as an additional 13 *Fusarium* isolates were used in validation studies of the developed *Focep* SCAR markers. The 13 reference isolates were kindly provided by G. Fourie (Department of Microbiology and Plant Pathology, University of Pretoria, South Africa).

Isolates were grown routinely on potato dextrose agar (PDA; Biolab, Gauteng, South Africa) supplemented with 0.04 g Streptomycin sulphate per litre agar (PDA$^+$). All isolates were stored in 15% glycerol at -80°C.

DNA extraction. *Fusarium* mycelia were harvested from 2-week-old PDA$^+$ plates. DNA was extracted with the Wizard® SV Genomic DNA Purification system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol, except that mycelia were first lysed using 0.5 g glass beads (2 mm in diameter) and 5 min of shaking on a Mixer Mill type MM 301 beater (Retsch Gmbh & Co. KG, Haan, Germany). DNA was eluted from the columns using 100 μl of sterile distilled water. DNA concentrations were determined using a NanoDrop-1000 (NanoDrop Technologies, Inc., DE, USA).

Two independent DNA extractions were made from each of the 79 *Fusarium* isolates and the six other formae speciales isolates. These DNA extractions were used to obtain RAPD fingerprint data for the unweighted paired group method with arithmetic averages (UPGMA) analyses. The two independent DNA extractions consisted of *F. oxysporum* culture plates that were grown at two different points in time. The duplicated DNA extractions were used to evaluate the reproducibility of the RAPD and IRAP-PCR analyses.

RAPD fingerprinting and UPGMA analysis. Twenty RAPD primers were evaluated that had previously been used for characterizing other formae speciales isolates of *F. oxysporum.* The RAPD primers were OPF-06, OPF-10, OPF-12, OPI-01, OPI-09 and OPI-18 (Jimenez-Gasco *et al.*, 2001), OPC-18 (Alves-Santos *et al.*, 2002), OPF-04, OPF-05, OPF-08 and OPF-13 (Freeman and Maymon, 2000), OPB-01 and OPB-02 (Vakalounakis *et al.*, 2005), OPB-08 (Chiocchetti *et al.*, 2001), OPA-01, OPA-02, OPA-04 and OPA-05 (Clark *et al.*, 1998) and OPB-07 and OPZ-12 (Lievens *et al.*, 2007). The polymorphic nature of the primers and their ability to differentiate between the main VCGs (0421 and 0425), SMVs and non-pathogenic *F. oxysporum* isolates were first evaluated on a subset of 18 *Fusarium* isolates. The group of 18 isolates consisted of seven South African *Focep* isolates, five Colorado *Focep* isolates, three non-pathogenic isolates from onion in South Africa, two other isolates representing formae speciales of *F.*

oxysporum (*F. oxysporum* f.sp. *cubense* and f.sp. *gladioli*) and one other *Fusarium* species [*Fusarium solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen].

All RAPD amplifications were conducted with AmpliTaq® DNA polymerase Stoffel fragment (Applied Biosystems, Foster City, CA, USA) to increase the reproducibility of RAPD amplifications. The AmpliTaq® DNA polymerase Stoffel fragment is highly thermostable and exhibits optimal activity over a broad range of magnesium ion concentrations. The enzyme also has a deletion of 289 amino acids at the N-terminal that results in increased stringency at lower ionic strengths and reduced misextensions (Applied Biosystems). Each RAPD amplification reaction consisted of 26 ng of genomic DNA, 1 µM RAPD primer (Operon Biotechnologies GmbH, Cologne, Germany), 400 µM of each dNTP, 0.104 mg/ml BSA Fraction V (Roche Diagnostics, Randburg, South Africa), 1 x Stoffel buffer (Applied Biosystems), 3 mM $MgCl_2$, and 1 U AmpliTaq® DNA polymerase Stoffel fragment (Applied Biosystems) in a total volume of 25 µl. Amplifications were performed in an Applied Biosystems 2720 Thermal Cycler (Foster City, CA) programmed for one cycle of 5 min at 95°C, followed by 44 cycles of 1 min at 95°C, 1 min at 35°C, 2 min at 72°C, and a final extension step of 6 min at 72°C. Amplification products were separated by electrophoresis on a 3% agarose gel (MS-8 agarose; Molecular screen, Hispanagar, Burgos, Spain) containing ethidium bromide and visualized under UV light.

Two RAPD primers [OPA-05 (5'-AGGGGTCTTG) and OPI-18 (5'-TGCCCAGCCT)] were used to generate RAPD fingerprints and a UPGMA dendogram for the 79 onion *Fusarium* isolates and six formae speciales isolates. These RAPD primers were the only two of the 20 evaluated RAPD primers that yielded amplicons that were specific for VCG 0425. RAPD reactions, amplifications and gel electrophoresis were conducted as described above. For each isolate, two amplifications were conducted from two independent DNA extractions in order to check for reproducibility. RAPD fingerprints obtained with amplifications using primers OPI-18 and OPA-05 were scored on the basis of the presence or absence of RAPD amplicons of the same electrophoretic mobility (Clark *et al*., 1998). The scoring results were converted to the nucleotide letters a, c, g and t within BioEdit 7.0.9 (Hall, Ibis Biosciences, Carlsbad, CA). The data were used to generate a UPGMA dendogram using the Molecular Evolutionary Genetics

Analysis (*MEGA*) 4.0 software (Tamura *et al.*, 2007). The model 'nucleotide p-distance' with 1 000 bootstraps were selected within *MEGA* 4.0.

IRAP-PCR fingerprinting. Four published LTR primers (Pasquali *et al.*, 2007) were evaluated to identify the most suitable primer pair combination for IRAP-PCR fingerprinting. The four LTR primers, Hani, Gagi, Hana and Gaga were screened in all possible combinations on the same subset of 18 *Fusarium* isolates that were used in the RAPD primer screening analyses, in order to identify the primer pair combinations that showed the best potential for revealing fingerprinting patterns and amplicons that were specific to the main *Focep* VCGs (VCG 0421 and 0425).

Each IRAP-PCR reaction consisted of 100 ng of genomic DNA, 0.40 μM of each of two LTR primers, 500 μM of each dNTP, 1 x AccuTaq^{TM} LA Buffer (Sigma-Aldrich, Kempton Park, South Africa), 1.5 mM MgCl$_2$, 1 μl Dimethyl Sulfoxide (DMSO) (supplied with AccuTaq^{TM}) and 1 U AccuTaq^{TM} LA DNA Polymerase (Sigma-Aldrich) in a total volume of 30 μl. Amplifications were performed in an Applied Biosystems 2720 Thermal Cycler programmed for one cycle of 30 s at 98°C, 40 cycles of 15 s at 94°C, 20 s at 55°C and 5 min at 68°C, followed by one cycle of 10 min at 68°C. Amplification products were separated by electrophoresis on a 2% agarose gel (MS-8 agarose) stained with ethidium bromide, and visualized under UV light.

One LTR primer pair, Hani (5'GAACCCTCCAACATTCAACA) and Gagi (5'TAACCGCTAGGGTCGTAACA) (Pasquali *et al.*, 2007), was used to generate IRAP-PCR fingerprints for the 79 onion *Fusarium* isolates and six formae speciales isolates. This primer pair combination was the only one that was able to differentiate VCG 0421, VCG 0425 and SMV 4 isolates from all the other VCGs, SMVs and non-pathogenic *F. oxysporum* isolates. IRAP-PCR reactions, amplifications and gel electrophoresis were conducted as described above. Amplification of each isolate was conducted twice, in order to confirm the reproducibility of the technique.

Cloning and sequencing of RAPD and IRAP PCR amplicons. Amplification and analyses of the 79 *F. oxysporum* onion isolates and six formae speciales isolates identified two RAPD-PCR amplicons (± 400-bp from OPA-05 and ± 370-bp from OPI-18) (Fig. 1) and three IRAP amplicons (± 400-bp, ± 780-bp and ± 1.35-kb) (Fig. 2) that

were specific for VCG 0425 isolates (RAPD analysis) and VCG 0421, VCG 0425 and SMV 4 isolates (IRAP analysis). These amplicons were cloned and sequenced from four South African *Focep* isolates (STE-U 6649, 6640, 6643 and 6656).

Cloning of the PCR amplicons was conducted by first excising each targeted PCR amplicon from a 2% agarose gel. The gel excised fragments were purified using the QIAquick Gel Extraction kit (Qiagen, Venlo, Valencia, CA, USA). The purified amplicons were ligated into vector pJET1.2 using the CloneJet[TM] PCR cloning kit (Fermentas International Inc., Ontario, Canada). The ligated plasmids were transformed into competent *Escherichia coli* cells using a standard protocol (Sambrook *et al.*, 1989). *E. coli* colonies containing inserts of the correct size were selected using PCR colony screening with forward and reverse pJET1.2 primers (Fermentas). Plasmids were extracted from positive colonies using the PureYield[TM] Plasmid miniprep system (Promega). The cloned amplicons were sequenced from the plasmids using the pJET1.2 primers. Sequence analyses were conducted by the Central Analytical Sequencing Facility at Stellenbosch University using the BigDye system (version 3.1 dye terminators, Applied Biosystems) and an ABI 3130XL Genetic Analyzer. Geneious Pro (Biomatters Ltd., Auckland, New Zealand) was used to view ABI trace files, and to obtain consensus double strand sequences for each cloned amplicon. Sequence data from selected isolates were submitted to Genbank (http://www.ncbi.nlm.nih.gov/Genbank/) as accessions GU120664 to GU120669. The identities of the cloned amplicons were investigated by conducting BLAST analyses in Genbank and the *Fusarium* comparative genome project at the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html).

Designing and evaluation of SCAR primers. SCAR primers were designed by selecting sequence regions within the cloned RAPD and IRAP amplicons that were 100% identical between the sequences of the four isolates used in the cloning experiments. The sequence of each of the designed primers was also analysed for specificity (amplification of other known sequences in GenBank and the *Fusarium* genome database), melting temperature, self-homology, cross-homology, internal stability, and compatibility with a complimentary primer using the NCBI Primer-BLAST website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index).

In total, 17 SCAR primers were designed from the cloned RAPD and IRAP amplicons (Table 1). The specificity of the designed primer pair combinations was tested using genomic DNA from a small subset of isolates, including four South African VCG 0425 isolates (STE-U 6641, 6657, 6660 and 6661), three Colorado VCG 0421 isolates (Foc 1, Foc 4 and Foc 8), one Colorado VCG 0424 isolate (Foc 23), four non-pathogenic *F. oxysporum* isolates (STE-U 6664, 6665, 6666 and 6676) and one *F. oxysporum* f.sp. *lycopersici* isolate (PPRI 5457). The specificity of all the possible primer pair combinations designed from each of the cloned sequences was tested using several different primer and $MgCl_2$ concentrations and annealing temperatures. By using various different PCR reaction and amplification conditions, it was found that the SCAR primers designed from the two RAPD sequences were not specific. However, three of the inter-retrotransposon (IR)-SCAR primer pairs that were designed from the IRAP sequences were specific when tested on the subset of isolates. These primer pairs were (i) HanO-1F and HanO-2R that amplifies a 359-bp amplicon, (ii) Hansec-1F and Hansec-2R that amplifies a 392-bp amplicon and (iii) HTH-1F and HTH-2R that amplifies a 244-bp amplicon.

The three IR-SCAR primer pairs (HanO-1F/HanO-2R, Hansec-1F/Hansec-2R and HTH-1F/HTH-2R) were evaluated on the 79 onion *F. oxysporum* and six formae speciales isolates. The PCR reactions were carried out using 30 µl reaction volumes containing 20 ng of genomic DNA, 200 µM of each dNTP, 1 mg/ml BSA Fraction V (Roche Diagnostics South Africa, Randburg), 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.65 unit BIOTAQ^{TM} (Bioline, London, United Kingdom), and 1 µM of each HanO-1F and HanO-1R primer or 0.5 µM of each HTH-1F and HTH-2R primer or 0.2 µM of each Hansec-1F and Hansec-2R primer. Thermal cycling conditions comprised an initial denaturation at $94^{o}C$ for 5 min, followed by 40 cycles of denaturation at $94^{o}C$ for 30 s, annealing at $60^{o}C$ for 30 s and elongation at $72^{o}C$ for 30 s, and a final elongation step at $72^{o}C$ for 7 min. Amplifications were performed in an Applied Biosystems 2720 Thermal Cycler. Five µl of the amplified products were electrophoretically resolved on a 3% agarose gel (MS-8 agarose) stained with ethidium bromide, and visualized under UV light.

Development of IR-SCAR multiplex PCRs. Two IR-SCAR multiplex PCR reactions were evaluated for detecting all VCG 0421, VCG 0425 and SMV 4 isolates in a single reaction. The first IR-SCAR multiplex PCR reaction consisted of amplifications

conducted with primers HanO-1F, HanO-2R, HTH-1F and HTH-2R. The second IR-SCAR multiplex PCR reaction consisted of amplifications conducted with primers Hansec-1F, Hansec-2R, HTH-1F and HTH-2R. These two multiplex reactions were tested on a total of 38 isolates, comprising the subset of 18 *F. oxysporum* isolates that were also used in the initial RAPD screening, two *F. oxysporum* f.sp. *melonis* isolates and an additional five to seven isolates that represented each of VCGs 0421 and 0425 and non-pathogenic isolates. Several different reaction conditions (MgCl$_2$ and primer concentrations) and amplification conditions (annealing temperatures) were evaluated.

The first IR-SCAR multiplex PCR amplifications using HanO-1F, HanO-2R, HTH-1F and HTH-2R were conducted on the 38 above-mentioned *F. oxysporum* isolates only. The IR-SCAR multiplex reaction consisted of 20 ng of genomic fungal DNA, 400 μM of each dNTP, 1 mg/ml BSA Fraction V (Roche Diagnostics South Africa, Randburg), 1 x PCR buffer, 2 mM MgCl$_2$, 0.65 unit BIOTAQ^{TM} (Bioline), 0.5 μM of each of HanO-1F and HanO-2R primer and 0.2 μM of each of HTH-1F and HTH-2R primer in a total volume of 30 µl. Thermal cycling conditions comprised an initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 45 s and elongation at 72°C for 30 s, and a final elongation step at 72°C for 7 min. Amplifications were performed in an Applied Biosystems 2720 Thermal Cycler. The amplified products (8 µl) were electrophoretically resolved on a 3% agarose gel (MS-8 agarose) stained with ethidium bromide, and visualized under UV light.

The second IR-SCAR multiplex PCR amplifications using primers Hansec-1F, Hansec-2R, HTH-1F and HTH-2R were conducted on 85 *F. oxysporum* isolates (the 79 *Focep* and six formae speciales isolates; Chapter 2). The IR-SCAR multiplex reaction consisted of 20 ng of genomic fungal DNA, 200 μM of each dNTP, 1 mg/ml BSA Fraction V (Roche Diagnostics South Africa), 1 x PCR buffer, 1.5 mM MgCl$_2$, 0.65 unit BIOTAQ^{TM} (Bioline),, 0.2 μM of each Hansec-1F and Hansec-2R primer and 0.5 μM of each HTH-1F and HTH-2R primer in a total volume of 30 µl. Thermal cycling conditions comprised an initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s, and a final elongation step at 72°C for 7 min. Amplifications were performed in an Applied Biosystems 2720 Thermal Cycler. The amplified products (8 µl) were electrophoretically

resolved on a 3% agarose gel (MS-8 agarose) stained with ethidium bromide, and visualized under UV light.

Isolation, identification and pathogenicity testing of a new collection of *F. oxysporum* isolates from onion bulbs in South Africa. *Fusarium* isolations were made from onion bulbs collected in the Koue Bokkeveld in 2007 and in the Boland in 2005. Isolations and morphological identification of *F. oxysporum* were conducted as previously described (Chapter 2). All *F. oxysporum* isolates were single-spored and stored in 15% glycerol at -80°C. In total, 88 *F. oxysporum* single-spored isolates were obtained that were deposited in the fungal culture collection at Stellenbosch University, Department of Plant Pathology as STE-U 6847 to 6934.

The pathogenicity of the *F. oxysporum* isolates was evaluated using an onion bulb inoculation method, as previously described (Chapter 2). Pathogenicity testing of each isolate was conducted using four onion bulbs, as well as control isolates consisting of two other *F. oxysporum* formae speciales isolates (*F. oxysporum* f.sp. *cubense* and f.sp. *lycopersici*), a pathogenic and non-pathogenic *F. oxysporum* isolate from onion, and a water control. Isolates that caused 70 to 100% necrosis were classified as highly virulent**.** When basal plate necrosis did occur, the pathogen was re-isolated from the basal plate onto PDA^{+} to fulfil Koch's postulates.

RAPD and IRAP-PCR fingerprinting of the new collection of *F. oxysporum* isolates from South Africa. RAPD (primers OPA-05 and OPI-18) and IRAP PCR (primer pair Hani and Gagi) fingerprints were generated for the new collection of 88 *F. oxysporum* isolates from onions, using the same protocols as previously described. Amplifications were only conducted using one DNA extraction, since fingerprints generated from independent DNA extractions were found to be reproducible. The RAPD OPA-05 and OPI-18 fingerprints of the 88 isolates were scored for the presence or absence of a ± 400-bp and ± 370-bp diagnostic VCG 0425 amplicon, respectively (Fig. 1). The IRAP fingerprint of each of the 88 isolates was also scored for the presence or absence of three diagnostic amplicons (± 400-bp, ± 780-bp and ± 1.35-kb) (Fig. 2).

Validation of the IR-SCAR multiplex PCR. The second IR-SCAR multiplex PCR (primers Hansec-1F, Hansec-2R, HTH-1F and HTH-2R) was further validated on a

total of 104 *Fusarium* isolates. These isolates included the new collection of *F. oxysporum* isolates (65 *Focep* and 23 non-pathogenic *F. oxysporum*) and 13 other *F. oxysporum* formae speciales isolates, as well as three other *Fusarium* species, namely *F. proliferatum* (Matsushima Nirenberg) (MRC 2301), *F. solani* (Martius) Saccardo (MRC 1873) and *F. graminearum* Schw. (*Gibberella zeae* Petch) (MRC 4927). The thirteen other *F. oxysporum* formae speciales isolates included f.sp. *lycopersici* (PPRI 5457 and CAV 315), f.sp. *gladioli* (CBS 137.97), f.sp. *lini* (CBS 259.51), f.sp. *radicis-lycopersici* (CAV 335), f.sp. *tulipae* (CAV 331), f.sp. *melonis* (PPRI 4954 and PPRI 4950), f.sp. *raphanai* (CAV 336), f.sp. *niveum* (CAV 332), f.sp. *vasinfectum* (CAV 337), f.sp. *dianthi* (CAV 327) and f.sp. *chrysanthemi* (CAV 345). The IR-SCAR multiplex reaction and amplification conditions were conducted as previously described.

RESULTS

RAPD fingerprinting and UPGMA analysis. Amplification of the 79 *F. oxysporum* isolates and six formae speciales isolates with RAPD primer OPA-05 yielded one to seven PCR amplicons. For amplifications with RAPD primer OPI-18, one to 13 PCR amplicons were obtained. Among the 85 *F. oxysporum* isolates, amplification with primer OPA-05 revealed a ± 400-bp amplicon for isolates in VCG 0425, but not for isolates in the other VCGs, those in SMVs and non-pathogenic *F. oxysporum* isolates (Fig. 1A). For RAPD primer OPI-18, a ± 370-bp amplicon was identified in all the *Focep* isolates that also contained the ± 400-bp OPA-05 amplicon, but not from the remaining isolates (Fig. 1B).

UPGMA cluster analysis of the combined RAPD data (OPA-05 and OPI-18 primers) of the 85 *F. oxysporum* isolates yielded a dendogram that contained two main clades (Fig. 3). Clade 1 contained all the VCG 0422, 0423 and 0424 *Focep* isolates from Colorado, the SMV 2 and HSI isolate (STE-U 6655) from South Africa and both VCG 0426 isolates from South Africa. Clade 1 also included the *F. oxysporum* f.sp. *lini* isolate (CBS 259.51) and two non-pathogenic isolates (STE-U 6673 and STE-U 6685), the latter from Limpopo and the Koue Bokkeveld, respectively. The sub-clades within clade 1 separated the Colorado isolates from the South African isolates. Clade 2 contained six sub-clades (2a to 2f). Sub-clade 2c included all *Focep* isolates from Colorado within

VCG 0421 and SMV 4. Sub-clade 2f contained all the *Focep* VCG 0425 isolates from South Africa. Non-pathogenic isolates were absent from these two sub-clades. Two SMV isolates (SMV 1 and SMV 3) from South Africa clustered in sub-clade 2e, together with non-pathogenic *F. oxysporum* isolates and two formae speciales of *F. oxysporum* other than *Focep* (CBS 317.97 and CAV 013). Sub-clades 2a, 2b and 2d only contained non-pathogenic *F. oxysporum* isolates from onion and isolates representing three other formae speciales of *F. oxysporum* (CAV 005, PPRI 5457 and CBS 412.90).

IRAP PCR fingerprinting. Amplification of the 85 *F. oxysporum* isolates with primer pair Hani and Gagi yielded zero to 13 PCR amplicons. Three amplicon sizes of approximately 400 bp, 780 bp and 1.35 kb were present in all VCG 0421 and VCG 0425 isolates. The SMV 4 isolate only contained the ± 400-bp and ± 1.35-kb amplicons. These diagnostic bands were absent from the other *Focep* VCGs and SMVs and from non-pathogenic *F. oxysporum* isolates (Fig. 2).

Cloning and sequencing of RAPD and IRAP PCR amplicons. Cloning and sequencing of the targeted IRAP and RAPD amplicons were successful. The sequences obtained from the IRAP ± 400-bp, ± 780-bp and ± 1.35-kb amplicons will hereafter be referred to as IRAP-Three, IRAP-Two and IRAP-One sequences, respectively. The RAPD ± 400-bp OPA-05 amplicon will be referred to as the OPA-05 sequence, and the two polymorphic OPI-18 ± 370-bp amplicons (see below) as the OPI-18A and OPI-18B sequences.

The sequences (GenBank accessions GU120664 to GU20669) of the cloned RAPD and IRAP amplicons that were obtained from the four investigated South African *Focep* isolates were sometimes not identical. The OPA-05 (GU120664), IRAP-One (GU120667), IRAP-Two (GU120668) and IRAP-Three (GU120669) sequences did not differ in the four investigated isolates. However, the OPI-18 sequence obtained from isolate STE-U 6649 (GU120666) differed from the sequences (GU120665) of the other three isolates, by having 13 single base pair polymorphisms and three single base pair deletions that were distributed across the length of the sequence. These two OPI-18 sequences will hereafter be referred to as OPI-18A and OPI-18B sequences (Table 2).

Some of the cloned *Focep* sequences were similar to sequences provided in GenBank and the *Fusarium* comparative genome project (*F. verticillioides, F. oxysporum* and *F. graminearum* genomes) at the Broad Institute. These included the IRAP-One, IRAP-Two and OPA-05 sequences. No significant matches, however, were found for the IRAP-Three, OPI-18A and OPI-18B sequences in either of the two databases (Table 2).

The IRAP-One sequence (1.348 kb) contained a 297-bp region (positions 21 bp to 318 bp) and a 464-bp region (positions 810 bp to 1274 bp) that had a 96% to 98% similarity to GenBank sequences submitted as *Fot1* transposons from *F. oxysporum* f.sp. *ciceris* (unpublished sequence AY039816), *F. oxysporum* (Daboussi *et al.*, 1992) and *Fusarium solani* var. *minus* (Daboussi *et al.*, 2002). In the *Fusarium* comparative genome database, there were 36 different regions with similarity to the IRAP-One sequence that were located on 15 different supercontigs of the *F. oxysporum* f.sp. *lycopersici* genome. The longest of these sequence matches consisted of a 465-bp region, whereas 22 of the matches were less than 104 bp in length. Only 117 bp (positions 1 to 21 bp and 319 to 415 bp) in the IRAP-One sequence had no similarity to sequences in any of the two sequence databases (Table 2). These regions were selected for designing SCAR primers.

The IRAP-Two sequence (783 bp) had no matches in GenBank, but many (169 regions distributed across 19 supercontigs) in the *F. oxysporum* f.sp. *lycopersici* genome that were not annotated. Most (156) of these matches were shorter than 100 bp in length, whereas the longest match consisted of a 227-bp region. Regions in the IRAP-Two sequence to which no similarity was found in the databases spanned a 261-bp region (positions 337 to 498) (Table 2).

Regions within the OPA-05 sequence (402 bp) had similarity in both sequence databases. The 3' region (base pairs 239 to 400) had 92% (151/165) sequence similarity to the 18S rDNA gene of several ascomycete fungi (Table 2). In the *Fusarium* comparative genome database, this same region of the sequence also had similarity to several supercontigs in the *F. oxysporum* f.sp. *lycopersici, F. verticillioides* and *F. graminearum* genomes. Sequences of the other cloned amplicons (IRAP-One, IRAP-Two, OPI-18A and OPI-18B) had no similarity to genomic regions in the *F. verticillioides* and *F. graminearum* genomes. SCAR primers designed for the OPA-05

region targeted the region (1 to 251 bp) for which no matches in sequence databases were found (Table 2).

Designing and evaluation of SCAR primers. None of the OPI-18 and OPA-05 SCAR primer pair combinations, when evaluated on a small subset of the isolates, could be optimized to detect all the *Focep* isolates of interest without detection of some of the non-pathogenic isolates. Therefore, these SCAR primers were not evaluated further.

Evaluation of the IR-SCAR primer pair combinations on the 79 *F. oxysporum* isolates showed that only three of the primer pair combinations were specific for all the VCG 0421, VCG 0425 and SMV 4 isolates. These IR-SCAR primer pair combinations consisted of primer pair (i) HanO-1F and HanO-2R designed from the IRAP-One sequence, (ii) Hansec-1F and Hansec-2R designed from the IRAP-Two sequence and (iii) HTH-1F and HTH-2R designed from the IRAP-Three sequence. The other IR-SCAR primer combinations either amplified DNA of the non-pathogenic isolates or of only some of the *Focep* isolates from VCGs 0421 and 0425.

Development of IR-SCAR multiplex PCRs. The multiplex reaction with IR-SCAR primers HanO-1F, HanO-2R, HTH-1F and HTH-2R was specific and amplified only the two major VCGs (VCG 0421 and 0425) and SMV 4 isolates when tested on a small subsample of 38 representative isolates. In the eight VCG 0421 isolates and one SMV 4 isolate that were evaluated, the expected 244-bp amplicon and an unexpected 390-bp amplicon were amplified. For the 15 evaluated VCG 0425 isolates, the expected 244-bp and 359-bp amplicons were amplified. However, an unexpected 390-bp amplicon, similar to that of the VCG 0421 isolates, was also amplified for some of the VCG 0425 isolates. The amplifications also yielded some additional light amplicons of different sizes for VCG 0421 and 0425 isolates. Two *F. oxysporum* f.sp. *melonis* isolates also yielded an amplicon, but this amplicon was slightly smaller than the 359-bp amplicon of the *Focep* isolates (Fig. 4A).

Amplification with the second IR-SCAR multiplex reaction (primers Hansec-1F, Hansec-2R, HTH-1F and HTH-2R) yielded the expected 392-bp and 244-bp amplicons for the isolates of interest, and this multiplex reaction was therefore evaluated on the larger set of 85 *F. oxysporum* isolates. Amplification of this set of isolates showed that the

IR-SCAR multiplex reaction was able to successfully identify isolates of the two major VCGs (0421 and 0425) as well as SMV 4. No amplification was obtained for isolates in the other VCGs and SMVs and for the non-pathogenic isolates. However, a 392-bp band was amplified from the two *F. oxysporum* f.sp. *melonis* isolates (Fig. 4B).

Isolation, identification and pathogenicity testing of *Fusarium oxysporum* isolates from onion bulbs in South Africa. In total, 88 *F. oxysporum* isolates were obtained from onion bulbs. Pathogenicity testing showed that 65 of the isolates (STE-U 6847 to 6911, and STE-U 6929) were highly virulent *Focep* isolates, since they caused more than 70% basal plate necrosis when inoculated on the onion cultivar Coastal Cream. The remaining 23 isolates (STE-U 6912-6934) were designated as being non-pathogenic *F. oxysporum* isolates and caused less than 10% basal plate necrosis on Coastal Cream.

RAPD and IRAP-PCR fingerprinting of the new collection of *F. oxysporum* isolates from onions in South Africa. RADP fingerprinting of the new collection of *F. oxysporum* isolates (65 *Focep* and 23 non-pathogenic *F. oxysporum*) from onion bulbs showed that 62 of the *Focep* isolates contained the diagnostic OPA-05 ± 400-bp and OPI-18 ± 370-bp amplicons that were previously identified as being specific to VCG 0425. These 62 *Focep* isolates thus belong to the South African VCG 0425. IRAP PCR fingerprinting showed that the 62 *Focep* isolates also contained the three IRAP PCR amplicon sizes (± 1.35-kb, ± 780-bp and ± 400-bp) that were previously shown to be diagnostic for VCGs 0421 and 0425. The remaining three *Focep* isolates (STE-U 6888, 6889, 6929) and 23 non-pathogenic *F. oxysporum* isolates did not contain any of the five aforementioned diagnostic amplicon sizes. Therefore, these three *Focep* isolates did not belong to VCG 0425. Two of the three *Focep* isolates (STE-U 6888 and 6929) may represent new VCGs or SMVs, since their RAPD and IRAP fingerprints did not match any of the known VCGs or SMVs. The RAPD and IRAP fingerprints of the third *Focep* isolate (STE-U 6889) corresponded to that of a known SMV, SMV 3 (Figs. 1 and 2).

Validation of the IR-SCAR multiplex PCR. Evaluation of the second IR-SCAR multiplex reaction (primers Hansec-1F, Hansec-2R, HTH-1F and HTH-2R) on a larger collection of 104 *Fusarium* isolates showed that the multiplex reaction was specific, and that it only detected the group of *Focep* isolates which contained the five diagnostic LTR and RAPD amplicons. However, amplification of two of the *F. oxysporum* f.sp. *melonis*

isolates (PPRI 4950 and PPRI 4954) yielded one of the two diagnostic amplicons (392-bp amplicon for which the primers were designed from the IRAP-Two amplicon). The IR-SCAR multiplex PCR thus confirmed that VCG0425 was the dominant *Focep* VCG in South Africa.

DISCUSSION

In this study, 79 previously characterized *F. oxysporum* onion isolates were used to develop a multiplex IR-SCAR PCR test for the rapid identification of the main *Focep* VCGs (0421 and 0425) and SMV 4 isolates in South Africa and Colorado. The multiplex IR-SCAR PCR test identifies the aforementioned isolates as a group, and does not differentiated between the VCGs. Therefore, two diagnostic RAPD amplicons (a ± 400-bp amplicon from OPA-05 primer, and ± 370-bp amplicon from OPI-18 primer) must be used to subsequently differentiate VCG 0425 isolates from VCG 0421 and the SMV 4 isolates. Using this approach, 65 previously uncharacterized *Focep* isolates from onions in South Africa were genotyped, which showed that 62 isolates belonged to VCG 0425. This confirmed that VCG 0245 is the dominant VCG in South Africa. Two of the three *Focep* isolates that were not VCG 0425 isolates are most likely new VCGs or SMVs. The third isolate (STE-U 6889) may be a SMV 3 isolate since it had the RAPD and IRAP fingerprints of this SMV. The SMV 3 identity of STE-U 6889 would have to be confirmed through VCG testing, and will determine whether the developed fingerprint methods may also be useful for identifying some other *Focep* VCGs and SMVs.

Focep VCG-specific SCAR markers or PCR multiplex tests could not be developed from some IRAP and RAPD amplicons. This may be due to the specific sequences encoded by these amplicons. Problems associated with the OPI-18 amplicon could be the presence of several polymorphisms (up to 13) and three indels in the cloned sequences of four isolates. Even though SCAR markers were developed that targeted conserved regions in the four sequences, the markers were not specific. It is thus likely that additional polymorphisms in this region are present among the larger set of onion *F. oxysporum* isolates. The reason for the failure to develop SCAR markers from the OPA-05 amplicon is unclear. VCG-specific SCAR markers could be developed from all the IRAP amplicons, but not all IR-SCAR markers were amendable for use in a multiplex

PCR. A multiplex PCR test is preferable, since it targets more than one genome region and thus increases confidence in the identification of the targeted group. The IRAP-One SCAR primer pair was not amendable for use in a multiplex PCR, since multiplex amplification with this primer pair resulted in the amplification of more than the expected two amplicons and unexpected amplicon sizes. This may be due to the IRAP-One SCAR primer pair annealing to too many regions in the *Focep* genome, since this sequence was represented by several copies in the *F. oxysporum* f.sp. *lycopersici* genome.

The IRAP-PCR fingerprinting method was more successful for the development of *Focep* VCG-SCAR markers than RAPD analysis. This could be due to the IRAP method targeting regions where transposable elements have inserted. Transposable elements have been hypothesized as being important in the evolution and virulence of *F. oxysporum* formae speciales (Daboussi and Langin, 1994; Hua-Van *et al.,* 2000; Daboussi *et al.*, 2002; Van der Does *et al.*, 2008). Some evidence for this is the successful development of several transposon-based markers for the identification of *F. oxysporum* forma specialis and races (Fernandez *et al*., 1998; Chiocchetti *et al*., 1999; Mes *et al*., 2000; Pasquali *et al*., 2004; Lievens *et al*., 2008). The importance of transposable elements in virulence in *F. oxysporum* was recently also suggested by Van der Does *et al.* (2008). Their investigation found that the putative dispensable genomic region associated with 'secreted in xylem' (*SIX*) virulence genes in *F. oxysporum* f.sp. *lycopersici* was enriched in transposable elements. It is, therefore, possible that the *Focep* pathogenicity and/or virulence-associated IRAP amplicons from this study may be useful tools for investigating (i) genomic regions possibly involved in virulence and pathogenicity in *Focep* and (ii) the evolution of VCGs, pathogenicity or virulence in *Focep*. For example, Jimenez-Gasco *et al.* (2004) used RFLP genotyping of genomic DNA with transposable element probes to formulate important hypotheses on the evolution of races within *F. oxysporum* f.sp. *ciceris*.

The usefulness of the IR-SCAR method for *Focep* identification has been demonstrated for the South African *Focep* population only. This is due to the small number of *Focep* isolates from Colorado that was available for analysis, and because of a lack of knowledge on *Focep* VCGs in other countries. The IR-SCAR method can be important for the rapid detection of *Focep* in onion seed and seedling transplants, since onion seed (Chapter 4) and seedlings (Schwartz and Mohan, 2008) have been indicated as

primary inoculum sources of *Focep*. However, the *Focep* IR-SCAR multiplex technique has some limitations. A major concern would be the inability of the markers to detect *Focep* isolates that do not belong to VCG 0425. The IR-SCAR test only identified 89% of the 95 tested South African *Focep* isolates and would, therefore, not detect all pathogens in primary inoculum sources. The value of the IR-SCAR test could also be reduced if new ecologically more fit and virulent *Focep* VCGs emerge, and if *Focep* genotypes on seed and seedlings differ from those on mature bulbs. The development of a PCR test that targets known host-specific virulence genes, therefore, may have been a better option for identifying inoculum sources (Lievens *et al*., 2008, 2009). However, for *Focep* and most *F. oxysporum* formae speciales, the genetic basis of pathogenicity and virulence is unknown. As yet, *F. oxysporum* f.sp. *lycopersici* is the only forma specialis for which diagnostic tests have been developed that target the host-specific *SIX* virulence genes (Lievens *et al.*, 2008, 2009).

The finer resolution of RAPD analysis for delineating VCGs and distinguishing pathogens from non-pathogens (Bentley *et al*., 1994; Kalc Wright *et al*., 1996; Chiocchetti *et al*., 1999) was also demonstrated for *Focep*. The *Focep* RAPD UPGMA analysis separated VCG 0421 and VCG 0425 from two non-pathogenic *F. oxysporum* isolates (STE-U 6687 and 6695) that were identical to the *Focep* isolates based on multi-gene sequence data (Chapter 2). Furthermore, RAPD analyses differentiated some VCGs and SMVs from each other that were previously identified as putative clonal lineages by multi-gene sequence data (Chapter 2). Two previously identified sequence-based clonal lineages included isolates from VCG 0421, VCG 0425, VCG 0426 (only one isolate) and SMV 4, SMV 2 and SMV 3, whereas the other lineage included isolates from VCGs 0422 and 0423. The RAPD analysis only supported the clonality of (i) VCG 0421 and SMV 4 isolates and (ii) most of the VCG 0422 and 0423 isolates (except for the one South African VCG 0423 isolate), since the isolates all clustered into the same RAPD sub-clades. The fact that isolates within these VCGs and SMVs are genetically similar to one another, although belonging to different VCGs, is most likely due to one of the groups accumulating a mutation in one or more of their vegetative incompatibility (*vic*) genes. For isolates to be vegetatively compatible, all their *vic* alleles must be identical (Correll, 1991).

RAPD analysis generally supported the distinct genetic differences between the two main *Focep* EF-mtSSu clades, clade 1 and 4 (Chapter 2). The EF-mtSSU-clade 1 is an ancestral clade that included all the VCG 0422, 0423 and 0424 isolates and one HSI isolate of the RAPD UPGMA clade 1 (Chapter 2). However, unlike the EF-mtSSU-clade 1, the RAPD UPGMA clade 1 also contained VCG 0426 isolates and an SMV 2 isolate. The divergent placement of these isolates between sequence phylogenies and the RAPD dendogram could be due to the RAPD primers being selected specifically to identify the main VCGs (VCG 0421 and VCG 0425) and to discriminate pathogens from non-pathogens. These primers can, therefore, not be considered to be neutral markers. Furthermore, the RAPD data contained too few polymorphisms and, therefore, are unlikely to reflect evolutionary and phylogenetic relationships (Jimenez-Gasco *et al*., 2004).

The current study was able to develop RAPD and IRAP fingerprint methods, as well as an IR-SCAR multiplex PCR test for the identification of important *Focep* VCGs (0421 and 0425) and SMV 4 in South Africa and Colorado. The dominance of VCG 0425 among isolates obtained from mature bulbs in South Africa, was also confirmed using these techniques. Although these PCR techniques can identify the current main VCGs (0421 and 0425), the dominance of these VCGs and, therefore, their importance in being identified may change in future if new ecologically more fit and virulent VCGs emerge. In addition to the development of tools for identifying important *Focep* VCGs, the study also identified sequences (three cloned IRAP amplicons that were specific to the main *Focep* VCGs) that could be important for future studies aiming to investigate virulence and the evolution of pathogenicity and VCGs within *Focep.*

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Table 1. Sequence characterized amplified region (SCAR) primers designed for amplification of *Fusarium oxysporum* f.sp. *cepae* vegetative compatibility groups 0421 and 0425

Amplicon[a]	SCAR primer name	SCAR primer sequence
RAPD OPA-05 amplicon (402 bp)	OPA-1F	5'-CTTGCCATGACAGGGCTC-3'
	OPA-1R	5'-GCAAATTCCAAGAGGCAGC-3'
	OPA-2F	5'-TGGACATGTACCACAGAAAGC-3'
	OPA-2R	5'-GCTATCCTCCTCGTTTCTCC-3'
RAPD OPI-18 amplicon (371 to 374 bp)	OPI-1F	5'-GCTAATATCTTGCTGGGGCA-3'
	OPI-2F	5'-CAGCCTAGAGGGGTTTTGCT-3'
	OPI-1R	5'-ATTCTGGATCCAATGAAGCG-3'
IRAP-One amplicon (1.35 kb)	HanO-1F	5'-GAACCCTCCAACATTCAACAC-3'
	HanO-1R	5'-CCTGCACCTCACCGATTTAT-3'
	HanO-2R	5'-CGAATCGATCAAATCGATT-3'
IRAP-Two amplicon (783 bp)	Hansec-1F	5'-TTATTGCCCTCATCGGAAAG-3'
	Hansec-1R	5'-ACCCTCCAACATTCAACAGC-3'
	Hansec-2R	5'-ACCAGCATGCAGCAACAGTC-3'
IRAP-Three amplicon (406 bp)	HTH-1F	5'-CATCGGAAGTGACATGGTTG-3'
	HTH-2F	5'-TAACCGCTAGGGTCGTAACA-3'
	HTH-1R	5'-TCTCTGCCAGTTCGGAGATT-3'
	HTH-2R	5'-AGGCTTTTCCAGCATTTGAA-3'

[a]Random amplified polymorphic DNA (RAPD) or inter-retrotransposon amplified polymorphism (IRAP) amplicons were used to design the SCAR primers.

Table 2. Similarity of *Fusarium oxysporum* f.sp. *cepae* random amplified polymorphic DNA (RAPD) and inter-retrotransposon amplified polymorphism (IRAP) PCR amplicons to sequences in GenBank and the *Fusarium* comparative genome project

Amplicon (GenBank accession)[a]	Length (bp) of cloned amplicon	BLAST analyses in GenBank				BLAST analyses in *Fusarium* comparative genome database			Nucleotide positions with no sequence similarity[d]
		E-value[b]	Nucleotide positions with sequence similarity[c]	Number identities / total number bp matches	Similarity to known genes	E-value[b]	Nucleotide positions with sequence similarity[c]	Number identities / total number bp matches	
OPA-05 (GU120664)	402 bp	3×10^{-55}	239-400	151/165	18S rDNA gene	5×10^{-51}	252-400	141/152	1-251
OPI-18A (GU120665)	374 bp	-	-	-	No sequence matches	-	-	-	1-374
OPI-18B (GU120666)	371 bp	-	-	-	No sequence matches	-	-	-	1-371
IRAP-One (GU120667)	1348 bp	0 / 0	21-318 / 810 to 1274	296/299 / 457/467	*Fot1* transposon	0 / 0	810-1274 / 416-786	451 to 456/465 / 362/372	1-21; 319-415
IRAP-Two (GU120668)	783 bp	-	-	-	No sequence matches	4×10^{-90} / 4×10^{-73} / 3×10^{-72}	8-234 / 13-200 / 11-200	213/227 / 176/188 / 177/190	337-498
IRAP-Three (GU120669)	406 bp	-	-	-	No sequence matches	-	-	-	1-406

[a]OPA-05 and OPI-18 refer to PCR amplicons from RAPD amplifications, whereas IRAP-One, IRAP-Two and IRAP-Three refer to amplicons from IRAP PCR amplifications.

[b]Only those sequence matches that had the highest E-values are shown. Except for the OPA-05 sequence, all sequences had similarity to the *F. oxysporum* f.sp. *lycopersici* (*Fol*) genome only.

[c]Nucleotide positions in the *Focep* sequence to which similarity was found in the specific database.

[d]Nucleotide positions in the *Focep* sequence to which no similarity was found in any of the databases.
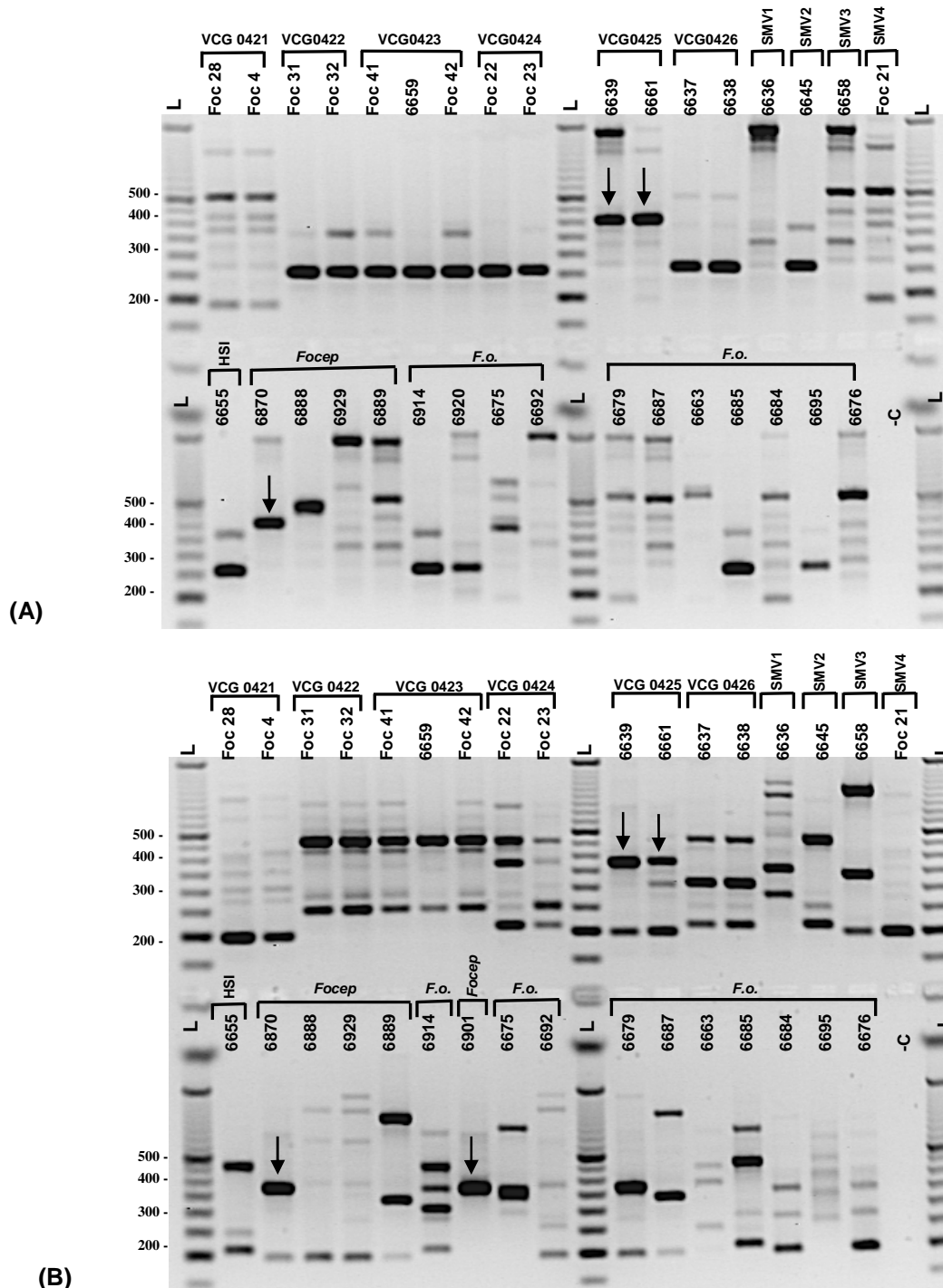
Fig. 1. Random amplified polymorphic DNA (RAPD) fingerprints obtained for onion *Fusarium oxysporum* isolates with RAPD primers (A) OPA-05 and (B) OPI-18. Codes for *F. oxysporum* f.sp. *cepae* (*Focep*) isolates from Colorado start with 'Foc', whereas the South African pathogenic and non-pathogenic (*F.o.*) isolates are indicated by numbers that represent their STE-U culture collection codes. Vegetative compatibility groups are referred to as VCGs, single-member VCGs as SMV, and heterokaryon self-incompatible isolates as HSI. Diagnostic amplicons for VCG 0425 isolates are indicated by arrows. The size fragments (bp) of a 50-bp Generuler[TM] ladder (L) are indicated on the left hand side of the figure. A negative water control (-C) was included in amplifications.
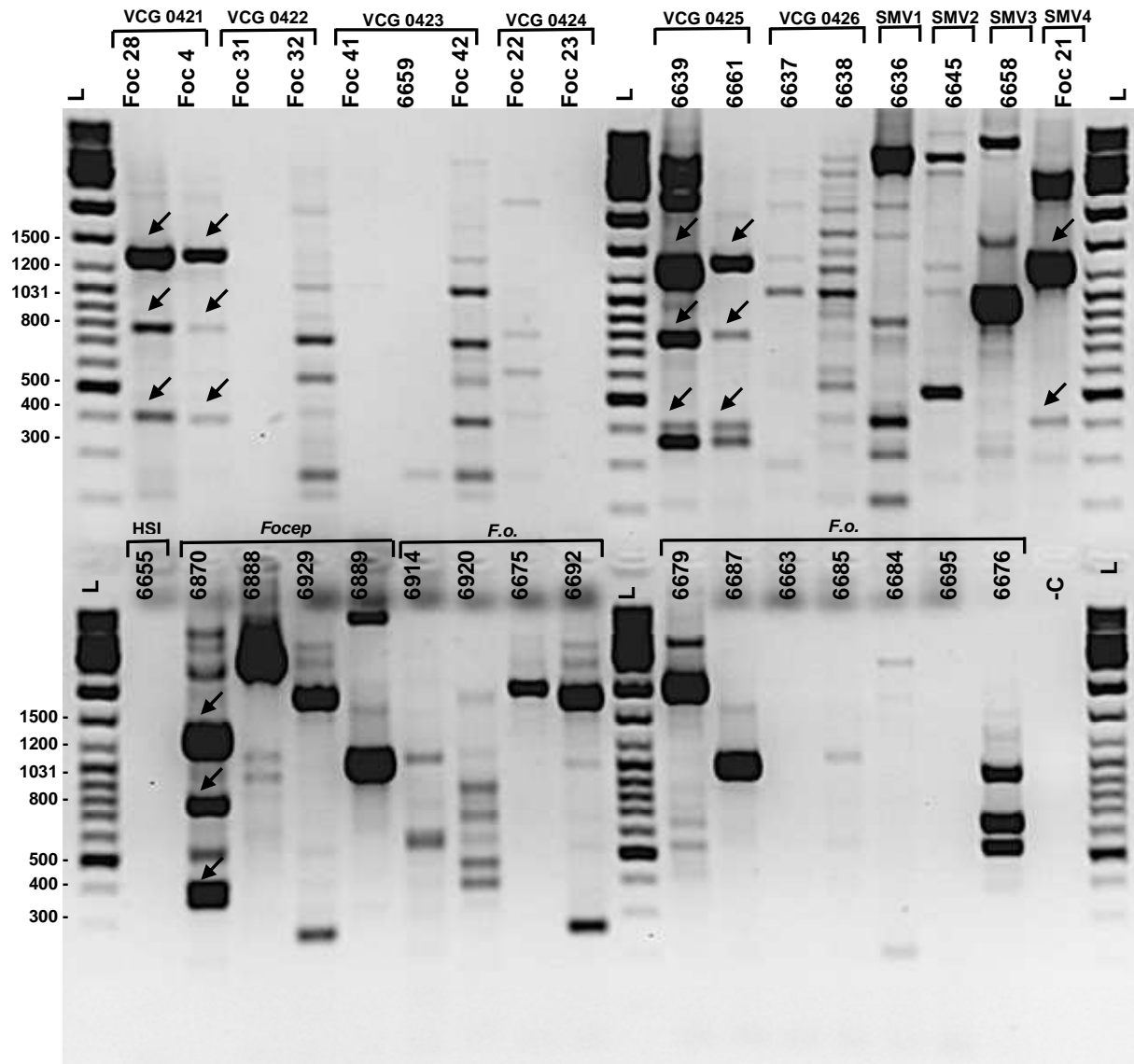
Fig. 2. Inter-retrotransposon amplified polymorphism (IRAP) fingerprints obtained with primers Hani and Gagi for *Fusarium oxysporum* isolates from onion. Codes for *F. oxysporum* f.sp. *cepae* (*Focep*) isolates from Colorado start with 'Foc', whereas the South African pathogenic and non-pathogenic (*F.o.*) isolates are indicated by numbers that are their STE-U culture collection codes. Vegetative compatibility groups are referred to as VCGs, single-member VCGs as SMV, and heterokaryon self-incompatible isolates as HSI. Three diagnostic amplicons for VCG 0421 and VCG 0425 isolates are indicated by arrows. The size fragments (bp) of a 100-bp Generuler[TM] ladder (L) are indicated on the left hand side of the figure. A negative water control (-C) was also included in amplifications.
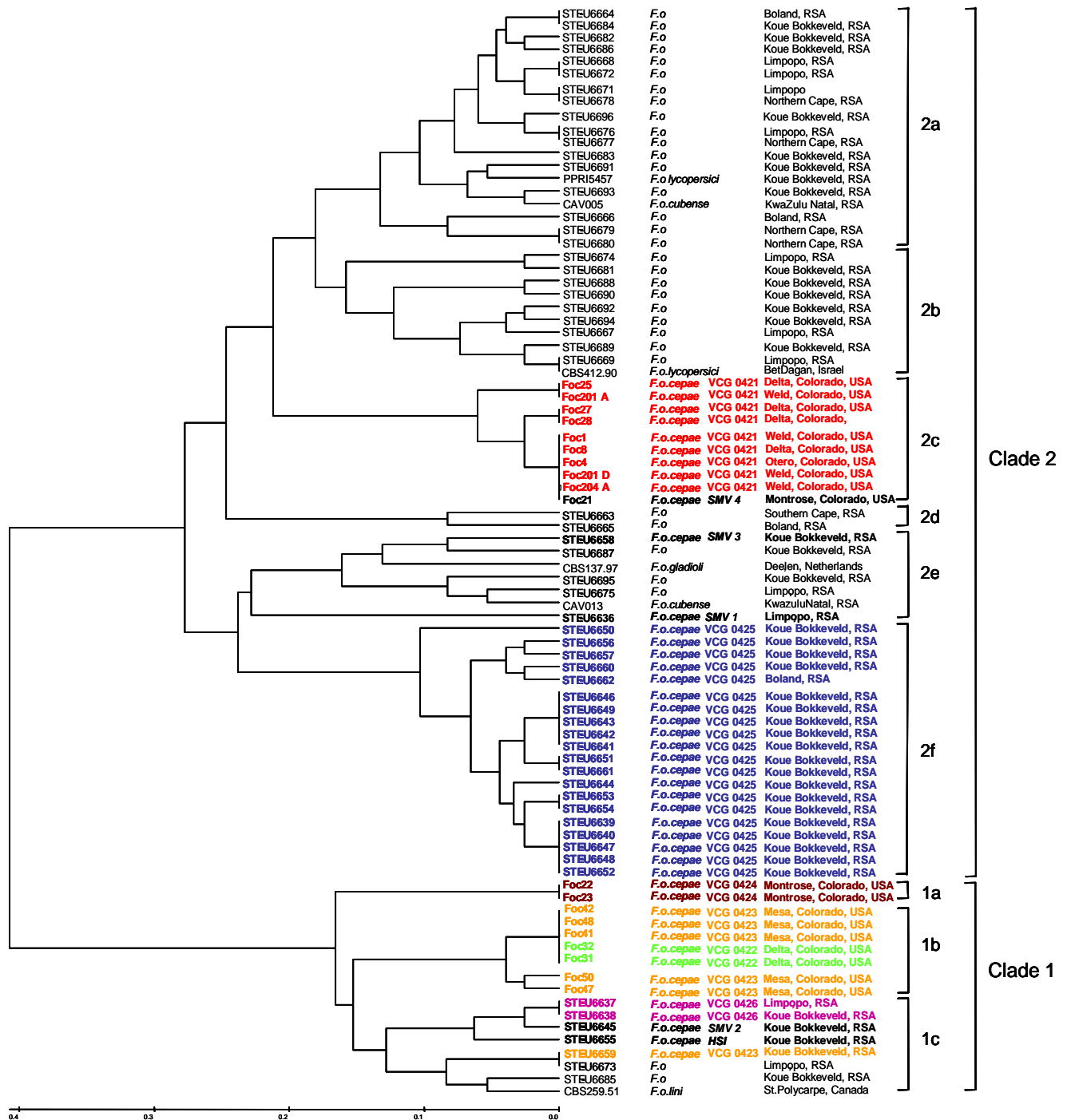
Fig. 3. Unweighted paired group method with arithmetic averages (UPGMA) dendrogram of *Fusarium oxysporum* (*F.o.*) isolates based on the combined random amplified polymorphic DNA (RAPD) data from primers OPA-05 and OPI-18. South African (RSA) *F. oxysporum* f.sp. *cepae* (*F.o.cepae*) and *F.o.* sequences have STE-U codes. United States (USA) Colorado *F.o.cepae* sequences are followed by "●". Each of the specific *F.o.cepae* vegetative compatibility groups (VCGs) are colour coded. Sequences of *F.o.cepae* single-member VCGs (SMV) and heterokaryon self incompatible (HSI) isolates are shown in bold. The place of origin of isolates is shown to the right of the dendogram. Nei and Li distances are shown below.
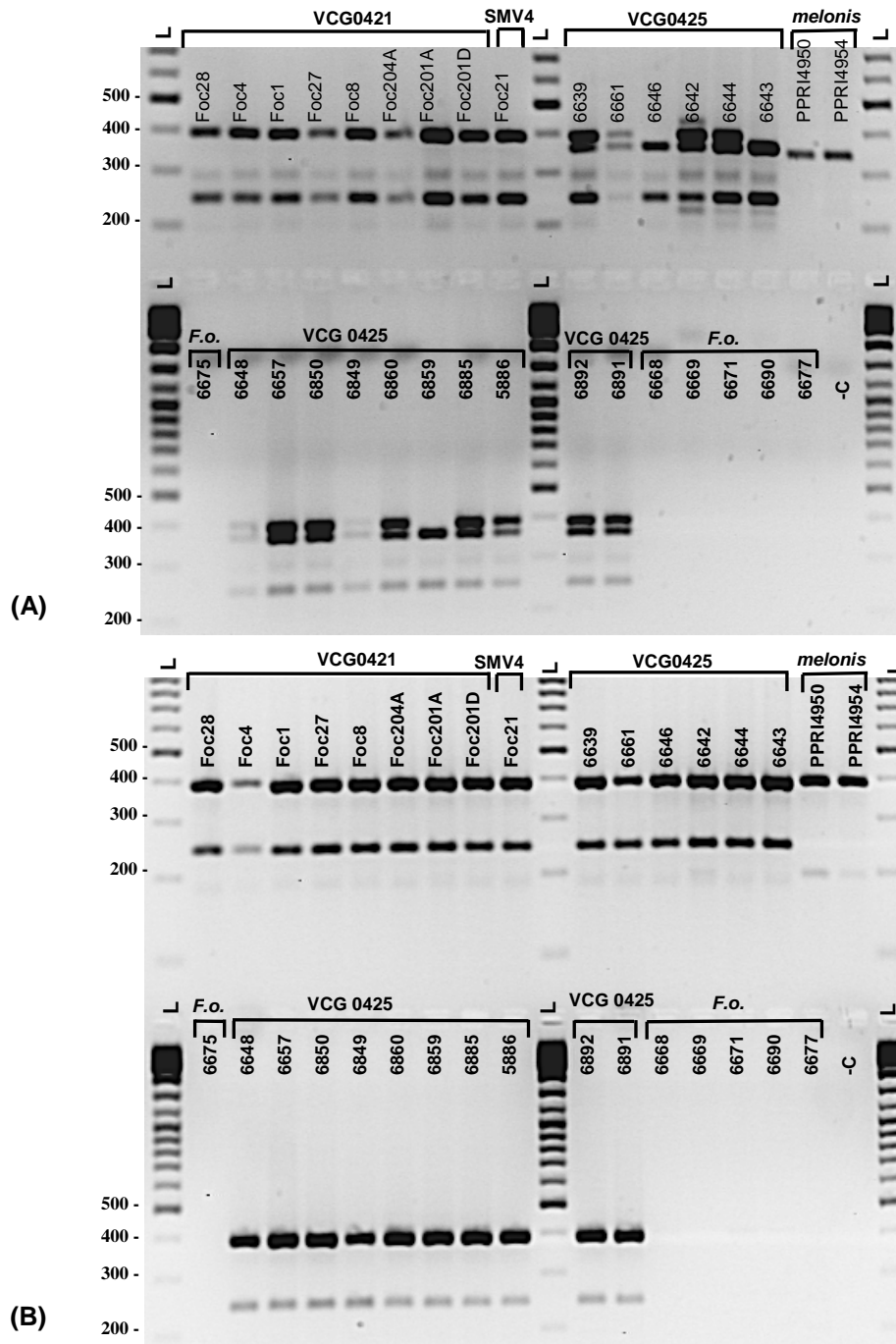
Fig. 4. PCR amplification products of *Fusarium oxysporum* isolates amplified with inter-retrotransposon sequence characterized amplified region (IR-SCAR) primers in multiplex reactions containing primers (A) HanO-1F, HanO-2R, HTH-1F and HTH-2R and (B) Hansec-1F, Hansec-2R, HTH-1F and HTH-2R. Codes for *F. oxysporum* f.sp. *cepae* (*Focep*) isolates from Colorado start with 'Foc', whereas the South African pathogenic and non-pathogenic (*F.o.*) isolates are indicated by numbers that are their STE-U culture collection codes. *Focep* vegetative compatibility groups are referred to as VCGs, and single-member VCGs as SMV. A negative water control (-C) was included in amplifications, as well as two *F. oxysporum* f.sp. *melonis* (*melonis*) isolates. The size fragments (bp) of a 100 bp Generuler[TM] ladder (L) are indicated on the left hand side of the figure.

4. INOCULUM SOURCES OF *FUSARIUM OXYSPORUM* F.SP. *CEPAE* IN THE WESTERN CAPE PROVINCE OF SOUTH AFRICA

ABSTRACT

Fusarium basal rot, caused by *Fusarium oxysporum* f.sp. *cepae* (*Focep*), has been identified as the leading cause of harvest and storage losses within the Western Cape onion industry in South Africa. The contribution of seed and seedlings as primary inoculum sources to these losses was unknown and was, therefore, investigated. The study included the isolation and pathogenicity testing of all *F. oxysporum* isolates obtained from seed and seedlings. Isolates that were identified as *Focep* were also genotyped to determine if they belonged to vegetative compatibility group (VCG) 0425, which is the main VCG associated with symptomatic mature bulbs in South Africa. The seedborne nature of *Focep* was further investigated using reporter gene-labelled *Focep* isolates in artificial inoculation studies. Seed isolation studies from 13 seed lots showed that seven of the seed lots were infected with either moderately or highly virulent *Focep* isolates. The infection frequency of seed lots was low (0.17 to 0.5%), and only two seed lots were infected by VCG 0425. The seedborne nature of *Focep* was confirmed by showing that a green fluorescent protein (GFP)-labelled *Focep* transformant could be transmitted from infected soil to the bulb, and from there to the seedstalks and seeds, where it infected 91.4% of the seeds. Investigations into the presence of *Focep*-infected onion seedling transplants in five nurseries showed that all the nurseries contained infected seedlings. Furthermore, the incidence of *Focep* infected seedling transplants increased from 2.7% at the 6-week-old growth stage to 5.7% at the 14-week-old stage. Highly virulent and moderately virulent *Focep* isolates were obtained from seedlings, but none of the isolates belonged to VCG 0425. Most (> 86%) of the *F. oxysporum* isolates from seed and seedlings were not *Focep* but non-pathogenic isolates. Altogether, the results indicate that onion seed and seedlings are important inoculum sources of *Focep* in the Western Cape Province, and that the main mature bulb associated *Focep* genotype (VCG 0425) is rarely associated with these sources.

INTRODUCTION

Fusarium basal rot of onion (*Allium cepa*) is a disease of world-wide importance, caused by *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *cepae* (H.N. Hans.) W.C. Snyder & H.N. Hans. (Schwartz and Mohan, 2008). In the Western Cape onion industry in South Africa, this disease has been identified as the leading cause of harvest and storage losses of intermediate day-length onion types. Losses are particularly severe when hot and dry conditions prevail during spring, the time at which bulb initiation and active bulb growth occur. Onion bulb producers establish their crop through onion seedling transplants obtained from nurseries, since direct seeding is not a successful crop production practice in the Western Cape Province (R.A. du Toit, Coordinating Committee for Onion and Potato, South Africa, personal communication).

Fusarium oxysporum f.sp. *cepae* (*Focep*) is a soilborne pathogen that survives in soil in the form of chlamydospores for long periods (Brayford, 1996; Sood, 1996; Cramer, 2000; Schwartz and Mohan, 2008). Due to its saprophytic ability, the pathogen can survive in the soil even when host plants are not available (Smith *et al*., 1988). Soilborne inoculum sources can infect onion seedlings and bulbs through direct penetration of the basal plate. Infection can also occur through natural wounds in the basal plate and on the roots, or through basal portions of bulb scales when inoculum levels in the soil are high (Sherf and Macnab, 1986; Cramer, 2000; Koike *et al*., 2007). Additionally, *Focep* infection can take place through maggot, smut or pink root-damaged onion tissue (Everts *et al*., 1985; Sherf and Macnab, 1986; Schwartz and Mohan, 2008). Following infection, symptom development and expression are influenced by environmental conditions. Fusarium basal rot symptoms include basal plate necrosis and rot of the inner basal scales, curving and yellowing of leaves and wilting at any plant growth stage (Cramer, 2000; Swift *et al*., 2002; Schwartz and Mohan, 2008; Dissanayake *et al.*, 2009b).

Due to the persistence of *Focep* in soil (Freeman *et al*., 2002; Flood, 2006), it is important not to (i) introduce the pathogen into pathogen-free soil or (ii) increase inoculum levels in already infested soil through the introduction of external inoculum. The latter is important because a direct correlation has been found between *Focep* inoculum density and damping-off of onion seedlings (Abawi and Lorbeer, 1972).

Possible primary inoculum sources of *Focep* that can result in the introduction or increase of pathogen populations to soil include onion seedlings and seed. It is well-known that *Focep* can successfully disseminate via infected onion seedlings over long distances, causing Fusarium basal rot symptoms on mature bulbs (Abawi and Lorbeer, 1972; Özer and Köycü, 2004; Schwartz and Mohan, 2008). However, the contribution of seed as an inoculum source is uncertain, since reports on the seedborne nature of *Focep* in onion have only been published in non-peer reviewed journals that are not readily available (Boff *et al*., 1995; El-Zawahry *et al*., 2000). In other crops, the seedborne nature of some *F. oxysporum* formae speciales has been shown. These formae speciales include *F. oxysporum* f.sp. *lactucae*, infecting lettuce (Garibaldi *et al.*, 2004; Pasquali *et al*., 2005, 2007); *F. oxysporum* f.sp. *basilici*, infecting sweet basil (Chiocchettti *et al.*, 2001; Pasquali *et al.*, 2006) and *F. oxysporum* f.sp. *radicis-lycopersici*, infecting tomato (Menzies and Jarvis, 1994). In sweet basil, it has specifically been shown that seeds collected from diseased plants contain *F. oxysporum* f.sp. *basilici*, and that these seeds can then produce diseased plants bearing infested seeds (Gamliel *et al*., 1996).

Focep is known to be a genetically diverse pathogen of onions (Swift *et* al., 2002; Galván *et al.*, 2008; Dissanayake *et al.*, 2009a, b; Chapter 2), but the *Focep* genotypes associated with different onion growth stages have not been investigated. Dissanayake *et al.* (2009a, b) only investigated the genetic diversity and virulence of pathogenic *F. oxysporum* isolates from *Allium fistulosum* (Welsh onion, also known as Japanese bunching onion). Some of these isolates were shown to also infect *A. cepa,* and were thus *Focep*. The Welsh onion isolates had a polyphyletic origin, and isolates also varied in virulence towards different Welsh onion cultivars (Dissanayake *et al.*, 2009a, b). Galván *et al.* (2008) also showed that *Focep* was polyphyletic, but were not specific whether their isolates obtained from onion roots and basal plates differed from each other. The study of Swift *et al.* (2002), which identified three *Focep* vegetative compatibility groups (VCGs) and two single member VCGs (SMVs), was not clear on whether these isolates were obtained from field grown plants or harvested bulbs. In South Africa, only *Focep* isolates from onion bulbs have thus far been characterized. This showed that the *Focep* populations were dominated by VCG 0425. The remaining bulb-associated isolates belonged to other VCGs that included highly virulent but also moderately virulent isolates (Chapters 2, 3).

The objective of the present study was to obtain a better understanding of the involvement of seed and seedlings as inoculum sources of *Focep*. The first aim of the study was to investigate whether onion seed and seedlings in the Western Cape Province of South Africa are infected with *Focep*. This was done by means of isolation and pathogenicity studies of *F. oxysporum* isolates obtained from seeds and seedlings. The second aim was to determine if the *Focep* isolates from seed and seedlings belonged to the main *Focep* genotype, VCG 0425, which is associated with post-harvest losses in mature onion bulbs. The VCG 0425 status of isolates was investigated using previously developed molecular genotyping techniques. The last aim of the study was to investigate the seedborne nature of *Focep* in onion through the use of green fluorescent protein (GFP) and red fluorescent protein (DsRed-Express) reporter gene-labelled *Focep* isolates in artificial inoculation studies under laboratory conditions.

MATERIALS AND METHODS

Analysis of onion seed for the presence of *F. oxysporum* and *Focep*.

Seed sampling. Onion seeds from two semi-commercial (HT49 and Cream Gold) and 11 commercial onion varieties were analyzed. In total, the varieties included three local and three international open-pollinated varieties, as well as seven *Filial 1* (F1) hybrid varieties (Table 1). Seeds were obtained from various local and internationally-based vegetable seed companies.

Isolation of F. oxysporum. For each variety, a total of 600 seeds were analyzed. Half of the seeds was rinsed for 30 s in sterile distilled water, surface sterilized for 60 s in 70% alcohol and dried in a laminar-flow cabinet. The other half was not surface sterilized, but were plated out with their original commercial fungicide treatment intact. Seeds were plated onto Petri dishes (90 mm in diameter) containing potato dextrose agar (PDA; Biolab, Gauteng, South Africa) supplemented with 0.04 g Streptomycin sulphate per litre agar (PDA$^+$), with 10 seeds per Petri dish.

The Petri dishes were incubated at 22ºC $\pm$ 4ºC on the laboratory bench, and were inspected on a weekly basis for fungal growth. Where conidial morphology was typical of *F. oxysporum*, the *Fusarium* colonies were purified using a hyphal tip and single-spore

culture technique (Leslie and Summerell, 2006). Single-spored isolates were stored in 15% glycerol at -80°C.

Identification of Focep isolates. The pathogenicity of *F. oxysporum* isolates from onion seed was evaluated using a previously described onion bulb inoculation method (Chapter 2). Pathogenicity testing of each isolate was conducted using four onion bulbs of the onion variety Coastal Cream. Each pathogenicity assay included negative controls consisting of a non-pathogenic *F. oxysporum* isolate and a water control, as well as a positive control consisting of a highly pathogenic *Focep* isolate. Isolates were identified as *Focep* if they caused between 20 and 100% necrosis of the cut basal plate. The *Focep* isolates were further sub-divided into highly virulent (70 to 100% necrosis) and moderately virulent (20 to 70% necrosis) isolates. Isolations were made from bulbs that developed more than 20% necrosis, to fulfil Koch's postulates. Isolates causing no or less than 10% necrosis of the cut basal plate section were considered non-pathogenic. Each trial was repeated once, in order to confirm the pathogenicity results.

Molecular identification of Focep isolates as VCG 0425 isolates. The presence of VCG 0425 isolates among the *Focep* isolates was investigated using a previously developed inter-retrotransposon sequence characterized amplified region (IR-SCAR) multiplex polymerase chain reaction (PCR) that identifies VCG 0425, VCG 0421 and SMV 4 isolates as a group. Results from the IR-SCAR PCR testing were also confirmed by inter-retrotransposon amplified polymorphism polymerase chain reaction (IRAP-PCR) fingerprinting of all the *Focep* isolates. This PCR shows the PCR amplicon sizes from which the IR-SCAR multiplex PCR test was developed. Isolates that gave a positive result in the IR-SCAR PCR test were further analysed with two RAPD primers that each yield one PCR amplicon that is specific for VCG 0425 isolates (Chapter 3). DNA extraction and PCR amplifications with the IR-SCAR primers (Hansec-1F, Hansec-2R, HTH-1F and HTH-2R), IRAP primers (Hani and Gagi) and RAPD primers (OPA-05 and OPI-18) were conducted as previously described (Chapter 3).

Analysis of onion seedlings for the presence of *F. oxysporum* and *Focep*.

Seedling sampling. During the winter of 2007, a total of 644 onion seedlings were collected from five Western Cape onion nurseries (WCN), WCN-1 to WCN-5. The nurseries were situated in the Sandveld (WCN-1), Koue Bokkeveld (WCN-2), Swartland

(WCN-3), Breede River (WCN-4) and Klein Karoo (WCN-5) regions (Fig. 1). These five nurseries represent five different geographical areas within the Western Cape Province of South Africa and collectively provided 68.5% of the onion seedlings transplanted in the Western Cape Province in 2007. In each nursery, seedlings were collected at two sampling times from 19 to 50 hectares of land (Table 2). Six-week-old seedlings were collected on 7 June 2007, and more established, 14-week-old seedlings were collected on 7 August 2007. In each nursery, two seedlings were randomly selected per hectare, which resulted in the sampling of 38 to 100 seedlings per nursery at each of the sampling times (Table 2).

Isolation of F. oxysporum and identification of Focep and VCG 0425 isolates. Small basal plate pieces that included the root plate and the root attachment region were aseptically removed from each seedling, washed in sterile water for 30 s, surface sterilized for 60 s in 70% alcohol and dried in a laminar-flow cabinet. A maximum of four basal plate pieces were placed onto a Petri dish (90 mm in diameter) containing PDA$^+$. *Fusarium oxysporum* isolates were sub-cultured, identified, purified and stored as described for the seed isolates. *Focep* isolates were investigated for the presence of VCG 0425 isolates and were also identified and sub-divided into highly virulent and moderately virulent isolates as described for the seed isolates.

Transformation of *Focep* isolates with two reporter genes and pathogenicity testing on mature onion bulbs.

Transformation of Focep isolates. Two *Focep* isolates were transformed with reporter genes, STE-U 6641 with GFP and STE-U 6650 with DsRed-Express. The two isolates were obtained from onion bulbs collected in the Koue Bokkeveld, and belonged to the highly virulent major *Focep* VCG in South Africa, VCG 0425 (Chapter 2). Transformation was conducted using a protoplast polyethylene glycol calcium chloride transformation method and vectors as described by Paparu *et al.* (2009). A concentration of 150 µg hygromycin/ mL PDA was used for selecting transformants, since this concentration suppressed all wild-type *Focep* growth. Putative transformants that grew on the selective medium were evaluated for fluorescence using epifluorescence microscopy as previously described (McLean *et al.*, 2009). For each of the isolates, three transformants that showed bright fluorescence were selected, and these were single-spored twice on selective media. From these isolates, transformant FocG1, derived from

isolate STE-U 6641, and transformant FocR1, derived from isolate STE-U 6650, were selected. Before being used in pathogenicity and colonization studies, the stable transformation of the two transformants was investigated by conducting several rounds of sub-culturing onto non-selective media, and evaluating whether the transformants retained their hygromycin and fluorescent phenotypes. Both isolates were stored at -20°C in 15% glycerol. Transformant FocG1 and FocR1 were submitted to the STE-U culture collection as STE-U 7227 and STE-U 7228, respectively.

Pathogenicity of transformants towards onion bulbs. Transformants FocG1 and FocR1 were evaluated for pathogenicity towards onion bulbs using the pathogenicity test described above for identifying *Focep* isolates. The wild-type strains of both of these isolates were also included as control treatments during the pathogenicity test. Some of the FocG1 inoculated bulbs were cut open to study the colonization of the basal plate and bulbs using epifluorescence stereo-microscopy.

Colonization of onion seedstalks and seed by reporter gene-labelled *Focep* isolates.

Inoculation of bolted onion bulbs. The inoculation of bolted onion bulbs was conducted by first inducing influorescence and seedstalk production under field conditions. These bulbs were then transplanted to trays in a laboratory where inoculation and seed production took place. Both reporter gene transformants (FocG1 and FocR1) were used in the inoculation study.

Disease-free bulbs of the onion variety Coastal Cream were planted during April 2008 at a local vegetable research station in soil that had had no *Allium* crop for 10 years. The soil was sandy, with a pH (H_2O) of 5.8, low levels of nutrients (2180 ohms) and a soil carbon level of 0.86%. Visually healthy bulbs were removed from the soil in groups of four during September 2008, with most of their root system and the soil around the root system still intact. By this time, the bulbs had bolted, forming healthy leaves and at least one seedstalk of 15 cm or longer. The bulbs and soil were transplanted into 5-L trays and transferred to a laboratory with an air temperature of 23 to 27°C. In total, 24 onion bulbs were transplanted into six 5-L trays, four bulbs per tray. Natural light conditions in the laboratory were supplemented with a photoperiod of 14 hours with cool white light that, together with the high air temperature, accelerated seedstalk growth. The plants were left

for 7 days to allow acclimatization to the laboratory, before inoculation with *Focep* reporter gene isolates.

The six trays containing four bolted onion bulbs with seedstalks were inoculated as follows: Four trays were inoculated with transformant FocG1, one tray with transformant FocR1, and the final tray served as a control, inoculated with un-colonized millet seed. The trays were inoculated by incorporating colonized millet seed into the top 3 cm of the soil surrounding the transplanted bulbs at a 5% v/v ratio. All trays were then irrigated thoroughly. Subsequently, the plants were irrigated once a week with municipal tap water supplemented with a balanced, water-soluble fertiliser at an electrical conductivity of 1.5 ms/cm (Hygroponic, Hygrotech Seed (Pty) Ltd., Strand, South Africa).

The first umbels with visible flowers were formed around the end of October 2008. After flower opening, pollination was aided by lightly stroking the top of the umbels with the palm of the hand. This was done on a regular basis until all the flowers withered. Small plastic bags were then fastened around the umbels, firstly to avoid *Fusarium* contamination and secondly to collect dislodged seed.

Isolations from basal plates, seedstalks and seed. When the onion seed was fully mature and started to dislodge from the umbels (end of January 2009), onion basal plates, seedstalks and onion seed were removed from the trays for isolation purposes. Five 3x3 mm pieces of each of the basal plate and the lower, middle and upper seedstalk area of each plant were plated onto PDA supplemented with 150 µg/ml hygromycin. The lower, middle and upper seedstalk areas represented a 5-cm seedstalk portion just above each onion neck, the centre of the seedstalk and just below the umbels, respectively. Half of the basal plate and seedstalk pieces were left unsterilized and the other half were washed with distilled water and then surface sterilized with 70% alcohol for 60 s. All the harvested onion seeds were plated out unsterilized. After 7 days, the *Fusarium* colonies that developed on the selective medium from each seed or onion plant piece were evaluated for fluorescence using epifluorescence microscopy. Images were captured with a Nikon digital camera DXM 1200 and Automatic Camera Tamer (ACT-1) computer software.

RESULTS

Analysis of onion seed for the presence of *F. oxysporum* and *Focep*. The total percentage of onion seed obtained from seed companies that was colonized by non-pathogenic *F. oxysporum* (0.9%) was far higher than those infected by *Focep* (0.13%). *Focep* was isolated from seven of the seed lots, with infections being low, ranging in averages from 0.17% to 0.5% for the different seed lots when sterilized and unsterilized isolations were considered. Among all the *Focep* seed isolates, half were highly virulent and the other half were moderately virulent. The highly virulent isolates were only identified in South African seed lots, but not in the international seed lots. Two of the South African seed lots (Australian Brown and Mikado) each contained one VCG 0425 isolate that was highly virulent. These two *Focep* isolates yielded the two diagnostic bands of the IR-SCAR multiplex PCR, as well as three VCG 0425 diagnostic IRAP-PCR amplicons and two RAPD amplicons. None of the other *Focep* isolates belonged to VCG 0425 based on IR-SCAR multiplex testing or IRAP-PCR fingerprinting (Table 1).

Focep was isolated from seeds that still had their fungicide treatment intact (unsterilized) as well as seeds that were sterilized. Highly virulent isolates, including VCG 0425 isolates, were only obtained from the unsterilized seed treatment. Surface sterilized seeds yielded only moderately virulent isolates (Table 1). Within the F1 hybrid and open-pollinated varieties, 0.07% and 0.19% of the seed tested in each category, respectively, were infected by *Focep*. The open-pollinated varieties Australian Brown and Caledon Globe had the highest average rates of *Focep* seed contamination, namely 0.5% and 0.3%, respectively (Table 1).

Analysis of onion seedlings for the presence of *F. oxysporum* and *Focep*. None of the onion seedlings showed any visible disease symptoms at the time of collection. However, *Focep* was isolated from all the nurseries, although at a much lower frequency than non-pathogenic *F. oxysporum* isolates. In general, the incidence of both of these groups increased from the first sampling date (6-week-old seedlings) to the second (14-week-old seedlings). Of the 6-week-old seedlings, 22.2% were colonized by non-pathogenic *F. oxysporum* isolates, whereas only 2.7% were infected with *Focep*. Only two of these *Focep* isolates were highly virulent, both collected from WCN-1. WCN-5 was the only nursery in which no 6-week-old seedlings were infected by *Focep*. Of the

14-week-old seedlings, a total of 39.8% were colonized by non-pathogenic *F. oxysporum* isolates, whereas 5.7% were infected by *Focep*. Highly virulent *Focep* isolates were isolated from 14-week-old seedlings in all nurseries, except from WCN-4, from which only moderately virulent *Focep* isolates were obtained. WCN-1 contained the highest percentage of seedlings infected with highly virulent *Focep* isolates (Table 2). None of the *Focep* isolates, highly or moderately virulent, belonged to VCG 0425, based on the IR-SCAR multiplex PCR and IRAP-PCR analyses. In total, 60% of the seedling associated *Focep* isolates were moderately virulent (Table 2).

When the results from both collection dates were combined, three of the five onion seedling nurseries had 5% or more *Focep*-infected seedlings (Table 2). These nurseries were WCN-1 (6.6%), WCN-3 (5%) and WCN-4 (5.3%). Lower infection rates were identified for WCN-2 (2.5%) and WCN-5 (1.5%). In addition, WCN-1, WCN-4 and WCN-5 showed an escalation of pathogenic isolates from the 6-week-old to the 14-week-old seedling growth stage. At the second sampling time, *Focep* infection in the WCN-1 nursery was the highest (10.7%) and included highly and moderately virulent isolates. For WCN-2 and WCN-3, the number of *Focep* isolates either decreased or remained the same during this period.

Transformation of *Focep* isolates with two reporter genes and pathogenicity testing on mature onion bulbs. The two selected reporter gene *Focep* transformants, FocG1 and FocR1, were both stably transformed, since they retained their fluorescence and hygromycin phenotypes after single-sporing and sub-culturing for several rounds onto non-selective media. The transformants showed bright green (FocG1) or red (FocR1) fluorescence in their mycelia as well as conidia when viewed using epifluorescent microscopy (Fig. 2). Pathogenicity testing of both transformants on onion bulbs showed that they were still pathogenic and caused severe *Fusarium* basal rot symptoms, only 4 weeks after inoculation. This was similar to the symptoms caused by the wild-type isolates of each transformant. Epifluorescence stereo-microscopy showed the progressive green fluorescent colonization of the basal plate in a V-shape for the FocG1 inoculated bulbs that were cut longitudinally (Fig. 3), but not in bulbs inoculated with the wild-type isolate of this transformant. This progressive colonization from the basal plate that decreases as the pathogen moves up in the bulb could also be seen in cross-sections of the FocG1 inoculated bulbs (Fig. 4), but not the wild-type inoculated bulbs. Visualization of

the FocR1 transformant in bulbs was not conducted due to the stereomicroscope not having the correct filters for viewing the DsRed-Express protein.

Colonization of onion seedstalks and seed by reporter gene-labelled *Focep* isolates.

Inoculation of bolted onion bulbs. After transplant of the field-grown bolted onion bulbs to trays in the laboratory, most of the foliage remained green and healthy. Subsequent to laboratory transplant of the bulbs, the combination of high air temperatures (23 to 27°C), a cool-white light supplemented photoperiod of 14 hours and weekly applications of the water-soluble fertilizer Hygroponic was used to ensure strong seedstalk growth. Upon transplant, most bulbs had more than one small (15 cm or longer) seedstalk. However, on average, less than one viable seedstalk and umbel was eventually formed per bulb.

Not all the transplanted bulbs yielded seedstalks that produced seed. Of the 16 FocG1 inoculated onion bulbs, 15 formed seedstalks and umbels, but only 10 of these umbels yielded viable flowers. In total, 116 seeds were harvested from these 10 flower heads. Although the four onion bulbs inoculated with the FocR1 transformant all formed seedstalks and umbels, rapid disintegration of these bulbs due to Fusarium basal rot negatively influenced flower initiation, pollination and fertilization. Consequently, no seed was harvested from this treatment. The four bulbs in the control treatment resulted in three viable seedstalks and umbels, but yielded only 17 onion seeds in total.

Isolations from basal plates, seedstalks and seed. Isolations from basal plates, seedstalks and seed yielded fungal growth on the hygromycin selective media for the *Focep* transformant treatments, but not from the un-inoculated control. The positive isolation of each of the inoculated transformants from plant tissue pieces was confirmed by (i) the presence of colonies growing on the selective hygromycin media and (ii) the fact that these colonies fluoresced red (FocR1) or green (FocG1) when viewed using epifluorescence microscopy. The un-inoculated control did not yield *Fusarium* growth on the selective media.

Isolation studies showed that the FocG1 and FocR1 transformants were able to colonize the basal plate and all three areas of the seedstalks (lower, middle and upper).

Isolations from onion plants showed that most of the inoculated bulbs were colonized in their basal plate by FocG1 and FocR1. The percentage of seedstalks (lower, middle and upper combined) colonized by FocG1 and FocR1 was higher in the unsterilized plant material than the sterilized plant material. For plants inoculated with the FocG1 transformant, the lower seedstalk area was more readily colonized than the middle and upper seedstalk areas. This trend, however, was not seen for the seedstalks of plants inoculated with the FocR1 transformant, where the upper sections (75%) of the seedstalks were more profusely colonized than the lower sections. The lower percentage isolations made from sterilized seedstalks is most likely due to this tissue type being very porous, resulting in the ethanol also penetrating the internal side of seedstalk tissue. The lower percentage isolations from sterilized versus unsterilized material was also evident when considering the percentage growth obtained from the 10 to 40 seedstalk pieces that were plated from each seedstalk area. The percentage tissue pieces (unsterilized) plated for each of the seedstalk areas, confirmed that for the FocR1 transformant, the middle and upper seedstalks were more colonized than the lower seedstalks (Table 3).

Plants inoculated with FocG1 and the control treatment were the only treatments that yielded seed. Isolation from seeds onto selective media of the 10 seedstalk-containing bulbs showed that, in total, 106 of the 116 unsterilized seeds (91.4%) yielded *Focep* growth that fluoresced bright green when viewed using epifluorescence microscopy. The fact that 10 plants, distributed across three different trays, all produced FocG1-infected seed supports the reproducibility of the results (Table 3).

DISCUSSION

Seed and seedlings used in the Western Cape onion production region have the potential to contribute to Fusarium field and post harvest losses. Both the seed and seedlings were found to be infected by moderately and highly virulent *Focep* isolates and can, therefore, result in the contamination of virgin soils or increase inoculum levels in soil. Most of the *Focep* genotypes identified in this study, however, did not belong to the highly virulent VCG 0425 based on molecular analysis. This VCG has previously been shown to be the most widely distributed genotype associated with mature symptomatic onion bulbs in South Africa (Chapters 2, 3). *Focep* VCG 0425 isolates were only

associated with two national onion seed lots (Australian Brown and Mikado), but not with seedlings.

The contribution of *Focep*-affected seed and seedlings to pre- and post-harvest losses of onion should be deliberated by considering (i) the ratio between moderately virulent and highly virulent isolates and (ii) the incidence of highly virulent isolates which are not of the VCG 0425 genotype. The moderately virulent isolates may be of some significance, since 1% of isolates from mature bulbs were previously also shown to be moderately virulent (Chapter 2). However, the incidence of moderately virulent isolates was much higher in seed (50%) and seedlings (60%). Although it is known that *Focep* isolates differ in virulence, and that a significant correlation exists between resistance to *Focep* in seedling and adult onion plants (Schwartz and Mohan, 2008), the virulence of specific isolates toward both growth stages has not been investigated. Therefore, in future, the pathogenicity of moderately virulent isolates from seeds and seedlings also needs to be determined on seedlings that are grown through to the bulb production and post-harvest phases.

The *Focep* genotypes, other than VCG 0425, detected on seed and seedlings may influence the evolution and aggressiveness of future pathogen populations in nurseries and field crops. This is of particular concern for imported seed, since moderately virulent *Focep* isolates were isolated from the varieties Pukekohe Long Keeper and Cream Gold. New *Focep* genotypes on seed and seedlings, especially those identified as highly virulent, may become more widespread if they have an advantage over VCG 0425 regarding fitness and virulence. The introduction of more diverse pathogen genotypes (highly or moderately virulent) may also create the potential for somatic recombination with local pathogen populations, yielding more virulent strains. The occurrence of somatic recombination has been hypothesized for some *F. oxysporum* formae speciales (Taylor *et al.*, 1999; O'Donnell *et al.*, 2009), and some circumstantial evidence for this has been found in *Focep* (Chapter 2).

The infection frequency of *Focep* from the seed lots was low (0.17 to 0.5%), but was comparable to those reported for *F. oxysporum* f.sp. *lactucae* (0.1 to 0.2%) (Garibaldi *et al.*, 2004) and *F. oxysporum* f.sp. *basilici* (0.9%) (Guirado Moya *et al.*, 2004). Whether *Focep*-infected seed has the potential to germinate and yield viable seedlings that can

serve as an inoculum source is unknown. This may be a possibility, since in sweet basil it has been shown that seeds collected from diseased plants contained *F. oxysporum* f.sp. *basilici*, and that these seeds could produce diseased plants bearing infested seeds (Gamliel *et al*., 1996). If all of the *Focep* genotypes identified in seed (both moderately virulent and highly virulent isolates) are able to serve as an inoculum source, this may result in substantial infections of nursery seed beds. At the detected *Focep* infection rate of 0.07% for F1 hybrid seed and 0.19% for open-pollinated varieties, between 2113 (F1 hybrid) and 6175 (open-pollinated) seeds out of every 1 625 000 seeds used for production of one hectare of onion transplants could be infected with *Focep*. From these infected seedlings, *Focep* inoculum can spread over short distances via irrigation water, soil and moving objects in a nursery situation (Sherf and Macnab, 1986; Howard *et al*., 1994; Gabor, 1996).

Isolation studies from seeds showed that *Focep* can colonize seeds internally, and that fungicide coating of seeds does not inhibit *Focep* growth. When *F. oxysporum* isolations were made from surface sterilized seed, an average *Focep* infection frequency of 0.1% was observed, and isolations from fungicide-coated unsterilized seeds yielded a comparable percentage (0.2%) of infected seed. Only moderately virulent *Focep* isolates were isolated from surface sterilized seeds, suggesting that these isolates can colonize seed internally, as has been reported for sweet basil seed infected with *F. oxysporum* f.sp. *basilici* (Vannacci *et al*., 1999; Chiocchetti *et al*., 2001). The highly virulent *Focep* isolates, including two VCG 0425 isolates, were only obtained from the fungicide-coated seeds. Due to the low infection frequencies, it cannot be said with certainty that these isolates only colonize seed externally. External colonization of sweet basil seed (Vannacci *et al*., 1999; Chiocchetti *et al*., 2001) and lettuce seed by *F. oxysporum* f.sp. *lactucae* (Garibaldi *et al.,* 2004) has been reported.

The seedborne nature of *Focep* VCG 0425 was demonstrated by using a GFP transformant inoculated into soil. This showed that, at least under laboratory conditions, the pathogen not only colonizes seedstalks but also seed. The high level of seed infection (91.4%) by transformant FocG1 was unexpected, but is most likely related to the favourable conditions for pathogen infection under laboratory conditions, and the very high inoculum concentration (5%) that was used. The seed transmission experiment was difficult to repeat for the following reasons: (i) bolted bulbs did not always form

seedstalks under laboratory conditions, (ii) some seedstalks produced no or very few seed and (iii) it is difficult to obtain inoculum concentrations and infection conditions that will cause disease, but not to such an extent that bulbs will not produce seed. The latter was evident when bulbs were inoculated with the red fluorescing transformant, FocR1. In this instance, infection did occur, but no seed was produced. The extensive colonization of the upper seedstalk section just below the umbels by FocR1 could have prevented seed formation. Some support for the reproducibility of the seed transmission experiment is provided by the fact that 10 plants, distributed across three different trays, all produced FocG1 infected seed. The colonization of seedstalks by the FocG1 and FocR1 transformants suggests that the pathogen spreads systemically from the basal plate through the seedstalk to the seeds.

Focep isolates were detected in all five Western Cape seedling nurseries, although the incidence differed as the season progressed. In comparison to onion seed, seedlings had a higher *Focep* infection frequency, and may thus be a larger primary inoculum source. It is, however, important to note that more than 50% of the *Focep* isolates were only moderately virulent, similar to what was found on seed. The average *Focep* seedling infection rate increased from 2.7% at the first sampling time to 5.7% at the second sampling time. This tendency towards an increase in the percentage of *F. oxysporum* isolates pathogenic to onion seedlings could have been caused by a significant increase in pathogenic *F. oxysporum* isolates in soil during the course of the production season, as reported by Everts *et al*. (1985), and pathogen proliferation on seedling roots. The tendency towards a higher infection rate at the second collection date, which was as high as 10.7% in WCN-1, is of greater importance since the seedlings are mature enough to be transplanted at the 16 week old stage. Under standard nursery conditions of 12 rows per seedling bed and 66 666 running meters of plant rows per hectare, a 5% seedling infection means that at least one seedling every 1.6 running meters could be infected if 55 kg of seed are sown per hectare. Historically, onion seedling transplants have been hypothesized to be the source through which *Focep* was distributed widely (Schwartz and Mohan, 2008). However, in view of the results from the current study, seed may also be an important method through which *Focep* genotypes can spread.

The current study showed that seed and seedlings are important inoculum sources of *Focep* in the Western Cape Province. A range of genotypes was detected that differed

in virulence, with the highly virulent and most common *Focep* genotype in commercial onion bulb production fields (VCG 0425) being detected at low levels from seed. Due to the uncertainty of the epidemiological impact of moderately virulent and highly virulent genotypes that do not belong to VCG 0425, future studies should investigate the pathogenicity and virulence of these isolates towards seedlings and older field-grown plants, in an attempt to elucidate their role in the epidemiology of the disease. A well-known risk associated with the introduction of new genotypes with planting material is the potential for increasing pathogen diversity and thus evolutionary potential. Therefore, future management practices should be aimed at reducing the risk associated with these inoculum sources. Pathogen-free seed and seedlings are important aspects of an integrated disease management strategy. Aspects of seed inoculum management that could be investigated in future, include introducing seed coating methods and fungicide options that are more effective than Thiram dusting and the slurry methods that are currently standard practice in the industry.

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Table 1. Percentage onion seeds that yielded non-pathogenic *Fusarium oxysporum* and *F. oxysporum* f.sp. *cepae* isolates in seed isolation studies

Onion variety	Variety type[a]	National or international supplier	Unsterilized[b]		Sterilized[c]		Average[d]	
			Percentage *F. o.*[e]	Percentage *Focep*[f]	Percentage *F.o.*	Percentage *Focep*	Percentage *F.o.*	Percentage *Focep*
Caledon Globe	Open-pollinated	National	1.3	0.67 (1HV;1MV)	1.3	0.33 (1MV)	1.3	0.50 (1HV; 2MV)
Australian Brown	Open-pollinated	National	1.7	0.67 (2HV)*	1.0	0	1.3	0.33 (2HV)
Coastal Cream	Open-pollinated	National	0.7	0	0.7	0	0.7	0
Pukekohe Long Keeper	Open-pollinated	International (New Zealand)	0.7	0	0.7	0.33 (1MV)	0.7	0.17 (1MV)
Cream Gold	Open-pollinated	International (Australia)	0.7	0	1.0	0.33 (1MV)	0.8	0.17 (1MV)
Early Lockyer Brown	Open-pollinated	International (Australia)	0.3	0	0.7	0	0.5	0
Orlando	*Filial 1* hybrid	National	1.3	0.33 (1HV)	1.0	0	1.2	0.17 (1 HV)
Ceres Gold	*Filial 1* hybrid	International (New Zealand)	2.0	0	0.7	0	1.3	0
Arnold	*Filial 1* hybrid	International (The Netherlands)	1.7	0	0.7	0	1.2	0
Mikado	*Filial 1* hybrid	National	0.7	0.33 (1HV)*	0	0	0.3	0.17 (1HV)
Red Beauty	*Filial 1* hybrid	International (The Netherlands)	0.7	0	0.3	0	0.5	0
Wellington	*Filial 1* hybrid	International (The Netherlands)	0.7	0	0	0	0.3	0
HT 49	*Filial 1* hybrid	National	1.7	0.33 (1MV)	0.7	0	1.2	0.17 (1MV)
Average percentages			1.1	0.2	0.7	0.1	0.9	0.13

[a] Variety type refers to the propagation method used to produce the seed. 'Open-pollinated' refers to pollination via natural mechanisms resulting in the production of new generations of seed from the same plant. '*Filial 1* hybrid' refers to the first generation seed of a natural cross between genetically different varieties.

[b] Three hundred seeds from each variety were analyzed with their original commercial fungicide treatment still intact.

[c] Three hundred seeds from each variety were analyzed in isolation studies by first rinsing the seeds for 30 s in sterile distilled water, followed by surface sterilization for 60 s in 70% alcohol before plating.

[d] Average indicates the average percentage of seed colonized from the unsterilized and sterilized treatment options.

[e] The percentage of seed yielding non-pathogenic *Fusarium oxysporum* (*F.o.*) isolates.

[f] The percentage of seed yielding pathogenic *F. oxysporum* f.sp. *cepae* (*Focep*) isolates. Highly virulent (HV) isolates caused 70 to 100% necrosis of the cut basal plate section of the susceptible variety Coastal Cream, whereas moderately virulent (MV) isolates caused only 20 to 70% necrosis. Isolates that were molecularly identified as VGC 0425 are followed by an "*".

Table 2. Percentage onion seedlings from five onion nurseries in the Western Cape region, which yielded non-pathogenic *Fusarium oxysporum* and *F. oxysporum* f.sp. *cepae* isolates

| | Onion seedling nurseries | | | | | | | | | | | | | | | | |
| | WCN-1 (42 ha.[a]) | | | WCN-2 (30 ha.) | | | WCN-3 (20 ha.) | | | WCN-4 (19 ha.) | | | WCN-5 (50 ha.) | | | Average[b] | |
Collection date	Nr.[c]	% *F.oxy*[d]	% *Focep*[e]	Nr.	% *F.oxy*	% *Focep*	Nr.	% *F.oxy*	% *Focep*	Nr.	% *F.oxy*	% *Focep*	Nr.	% *F.oxy*	% *Focep*	% *F.oxy.*	% *Focep*
07/07/2007	84	29.7	2.4 (2HV)	60	13.3	3.3 (2MV)	40	37.5	5.0(2MV)	38	26.3	2.6 (1MV)	100	4.0	0	22.2	2.7
07/08/2007	84	58.3	10,7 (4HV;5MV)	60	8.3	1.7 (1HV)	40	60.0	5.0(2HV)	38	47.4	7.9 (3MV)	100	25.0	3 (1HV;2MV)	39.8	5.7
Average %		44.9	6.6		10.8	2.5		48.8	5.0		36.9	5.3		14.5	1.5	31.0	4.2

[a] The number of hectares from which onion seedlings were collected in each of the five Western Cape nurseries (WCN).

[b] Average indicates the average percentage seedlings colonized in all five nurseries.

[c] The number of onion seedlings from which isolations for *F. oxysporum* were made. Two seedlings were randomly selected from each hectare at each of the collection dates.

[d] Percentage seedlings yielding non-pathogenic *Fusarium oxysporum* (*F. oxy.*) isolates.

[e] Percentage seedlings yielding *Fusarium oxysporum* f.sp. *cepae* (*Focep*) isolates. Highly virulent (HV) isolates caused 70 to 100% necrosis of the cut basal plate section of the susceptible variety Coastal Cream, whereas moderately virulent (MV) isolates caused only 20 to 70% necrosis.

Table 3. Colonization of onion basal plates, seedstalks and seeds by two reporter gene (GFP and DsRed-Express) labelled *F. oxysporum* f.sp. *cepae* (*Focep*) isolates that were inoculated into soil surrounding bolted onion bulbs

| Plant part | Transformants | | | | Control[c] | |
| | Percentage of plants (percentage tissue pieces) yielding FocG1[a] | | Percentage of plants (percentage tissue pieces) yielding FocR1[b] | | | |
	Unsterilized[d]	Sterilized[d]	Unsterilized	Sterilized	Unsterilized	Sterilized
Basal plate	75.0 (52.5)	81.3 (85.0)	75.0 (70.0)	50.0 (50.0)	0 (0)	0 (0)
Lower seedstalk[e]	60.0 (55.0)	33.3 (32.5)	50.0 (50.0)	25.0 (10.0)	0 (0)	0 (0)
Middle seedstalk[e]	46.7 (45.0)	13.3 (17.5)	75.0 (80.0)	25.0 (10.0)	0 (0)	0 (0)
Upper seedstalk[e]	46.7 (42.5)	6.7 (7.5)	75.0 (80.0)	25.0 (30.0)	0 (0)	0 (0)
Onion seed	100.0 (91.4)	Not done	No seed produced	No seed produced	0 (0)	Not done

[a] FocG1 is a green fluorescent protein (GFP) *Focep* transformant. Percentage of plants of which the basal plate (16 plants analysed), seedstalks (15 plants analysed) and seeds (10 plants analysed) yielded FocG1 growth on 150 μg/mL hygromycin are shown. In brackets, the percentage of tissue pieces (total of 40 plated) and seeds (total of 116) yielding FocG1 growth is shown. The colonies that grew on the selective media all fluoresced green when viewed with epifluorescence microscopy.

[b] FocR1 is a *Focep* DsRed-Express transformant. The percentage of plants (4 plants analysed) is shown for which the basal plate and seedstalk yielded FocR1 growth on 150 μg/mL hygromycin. In brackets, the percentage of tissue pieces (total of 10 plated) yielding FocR1 growth is shown. The colonies that grew on the selective media all fluoresced red when viewed with epifluorescence microscopy.

[c] The control consisted of bulbs inoculated with un-colonized millet seed. A total of 4 plants were analysed; 10 tissue pieces were plated per tissue type; 17 onion seeds were plated.

[d] Plant parts were plated onto selective media containing hygromycin without surface sterilization (unsterilized) or by first surface sterilizing plant parts in 70% ethanol for 60 s (sterilized).

[e] The lower, middle and upper seedstalk areas represented a 5 cm seedstalk length just above each onion neck, in the centre of the seedstalk and just below the umbels, respectively.
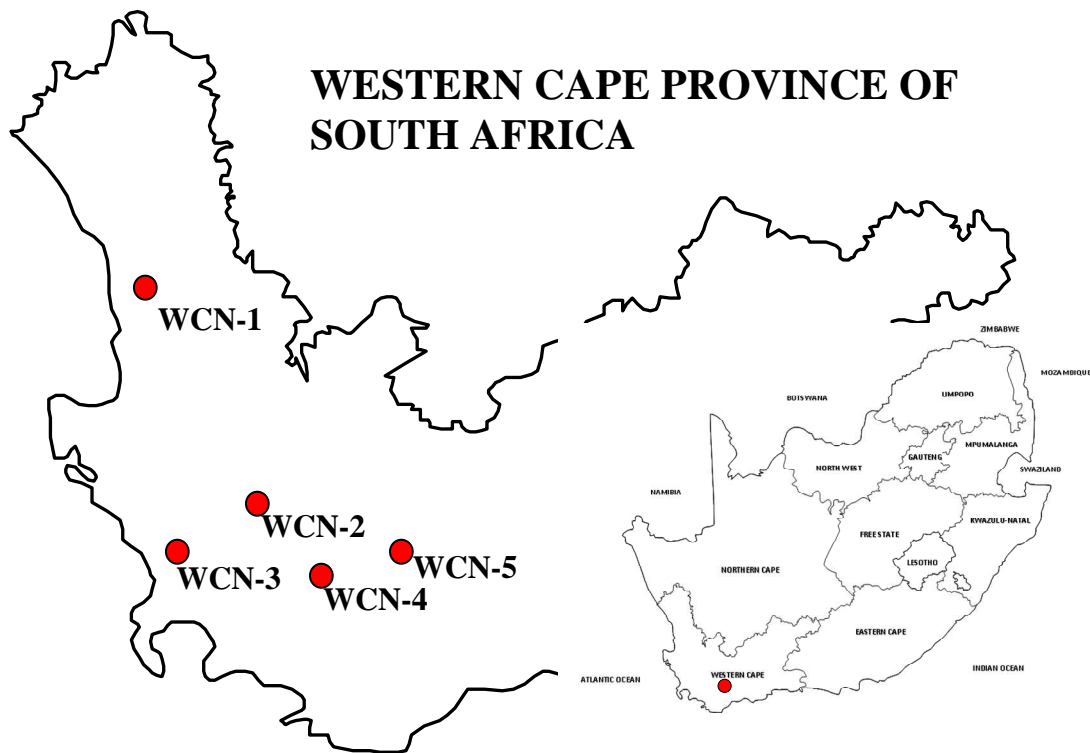
Fig. 1. Western Cape Nurseries (WCN) from which onion seedlings were collected for *Fusarium oxysporum* isolations. The nurseries were situated in the Sandveld (WCN-1), Koue Bokkeveld (WCN-2), Swartland (WCN-3), Breede River (WCN-4) and Klein Karoo (WCN-5) regions, representing five different geographical areas within the province.
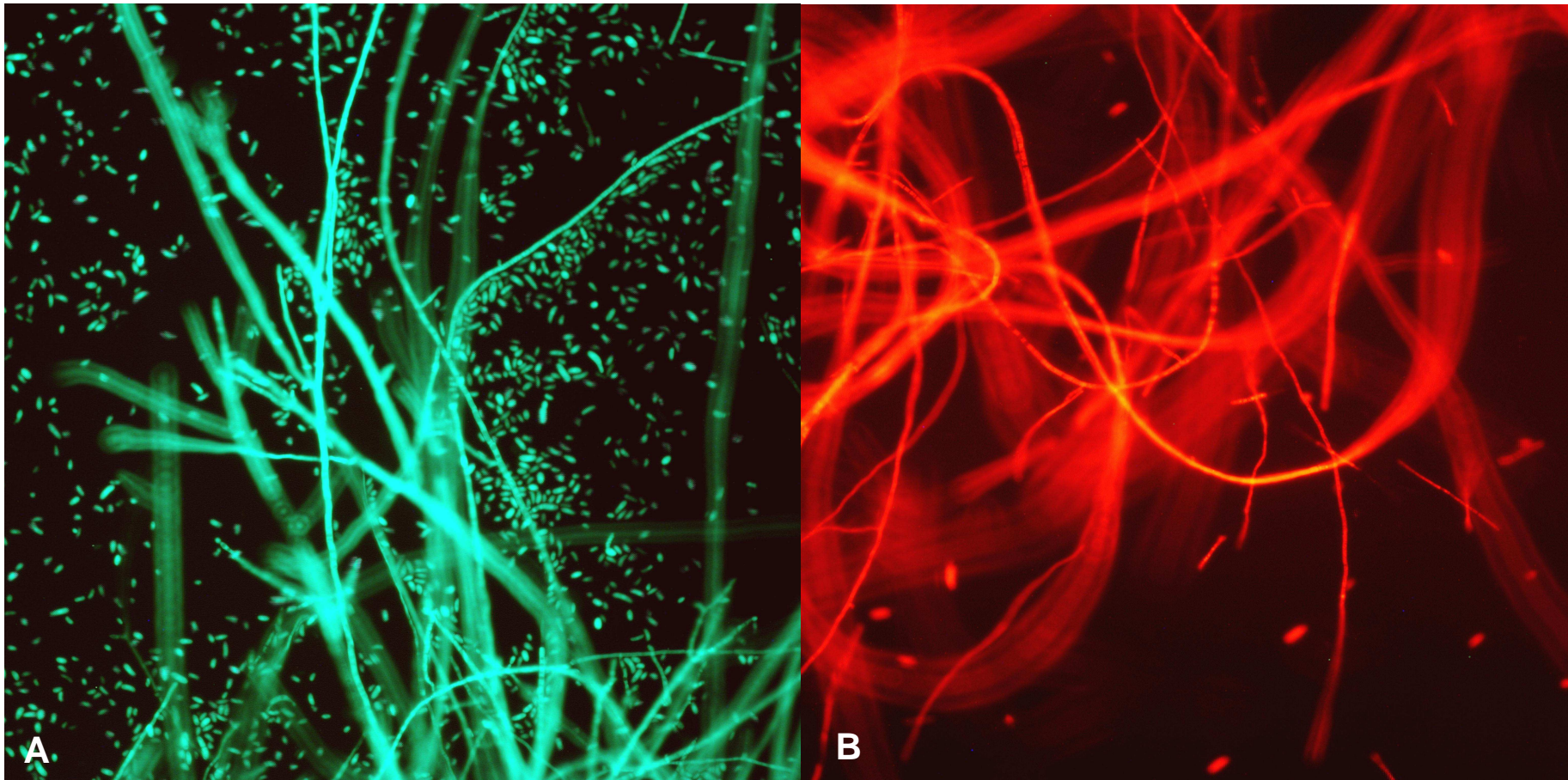
Fig. 2. Fluorescing conidia and mycelia of *Fusarium oxysporum* f.sp. *cepae* transformants (A) FocG1 that were transformed with the green fluorescent protein and (B) FocR1 that was transformed with the DsRed-Express gene. Fluorescence was viewed using epifluorescence microscopy.
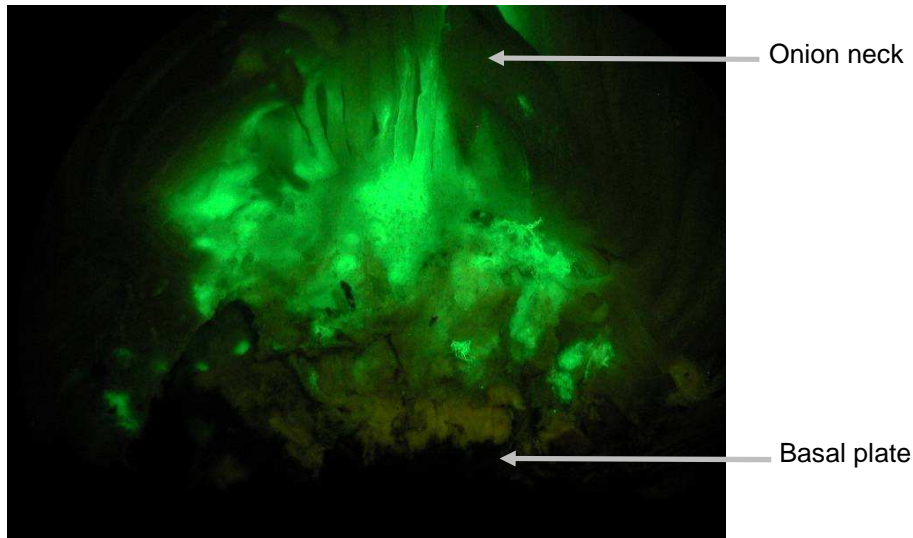
Fig. 3. Epifluorescence microscopy of an onion bulb that was cut open longitudinally, 21 days after the basal plate was inoculated with a green fluorescence protein-labelled *Fusarium oxysporum* f.sp. *cepae* transformant (FocG1).
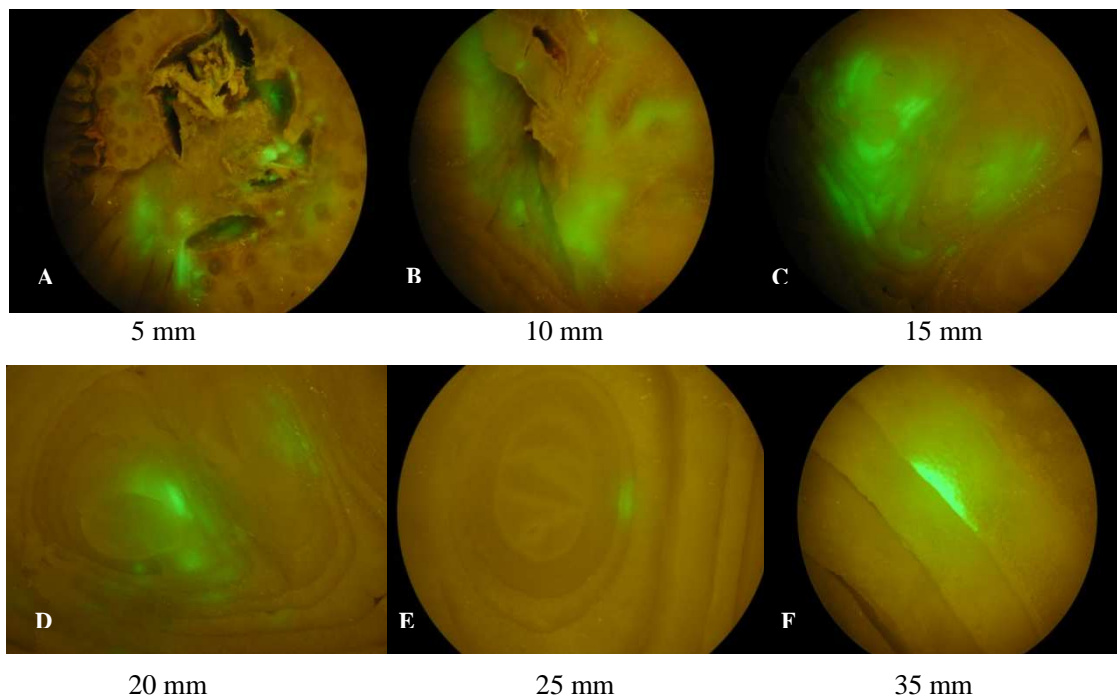
Fig. 4. Epifluorescence photographs of an onion bulb, 21 days after inoculation with a green fluorescent protein-labelled *Fusarium oxysporum* f.sp. *cepae* transformant (FocG1). A to F shows transversal sections of the bulb that were first cut at a depth of 5 mm into the basal plate (A), followed by deeper sections towards the neck of the onion (B to F).