Characterisation of both *Hoodia gordonii* and the associating wilt causing pathogen *Fusarium oxysporum*

by

Onoufrios Agathoclis Philippou

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Promoter:
Dr A van Biljon

Co-promoters:
Dr A Minnaar-Ontong
Prof WJ Swart

Declaration

“I, Onoufrios Agathoclis Philippou, do hereby declare that the thesis hereby submitted by me for the degree Philosophiae Doctor in Plant Breeding/Plant Pathology at the University of the Free State represents my own independent work and has not previously been submitted by me at another University/faculty.

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....	……...
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Quote

"*Do not spoil what you have by desiring what you have not; remember that what you now have was once among the things you only hoped for.*" Epicurus

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List of abbreviations

AKA	also known as
AFLP	Amplified fragment length polymorphism
AMM	ammonium medium
AP-PCR	Arbitrarily Primed PCR
ARC-PPRI	Agricultural Research Council - Plant Protection Research Institute
ATP	Adenosine 5'-triphosphate
β-tub	β-tubulin
bp	Base pairs(s)
BM	Basal medium
CAM	crassulacean acid metabolism
CBS-KNAW	Centraalbureau voor Schimmelcultures - an institute of the Royal Netherlands Academy of Arts and Sciences
CLA	Carnation leaf agar
CMD	Calmodulin
CSIR	Council for Scientific and Industrial Research
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
dsRNAs	double-stranded RNAs
EDTA	Ethylene-diaminetetraacetate
ETS	external transcribed sequence
FOSC	*Fusarium oxysporum* species complex
f. sp.	*formae specialis*
GPS	global positioning system
HMM	minimal medium containing hypoxanthine
IGS	Intergenic spacer region
ITS	Internal transcribed spacers
$KClO_3$	potassium chlorite
masl	meters above sea level
MAT	Mating type
mDNA	Mitochondrial DNA
$MgCl_2$	Magnesium chloride
MM	minimal medium
MMC	minimal medium containing chlorite

MRC	Medical Research Council
MtR	mitochondrial
MtSSU	mitochondrial small subunit
NaCl	Sodium chloride
$NaNO_3$	Sodium Nitrate
NCBI	National Centre for Biotechnology Information
NCSS	Number Cruncher Statistical System
NIR	Nitrate Reductase Coding Region
***nit* mutants**	Nitrate-nonutilising mutants
NMM	minimal medium containing nitrate
NTSYSpc	Numerical taxonomy and multivariate analysis system
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDC	PDA containing chlorite
PIC	Polymorphic information content
r	Goodness of fit
®	Reserved
RAPD	Random amplified polymorphic DNA
RBAM	rose bengal amended medium
RCBD	randomised complete block design
RFLP	Restriction fragment length polymorphism
RNAse	Ribonuclease
rRNA	Ribosomal ribonucleic acid
SAHN	sequential agglomerative hierarchical nested cluster analysis
SANBI	South African National Biodiversity Institute
SD	standard deviation
SNA	Spezieller Nährstoffarmer Agar
spp	Species
SSR	Simple sequence repeat
Taq	*Thermus aquaticus*
TEF	Elongation factor-1 alpha
™	Trade Mark
TAE	Tris-acetate-EDTA
TBE	Tris- Cl/borate/EDTA
TE	Tris-Cl/EDTA
Tris-Cl	Tris (hydroxymethyl) aminomethane

TSS	Total soluble solids
UAM	Uric acid medium
UK	United Kingdom
UPGMA	Unweighted pair-group method using arithmetic averages
USA	United States of America
UV	Ultraviolet
VCG	vegetative compatible group
VWA	Van Wyk's agar
WA	Water agar

List of SI units

℃	Degrees Celsius
cm	Centimetre
g	Gram(s)
h	Hour(s)
kg	Kilogram
km	Kilometre
L	Litre
m	meter(s)
M	Molar(s)
min	Minute(s)
mg	Miligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar(s)
ng	Nanogram
pH	Power of hydrogen
pmol	Picomole(s)
r/s	Revolutions per second
s	Second(s)
U	Unit(s)
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar(s)
V	Volt(s)
v/v	Volume per volume
W	Watt(s)
w/v	Weight per volume

Chapter 1
General Introduction

Semi-arid regions in South Africa, Namibia and Botswana are a challenge for conventional cropping systems because of limited or irregular rainfall, poor soils and high temperatures (Le Houérou, 1996; Mula and Saxena, 2010). Therefore, the cultivation of conventional crops such as wheat, maize, and rice in these areas has proven to be agriculturally unproductive without the aid of irrigation. However, productivity in these areas can be increased by the cultivation of adapted crops such as *Hoodia gordonii* (Masson) Sweet ex Decne. These plants can survive in the semi-arid regions and for hundreds of years *H. gordonii* has been used by the San people for the prevention of dehydration and as an appetite suppressant while on long hunting trips (Rader *et al.* 2007). Presently, the plant has been classified as endangered and is been illegally harvested in the wild for the sought after main ingredient (P57 compound) used in diet products (Avula *et al.*, 2007).

To cultivate *H. gordonii* as a future cash crop for pharmaceutical companies and to start a breeding programme in the drought prone regions in South Africa, many aspects will have to be assessed. Initial assessments would be based on location, morphology, chemical analysis, pests and pathogens associated with the plant. Location is important as the plant only grows in semi-arid areas. There are large areas in South Africa that are semi-arid (24.6%) and receive 401-600 mm of rainfall annually (Palmer and Ainslie, 2005; Mula and Saxena, 2010) that may be utilised for cultivation of this new cash crop. Morphology of the plant is an important trait in all major commercial crops, and an understanding of their qualitative and quantitative traits will aid in selecting plants for future breeding programmes (Brown and Caligari, 2008) and disease management stratagies (Narayanasamy, 2013). A study done by Avula *et al.* (2006) determined the percentage of the P57 compound (oxypregnane steroidal glycoside) using HPLC (Rader *et al.*, 2007), together with the analysis of other sugars could assist in selection of plants for future breeders.

Currently, special permits are required to grow the plants. Nurseries exist throughout the region where *H. gordonii* grows in the wild and large areas have been planted in Pofadder (29°7' 46.3" S; 19°23' 37.2" E) and Kaka mas (28°47' 41.64" S; 20°37' 48" E).
However, in 2004 an observation of wilt disease was observed in a nursery that caused damage up to 90% of the crop in the nursery (pers. comm. WJ Swart). Although other

pests and diseases have been observed and reported on *Hoodia* (Lamprecht *et al.*, 2008; Swart, 2008), these disease/s are less significant than wilt disease.

In *H. gordonii* nurseries located in South Africa, plants are grown from seed. However, the varieties have not been fully characterised, hampering research and breeding efforts directed at the development of improved varieties. It is also difficult to pollinate the plant as flowering does not occur simultaneously. In addition, few published records of qualitative and quantitative traits, such as: morphology, biochemical analysis (sugar composition and % P57 compound) and diseases are available (Mulej and Strlič 2002; Avula *et al.,* 2007; Lamprecht *et al.*, 2008).

Commercially, *H. gordonii* is mainly cultivated in summer rainfall areas, most of which are prone to hail and sand storms. Physical damage caused by hail or sand can facilitate the entry of pathogenic fungi (Lamprecht *et al.*, 2008). Varieties currently being cultivated have not been screened for resistance to fungal diseases although reports of new diseases and associated financial losses due to fungal pathogens are available (Lamprecht *et al.*, 2008; Swart, 2008; Philippou *et al.*, 2013).

Wilt disease, caused by a soilborne pathogen, has a far reaching effect since it spreads via the soil through the root system and through irrigation water. No reports have been published to determine the effectiveness of fungicides on the disease.

Given the aforementioned problems confronting commercial cultivation of *Hoodia*, a study was undertaken to firstly, identify the wilt causing agent and secondly, to characterise the pathogen and host using different molecular and biochemical methods.

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Chapter 2

Importance of the endangered *Hoodia gordonii* and the wilt causing pathogen *Fusarium* oxysporum

2.1 Introduction

For hundreds of years the San people from the Kalahari Desert in Southern Africa used *Hoodia gordonii* as an appetite suppressant and for the prevention of dehydration while they were on long hunting trips (Rader *et al.*, 2007). Marloth (1932) first recorded the use of *Hoodia* species to suppress hunger and thirst, which was an ancient widespread practise used by Khoi-San people. Presently, the traditional use of *H. gordonii* reported by communities is both the cause and effect of this natural resource that has become scarce. In natural ecosystems, these plants are a minor source of food and moisture to wildlife with the stems providing shelter and breeding sites for small animals and insects (Mulej and Strlič 2002; Swart, 2008). Of all the *Hoodia* species, *H. gordonii* is the only commercially sought-after species primarily due to claims of its anorectic activity (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996; van Wyk *et al.*, 1997; van Wyk and Gericke, 2000; van Wyk and Wink, 2004; van Wyk, 2008a). Out of an estimated 3000 medicinal plant species which are commonly used in traditional medicine in South Africa, *H. gordonii* is one of only 38 indigenous species that has been commercialised (i.e. available as processed materials in packaging and in various dosage forms as teas, supplements, tinctures, tablets, capsules or ointments) (Cunningham, 1988; Mander, 1998; Williams *et al.*, 2000; van Wyk, 2008a).

The plant has become extremely important to pharmaceutical companies because of an appetite suppressant patented oxypregnane steroidal glycoside compound known as P57 that is extracted from stem sap (van Heerden *et al.*, 1998; Rader *et al.*, 2007). In 1995, the Council for Scientific and Industrial Research (CSIR) in South Africa isolated this active compound (P57). In 1998, a patent was granted by the World Intellectual Property Organisation on pharmaceutical compositions with appetite suppressant activity. In the same year the CSIR licensed the rights for further production and development of P57 to Phytopharm in the UK. In 1998 Phytopharm UK in turn sub-licensed the rights to Pfizer for the development and worldwide commercialisation. During 2001 and 2002, in terms of a benefit sharing agreement with the CSIR, all the

San communities in those states encompassing the range of the plant would benefit from the development of P57 (van Heerden, 2008; Glasl, 2009; Wynberg *et al.*, 2009).

In 2000, clinical development of P57 commenced. Clinical development of such products can take seven to ten years. The cost to the pharmaceutical companies to fully develop a prescription drug typically exceeds $500 million from start to commercialisation (Stephenson, 2003). In 2003 the USA-based company Pfizer merged with Pharmacia Corporation and announced that they were not proceeding with clinical trials to develop an anti-obesity drug (van Heerden 2008; Glasl, 2009; Wynberg *et al.*, 2009). Unconfirmed reports stated reasons for terminating the research on *H. gordonii* which included difficulty in synthesising the P57 molecules and that the synthetic molecules were not as effective as the natural molecules (Holt and Taylor, 2006; Glasl, 2009). In 2004, the patent was licensed to Unilever for the commercialisation of *H. gordonii* extracts into food products to produce functional foods. Although large sums of money were invested into this project for the development of an extraction facility, these plans were abandoned in December 2008 due to safety and efficacy concerns (Rader *et al.*, 2007; Avula *et al.*, 2008; Swart, 2008). Although Unilever terminated all activities related to *Hoodia* in South Africa on 31 March 2009, Phytopharm remained optimistic about the *Hoodia* programme and insisted that it would find other partners for further development of what promised to be a very lucrative industry (Wynberg *et al.*, 2009; Vermaak *et al.*, 2011).

2.2 Taxonomic background on *Hoodia gordonii*

Hoodia gordonii known as *Hoodia*, “Bitterghaap” “Bobbejaangghaap” “Jakkalsghaap” or “Wildeghaap”, is part of the genus *Hoodia* classified as stapeliads within the subfamily Asclepiadoideae belonging to the Apocynaceae family. It was previously classified as part of the genus *Trichocaulon* (Bruyns, 2005). A complete botanical name *H. gordonii* (Masson) Sweet ex Decne was based on the discoveries of several people. In 1774, Carl P. Thunberg and Francis Masson discovered the first *Hoodia* species (*H. pilifera*). In 1779, Robert J. Gordon made a drawing of a *Hoodia* species which was published by Masson described as *Stapelia gordonii* Masson. In 1830, Robert Sweet of England placed *gordonii* into a new genus. The genus was named *Hoodia*, after Mr. Hood, a renowned succulent grower in Britain. In 1844, Joseph Decaisne first published *H. gordonii* after the previous generic names were declared invalid (Bruyns, 2005; van Heerden, 2008). White and Sloane (1937) taxonomically revised this genus and later

Bruyns (1993) and Plowes (1992, 1996) contributed to the nomenclature. Bruyns (2005) published a book illustrating and describing the genus and species in detail.

The taxonomy of *Hoodia* is still in debate. After nearly 240 years since *Stapelia gordonii* was first described, 13 species have been identified within the *Hoodia* genus found in Southern Africa. Issues have arisen due to previous taxonomic classification, as well as numerous morphological and physiological attributes. Based on a report by CITES (2004), 13 species and four subspecies of *Hoodia* are found in the south western parts of Southern Africa. These are *H. alstonii* (N.E. Br.) Plowes, *H. flava* (N.E.Br.) Plowes, *H. currorii* (Hook.) Decne. subsp. *currorii, H. currorii* (Hook.) Decne. subsp. *lugardii* (N.E.Br.) Bruyns (endemic to Namibia), *H. dregei* N.E.Br., *H. gordonii* (Masson) Sweet ex Decne, *H. juttae* Dinter (endemic to Namibia), *H. mossamedensis* (L.C.Leach) Plowes (endemic to Angola), *H. officinalis* subsp*. delaetiana* (Dinter) Bruyns, *H. officinalis* subsp. *officinalis* (N.E. Br.) Plowes, *H. parviflora* N.E.Br., *H. pedicellata* (Schinz) Plowes, *H. pilifera* (L.f.) Plowes subsp. *annulata* (N.E.Br.) Bruyns, *H. pilifera* (L.f.) Plowes subsp. *pilifera*, *H. pilifera* (L.f.) Plowes subsp. *pillansii* (N.E.Br.) Bruyns, *H. ruschi* Dinter and *H. triebneri* (Nel) Bruyns (Bruyns, 1993; Germishuizen and Meyer, 2003; Avula *et al.*, 2008).

Hoodia gordonii has had many previously unjustified classified synonyms. These include *H. albispina* N.E.Br., *H. bainii* Dyer, *H. barklyi* Dyer, *H. burkei* N.E.Br., *H. husabensis* Nel, *H. langii* Oberm. and Letty, *H. longispina* Plowes, *H. pillansii* N.E.Br., *H. rosea* Oberm. and Letty, *H. whitesloaneana* Dinter. Although there was no clear distinction between *H. gordonii* and the aforementioned synonyms, taxonomists have agreed with current taxonomic classification systems and included all synonyms into one species. To date, only a select few taxonomists have enough experience with this taxon to comment on the inclusion or separation of *H. gordonii* (Liede-Schumann and Meve, 2006). To assist future taxonomists with this genus, specimens of *H. gordonii* are stored among other plant species specimens in herbarium collections such as Royal Botanic Gardens, Kew (K), (K000306199) and University of the Free State (BLFU) and can be compared with other specimens and species (Figure 2.1 and 2.2). However, considering the diverse morphological differences in the genus, this taxon can easily be distinguished from different species within the genus based solely on its morphological characteristics (Bruyns, 2005).

Figure 2.1 *H. gordonii* (South Africa) is stored in Herbarium collections at Royal Botanic Gardens, Kew..

Figure 2.2 *H. gordonii* (Namibia) is stored in Herbarium collections at University of the Free State.

2.3 Morphology

Hoodia spp. are slow-growing, spiny stemmed, succulent flowering perennial plants which can reach up to one meter in height and have large flowers ranging in colour and size with a strong pungent carrion-like smell (Jürgens et al., 2006; Rader et al., 2007). The thorny stems are fleshy and contain a sticky clear watery sap which is released when the plant is injured. The sap has a bitter taste but despite this, the plant is consumed by the native inhabitants and insects (Mulej and Strlič, 2002), although the bitter taste serves as a deterrent to certain herbivores (Swart, 2008). The pale green stems are cylindrical, smooth and erect, growing up to 25 to 60 mm thick and are covered with projected obtuse tubercles from which thorns arise. The transverse section of the stem is angled or round, which has prominent ribs constituting tubercles which are vertically arranged into 11 to 31 mm long abundant amounts of ribs (Mulej and Strlič, 2002). Only one stem is produced during the initial growth stage, as the plant matures the plant branches out near ground level. Mature plants may produce as many as 50 or more individual branches (Barkhuizen, 1978). At the apex and upper regions of the

stems, peduncles are produced, forcing the peduncle to grow in a lateral position. The peduncle is always located between the tubercles. The green thorns which are transmuted leaves are a distinctive characteristic in the H. gordonii plant (Mulej and Strlič, 2002). Thorns are found in rows running vertically along the length of the stems and serve as a minor deterrent against herbivores (Swart, 2008).

Flowers are normally produced during early Spring and can grow as large as 110 mm in diameter (Barkhuizen, 1978; Swart, 2008). They appear in large numbers opening successively and usually form at the apex of the stem (Mulej and Strlič, 2002). The calyx lobes that overlap slightly at the broad base are smooth. The corolla varies in diameter from 8 to 170 mm, which are lobed to large, flat and saucer to shallowly cup-shaped, lobes valvate in the bud, smooth on the outside, papillate to smooth within. The corona occurs in two rows which develop from the stamina column and are smooth. The outer corona is basally copular and forms into two lobes towards the apex. The inner corona has 5 distinct dorsal and ventral flattened lobes present on the back parts of the anthers. Anthers are dorsally connected to the outer lobes. The staminal column forms near the base of the corolla tube. Anthers have two locular present on top of style head which are sub-quadrate and without apical appendage. Style head do not produce beyond anthers and they are truncate and depressed at the apex. Pollinia are approximately fusiform, thin, paired with horn like structures somewhat diverging, uniformly coloured and smooth (Mulej and Strlič, 2002).

The flowers are pollinated by various types of flies, blowflies (*Musca domestica* and *Calliphora* species), which are attracted to the colour and an unpleasant carrion-like smell (Bruyns, 1993; Meve and Liede, 1994; Vermaak *et al.*, 2011). The complicated structure of the corona and rigid hairs on the corolla limit the fly's access to the nectar producing glands, although it brushes itself against the proboscis near the slots towards the mouth of the flower (Mulej and Strlič, 2002). By doing this, the hairs or bristles of the head or legs of the fly are often stuck in the guide rails and with one exit point which is upwards towards the stamina lock (Barad, 1990; Mulej and Strlič, 2002), which is connected to the jag of the corpusculum of the pollinarium. The fly then pulls out the entire pollinarium and moves to the next flower where it attaches to the germination crest. The germination crest latches onto the guide rail where the pollinium lodges itself. The pollinium remains in the style and the pollen germinates from the germination crest. After a few days the corolla with the gynstegium dries up and falls off leaving two carpels that develop into a fruit (Barad, 1990; Mulej and Strlič, 2002).

2.4 Geographical distribution

Hoodia gordonii is found in a geographical region spanning four southern African countries (Albers and Meve, 2004), predominantly in the western parts of Southern Africa. These regions include, South Africa north-western summer rainfall regions, winter rainfall regions of south-western Namibia regions and Botswana (MET, 2002; Swart, 2008; Vermaak, 2011). The plant grows in plains and rocky areas (Albers and Meve, 2001; Pawar *et al.*, 2007). In South Africa the species is found in the Western Cape and the north and north western regions of the Northern Cape as far as Kimberley (Bruyns, 2005). The plant can survive extreme heat (> 40℃) as well as relatively low temperatures (-3℃) but is very susceptible to fros t (Olivier, 2005; Holt and Taylor, 2006).

2.5 Growth and habitat

Plants are found in a variety of habitats such as arid sandy plains, rocky slopes or barren, flat landscapes, gravel plains, steep rock strewn mountains and dunes along the coast. Wild plants and commercially grown plants have been noted to mature slowly. Plants grown commercially require a great deal of effort to create an environment similar to that of wild plants (Bruyns, 2005; Olivier, 2005; Holt and Taylor, 2006; Vermaak, 2011). As the seedling germinates it produces a primary stem and side shoots could arise from the base of the primary stem and develop further from the lower part of the stem. Some side shoots develop roots and become independent from the primary root system when it comes in contact with soil (Mulej and Strlič, 2002). Under ideal conditions plants can reach one meter at full maturity (Barkhuizen, 1978) and then the matured and older stems in the centre senesce, making way for stems to spread and form new plants (Mulej and Strlič, 2002). A fully developed plant can weigh as much as 30 kg (Barkhuizen, 1978). The plant’s habitat over the past few decades has been exploited by human development, thus restricting distribution of the plant, which is currently found in small dispersed populations (Lamprecht *et al.*, 2008).

2.6 Threats to *Hoodia gordonii*

Hoodia gordonii is considered an endangered species due to its limited habitat and very slow growth rate (Rader *et al.*, 2007). As a result, *H. gordonii* is listed as an endangered species and export out of Southern Africa is strictly controlled (CITES, 2004). Unfortunately, because of the huge demand for *H. gordonii* products in the United States, the supply of approved and controlled *H. gordonii* products cannot match this,

thereby posing a threat to the endangered plant and its communities (Avula *et al.,* 2007). Therefore, it is fundamentally important to understand both human and natural threats which have a negative impact on the species' survival in the wild (Lamprecht *et al*., 2008; Swart, 2008).

Human threats include commercial wild harvesting (illegal harvesting) and habitat destruction which involves over grazing, trampling by livestock, crop cultivation, road construction, off-road driving, urban development and mining. Although trade of *Hoodia* is illegal without permits, in terms of regulations in Southern African countries, it may also infringe on patent rights and benefit sharing agreements in the future, due to the illegal wild harvesting (Foden, 2010). Swart (2008) described various natural threats that control *Hoodia* in the wild. When natural die-back occurs it is followed by recruitment events that ensure the next generation of plants in the wild. Other threats to *Hoodia* are insects such as, the African Monarch butterfly caterpillar (*Nymphalidae, Danaus chrysippus*) which feeds on the flowers and thus impacts negatively on seed production. Caterpillars feed on the inner parts of the stems, causing the plant to fall over and die. The milkweed bug (*Spilostethus pandurus*) lays its eggs in thorn follicles, while snout beetles (*Paramecops stapeliae*), mites (*Tetranychus urticae*) and fruit flies (*Dacus bistrigulatus*) lay their eggs on stems (Swart, 2008).

In 2005, a new *Fusarium dimerum*-like fungus, later identified as *Fusarium delphinoides*, was isolated from diseased *H. gordonii* stems collected from a commercial planting in the Clanwilliam district of the Western Cape Province (Lamprecht *et al*., 2008). The symptoms on *Hoodia* stems included black and blistered lesions and dry-rotten stems. It was suspected that the inoculum source was soil and that injury to plants may be caused by sand storms, thus facilitating infection. The distribution and importance of this disease in wild populations and commercial plantings is unknown and further research needs to be done based on the popularity of this plant and its commercial value (Lamprecht *et al*., 2008; Schroers *et al*., 2009). During the period of 2004 to 2007, a wilt disease, which destroyed up to 90% of *H. gordonii* plants, was observed in experimental plantings in Kakamas and Pofadder, South Africa (pers. comm. WJ Swart). Subsequent isolation of the pathogen revealed that *F. oxysporum* was the causal pathogen (Philippou *et al*., 2013). Although *F. oxysporum* causes wilt in major crops and is a common soilborne fungus (Kistler, 1997), no prior knowledge of the effect of this fungus on *H. gordonii* had been documented.

2.7 The genus *Fusarium*

Fusarium is a filamentous fungus found in various substrates and is associated with different diseases in numerous crops (Leslie *et al.*, 1990; Pitt *et al.*, 1994). The taxonomic system for the classification of *Fusarium* species is very complex. Although more than 80 species have been identified, a problem persists in identifying many *Fusarium* species morphologically due to the various systems used by researchers globally and an absence of morphological variation in many species (Leslie and Summerell, 2006).

Fusarium has been regarded as one of the most important group of fungi, because of the diversity within the genus consisting of predominantly pathogenic, facultative parasites or saprophytes depending on their host (Fincham *et al.*, 1979; Snyder and Hansen, 1981). Diverse *Fusarium* species are found in fertile cultivated and forest soils (Burgess *et al.*, 1975; Burgess *et al.*, 1988; Jeschke *et al.*, 1990). A high degree of variation in morphological and physiological characteristics allows certain species such as *F. oxysporum* and *F. equiseti* to occupy many different habitats (Burgess *et al.*, 1989). *Fusarium* species can survive in soils as parasites or as saprophytes in plant residues and organic matter. The production of dormant spores, predominantly chlamydospores, are formed as a survival mechanism in soil, remaining viable for many years before being induced to develop during favourable environmental conditions (Gordon, 1959; Booth, 1971; Fincham *et al.*, 1979; Burgess, 1981).

The genus *Fusarium* contains many pathogenic species (Taylor *et al.*, 2000) that are widely distributed in soils (Burgess, 1981, Nelson *et al.*, 1994). These pathogenic species cause diseases of numerous economically important crops, as well as human infections, animal toxicity and mycoses (Nelson *et al.*, 1981, Liddell, 1991, Nelson *et al.*, 1994; Summerell *et al.*, 2003). In cereal crops, certain *Fusarium* species are known to produce mycotoxins that can affect the health of humans and animals (de Hoog *et al.*, 2000).

The genus is of significant importance to agriculture and forestry (Toussoun, 1981) and most cultivated plants have at least one disease associated with *Fusarium* (Leslie and Summerell, 2006). Plant diseases caused by this genus are crown rot, head blight or scab, vascular wilt, root rot and canker. The most devastating disease caused by *Fusarium* species was the infection of *F. oxysporum* f. sp. *cubense* on banana in Panama, known as Panama disease (Ploetz, 1990) affecting all economic sectors in

Panama's agricultural industry. Panama disease causes economic losses to banana plantations in Asia and Australia (Hwang and Ko, 2004). Another major disease caused by this genus is *Fusarium* head blight on wheat and barley caused by *F. graminearum*, *F. pseudograminearum*, *F. avenaceum* and *F. culmorum* in the United States (Windels, 2000). There are other *Fusarium* associated diseases that present problems to agriculture in South Africa such as *Fusarium* head blight on wheat (Boshoff, 1996) and barley, and Gibberella ear rot of maize in the Northern Cape Province, South Africa (Boutigny *et al*., 2012). *Fusarium* head blight is caused by several *Fusarium* species, which include *F. avenaceum*, *F. graminearum*, *F. crookwellense*, *F. culmorum*, *F. langsethiae*, *F. poae*, *F. sporotrichioides*, and Microdochium nivale (Fr.) Samuels and Hallett (Dill-Macky, 2010). The most common *Fusarium* species found in soil in the Western Cape of South Africa were *F. chlamydosporum*, *F. solani* and *F. oxysporum* (Bushula, 2008). In the North Western Province of South Africa *Fusarium* wilt of cultivated *Protea* spp. was first reported by Swart *et al.* (1999) on six cultivars (*P. aristata* x *repens* cv. Venus, *P. cornpacta* x *susannae* cv. Pink Ice, *P. cynaroides*, *P. eximia* x *susannae* cv. Cardinal, *P. eximia* x *susannae* cv. Sylvia, *P. magnijka* x *susannae* cv. Susara and *P. repens* cv. Sneyd).

2.7.1 Taxonomic history of *Fusarium*

In 1809, Link began a taxonomic study on *Fusarium* (Link, 1809; Snyder and Toussoun, 1965) which has since been fundamentally debated. This is largely due to variation in the morphology of isolates and the lack of a universal species concept used on species within the genus (Szécsi and Dobrovolszky, 1985; Leslie and Summerell, 2006). Some taxonomists emphasise subtle differences and consequently recognise more species whereas others emphasise similarities and therefore recognise less species (Leslie and Summerell, 2006).

Early workers such by, Wollenweber and Reinking (1935) classified approximately 1000 isolates into 16 sections, 65 species, 55 varieties and 22 forms based on the structure of sporodochia on plant tissue (Burgess *et al*., 1994). A publication of *Die Fusarien* in 1935, was a fundamental stepping stone which established the then *Fusarium* taxonomic status. Thereafter, the establishment of a standard reference work (Nelson *et al*., 1983) helped to support this *Fusarium* taxonomic system. In the development of a taxonomic system for *Fusarium*, many researchers based their studies specifically on the morphological characteristics and created various classification methods. The splitter group consisted of Wollenweber and Reinking (1935), Raillo (1950), Bilai (1955),

Gerlach and Nirenberg (1982), and Joffe (1986). Recommendations by the group separated the genus *Fusarium* into species, varieties, and forms. Gerlach and Nirenberg (1982) later introduced 78 species into the genus. However, their system was difficult and complex since species were identified based on differences only and excluded similarities between each strain which lead to many new species or varieties (Nelson *et al.*, 1983). Thereafter, Raillo (1950) and Bilai (1955) proposed their systems, which were not understood well. For example, they combined section *Liseola* with section *Elegans* and then combined section *Gibbosum* with *Discolor*.

Snyder and Hansen (1940) began their studies of *Fusarium* taxonomy in 1930's and presented their results a decade later. Snyder and Hansen (1940; 1941; 1945) classified and placed all the species from Wollenweber and Reinking (1935) into nine species. They combined sections Arthrosporiella, Discolor, Gibbosum, and Roseum into *F. roseum*. This was based primarily on the morphology of the macroconidia and cultural variations. Snyder and Hansen (1945) contributed significantly with the use of single spore cultures and excluded cultures deemed as degenerate variants from taxonomic consideration (Nelson, 1991, Burgess *et al.*, 1994, Leslie and Summerell, 2006). However, this system of placing the sections in this manner was confusing and not accepted by all *Fusarium* taxonomists. Nevertheless, Snyder and Hansen (1940) are highly respected for their work on analysing the species through single conidium cultures. Their research on the variation of *F. solani* and *F. oxysporum,* in particular, is greatly acknowledged among taxonomists. Messiaen and Cassini (1968) as well as Matuo (1972) are also considered part of the lumpers group. Nelson (1991) however, stated that neither group (splitters and lumpers) produced an ideal and practical identification system for *Fusarium* species since Wollenweber's system was too complex and Snyder and Hansen's system was too simple.

An intermediate group of taxonomists, the 'moderates', (Gordon and co-workers, 1944; 1952; 1954; 1956; 1959; 1960), classified *Fusarium* based on conidiogenous cells, particularly those producing macroconidia, as a primary taxonomic characteristic (Booth, 1971, Leslie and Summerell, 2006). This system reduced the number of species suggested by the splitters, but was less extreme than that of the lumpers. Nelson (1991) stated that no general agreement as to which classification system was most suitable for *Fusarium* had been approved. However, the development of a practical classification method, which uses data from historical classification systems in addition to that collected from current research, has still not materialised (Summerell *et al.* 2003; Leslie and Summerell, 2006).

Gordon's taxonomic system (Gordon, 1944) is closely related to Wollenweber and Reinking (1935) and pays consideration to Snyder and Hansen's system (Snyder and Hansen, 1945) as well. Booth's (1971) taxonomic system then modified Gordon's system (Gordon, 1944) by amending the perfect stage description and promoting the use of conidiophores and conidiogenous cells as morphological characters. Booth (1971) added new criteria which separated the species in different sections based on the presence of monophialides and polyphialides. Thereafter, Nelson *et al.* (1983) combined all the above mentioned systems with their findings to create an ideal and practical approach to the identification and classification of *Fusarium*. Due to Nelson *et al.* (1983) the system reduced the number of species and combined the various varieties and forms into suitable species (Snyder and Toussoun, 1965; Nelson 1991; Nelson *et al.*, 1983; Burgess *et al.*, 1994; Nelson *et al.*, 1994; Summerell *et al.*, 2003; Leslie and Summerell 2006).

This simple concept by Nelson *et al.* (1983) and Burgess *et al.* (1994) was accepted by many researchers. The *Fusarium* laboratory manual published by Leslie and Summerell (2006) combined the entire taxonomical system with the most recent procedures and methods for species identification. Furthermore, Leslie and Summerell (2006) incorporate the morphological, biological, and phylogenetic species concepts. The problems associated with *Fusarium* taxonomical system complexities is due to the inability to make a direct link with anamorph-teleomorph, section relationships, species delimitation, mutational variants, and subgroup identification (Windels, 1991). In addition, many scientists working with *Fusarium* species have created difficulties in a global conformity of methodical *Fusarium* taxonomy (Liddell, 1991; Summerell *et al.*, 2003).

In the past the genus *Fusarium* was comprised of species, it is generally accepted to group them into sections based on their morphology and physiological similarities (Wollenweber and Reinking, 1935; Nelson *et al.*, 1983). However, in the last decade publications describing new species (Nirenberg, 1995; Aoki and O'Donnell, 1998; Aoki and O'Donnell, 1999; Skovgaard *et al.*, 2003; Torp and Nirenberg, 2004) and using molecular methods (Waalwijk *et al.*, 1996; Benyon *et al.*, 2000), have highlighted a need for a revision of the current classification. To complicate matters even more, within individual species they have been grouped into *formae specialis* based on the plant host they infect (Summerell *et al.*, 2003). One of many examples of this is *F. oxysporum* species complex belonging to section *Elegans* e.g. *F. oxysporum* f. sp. *vasinfectum* (Kim *et al.*, 2005), some strains of pathogenic *F. oxysporum* are included in Table 2.1.

Table 2.1 *Fusarium oxysporum formae speciales* strains on various crops

Formae specials	**Host**	**Disease name**	**References**
F. oxysporum f. sp. *anethi*	Dill	Vascular wilt	Gordon (1965)
F. oxysporum f. sp. *apii*	Celery	Celery wilt, Yellows	(Nelson and Sherbakoff) Snyder and Hansen (1940)
F. oxysporum f. sp. *asparagi*	Asparagus	*Fusarium* yellows	Cohen (1946)
F. oxysporum f. sp. *batatas*	Sweet potato	Stem rot	(Wollenweber) Snyder and Hansen (1940)
F. oxysporum f. sp. *betae*	Cabbage	Cabbage wilt	(Stewart) Snyder and Hansen (1940)
F. oxysporum f. sp. *callistephi*	China aster	*Fusarium* wilt	(Beach) Snyder and Hansen (1940)
F. oxysporum f. sp. *cassiae*	Cassia	*Fusarium* wilt	Gordon (1965)
F. oxysporum f. sp. *cepae*	Onion	Damping-off (basal rot)	(Hansen) Snyder and Hansen (1940)
F. oxysporum f. sp. *chrysanthemi*	Chrysanthemum	Vascular wilt	Armstrong *et al.* (1970)
F. oxysporum f. sp. *conglutinans*	Cabbage	*Fusarium* wilt	(Wollenweber) Snyder and Hansen (1940)
F. oxysporum f. sp. *cubense*	Banana	Panama disease	(Smith) Snyder and Hansen (1940)
F. oxysporum f. sp. *dianthi*	Carnation	*Fusarium* wilt	(Prill. & Delacr.) Snyder and Hansen (1940)
F. oxysporum f. sp. *gladioli*	Gladiolus	Vascular wilt	(Massey) Snyder and Hansen (1940)
F. oxysporum f. sp. *lilii*	Lilly	*Basal rot*	Imle (1942)
F. oxysporum f. sp. *mathioli*	Alfalfa	*Fusarium* wilt	Baker (1948)
F. oxysporum f. sp. *melongenae*	Eggplant	*Fusarium* wilt	Matuo and Ishigami (1958)
F. oxysporum f. sp. *narcissi*	Daffodil	Basal rot	(Cooke & Massee) Snyder and Hansen (1940)
F. oxysporum f. sp. *nicotianae*	Tobacco	*Fusarium* wilt	(Johnson) Snyder and Hansen (1940)
F. oxysporum f. sp. *niveum*	Watermelon	*Fusarium* wilt	(Smith) Snyder and Hansen (1940)
F. oxysporum f. sp. *phaseoli*	Bean	*Fusarium* yellows	Kendrick and Snyder, 1942
F. oxysporum f. sp. *pisi*	Pea	*Fusarium* wilt	(Hall) Snyder and Hansen (1940)
F. oxysporum f. sp. *radicis-cucumerinum*	Cucumber	*Fusarium* wilt	Vakalounakis (1996)
F. oxysporum f. sp. radicis-lycopersici	Tomato	Crown and Root rot	Jarvis and Shoemaker (1978)
F. oxysporum f. sp. *tracheiphilum*	Cowpea	*Fusarium* wilt	(Smith) Snyder and Hansen (1940)
F. oxysporum f. sp. *tuberosi*	Potato	Vascular wilt	Snyder and Hansen (1940)
F. oxysporum f. sp. *tulipae*	Tulip	Vascular wilt	Snyder and Hansen (1940)
F. oxysporum f. sp. udum	Pigeon pea	Pigeon pea wilt	(Butler) Snyder and Hansen (1940)
F. oxysporum f. sp. vanillae	Vanilla	*Fusarium* wilt	(Tucker) Gordon 1965
F. oxysporum f. sp. *vasinfectum*	Cotton	*Fusarium* yellows	(Atk.) Snyder and Hansen (1940)

2.8 Fusarium oxysporum as a causal agent of Fusarium wilt

The F. oxysporum is an active saprophyte found abundantly in soil and organic matter, with some formae specialis that are pathogenic (Smith et al., 1988). Its saprophytic ability allows it to survive in the soil between crop rotations in infected plant debris. The fungus can survive either as mycelium or any of its three different spore types (macroconidia, microconidia and clamydospores). F. oxysporum infects healthy plants if surrounding soil is contaminated with inoculum of the pathogen. The fungus invades the plant's roots either with its sporangial germ tube or by means of mycelial fragments. Direct infection may occur through the root tips, wounds in the roots or at the formation point of lateral roots. Once inside the plant, the mycelium grows through the root cortex intercellulary and as soon as it reaches the xylem, it invades the adjacent vessels through the pits. The mycelium remains in the vessels where it usually moves toward the stem and crown of the plant eventually developing microconidia, which are carried upward within the the sap flow.The microconidia germinate and the mycelium can penetrate the upper wall of the xylem vessel allowing more microconidia to be produced in the next vessel. As the pathogen develops further, it restricts the plant's vascular tissue and prevents water flow which permits the stomata to close thereby causing the plant to wilt and eventually die. Once the plant is dead the fungus invades the plant's parenchymatous tissue until it reaches the surface of the dead tissue where it sporulates abundantly. The resulting spores generate new inoculum for further spread of the fungus (Agrios, 1988; Leslie and Summerell, 2006).

2.8.1 Disease spread

The primary spread of *F. oxysporum* over short distances is facilitated by irrigated water and contaminated farm equipment (Agrios, 1988). The fungus can be dispersed by many different means: spores dispersed by wind and spread over long distances, by infected transplants in soil, seeds, or infected planting material (Garibaldi *et al.*, 2004). Although the fungus may infect and contaminate seed, dispersal by means of seed is extremely rare (Agrios, 1988).

2.8.2 Wilt symptoms on various crops

F. oxysporum consists of many different formae specialis that are plant pathogenic strains often causing vascular wilt diseases (Beckman, 1987; Nelson *et al.*, 1981; Summerell and Rugg, 1992), damping-off (Nelson *et al.*, 1992) and crown and root rot

(Jarvis and Shoemaker, 1978). In plants infected with the vascular wilt pathogen, xylem vessels become clogged due to gels composed of neutral sugars commonly found in the host plant's cell wall (van der Molen, 1986; Opperman and Wehner, 1994). The most important species of *Fusarium* causing vascular wilt is *F. oxysporum* although some specialised strains of *F. oxysporum* may also induce yellows, root necrosis, rot and damping-off, instead of the more severe vascular wilt (Agrios, 1988; Smith *et al.*, 1988).

Vascular wilt on infected crops caused by *F. oxysporum* initially appear as small vein clearing on the outer portion of the younger leaves, followed by a downward drooping of the older leaves (epinasty). During the seedling stage the *F. oxysporum* infected plants may wilt and die shortly after symptoms appeared. In mature plants vein clearing and leaf epinasty are often followed by stunting, lower leaves begin to turn yellow, formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant (Agrios, 1988). Vascular tissue becomes clogged and turns colour from clear to brown which is strong evidence of *Fusarium* wilt. In fully matured plants the symptoms usually become more apparent during blossoming and fruit maturation (Jones *et al.*, 1982; Smith *et al.*, 1988).

2.8.3 Disease control and management

Fusarium oxysporum and its many *formae speciales* and races affect numerous plant hosts (Ploetz and Correll, 1988), involving many different methods in controlling and managing due to varying pathosystems involved (Jones *et al.*, 1982, Agrios, 1988, Smith *et al.*, 1988). Although numerous disease management and control programmes exist, they are often not adequate enough in controlling *Fusarium* wilt diseases (Borrero *et al.*, 2006, Nel *et al.*, 2007). An efficient method used in controlling and managing *F. oxysporum* includes disinfestation of the soil and planting material with fungicidal chemicals, crop rotation with non-hosts of the fungus (Jones *et al.*, 1982; Agrios, 1988; Smith *et al.*, 1988). Economically important plant pathogenic strains can be recovered from non-host and usually native plants (Helbig and Carroll, 1984; Wang *et al.*, 2004). Identifying how plants protect themselves at molecular level is an important step towards producing resistant plants where resistance is not readily available in closely related species and wild varieties.

2.9 *Fusarium* oxysporum species complex

Fusarium oxysporum Schlechtendal (emend. Snyder and Hansen), is a species complex which has been subject to considerable debate. This is due to the application of different taxonomic systems. Wollenweber (1913) originally placed *F. oxysporum* together with six other *Fusarium* species in the Section *Elegans*. Snyder and Hansen (1940) later accepted the six taxa as indistinguishable from *F. oxysporum*. Currently, *F. oxysporum* has a diversity of morphologically similar fungi with several phylogenetic origins made up of at least three known clades (O'Donnell *et al.*, 1998b, Baayen *et al.*, 2000a, Bogale *et al.*, 2007).

Problems associated with the taxonomy of *F. oxysporum* have forced researchers to search for alternative techniques that are fast and reliable in identifying and classifying the species. Researchers use these alternative (molecular) techniques either to complement or to substitute those based solely on pathogenicity.

2.10 Morphological characterisation of *Fusarium* oxysporum

A major obstacle to the taxonomic study of *Fusarium* has been the confusing and incorrect application of species names to toxigenic and pathogenic isolates, due to the fundamental limitation of morphological species identification and its application (Geiser *et al.*, 2004).

2.10.1 Morphology

Three types of asexual spores (microconidia, macroconidia, and chlamydospores) are produced by *F. oxysporum*. The most abundant and frequently produced spore by the fungus under all conditions, and even within infected plant vessels, is the microconidium. Microconidia are one or two celled and macroconidia are three to five celled which are slightly pointed and curved toward the ends. Macroconidia are commonly found on dead plant material and in sporodochia. Chlamydospores are round and thick-walled spores which are produced either terminally or intercalary on older mycelium or in macroconidia. Chlamydospores are either one or two celled (Leslie and Summerell, 2006). Morphological characteristics that are particularly important in identifying *F. oxysporum* species are the ability to produce microconidia, chlamydospores and the shape of the macroconidia and the microconidia. The hyphae branch-off producing short monophialedes on which false heads, which later bear microconidia, develop. Single

gene mutations can drastically transform the number and morphology of microconidia and macroconidia (Ohara and Tsuge, 2004; Ohara *et al.*, 2004).

Morphologically, *F. oxysporum* may be confused with *F. solani* as it also produces microconidia in false heads on elongated monophialides formed on the hyphae (Booth, 1971; Nelson *et al.*, 1981). *F. oxysporum* may also be confused with *F. subglutinans* which also produces microconidia from polyphialides and does not produce chlamydospores. In some isolates of *F. oxysporum* chlamydospores are formed slowly and the presence of chlamydospores and the short microcondia producing monophialides in *F. oxysporum* differentiates it from *F. solani* and *F. subglutinans*, respectively (Booth, 1971; Nelson *et al.*, 1981). Mutations occur frequently with *F. oxysporum*, where they produce either more mycelium, or pionnotal, where aerial mycelium is suppressed (Waite and Stover, 1960). A researcher with little experience would find it very difficult to identify these isolates (Bogale *et al.*, 2006). On potato dextrose agar (PDA) the different *formae specialis* of *F. oxysporum* may have varying appearances. Generally, the aerial mycelium initially appears white and then changes in colour ranging from violet to dark purple depending on the strain of *formae specialis*. The colony may appear cream or orange in colour if sporodochia are abundant (Smith *et al.*, 1988).

2.10.2 Heterokaryosis and the parasexual cycles

Heterokaryosis was first termed in 1912 to describe the relationship of nuclei of opposite mating in the same hyphae in *Phycomyces nitens* (C. Agardh) Kunze (Parmeter *et al.*, 1963). The term hetrokaryosis was used for all fungal systems where different nuclei have common vegetative hyphae. The formation of heterokaryons among unlike fungal individuals is an essential part of many fungal life cycles and may possibly serve as the initial step in the parasexual cycle and the transmission of hypovirulent factors such as dsRNA (Leslie, 1993). Puhalla (1984) observed anastomosis in *F. oxysporum* f. sp. *apii* by pairing colour mutants. Puhalla (1984) identified no nuclear migration within the heterokaryon and observed that hyphal tips, 2-3 mm ahead of the anastomosed cells, were homokaryotic. Puhalla and Spieth (1983) reported genetic complementation in *F. verticillioides* (Saccardo) Nirenberg which was limited to the anastomosed cells and products produced were translocated to the homokaryotic regions of the mycelium. This occurrence of heterokaryon formation may be a molecular tool for releasing genetic diversity found in the heterokaryon. The formation of heterokaryons has important benefits of mitotic genetic exchange (parasexual cycle) or may increase growth in

biomass for mutual physiological support for nutritional resource utilisation or asexual/sexual reproduction (Leslie, 1993; Di Primo *et al.*, 2001).

Pontecorvo *et al.* (1953) first used the term 'parasexual cycle' to describe the genetic process in *Aspergillus nidulans* (Eidam) G. Winter, which involved heterokaryon formation, fusion of two different haploid nuclei to produce a diploid heterozygous nucleus, and mitotic recombination. Genetically unlike nuclei are combined within a cytoplasm where nuclear fusion may occur. When genotypically different haploid nuclei pair together a heterozygous diploid nucleus is produced. During the duplication of such nuclei, mitotic crossing over occurs to produce spores which have diploid nuclei homozygous for certain genetic markers, or haploidisation occurs and spores produced are haploid nuclei containing new combinations of genetic markers, or both types of the above mentioned occurs (Tuveson and Garbere, 1961; Leslie, 1993).

Parasexual recombination has been identified in many ascomycetes, basidiomycetes and deuteromycetes, indicating that the parasexual process is common in fungi (Leslie and Klein, 1996; Glass *et al.*, 2000). Leslie (1993) proposed using the parasexual cycle in *F. oxysporum* f. sp. *pisi* to determine underlying reasons for pathogenicity. Heterokaryosis in imperfect fungi may be a particularly important mechanism for parasexual recombination. One hypothesis is that parasexual recombination explains the diversity found in many fungi and particularly the origin of new races of plant pathogenic fungi (Leslie and Klein, 1996). Hypothetically, parasexual recombination between different races of a pathogen may possibly give rise to new combinations of virulence and avirulence genes (Pontecorvo *et al.*, 1953; Gomez-Gomez *et al.*, 2002).

2.10.3 Vegetative compatibility groups

Sexual and vegetative heterokaryons in most fungi are quite distinct from one another (Leslie 1993). Strains that are able to form a sexual heterokaryon may not be able to form a vegetative heterokaryon and *vice versa* (Di Primo *et al.*, 2001). Strains that are able to form vegetative heterokaryons are termed vegetatively compatible (Correll *et al.*, 1986a; 1986b; 1987). Vegetative compatibility is thus the ability of two fungal isolates to fuse by means of hyphae at the contact zone when grown in close proximity. Vegetative compatibility groups (VCGs) are defined as groups of isolates that share identical alleles at corresponding loci. Therefore, *F. oxysporum* which is an asexual organism uses vegetative compatibility as an important method of creating genetic diversity and vegetative compatibility analysis is an important tool in identifying genetic relatedness of

isolates (Glass *et al.*, 2000). Strains that are vegetatively compatible with one another are normally described as members of the same VCG (Leslie, 1993).

Sexual compatibility is generally controlled by one or more mating type loci that may have two or more alleles. Vegetative compatibility is controlled by various vegetative incompatibility loci (*vic* loci) (Leslie, 1993). Generally, vegetative compatibility is homogenic, where two fungal isolates are vegetative compatible they share identical alleles at each of their corresponding loci. Therefore, vegetative compatible strains are normally more genetically similar than incompatible strains (Puhalla 1985). Correll *et al.* (1986b) observed that characteristics of fungal isolates, which were vegetatively compatible, shared certain traits such as, virulence, colony size, isozyme profiles and antibiotic production.

The use of VCG analysis has several advantages. Firstly, it is a useful method to determine genetic diversity and can be used in population studies (Elias and Schneider, 1992, Smith-White *et al.*, 2001). Since 1985, there has been an increase in the number of *formae speciales* reported that have been characterised using the VCG method, following the approach and method described by Puhalla (1985) and Leslie (1993). Secondly, it is cheaper and relatively easy to perform on a large number of isolates compared to genetic analyses (Morita *et al.*, 2005), such as DNA fingerprinting (Fernandez *et al.*, 1994; Kawabe *et al.*, 2005), random amplified polymorphic DNA (RAPD) (Kalc-Wright *et al.*, 1996; Kerényi *et al.*, 1997) and amplified fragment length polymorphisms (AFLP) (Minnaar-Ontong *et al.*, 2008). VCG analysis can be used in the constructing genetic maps and in the future will be used as a novel target for antifungal agents (Leslie and Klein, 1996).

In addition, spontaneous vegetative compatibility allows for the characterisation of non-pathogenic sections within a population. *Fusarium oxysporum* is found in abundance in both nature and cultivated soils. The presence of non-pathogenic *F. oxysporum* isolates has the potential for natural biological control (Fravel *et al.*, 2003). A sound understanding of non-pathogenic *F. oxysporum* isolates and their potential for biological control is to be encouraged. A proposal by leading researchers in the field of VCGs strongly noted the importance of using nitrate non-utilising (*nit*) mutant isolates generated from VCGs for the identification of non-pathogenic *F. oxysporum* strains in the development of biological control agents in the future (Correll *et al.*, 1987; Liu *et al.*, 1995).

Vegetative compatibility analysis has been used in the characterisation of populations of *F. oxysporum* (Elias and Schneider, 1991; Appel and Gordon, 1994). Fungal strains able to form heterokaryons through anastomosis are assigned to the same VCG. Without sexual recombination the members of each VCG would be genetically isolated subpopulations which would be subjected to population genetic evolution, such as selection, mutation, migration and drift. Furthermore, members from the same VCG have the ability to exchange genetic material through parasexual recombination (Pasquali *et al*., 2005). Puhalla (1985) first attempted by using VCGs to differentiate and classify strains of *F. oxysporum* genetically rather than morphologically and/or host range. Puhalla used a technique previously applied to *Aspergillus* to created auxotrophic mutants unable to use nitrate as a sole nitrogen source. The *nit* mutants recovered were affected at different steps in the pathway involved in nitrogen reduction. *Nit* mutants were then paired on minimal medium. If isolates paired to produce a heterokaryon they fused at the contact zone and the complementation resulted in wild type growth. Such isolates were determined to be in the same VCG. Puhalla (1985) proposed a four or five digit numerical system for VCG designation which is currently used presently. The use of a numerical system for VCGs in *F. oxysporum* was supported by Kistler *et al*. (1998), Katan (1999) and Di Primo *et al*. (2001). A total of 38 *formae speciales* of *F. oxysporum* have been subjected to VCG analysis Katan (1999) and Di Primo *et al*. (2001).

2.10.3.1 Analysis of *nit* mutants

Nitrate as a nitrogen source, when reduced to ammonium via nitrate reductase and nitrite reductase, can be utilised by most fungi (Fig. 2.3). Although a few fungi are not capable of utilising nitrate, such as the higher Basidiomycetes, Saprolegniaceae and Blastocladiales, this is presumably due to their inability to synthesise nitrate reductase. Chlorate, a substitute substrate of nitrate, has aided enormously in the study of nitrate assimilation in fungi as well as bacteria, algae and plants. The reduction of chlorate to chlorite by nitrate reductase may result in chlorate toxicity in these organisms. Normally, chlorate sensitive strains are able to reduce nitrate to nitrite, although chlorate resistant strains are not (Correll *et al*., 1987). *F. oxysporum* is able to produce *nit* mutants without mutagen treatment and VCG analysis can therefore be used to characterise these *nit* mutants to determine heterokaryon formation and vegetative compatibility.

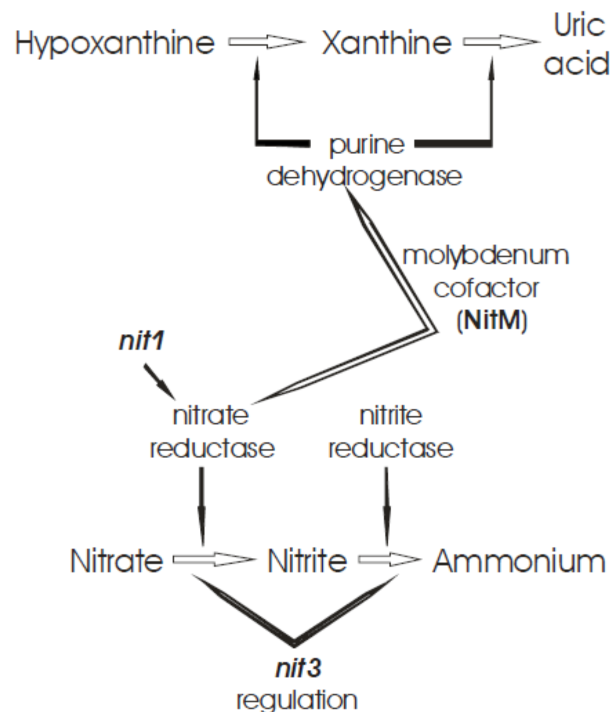

Figure 2.3 NO_3 metabolism pathway as it relates to the generation and classification of *nit* mutants for VCG testing (as depicted in The *Fusarium* Laboratory Manual, Leslie and Summerell, 2006).

Nit mutants from a number of fungi have been produced, including *F. oxysporum, Neurospora crassa* Shear and B.O. Dodge; *Ustilago maydis* (DC.) Corda; *A. nidulans* G Winter; *A. flavus* Johann Heinrich Friedrich Link; *F. verticillioides* and *F. solani* (Martius) Appel and Wollenweber emend. Snyder and Hansen. All the *nit* mutants recovered from *F. oxysporum* were divided into three phenotypic categories. These categories are characterised by a mutation on the nitrate reductase structural locus (*nit*1), or a nitrate assimilation pathway specific regulatory locus (*nit*3) or loci that influences the assembly of a molybdenum containing cofactor necessary for nitrate reductase activity (NitM) (Correll *et al.*, 1987).

To date, no sexual stage is known for *F. oxysporum*, thus the only method to transfer genetic material would be by means of parasexuality. Since parasexuality in nature relies on heterokaryosis, it could only occur between members of the same VCG. Therefore, each VCG generated is a genetically isolated population. VCGs have shown that it is possibile to correlate its presence with *formae speciales* or different races of a pathogen. VCG analysis easy to perform experimentally and would avoid the time consuming and unreliable pathogenicity tests (Correll *et al.*, 1986b; Correll, 1991; Fernandez *et al.*, 1994; Kondo *et al.*, 1997; Elena and Pappas, 2002).

Alternatively it is advantageous if vegetative incompatibile phenotypes that are genetic markers and occur naturally could be used to differentiate strains of *F. oxysporum*. To determine if two isolates are vegetatively compatible, they must be tested with complementary mutants so that heterokaryotic growth can be detected on a selective medium.

2.10.4 Pathogenicity used to identify *Fusarium oxysporum*

There are many variants of *F. oxysporum* with respect to host and plant specifications (Snyder and Hansen, 1940). Most isolates of *F. oxysporum* are non-pathogenic soil inhabitants, thus a need to distinguish these morphological indistinguishable species arose by determining which isolates caused disease with various types of plants (Kistler, 1997). Although *F. oxysporum* causes wilt in many different crops, pathogenic strains within the species have limited host ranges. *F. oxysporum* was subdivided into *formae speciales* based on virulence of pathogenic strains ability to cause disease in a specific host or group of hosts (Snyder and Hansen, 1940). Due to specificity, these *formae speciales* are based on a strain's ability to infect a host plant, or a variety of different host plants and cause disease (Nelson *et al.*, 1981; Nelson, 1991). Some *formae speciales* are further subdivided into races based on pathogenicity to a set of differential cultivars within the same plant species. There are over 120 described *formae specialis* and races of *F. oxysporum*, causing vascular wilt diseases of many agricultural crops (Armstrong and Armstrong, 1981; Correll, 1991).

Although *F. oxysporum* distribution is worldwide, different *formae speciales* of *F. oxysporum* often have varying degrees of distribution depending on where the host plant is grown (Raabe *et al.*, 1981). For example, *F. oxysporum* f. sp. *cubense,* which causes Panama disease on bananas, only occurs where its host is grown (Ploetz, 2000). Although differentiating strains of *F. oxysporum* using virulence as characteristic has been extremely useful, there are problems since the characterisation of some strains is based solely on their pathogencity (Correll, 1991). Based on differences among *formae speciales* and races in terms of their ability to infect specific crop species and to differentiate between them is of great economic and scientific importance. However, identification based solely on pathogenicity is costly and labour intensive (Ploetz and Correll, 1988; Bogale *et al.*, 2006). Furthermore, results of pathogenicity tests are often questionable because they are affected by a variety of factors, such as host genotype (Armstrong and Armstrong, 1981), host growth conditions (Correll, 1991), growth stage of host (Hart and Endo 1981), and method of infection (Kraft and Haglund, 1978).

Taxonomy based solely on virulence excludes the identification of non-pathogenic strains, which represent a major component of the species complex (Correll, 1991; Bogale *et al.*, 2006).

2.11 Molecular identification of *Fusarium* species

Fusarium identification based on morphological studies requires substantial knowledge and is not always accurate. However, molecular techniques can assist with the identification of fungi within disease complexes. Presently, the use of DNA polymorphisms as a trait has aided in the recognition and classification systems of fungi that are genetically different (Leslie and Summerell 2006). DNA based methods for taxonomy using fingerprinting techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), AFLP (Baayen *et al.*, 2000a; Jurgenson *et al.*, 2002; Zeller *et al.*, 2003a; Schmidt *et al.*, 2004; Bentley *et al.*, 2008; Bogale *et al.*, 2009), sequence analyses using RNA and DNA gene sequences examining variations in specific protein coding gene regions. Molecular methods overcome all the confinements related with the use of morphological identification of sub-species taxa, vegetative compatibility and pathogenicity. If correlated to pathogenicity, they could also be useful in identifying virulence groups (Woo *et al.*, 1996). DNA based techniques are also important for use in phylogenetic studies. For the purpose of this review, the DNA based techniques are broadly grouped into fingerprint techniques: RAPD, RFLP, AFLP and DNA sequence analyses. A description of these genetic methods and their use in the taxonomy of *F. oxysporum* isolates are listed below.

2.11.1 Fingerprinting techniques

Most fungal taxonomic studies conducted today use DNA based genetic markers (McDonald, 1997). Various DNA based genotyping techniques have been widely used to analyse genetic differences for cultivar and isolate genotyping such as RFLP, RAPD, AFLP and simple sequence repeat (SSR). Useful information is provided by using fingerprinting techniques spanning the entire genome of an organism, while DNA sequencing targets a specific single gene region. DNA fingerprinting can be used to identify clonal lineages within a population (McDonald and McDermott, 1993).

2.11.2 Restriction fragment length polymorphisms

RFLPs were first described by Jeffreys *et al.* (1985). As a molecular technique RFLPs produce restriction enzyme fragments of different length for evaluating variations among and within species. RFLPs detect differences in both coding and non-coding regions and rely solely on DNA to DNA hybridization within the genome (McDonald and McDermott, 1993). RFLPs are often used in creating genetic maps of chromosomes and have the ability to identify specific locations within the genome using selective co-dominant markers (Kohn, 1992). Although this technique is used in pathogenic fungi studies, RFLPs are primarily limited to mitochondrial DNA, however higher polymorphisms are observed when using nuclear DNA which gives higher resolution and information in these studies (McDonald, 1997).

The use of this technique has been widely used for identification and characterisation of *Fusarium* species (Edel *et al.*, 1995; Bentley *et al.*, 1998; Bogale *et al.*, 2007; Leong *et al.*, 2009). RFLPs are helpful in evaluating intraspecific relationships in *F. oxysporum*, particularly among pathogenic and non-pathogenic races and estimating the phylogenetic relationships between *Fusarium* groups (Appel and Gordon, 1995; Konstantinova and Yli-Mattila, 2004). However, no reports using PCR based RFLP groups correspond with VCGs or pathogenicity groups. However, various studies using RFLP have shown some degree of correlation between VCGs and RFLPs. For example, Rosewich *et al.* (2003) showed IGS haplotypes and VCGs corelated among 57 *F. oxysporum* f. sp. *lycopersici* isolates represented from five VCGs. Restriction analysis of the IGS region using *Eco*RI, *Rsa*I, and *Hae*III resulted in four haplotypes which fully corresponding to four VCGs. However, VCGs 0032 and 0030 shared a single haplotype. Appel and Gordon (1995) had similar results with a close correspondence in *F. oxysporum* f. sp. *melonis*. Although there was correlation with VCGs and RFLPs, this variation was not correlated with VCGs, pathogenicity or geographical groups (Majer *et al.*, 1996).

Although this technique is reproducible, RFLPs are limited due to low levels of polymorphism (McDonald and McDermott, 1993; McDonald, 1997). The technique requires large amounts of pure DNA, it is time consuming and expensive for differentiating large numbers of samples. RFLPs technique poses a problem as they do not correspond to VCGs or pathogenicity tests and the taxonomy of *F. oxysporum* due to its inability to recognize alleles (Majer *et al.*, 1996). RFLPs have several disadvantages in addition to the lack of correspondence with VCGs, pathogenicity or geographical

groups. Firstly, the number and types of RFLPs groups formed are dependent on the number and type of restriction enzyme and/or restriction enzyme probe combination used. Secondly, a single nucleotide substitution that results in a loss or gain of a restriction site, therefore different fragment sizes between strains, could be scored more than once resulting in an overestimation of variation during phenetic analysis. Thirdly, it is difficult to detect and separate mitochondrial plasmids that may be co-isolated with mDNAs of some strains. Lastly, RFLPs depends on the size and variability of the DNA segment amplified. For example, conserved sequences found in the large and small subunit genes of the rDNA are more useful in the taxonomy of distantly related fungi (Bowman *et al.*, 1992; Bruns *et al.*, 1992), whereas spacer regions of the rDNA, which evolve more rapidly, are more appropriate in the study of intra-specific relationships (Baura *et al.*, 1992; Lee and Taylor, 1992; O'Donnell, 1992). These limitations therefore limit the use of the method in studying the taxonomy of *F. oxysporum*.

2.11.3 Random amplified polymorphic DNA

Due to limitations of RFLPs, the development of RAPDs was then used for identification and characterisation of clones and/or clonal lineages of fungal species, and numerous studies were done on *Fusarium* spp. RAPD (Williams *et al.*, 1990) or the Arbitrarily Primed PCR (AP-PCR; Welsh and McClelland, 1990) and its variation, use single short primers of random sequence to generate DNA fragments that were developed to assist in constructing genetic maps and for evaluating genetic diversity (Cateno-Anollés *et al.*, 1991). The use of RAPDs as genetic markers for the use in characterising genetic variability has increased in popularity due to its speed, low cost, low DNA concentration needed for screening purposes, lack of radioactivity and there is no need for prior sequence information about the target DNA. DNA can be extracted from any organism using the RAPDs technique (Welsh and McClelland, 1990; Williams *et al.*, 1990). It is also applicable to large numbers of strains and allows for analysis of variation at more than one locus (Bentley *et al.*, 1995). Thus the RAPD technique is a useful molecular tool for the characterisation of *F. oxysporum* strains.

Research has demonstrated a correlation between pathogenicity and/or VCGs using RAPDs. Wang *et al.* (2001) tested 24 strains from 13 *formae speciales* using seven primers chosen from an initial 132 primers. Results showed RAPDs were specific to each *forma specialis* and that probes developed from *forma specialis*-specific RAPD bands showed different specificity to the 13 *formae speciales* after Southern hybridisation (Southern, 1975). The results thus showed that the markers based on various

fingerprints are potentially useful for the identification of *formae speciales* without the need for pathogenicity tests. Similar studies revealed that RAPDs corresponded with VCGs (Bentley *et al.*, 1995; Nelson *et al.*, 1983), *formae speciales* (Vakalounakis and Fragkiadakis, 1999; Pasquali *et al.*, 2003), and/or races (Manulis *et al.*, 1994). However, only one to three representative strains from each *formae speciales* were used in this study.

RAPDs are unable to differentiate strains based on pathogenicity or geographical origin. This was demonstrated in a study by Cramer *et al.* (2003) using 34 strains including *F. oxysporum* f. sp. *phaseoli*, *F. oxysporum* f. sp. *betae*, non-pathogenic *F. oxysporum* and an isolate of *F. solani*. From the 12 primers selected, UPGMA analysis showed that only *F. oxysporum* f. sp. *phaseoli* isolates collected from the same geographical area clustered together. However, this cluster was comprised of races 1 and 4. No other clustering by race or geographical area was observed. Results indicated that a few *F. oxysporum* f. sp. *betae* strains were more closely related to non-pathogenic *F. oxysporum* and the out-group *F. solani*. Cramer *et al.* (2003) concluded that RAPD analysis was not suitable for such a study. Therefore, RAPDs could not differentiate strains based on pathogenicity or geographical origin, as was reported in other studies (Woo *et al.*, 1996; Vakalounakis *et al.*, 2004).

The RAPD technique has a few disadvantages when used for grouping *F. oxysporum* strains. The method may differentiate between *formae speciales* and fail to differentiate between VCGs and/or races (Vakalounakis and Fragkiadakis, 1999). Although, it may differentiate between VCGs it may fail to differentiate between races (Bentley *et al.*, 1995). RAPDs cannot differentiate between pathogenic and non-pathogenic isolates (Woo *et al.*, 1996). In addition for RAPDs to be effective a large number of primers need to be screened since not all primers can resolve strains (Wang *et al.*, 2001). Additional disadvantages of RAPDs include high homoplasy when generating data using RAPD primers, the lack of co-dominance, as well as the lack of DNA sequences identified by the primer. Therefore, the quantity of polymorphisms detected by an individual primer might be limited (Kerényi *et al.*, 1997; Belabid *et al.,* 2004; Nagarajan *et al.,* 2004). Due to problems associated with experimental repeatability, studies based on taxonomy and genetic relatedness requires the use of more reliable markers.

2.11.4 Amplified fragment length polymorphisms

Due to limitations of RAPDs, AFLPs have been the main fingerprinting technique used in *Fusarium* species identification (Leslie and Summerell, 2006). AFLP is a DNA fingerprinting technique which is able to distinguish and detect genomic restriction DNA fragments by PCR amplification (Zabeau and Vos, 1993, Vos *et al.*, 1995, Blears *et al.*, 1998, Savelkoul *et al.*, 1999). Genomic DNA is simultaneously digested with a six-base cutter (commonly *Eco*RI) and a four-base cutter (commonly *Mse*I or *Msp*I) restriction enzymes. Adaptors are ligated to the ends of the restricted fragments, which are then amplified using adaptor-specific primers with one (+1) or no (+0) selective nucleotides at their 3'-ends. In a second round of PCR fewer fragments are selectively amplified using adaptor-specific primers with two (+2) or three (+3) selective nucleotides. AFLP analysis has several advantages, it is highly reproducible, sensitive, provides resolution of the whole genome and requires no prior sequence knowledge and specific co-amplification and detection of a large number of restriction fragments is possible. The use of this method is relatively quick and cost effective. AFLPs are readily adapted to screen a large number of individuals (Vos *et al.*, 1995; Meudt and Clarke, 2007). AFLPs provide neutral markers, not subject to natural selection and allows for analysis of variation at more than one locus (Majer *et al.*, 1996; Baayen *et al.*, 2000a). Markers produced from AFLPs are found throughout the genome and allow for the testing of the whole genome of an organism (Vos *et al.*, 1995; Blears *et al.*, 1998; Mueller and Wolfenbarger, 1999; Meudt and Clarke, 2007). The AFLP technique is able to detect polymorphisms in different genomic regions. These polymorphisms are in the sequences between the restriction sites and are caused by mutations, insertions, deletions, and by mutations in the primer binding sites (Vos *et al.*, 1995; Blears *et al.*, 1998; Savelkoul *et al.*, 1999).

AFLP primer combinations that are used for fingerprinting generate AFLP fragments which correspond to unique positions on the genome and they can be used as markers in genetic and physical maps (Vos *et al.*, 1995). Over the past few decades AFLP analysis has been used in the taxonomy and phylogeny of different fungi (Majer *et al.*, 1996; Rosendahl and Taylor, 1997; Baayen *et al.*, 2000a; Kiprop *et al.*, 2002; Abdel-Satar *et al.*, 2003). However, it has not been used excessively in the *F. oxysporum* species complex. Sivaramakrishnan *et al.* (2002) used AFLPs to differentiate among races although the study showed the diversity within each race was not conclusive in terms of whether any overlaps existed between races or whether clusters existed. A lack of groupings based on UPGMA analysis of the same data matrix, ultimately shows that AFLPs did not actually distinguish between races.

The main disadvantage of AFLPs is that they do not correspond with vegetative compatibility, pathogenicity and/or geographical origin (Bogale *et al.*, 2006). Baayen *et al.* (2000b) demonstrated this in a study were 89 strains representing eight *formae specialis* were used. Non-pathogenic strains and a reference strain from each of the three main clades identified by O'Donnell *et al.* (1998b) were also included. The result proved to be very complex based on the relationship between AFLP based groups, VCGs and *formae specialis*. Other studies conducted by Bao *et al.* (2002), Kiprop *et al.* (2002), Abdel-Satar *et al.* (2003) determined that there was no correspondence between AFLPs and pathogenicity, geographic origin and/or cultural characteristics. The above mentioned studies proved that despite AFLPs advantages, AFLP alone does not give enough resolution for taxonomic and phylogenetic studies of *F. oxysporum*. Other disadvantages of AFLP markers are that they are dominant markers since alleles are not easily recognised and the technique does not differentiate between homozygous dominant and heterozygous individuals (Majer *et al.*, 1996; 1998; Mueller and Wolfenbarger, 1999). The technical disadvantage of AFLPs include, the incomplete restriction of DNA samples which can result in detection of differences in banding patterns, that do not reflect true DNA polymorphisms. The type of frequent cutter primer used can reduce the resolution of the technique (Vos *et al.*, 1995); therefore it is important to design primers and restriction enzymes.

The advantages of AFLPs are listed below. Fungi, including *F. oxysporum*, are haploid and this is irrelevant when haploid strains are studied using AFLPs. Generating AFLP fragments assists in determining species that are morphologically impossible to differentiate. Even though, at genus or family level the use of AFLP analyses are not informative, the technique has been used successfully in determining phylogenetic relationships among individuals such as bacteria (Huys *et al.*, 1996) and fungi (Majer *et al.*, 1998; Baayen *et al.*, 2000a; Schmidt *et al.*, 2004; Bogale *et al.*, 2009). In assessing the population structure, intraspecific and interspecific genetic variation is incorporated (Savelkoul *et al.*, 1999). AFLPs is strong, consistent and repeatable method for genetic analysis which has been successfully used for intraspecific genetic diversity analysis of *Fusarium* species (O'Donnell *et al.*, 2000; 2004; Zeller *et al.*, 2003a; Schmidt *et al.*, 2004; Bentley *et al.*, 2008; O'Donnell *et al.*, 2008; Bogale *et al.*, 2009). The characterisation of *F. udum* Butler (Kiprop *et al.*, 2002) and the identification of a new *Fusarium* species in the *G. fujikuroi* species complex were done with the aid of AFLPs (Zeller *et al.*, 2003b). AFLP studies of *F. oxysporum* were able to evaluate the integrity of the phylogenetic lineages. Investigations of the genetic variability of this wilt causing pathogen have assisted in resolving the taxonomic complexity of the species (Bogale *et al.*, 2006).

2.12 DNA sequencing analyses

The development of sequencing has become an important molecular tool in accurately identifying relationships between fungal groups and fungal species within these groups using phylogenetic markers to evaluate them, which previously was based solely on morphology (Roger *et al.*, 1999). In the past, phylogenetic relationships amongst some related *Fusarium* have been examined using rRNA gene sequences (Guadet *et al.*, 1989; Logrieco *et al.*, 1991). Due to the problems associated with rRNA gene sequences, scientists have focused on sequencing of protein coding genes (Roger *et al.*, 1999). Nucleotide sequences from certain gene or DNA regions assist in determining phylogeny at various taxonomic levels. Therefore, relatively large numbers of loci have been sequenced from one or more *Fusarium* species whose entire or partial genome sequences are available (in Genbank), among them there are: β-tubulin (β-tub), translantion elongation factor 1 alpha (TEF), Internal transcribed spacer (ITS), mitochondrial small subunit (mtSSU) and calmodulin (Cmd) (Genbank: ncbi.nlm.nih.gov). Other important genes regions such as the the Mating Type (MAT), the intergenic spacer (IGS), the Nitrate Reductase Coding Region (NIR), have been extensively used in the taxonomy and phylogeny of *Fusarium* species (Leslie and Summerell, 2006). Due to the variability of these highly conserved regions found mainly in the introns they provide high resolution at the sub-species level. A few of these important genes and their importance will be discussed bellow.

Internal transcribed spacers ITS1, ITS2 ITS3 and ITS4 are the spacer regions located between the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS. The ITS region is the most widely sequenced DNA region in fungi (Peay *et al.*, 2008). More variation was observed in the ITS1 and ITS2 region within closely related species and the main reason they are used for taxonomic studies (O'Donnell, 1992). Analysis of phylogenetic studies based on nucleotide sequences of rRNA genes such as ITS regions, can assist in determining relationships at a species level (White *et al.*, 1990; Young-Mi *et al.*, 2000). The ITS region has proven especially useful for identifying geographic races and relationships amongst similar species and closely related genera in *Fusarium* (White *et al.*, 1990). Using the ITS2 region sequence assisted in the identification of *F. solani*. However, *Fusarium* species in the *Sporotrichiella* section, *F. langsethiae* and its closely related species, *F. sporotrichioides* failed to differentiate (Yli-Mattila *et al.*, 2004). Similar results were seen by Turner *et al.* (1998) where the use of ITS-RFLP failed to differentiate between *F. avenaceum* and *F. tricinctuin*. Although there are shortcomings, the use of

ITS1+ITS4 primers have been described that allow selective amplification of fungal sequences including *F. oxysporum* (Gardes and Bruns, 1993; Jeeva *et al.*, 2008). Due to the limitation of sequence based phylogeny, certain ITS regions of the DNA are cladistically uninformative and even misleading, as is the case of O'Donnell and Cigelnik (1997), work on the phylogeny of *F. oxysporum* and the *G. fujikuroi* species complexes. O'Donnell and Cigelnik (1997) determined that half of the species of the *G. fujikuroi* and *F. oxysporum* lineages studied had either type I or type II ITS2 sequences as the major ITS2 type. The divergence between the two ITS2 types was greater than that observed within each type.

The mating type gene is controlled by a single locus with two idiomorphic alleles (MAT-1 or MAT-2). The alleles each have a conserved α box domain and a high mobility group box domain. Both sexual and asexual *Fusarium* species carry these MAT-1 and MAT-2 idiomorphs which are closely linked together (Yun *et al.*, 2000). Arie *et al.*, 2000 reported mating type tests from various races of *F. oxysporum* f. sp. *lycopersici* from different lineages (O'Donnell *et al.*, 1998b) carried the MAT-1 gene. Recently, the mating type locus, MAT1 cloned from *F. oxysporum* was found in all tested isolates (Arie *et al.*, 2000). Each isolate had either MAT-1 or MAT-2 idiomorphs. Previous studies showed, other than phylogenetic analyses based on other genes, mating type (MAT) based phylogenetic analyses could assist in the evolution study of closely related fungi (Pöggeler, 1999; Barve *et al.*, 2003), including *formae speciales* of *F. oxysporum* (Kawabe *et al.*, 2005; Kawabe *et al.*, 2007).

Elongation Factor 1 Alpha gene (TEF) sequences (Geiser *et al.*, 2004), is a short and highly conserved region which synthesises a protein that is important in the role of protein synthesis, which has been the most widely used for sequencing *Fusarium* species (Silar and Picard, 1994; Rösel and Kunze, 1995; Leslie *et al.*, 2007) and has the highest phylogenetic effectiveness for identification of *Fusarium* species (Geiser *et al.*, 2004). The sequences produced by the genes have been used to analyse phylogenetic trees in phylogenetic studies, since these phylogenies are similar with other genetic phylogenies. Compared to rRNA gene, sequences from the TEF gene provide more phylogenetic information (O'Donnell *et al.*, 1998b; Knutsen *et al.*, 2004). These sequences have been used to classify species, deduce phylogenetic relationships between species and to evaluate inter- and intraspecific variation (O'Donnell *et al.*, 1998b; Baayen *et al.*, 2000a; 2001). However, the gene generates a low resolution of relationships among and within lineages (Knutsen *et al.*, 2004). Bogale *et al.* (2007) used RFLP analyses of PCR amplified TEF to identify and distinguish between *F. redolens*

and members of the three phylogenetic clades of *F. oxysporum*. There were three TEF-RFLP patterns among *formae speciales* of *F. oxysporum* and these patterns corresponded with the three clades. A study by Coutinho *et al*. (2007) used morphological features and the sequences of TEF and β-tub genes to identify *F. circinatum* which causes pitch canker in a species of pine in South Africa. Another study by O'Donnell *et al*. (1998b) used DNA sequences of nuclear TEF and mtSSU rRNA genes to determine whether lineages of *F. oxysporum* f. sp. *cubense* have a monophyletic origin. Phylogenetic trees generated from the combined dataset showed five lineages corresponding to *F. oxysporum* f. sp.cubense with a large dichotomy between two taxa represented by strains commonly isolated from diseased bananas. The result revealed that Panama disease on banana is caused by *Fusarium* with independent evolutionary origins. Studies using this TEF sequence make this gene region an important tool in resolving taxonomic positions in *Fusarium* species and sub-species.

Beta tubulin gene is a highly conserved region and is an excellent tool for studying phylogenetic relationships at all taxonomic levels. The β-tub gene is more informative than the rRNA genes and was proposed by O'Donnell *et al.* (1998b) to study phylogenetic relationships within and amongst groups of *Fusarium* spp. Knutsen *et al.* (2004). This protein coding gene has been effectively used to determine both intra and interspecific relationships. A well-known example where it has been used at the interspecies level for the molecular characterisation of *Fusarium* species (O'Donnell *et al.*, 1998a; Aoki and O'Donnell, 1999; O'Donnell *et al.*, 2000), even though it presents low resolution within and amongst lineages, it suggests the use of genes which contain even more phylogenic information. This marker became a less popular phylogenetic indicator to determine relationships within and amongst *Fusarium* groups since it was observed that β-tub lacks the ability to identify differences in the 5'-end of the nuclear 28S rDNA (Knutsen *et al.*, 2004). Understanding the relationships within and amongst different *Fusarium* groups using β-tub gene, the use of multiple genes sequences should be used in gene genealogical studies. O'Donnell *et al.* (2000; 2004) and Starkey *et al.* (2007), have demonstrated highly efficient method in identifying phylogenetic species using multiple genes sequences (Taylor *et al.*, 2000) and perhaps assist in accurately defining the taxa. The combination of multiple genes also provides better resolution of relationships within and amongst lineages in a fungal group e.g. the *F. oxysporum* species complex (FOSC) (Baayen *et al.*, 2000a).

Calmodulin (Cmd) is a conserved region that is present in fungi (Charbonneau *et al.*, 1980; Marshak *et al.*, 1984). However, the Cmd gene provides little resolution among and within lineages which suggests the use of genes which contained even more phylogenetic information for inferring of relationships within and between *Fusarium* groups. However, Cmd gene sequences used by O'Donnell *et al.*, 2000 showed a very reliable use for phylogenetic analysis on the *G. fujikuroi* complex and *Fusarium* related species. Mulè *et al.* (2003) reported the first report on species specific primers for two *Fusarium* species obtained from DNA sequences of a Cmd gene, designed in the intron regions. Although they display a very poor level of sequence conservation, and mutational changes are misleading, this proved that the intron variation between species is greater than the exon variation (Boucher *et al.*, 1996; Donnelly *et al.*, 1999). Although, *F. oxysporum* is considered a morphologically and phylogenetically related species of the so called *G. fujikuroi* complex, both species are identical when comparing them with Cmd sequences only (Nelson *et al.*, 1983; O'Donnell *et al.*, 2000).

O'Donnell *et al.* (1998b) used a nucleotide sequence based phylogeny to present their study using a variety of gene regions (Internal Transcribed Spacer (ITS), the intergenic spacer (IGS), the Nitrate Reductase Coding Region (NIR), the mitochondrial Small Subunit (mtSSU), the TEF and β-tub partial genes in *F. oxysporum which* provided a high resolution at the sub-species level, where isolates of *F. oxysporum* f. sp. *cubense* were shown to have polyphyletic origins. The study included 47 *F. oxysporum* f. sp. *cubense* isolates found in 12 VCGs, 10 other *formae speciales*, one *F. inflexum* and two unknown *Fusarium* isolates. The results showed that the *F. oxysporum* f. sp. *cubense* isolates grouped into two separate clades, clades 1 and 2. Some *formae speciales* also grouped into one clade demonstrating that the clades did not correspond to *formae speciales*. Based on the sequence data from 12 VCGs of *F. oxysporum* f. sp. *cubense*, they were separated into five clonal lineages, with lineages 1, 3 and 5 in clade 2, and lineages 2 and 4 in clade 1. This showed that there was no direct comparison between VCGs and clonal lineages. Similar results were also observed as well in a study by Baayen *et al.* (2000a) that used TEF and mtSSU partial gene sequences which included 101 *F. oxysporum* isolates from eight *formae speciales* and 12 non-pathogenic strains, and found no relationship between clades and pathogenicity. Nucleotide sequence data from other gene regions, such as the NIR, ITS 1 and 2, and β-tub, has also not solved the problem of differentiating relationships between different *formae speciales* (Skovgaard and Rosendahl, 1998; Bao *et al.*, 2002).

O'Donnell *et al.* (1998b), Skovgaard and Rosendahl (1998), Baayen *et al.* (2000a), Bao *et al.* (2002) demonstrated that sequencing is a useful tool to sort strains of *F. oxysporum* into sub species taxa based on phylogeny. However, these phylogenetic groups do not always correspond with groups based on the race groups determined by pathogenicity and VCGs. Due to the lack of similarities the sequencing cannot substitute for pathogenicity tests for grouping strains into *formae speciales* and races. However, phylogenetic groups are very useful since they demonstrate the evolutionary relationships among strains within a *forma specialis* or a race, and also between *formae speciales* and/or races. Phylogenetic groups aid in identifying genetic relationships between pathogenic and non-pathogenic strains, and study the diversity within each of these sub-species groups.

An important disadvantage of sequence based phylogeny is that certain regions of the DNA are cladistically uninformative and even misleading. O'Donnell and Cigelnik (1997) revealed this in a study on *F. oxysporum* and the *G. fujikuroi* species complexes to determine phylogeny where they found that all the strains studied had two non-orthologous rDNA ITS2 types. Half of the species of the *G. fujikuroi* and *F. oxysporum* lineages used in the study possessed either type I or type II ITS2 sequences as the major ITS2 type. The differences between the two ITS2 types were greater than that examined within each type. ITS2 trees were therefore not comparable with trees inferred from the partial β-tub gene, mtSSU rDNA, nuclear 28S rDNA and nuclear rDNA ITS regions. Presently, the taxonomy of *Fusarium* species, using the combinations of all three nucleotide sequences (TEF, β-tub and Cmd genes) has become very popular for phylogenetic studies (pers. comm. PW Crous). Although various techniques have been developed for taxonomic purposes to charecterise, identify and classify *F. oxysporum* the above mentioned sections have been grouped accordingly into VCG tests, pathogenicity tests and DNA based techniques. Examples of advantages and disadvantages of each technique are described below (Table 2.2). Presently, phylogeny of strains can be assessed using DNA based methods since they have better resolution at the sub-species level. If based on appropriate number and types of genes and sequence analysis is a better choice for phylogenetic studies in the *F. oxysporum* species complex. However, since each technique has some advantages over others, the use of a combination of techniques should allow the development of more reliable approach to providing a better classification system.

Table 2.2 A summary of advantages and disadvantages of tools used in the taxonomy and phylogeny of the *F. oxysporurn* species complex

Tool	Advantage	Disadvantage
Pathogenicity tests	- Have a practical importance in terms of agriculture and forestry	- Time intensive and high costs due to the large number of formae speciales and races available.
		- Results rely on the interaction among the host, the pathogen and the environment
		- Tests exclude classification of non-pathogenic members.
Vegetative compatibility tests	- Not influenced by environmental factors.	- VCGs may lack association with pathogenicity groups.
	- Classification of non-pathogenic members.	- Self incompatible isolates do not relate to the test.
	- Provide information on genetic relatedness of compatible strains.	- Genetic relatedness between VCGs cannot be determined.
DNA based techniques	- Allow classification of non-pathogenic members.	- May lack correspondence with pathogenicity groups.
	- Provide unbiased markers.	- RAPDs and AFLPS are dominant markers, and retrieve high homoplasy.
	- Provide resolution at sub species level.	- RAPDs have low repeatability.
	- Extensively used in phylogenelic studies.	- RFLP haplotypes depend on the number and type of restriction enzyme and/or probe used.
	- RAPDs. RFLPs and AFLPs allow multilocus analysis of variation.	- Insufficient resolution and aneuploidy can misleading result of chromosome number and wrong karyotyping.
	- RFLPs provide co-dominant markers.	- There are no SSR markers developed: and AFLPs have not been used extensively.
		- Sequences from certain regions of the DNA are cladistically uninformative and misleading.

The objective of the current study is based on the importance and value of the aforegoing information and the applicability of *F. oxysporum* on *H. gordonii,* which is necessary in understanding both plant pathogen and host using different methods to characterise them.

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Chapter 3

Morphological and genetic characterisation of *Fusarium oxysporum* associated with wilt of *Hoodia gordonii*

3.1 Introduction

Hoodia gordonii, a succulent plant, is widely scattered in semi-arid areas of South Africa, Namibia and Botswana (Albers and Meve, 2001; Pawar *et al.*, 2007). Due to the presence of an appetite suppressive compound referred to as P57 (Pawar *et al.*, 2007; van Heerden *et al.*, 2007), the plant has been harvested in its wild state to the extent where its survival is seriously threatened. In 1998, Phytopharm Pharmaceuticals (United Kingdom) obtained a permit and sublicensed the pharmaceutical company, Pfizer to develop and commercialise the product production of P57 from *H. gordonii* (Vermaak *et al.*, 2011). In 2005, a dry rot disease was observed on *H. gordonii* stems in a commercial planting site in the Clanwilliam district of the Western Cape Province (Lamprecht *et al.*, 2008; Schroers *et al.*, 2009). The symptoms included black blistered lesions on the stems. Isolates from the *F. dimerum* species group were isolated from diseased *H. gordonii* plants and pathogenicity tests proved that *F. delphinoides Schroers, Summerb., O'Donnell & Lampr.* caused the disease (Schroers *et al.*, 2009). During 2004, extensive wilting was observed in a *H. gordonii* commercial plantation near Kakamas (Northern Cape, South Africa) and subsequently, in 2007, in a *H. gordonii* commercial plantation near Pofadder (Northern Cape, South Africa). In both instances, *F. oxysporum* was isolated from diseased plants (Philippou *et al.*, 2013).

Morphologically, *F. oxysporum* is identified by the production of microconidia in false heads, shape of its macroconidia, chlamydospores and the presence of short monophialides. The shape of macroconidia may be slightly sickle shaped with a thin walled apical cell and a foot shaped basal cell produced in abundant sporodochia (Leslie and Summerell, 2006). Morphologically, isolates of the fungus are indistinguishable from each other despite the existence of pathogenic isolates that are divided into at least 150 different *formae speciales*, based on virulence to a particular host or group of hosts (Correll, 1991). Many *formae speciales* show a race structure and are further subdivided into races based on virulence to a particular set of different host cultivars that vary in disease resistance (Armstrong and Armstrong, 1981; Correll, 1991). The development of appropriate disease management strategies that will improve the durability of *Fusarium*

wilt resistance therefore needs a sound understanding of the different races. Although it is not possible to morphologically distinguish one pathogenic *formae speciales* from another based solely on physical morphology on various mediums and spore morphology, the use of a suitable host plant can aid in differentiating them (Belabid *et al.*, 2004). Virulence tests have assisted in characterising different isolates of *F. oxysporum* although they require large numbers of isolates from various sources which have to be screened for pathogenicity using plants grown in a standardised environment (Leslie and Summerell, 2006). One issue associated with characterising isolates based solely on pathogenicity is races and the ability of these races to have various levels of infection based on the age of a host and prevailing environmental conditions. Vegetative compatibility groups (VCGs) may present an alternative method to understand the various races and their relationships to plant hosts. VCGs are commonly used to characterise a set of isolates from a novel population. Such populations may be subsets of larger populations that are uncharacterised but thought to be composed of only one or a few VCGs (Harveson and Rush, 1997).

The objectives of this study were to identify the morphological species causing wilt on *H. gordonii* and to evaluate the number of different vegetatively compatible groups present among isolates collected from commercial *Hoodia* plantings in the Northern Cape Province of South Africa. It was hypothesised that 1) *F. oxysporum* was the dominant causal species of wilt on *H. gordonii*, 2) the *F. oxysporum* isolates collected consist of pathogenic and non-pathogenic isolates and 3) a diverse number of VCGs could exist among the *F. oxysporum* isolates collected from *H. gordonii*.

3.2 Materials and methods

3.2.1 Collection of field samples

Eighty nine *Fusarium* isolates were isolated from *H. gordonii* seeds, diseased vascular tissue of stems and roots as well as associated soil. Samples were collected in 2004, 2007 and 2010 from four different geographic locations in the Northern Cape Province, namely Kakamas, Klein Pella, Pofadder and Prieska (Figure 3.1). Eighteen isolates were recovered from seeds and the vascular tissue of roots respectively, 16 from the vascular tissue of stems and 37 from soil. Locations were selected based on the occurrence of wilted *Hoodia* plants in commercial plantations. No wilt of *H. gordonii* was observed in wild populations although many dead plants were encountered in various locations in the Northern Cape Province.

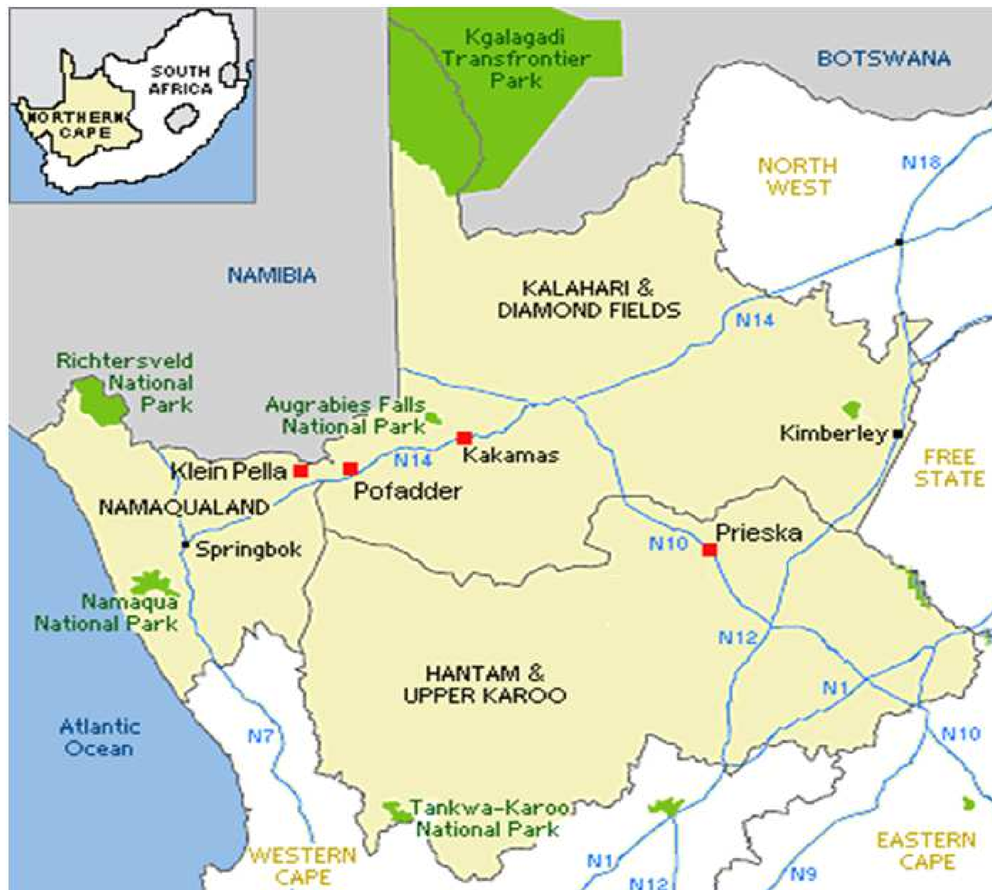

Figure 3.1 Sampling locations (red blocks) from *H. gordonii* commercial plantations in the Northern Cape Province of South Africa.

3.2.2 Identification of fungal isolates

Fusarium isolates were obtained from soil and plant material associated with both healthy and wilted *Hoodia* plants (Table 3.1). Soil samples (50 mg) were collected from the rhizosphere region of healthy and infected plants and isolation of the fungus was conducted by means of dilution plating. Seeds, roots and stems were surface sterilised by immersing plant material into a 1.25% (v/v) sodium hypochlorite solution for 3 min, rinsing three times for 30 s with sterilised dH_2O, and then plating onto a semi-selective medium for *Fusarium* (van Wyk *et al.*, 1986) (Appendix I). Plates were incubated under a 12 h light/dark cycle for 4-7 days at 25℃. After 4 -7 days fungal hyphae were transferred from developing colonies to potato dextrose agar (PDA; Appendix I) plates and incubated in a sterile growth chamber with a 12 h light/dark cycle at 25℃ for an additional 4-7 days. Fungal isolates were then sub-cultured on Spezieller Nährstoffarmer agar (SNA; Appendix I) (Nirenberg, 1976) until conidia formed.

Table 3.1 Isolates of *Fusarium* species collected from *H. gordonii*

Study code	Location	Substrate	Study code	Location	Substrate
OPC001	Prieska	Soil	OPC047	Pofadder	Soil
OPC002	Prieska	Soil	OPC048	Pofadder	Soil
OPC003	Prieska	Soil	OPC049	Pofadder	Soil
OPC004	Prieska	Soil	OPC050	Pofadder	Soil
OPC005	Prieska	Soil	OPC051	Pofadder	Soil
OPC006	Prieska	Soil	OPC052	Pofadder	Soil
OPC007	Prieska	Soil	OPC053	Pofadder	Soil
OPC008	Prieska	Soil	OPC055	Prieska	Stem
OPC009	Prieska	Soil	OPC056	Pofadder	Stem
OPC010	Prieska	Soil	OPC057	Pofadder	Stem
OPC011	Prieska	Soil	OPC058	Pofadder	Stem
OPC012	Prieska	Soil	OPC059	Pofadder	Stem
OPC013	Prieska	Soil	OPC060	Pofadder	Stem
OPC014	Prieska	Soil	OPC061	Pofadder	Stem
OPC015	Prieska	Soil	OPC062	Pofadder	Stem
OPC016	Prieska	Soil	OPC063	Klein Pella	Stem
OPC017	Prieska	Soil	OPC064	Klein Pella	Stem
OPC018	Prieska	Soil	OPC065	Klein Pella	Stem
OPC019	Prieska	Soil	OPC066	Klein Pella	Stem
OPC020	Prieska	Root	OPC067	Klein Pella	Stem
OPC021	Prieska	Root	OPC068	Klein Pella	Stem
OPC022	Prieska	Root	OPC098	Klein Pella	Root
OPC023	Prieska	Root	OPC099	Klein Pella	Root
OPC024	Prieska	Root	OPC100	Klein Pella	Root
OPC025	Prieska	Root	OPC101	Kakamas	Stem
OPC026	Prieska	Root	OPC102	Kakamas	Stem
OPC027	Prieska	Root	OPC103	Klein Pella	Seed
OPC028	Klein Pella	Root	OPC104	Klein Pella	Seed
OPC029	Prieska	Root	OPC105	Klein Pella	Seed
OPC030	Prieska	Root	OPC106	Klein Pella	Seed
OPC031	Prieska	Root	OPC107	Klein Pella	Seed
OPC032	Prieska	Root	OPC108	Klein Pella	Seed
OPC033	Prieska	Root	OPC109	Klein Pella	Seed
OPC034	Klein Pella	Root	OPC110	Klein Pella	Seed
OPC035	Pofadder	Soil	OPC111	Klein Pella	Seed
OPC036	Pofadder	Soil	OPC112	Klein Pella	Seed
OPC037	Pofadder	Soil	OPC113	Prieska	Seed
OPC039	Pofadder	Soil	OPC114	Prieska	Seed
OPC040	Pofadder	Soil	OPC115	Prieska	Seed
OPC041	Pofadder	Soil	OPC116	Prieska	Seed
OPC042	Pofadder	Soil	OPC117	Prieska	Seed
OPC043	Pofadder	Soil	OPC118	Prieska	Seed
OPC044	Pofadder	Soil	OPC119	Prieska	Seed
OPC045	Pofadder	Soil	OPC120	Prieska	Seed
OPC046	Pofadder	Soil			

3.2.3 Single spore cultures

Cultures originating from SNA plates were purified by sub-culturing single spores for identification purposes following dilution plating onto fresh SNA (Leslie and Summerell, 2006). Macroconidia from sporodochia on SNA plates were suspended in 10 ml sterile distilled water to obtain a spore suspension. One ml of the spore suspension was used to make three successive 1:10 serial dilutions in sterile water. One ml of each (10^{-1}, 10^{-2} and 10^{-3}) spore suspensions was spread on separate water agar plates (WA, Appendix I). Plates were incubated overnight in the dark at 25°C. Agar blocks with a single germling were cut from the WA plates with a sterile needle at 45x magnification and transferred to PDA plates. Plates were incubated for 7-10 days with a 12 h light/dark cycle, under ultraviolet light at 25°C before subculturing on PDA, SNA and carnation leaf agar (CLA, Appendix I) (Fisher *et al*., 1982) for identification purposes. Morphological identification followed the protocol of Summerell *et al.* (2003). All macroconidia used for identification purposes were obtained from sporodochia with the exception of *F. dimerum* isolates as sporodochia were not distinct or absent. Morphological characterisation criteria for the identification of *Fusarium* isolates are summarised in Table 3.2.

3.2.4 Pathogenicity tests

3.2.4.1 Plant materials

Pathogenicity tests were conducted in a greenhouse maintained at temperatures of 22°C and 18°C, day and night temperatures, respectively. Plants used for pathogenicity tests were grown from *H. gordonii* seeds collected from Pofadder, Northern Cape Province. Other species included as control treatments were *H. pilifera* (L.f.) Plowes subsp. *annulata* (N.E.Br.) Bruyns and carnation (*Dianthus caryophyllus*). Seeds were surface sterilised by immersing them into a 1.25% (v/v) sodium hypochlorite solution for 3 min and then rinsing three times for 30 s with sterilised dH_2O. Seeds were then air dried and sown into seedling trays containing sterilised soil and grown in a greenhouse at 22°C day and 18°C night temperatures. When *Hoodia* plants were six months old and carnation seedlings had each developed four leaves, they were transplanted into 25 cm plastic pots. The experiment was arranged in a randomised complete block design (RCBD) and was run in duplicate.

Table 3.2 Morphological criteria for the identification of *Fusarium* isolates (Leslie and Summerell 2006)

Species	Colony colour	Sporodochia	Macroconidia			Microconidia			Clamydospores
	Pigment	CLA	P	S	BC	P	S	Ph	P
F. culmorum	Carmine red	Orange to brown	Yes	Thick, short, 3-4 septa	Slight notch	No	-	-	None
F. dimerum	Salmon	absent	Yes	Very short, wide, 0-2 septa	Blunt, slightly notched	No	-	-	Rare and difficult to find
F. equiseti	Brownish	Orange	Yes	Long, slender, 5-7 septa	Long distinct	No	-	-	Yes, yellowish brown
F. nelsonii	Carmine red	Pale cream	Yes	Straight or falcate 3-5 septa	Foot shaped	No	-	-	Terminal
F. oxysporum	Purple	Pale Orange	Yes	Short to medium 3-5 septa	Foot shaped	Yes	Slightly curved	Short, Monophilades	Yes
F. solani	Redish brown	Cream, blue or green	Yes	Wide and straight, 5-7 septa	Straight to almost cylindrical	Yes	Oval, 0-2 septa	Long, Monophilades	Terminal

CLA – Carnation leaf agar P – Present S – Shape BC – Basal cell Ph – Phialide

3.2.4.2 Pathogenicity tests

Ten *F. oxysporum* isolates were selected for inoculation with the following criteria as it was impossible to distinguish between them morphologically. Table 3.3 represents isolates that were different based on (1) location, (2) colony colour (two isolates were observed on PDA with dark purple mycelium, two isolates with light purple mycelium and the rest of the isolates were purple mycelium), (3) the fastest growing isolates on PDA in terms of the time taken to cover the total plate surface area from a single spore; fast growth rate is associated with a pathogen's ability to compete successfully with other fungal species (Minerdi *et al.*, 2008) and (4) the year in which they were collected from various and geographic location (commercial locations were *H. gordonii* was grown).

Table 3.3 Isolates of *F. oxysporum* used for inoculation of *Hoodia* plants to test pathogenicity

Treatment	Study code	Location	PDA	Growth (Days)	Year	Species
1	OPC001	Prieska	Purple	7	2007	*F. oxysporum*
2	OPC002	Prieska	Purple	7	2007	*F. oxysporum*
3	OPC003	Prieska	Purple	14	2007	*F. oxysporum*
4	OPC004	Prieska	Purple	14	2007	*F. oxysporum*
5	OPC007	Prieska	Light Purple	14	2007	*F. oxysporum*
6	OPC028	Prieska	Light Purple	7	2009	*F. oxysporum*
7	OPC039	Pofadder	Dark purple	14	2007	*F. oxysporum*
8	OPC040	Pofadder	Dark purple	14	2007	*F. oxysporum*
9	OPC055	Prieska	Purple	14	2010	*F. oxysporum*
10	OPC101	Kakamas	Purple	7	2004	*F. oxysporum*
11*	OPC224	Bet Dagan, Isreal	Purple	7	1990	*F. oxysporum* f. sp. *dianthis*
12**	None	-	-	-	-	-

*Positive control **Negative control (Sterile oat grains)

Isolates used in the pathogencity tests were deposited at the Centraalbureau voor Schimmelcultures - an institute of the Royal Netherlands Academy of Arts and Sciences (CBS-KNAW) and Agricultural Research Council - Plant Protection Research Institute (ARC-PPRI) culture collection in Pretoria.

Inoculum was produced using oats (*Avena sativa* L.) grains (Dill-Macky, 2003). Oats (200 g) were soaked in 100 ml dH_2O glass flasks for 20 hours, to allow time for the oats to absorb the water. Excess water was drained from the flask and then sealed. The grain was autoclaved at 121℃ for 1 hour and allowed to c ool down to room temperature. Sterilisation by autoclaving was repeated for two consecutive days. The sterile oat grains were inoculated with mycelium plugs from a seven day old culture of *Fusarium*. *Fusarium oxysporum* f. sp. *dianthis* (CBS 416.90) was used as a positive control since the *forma specialis* is a specialised pathogen on carnations and the sterile oat grain was used as a negative control. Each isolate was incubated for one week at 25℃ after which

the colonised oat grains were dried in an oven at 30℃ for 72 hours. The inoculum was ground into a fine powder with a coffee grinder while making provision for contamination and stored at 5℃.

The prepared inoculum was applied to sterile soils separately with a control consisting of sterile oats powder. Healthy plants were transplanted into the inoculated soil medium. Six *H. gordonii*, six *H. pilifera* subsp. *annulata* and six carnation plants were used for testing each isolate. The control plants that were selected based on *H. pilifera* subsp. *annulata* been related species within the genus *Hoodia* and carnation as one of the control treatments was a known pathogen on carnations. Twelve treatments in total were set up for the pathogenicity tests, ten of which were the ten *F. oxysporum* isolates (Table 3.3), in addition to one positive control and one negative control to determine if the *F. oxysporum* isolates were pathogenic or non-pathogenic. Treatments were arranged in a randomised complete block design (RCBD) with six replications. A RCBD was used for its ability to take advantage of grouping similar uniform experimental units into replicates to determine observed differences between treatments. Plants were watered every two days with 50 ml of water as a standardised method to keep conditions in the greenhouse similar. Pathogenicity was determined based on seedling mortality. Isolations were made from symptomatic tissue on diseased plants to prove Koch's postulates. Comparisons were made with the original inoculated isolate based on colony morphology.

Disease severity was rated three weeks post inoculation using a two point scale. Where plants showed no symptoms a score of 0 was given and when symptoms were observed, a score of 2 was given. Data were analysed using Xlstat (2009) for Microsoft Excel. Analysis of variance (ANOVA) was performed to compare treatment means.

3.2.5 Vegetative compatibility

3.2.5.1 Isolates

Nitrate non-utilising (*Nit*) mutants, including both *nit1* and NitM, were generated for each isolate. Representative testers were selected based on non-compatibility with any initial matches and one identified non-pathogenic isolate. At least one, and preferably two to three, *nit1* and NitM mutants were selected for each isolate as prescribed by Leslie and Summerell (2006). These provided back-up mutants which were used when isolates reverted during the analysis. VCG control tester isolates of *F. oxysporum* examined in this study included 22 isolates from the Plant Pathology Culture Collection, University of

the Free State, Bloemfontein, South Africa, two isolates from the Medical Research Council, Tygerberg, South Africa and one isolate from the CBS-KNAW, Netherlands (Table 3.4). The batch included a total of 24 isolates from South Africa and one from Israel. These isolates were obtained from various hosts and substrates. Four (NitM) testers were generated and selected from the 45 isolates associated from *H. gordonii* and were selected for use as representatives VCGs.

Table 3.4 VCG NitM control testers used in this study

Study code	Isolate ID	Location	Year	Host or substrate	Species	VCG code
OPC200	CCP 7	Tzaneen	2005	Pine tree	*F. oxysporum*	NA
OPC201	CCP 119	Douglas	2005	Muskmelon	*F. oxysporum* f. sp. *melonis*	0134
OPC202	CCP 121	Douglas	2005	Soil	*F. oxysporum*	NA
OPC203	CCP 122	Douglas	2003	Soil	*F. oxysporum*	NA
OPC204	CCP 123	Douglas	2003	Soil	*F. oxysporum*	NA
OPC205	MRC 6264	OFS, RSA	2005	Wheat rhizosphere	*F. oxysporum*	NA
OPC206	CCP 331	Ficksburg	2003	Asparagus	*F. oxysporum* f. sp. *aspargi*	1001
OPC207	CCP 337	Ficksburg	2003	Asparagus	*F. oxysporum* f. sp. *aspargi*	1002
OPC208	CCP 338	Ficksburg	2003	Asparagus	*F. oxysporum* f. sp. *aspargi*	1007
OPC209	CCP 339	Roodeplaat	2004	Potatoes	*F. oxysporum* f. sp. *tuberosi*	NA
OPC210	CCP 448	Tzaneen	2003	Banana	*F. oxysporum* f. sp. *cubense*	0120
OPC211	CCP 551	Prieska	2005	Sorghum	*F. oxysporum*	NA
OPC212	CCP 553	Prieska	2005	Sorghum	*F. oxysporum*	NA
OPC213	GAS	Barkley West	2003	Onion	*F. oxysporum* f. sp. *cepae*	0425
OPC214	S116911	Bethlehem	2003	Soil	*F. oxysporum*	NA
OPC215	S13811	Bethlehem	2003	Soil	*F. oxysporum*	NA
OPC216	S1450	Bethlehem	2003	Soil	*F. oxysporum*	NA
OPC217	S14811	Bethlehem	2003	Soil	*F. oxysporum*	NA
OPC218	S231213	Bethlehem	2003	Soil	*F. oxysporum*	NA
OPC219	S6912	Bethlehem	2003	Soil	*F. oxysporum*	NA
OPC220	Srr10	Orania	2003	Soil	*F. oxysporum*	NA
OPC221	CCP 125	Pretoria	2005	Bean	*F. oxysporum* f. sp. *phaseolus*	0161
OPC222	S13911	Bethlehem	2003	Soil	*F. oxysporum*	NA
OPC223	MRC 8435	OFS, RSA	2005	Soil	*F. oxysporum*	NA
OPC224	CBS 416.90	Bet Dagan, Isreal	1990	Carnation	*F. oxysporum* f. sp. *dianthi*	0202

NA = Not assigned

3.2.5.2 Generation of *Nit* mutants

Ten minimal medium containing potassium chlorite (MMC; Appendix I) plates were used for each isolate. Each plate had three to four colonies per isolate to obtain a series of *nit* mutants over a 14 day period. Where a NitM isolate was not observed for all of the wild type isolates, a *nit3* mutant may be used to substitute. Isolates were characterised based on vegetative compatibility using the method of Puhalla (1985). This method allocates isolates to VCGs based on heterokaryon formation between complementary nitrate non utilizing (*nit*) mutants. Pairings of *nit1* and *nit3* isolates were placed in daisy configuration of five agar plugs per plate (Puhalla, 1985).

A small 3 mm^3 block of mycelia from PDA cultures of each isolate was inoculated in the centre on to 9-cm-diameter plates containing PDA amended with chlorite (PDC;

Appendix I), minimal medium containing chlorite (MMC) or rose bengal amended medium (RBAM; Appendix I) and incubated at 27℃ in the dark (Bell and Crawford, 1967; Puhalla, 1985; Correll *et al.*, 1987; Elias and Cotty, 1995; Glass, 2000). Spontaneous fast growing chlorate resistant sectors were recovered from some *F. oxysporum* isolates when cultured on MMC medium with 1.5%, 2.0%, 2.5%, 3.0% or 3.5% chlorate as prescribed by Leslie and Summerell (2006). Isolates which did not produce chlorate resistant sectors were then inoculated on PDC medium with 1.5%, 2.0%, 2.5%, 3.0% or 3.5% chlorate. If mutants were not recovered on this medium then the isolates were incubated on RBAM medium. The plates were examined daily for the appearance of quick growing sectors from the initially restricted colony. Chlorate resistant sectors which were selected were transferred to a minimal medium containing sodium nitrate ($NaNO_3$) as the sole nitrogen source and incubated at 27℃ for 7 days.

3.2.5.3 Identification of *Nit* mutant phenotypes

Basal medium (BM) was used to prepare other media needed to determine VCGs (Correll *et al.*, 1987). The *nit* mutants were placed in different phenotypic mutant types (*nit1*, *nit3*, and NitM) based on their growth on media containing one of three different nitrogen sources: minimal medium containing nitrite (MM; Appendix I), minimal medium containing nitrate (NMM; Appendix I) and minimal medium containing hypoxanthine (HMM; Appendix I). A positive control was used by placing *nit* mutants on ammonium medium (AMM; Appendix I). Uric acid medium (UAM; Appendix I) was used to determine whether *nit3* mutants where positive or negative based on a purple colour secretion on the medium. Phenotype was determined by placing a mycelium transfer (3 mm^3 MM block) of the *nit* mutant on each of the three media (Correll *et al.*, 1987). Phenotypes were determined by colony morphology on media containing nitrate, nitrite or hypoxanthine as the sole nitrogen sources. Plates were incubated at 27℃ in the dark for 7 days.

3.2.5.4 Compatibility tests

The pairing of *nit* mutants performed on MM medium, were used to determine vegetative compatibility. Heterokaryon formation between mutants was identified by the formation of a wild type mycelium growth at the contact zone. Mycelia plugs from each *nit* mutant were placed in a daisy wheel formation 3 cm apart on MM plates. The plates were incubated at 27℃ for 7 days and cultures were obse rved daily over 4 weeks. At least two compatible NitM mutants from each of the isolates participated in the compatibility

parings with *nit1*, *nit3* or NitM type mutants from other isolates in all possible combinations to determine the number of VCGs present among 45 isolates of *F. oxysporum* associated with *H. gordonii*. Compatibility between isolates was observed by the formation of dense mycelium growth where the mutants had anastomosed to form hetrokaryons. Heterokaryon formation was evaluated and recorded for 4 weeks. Compatibility pairings were repeated twice for each isolate with different *nit* mutants.

3.3 Results

3.3.1 Morphological identification

Eighty nine isolates were recovered and morphologically characterised. These isolates included six *Fusarium* species, namely *F. culmorum* (3.4%), *F. dimerum* (22.5%), *F. equiseti* (12.4%), *F. nelsonii* (11.2%), *F. oxysporum* (49.4%) and *F. solani* (1.1%). *Fusarium oxysporum* was present at high frequencies at all locations sampled, where wilt of *H. gordonii* was observed. Although the other five *Fusarium* species were isolated from four locations, none of these species had been documented to cause wilt. *Fusarium oxysporum* was isolated at relatively high frequencies (Table 3.5) at all locations sampled, except Klein Pella (10%). *F. equiseti* (33%) was the dominant *Fusarium* species recovered at Klein Pella. The lowest percentage (4%) of *F. culmorum* was identified in Pofadder and occurred at two of the five locations sampled. Kakamas was the only location where *F. solani* (50%) was recovered. *Fusarium dimerum* occurred at three of the five locations, with the lowest frequency at Prieska (20%) and the highest at Klein Pella (29%). *Fusarium nelsonii* occurred at two of the five locations sampled with the lowest frequency (9%) at Prieska and highest at Klein Pella (29%).

The composition of *Fusarium* isolates collected at each individual location differed. Klein Pella and Prieska were similar with four species detected at each locality, except for *F. culmorum* which was present in low frequencies at Prieska. Pofadder isolates had the highest percentage (72%) of F. *oxysporum*. At Kakamas, only two species were recovered, *F. oxysporum* and *F. solani* (Table 3.5). At Pofadder only three species were recovered, *F. culmorum*, *F. dimerum* and *F. oxysporum* (Table 3.5). The Prieska nursery had the highest number of morphospecies present as well as the highest observed frequencies fo*r F. oxysporum* and *F. dimerum* (Table 3.5).

Table 3.5 Frequency (%) of *Fusarium* species detected at locations sampled

	F. culmorum	F. dimerum	F. equseti	F. nelsonii	F. oxysporum	F. solani
Kakamas	-	-	-	-	50	50
Klein Pella	-	29	33	29	9	-
Pofadder	4	24	-	-	72	-
Prieska	5	19	10	10	56	-

3.3.2 Pathogenicity tests

Out of all *Fusarium* isolates collected from commercial *H. gordonii* plantings, *F. oxysporum* was the only species known to be associated with wilt in other plant hosts. Pathogenicity tests indicated that of the suspected wilt-causing fungi under investigation, *F. oxysporum* had obvious ability to cause wilt to inoculated *H. gordonii* plants. It was also shown to cause wilt to another species in the genus, namely *H. pilifera* subsp. *annulata*. After 14 days, *H. gordonii* plants, planted in inoculum infested soils showed typical wilt disease symptoms. However, plants in soil infested with *F. oxysporum* isolates (OPC004 and OPC028), *F. oxysporum* f. sp. *dianthi* and plants planted in the sterile soil, were disease free. The highest percentage of wilt on the *H. gordonii* plants was observed as 100% wilt where the soil was infested with *F. oxysporum* isolates OPC007, OPC040, OPC055 and OPC101. The lowest percentage of wilt on the *H. gordonii* plants was observed as 33.3% wilt where the soil was inoculated with *F. oxysporum* isolates OPC002, OPC003 and OPC039. The *H. pilifera* subsp. *annulata* plants, planted in infested soils showed similar wilt disease symptoms as observed on *H. gordonii*. However, plants where soil was infested with three *F. oxysporum* isolates (OPC003, OPC028 and OPC039) and *F. oxysporum* f. sp. *dianthi* along with the plants planted in the sterile soil, were disease free. The highest percentage (100%) wilt on *H. pilifera* subsp. *annulata* was observed with isolate OPC101. The lowest percentage (50%) wilt on this species was observed with isolates OPC001, OPC007, OPC040 and OPC055. The positive control (*F. oxysporum* f. sp. *dianthi*), tested on *H. gordonii* and *H. pilifera* subsp. *annulata* caused no wilt, although when tested on carnations, it caused wilt of all the inoculated plants (Table 3.6).

3.3.2.1 Symptoms on inoculated plants

Seven days after the plants were planted in the inoculum infested soil, the onset of wilting gradually increased which caused stems to rot at the base and shrivel up, causing plants to collapse and eventually die. Affected plants also exhibited discolouration of the vascular system (Figure 3.2 and 3.3).

Table 3.6 Percentage of survivors and wilt associated with *F. oxysporum* isolates

Treatment	Inoculums	H. gordonii	*H. pilifera* subsp. *Annulata*	Carnation
		Wilt %	Wilt %	Wilt %
1	OPC001 [(a, b)]	50.0	50.0	0.0
2	OPC002 [(a, b)]	33.3	66.3	0.0
3	OPC003 [(a)]	33.3	0.0	0.0
4	OPC004 [(a, b)]	0.0	83.3	0.0
5	OPC007 [(a, b)]	100.0	50.0	0.0
6	OPC028	0.0	0.0	0.0
7	OPC039 [(a)]	33.3	0.0	0.0
8	OPC040 [(a, b)]	100.0	50.0	0.0
9	OPC055 [(a, b)]	100.0	50.0	0.0
10	OPC101 [(a, b)]	100.0	100.0	0.0
11*	CBS 416.90 (c)	0.0	0.0	50.0
12**	None	0.0	0.0	0.0

*Positive control, **Negative control (Sterile oat grains), Pathogenicity is considered positive (+) when at least 33.3% of the *Hoodia gordonii* (a)*, H. polifera subsp. annulata* (b) *and* carnation (c) plants were dead at the end of the trial and all plants showed at least mild symptoms; it is considered negative (–) when below 33.3% of dead *Hoodia gordonii* plants and at least one plant showed no symptoms at all

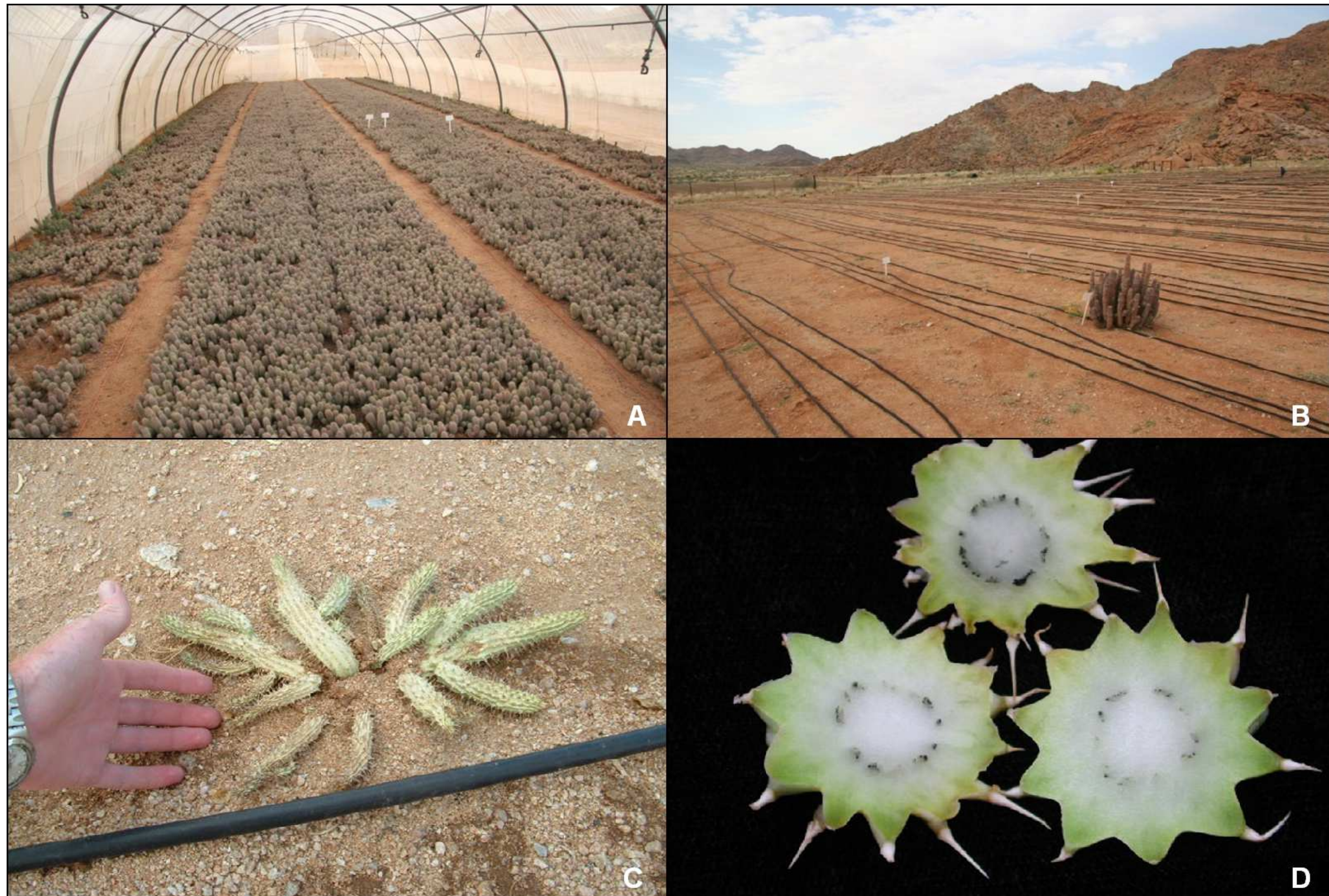

Figure 3.2 **(A) Before wilt infection: *H. gordonii* cultivation in Kakamas, (B) After wilt infection: *H. gordonii* cultivation in Kakamas, (C) *H. gordonii* wilt in cultivated nursery in Kakamas and (D) Wilt infected stem *H. gordonii.***

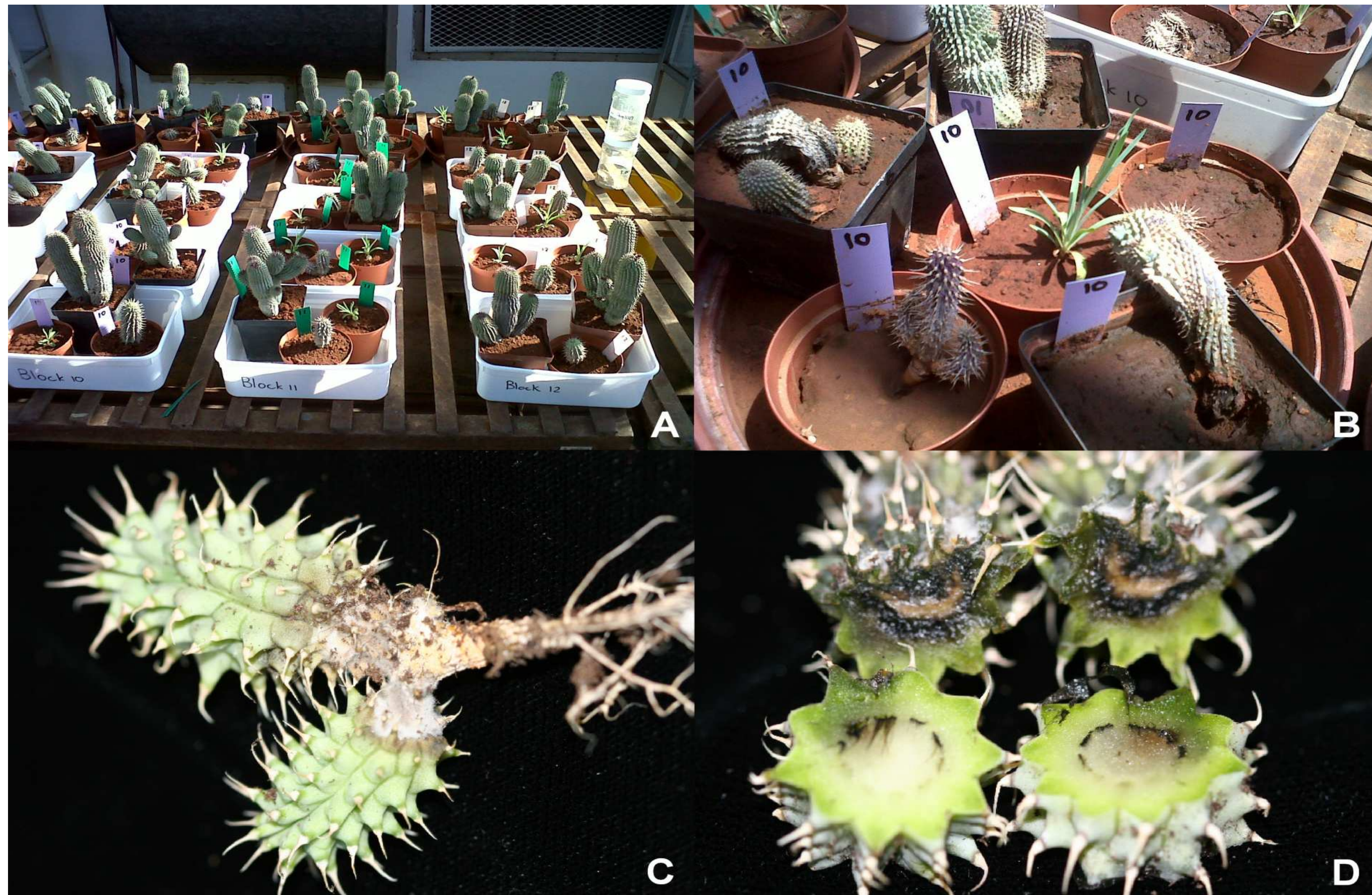

Figure 3.3 **(A) Before wilt infection: *H. gordonii* pathogenicity tests in greenhouse, (B) After wilt infection: *H. gordonii* pathogenicity tests in the greenhouse, (C) *H. gordonii* wilt resulting from inoculation in the greenhouse with mycelium, discolouration and fruiting bodies visible at base of the stem and (D) Cross-section through wilt infected stem of *H. gordonii.***

3.3.3 Analysis of VCG's

3.3.3.1 Generation of *Nit* mutants

Chlorate resistant mutants were readily obtained from all *F. oxysporum* isolates. Most of the fast growing chlorate resistant sectors recovered were not able to utilise nitrate as a sole nitrogen source (Figure 3.4) and as a result mutants grew as thin expansive colonies with no aerial mycelium on MM (Figure 3.5). The sectors with no aerial mycelium were cut out then plated on MM and labelled *nit* mutants. A small number of sectors were recovered from each isolate that were chlorate resistant but able to utilise nitrate.

The number of chlorate resistant sectors produced depended upon each individual isolate and the amount of chlorate used in the medium. Thin growth with little or no aerial mycelium on MM, indicated that the sectors were also unable to reduce nitrate and were considered to be *nit* mutants. Generally, *nit* mutants were readily produced although this was not the case with a few isolates. Those few isolates that struggled to produce *nit* mutants were placed on medium with adjusted chlorate volumes as described by Leslie and Summerell (2006).

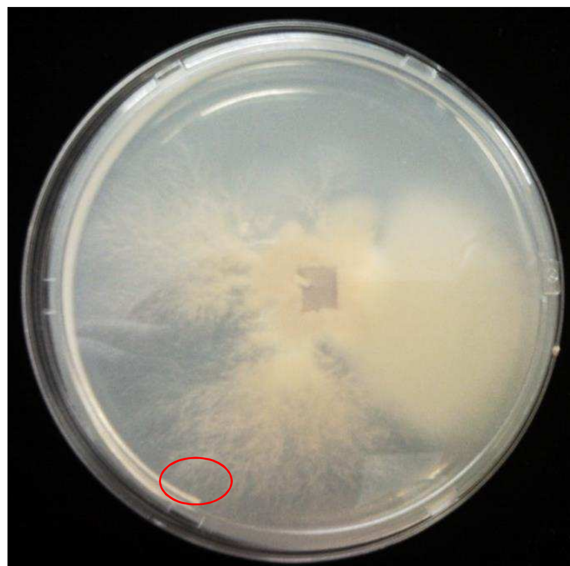

Figure 3.4 Growth of isolate OPC008 on PDA with 1.5% chlorate (PDC) after 7 days of incubation at 27°C. Red elliptical area is the chlo rate-resistant sectors with no aerial mycelium.

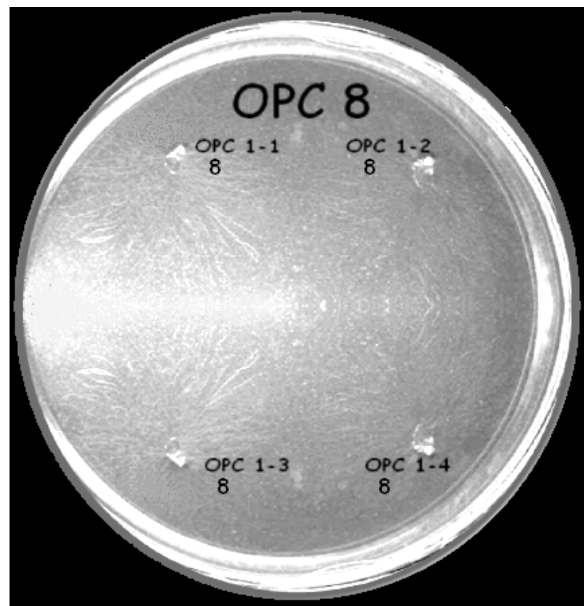

Figure 3.5 Growth of *nit* mutants from isolates OPC008 on basal medium after 7 days of incubation at 27℃. Note the normally expansive, bu t very thin growth.

A total of 432 nitrate non-utilizing (*nit*) mutants were recovered from 45 isolates of *F. oxysporum* cultured on three media, PDC, MMC, or RBAM and in the majority of cases more than one mutant was produced for each isolate. This allowed for extensive testing to determine more accurately which VCGs the *nit* mutants belonged to as compared to one isolate being used only. Nearly 52% of *nit* mutants were generated by isolates inoculated on PDC medium; 34% *nit* mutants on MMC medium; and 14% on RBAM medium.

3.3.3.2 Phenotypes identification from *Nit* mutants

The phenotypes of the *nit* mutants generated from *F. oxysporum* isolates were determined by evaluating colony morphology on media containing nitrate, nitrite or hypoxanthine as sole nitrogen sources. The *nit* mutants were divided into three phenotypic classes: *nit1*, *nit3*, and NitM. Growth was assessed as being wild type or thin expansive colonies with no aerial mycelium. The three *nit* mutant classes were not generated with the same frequency with the majority of *nit* mutants recovered being *nit1* mutants irrespective of which media was used. The *nit1* mutants were approximately three times as common as the NitM and *nit3* mutants (Figures 3.6, 3.7 and 3.8). Among the 432 *nit* mutants, there were 305 *nit1* mutants, 89 *nit3* mutants, and 41 NitM mutants obtained (Table 3.7). Not all three phenotypes were recovered from all isolates, and the frequency of occurrence of each mutant type varied from isolate to isolate.

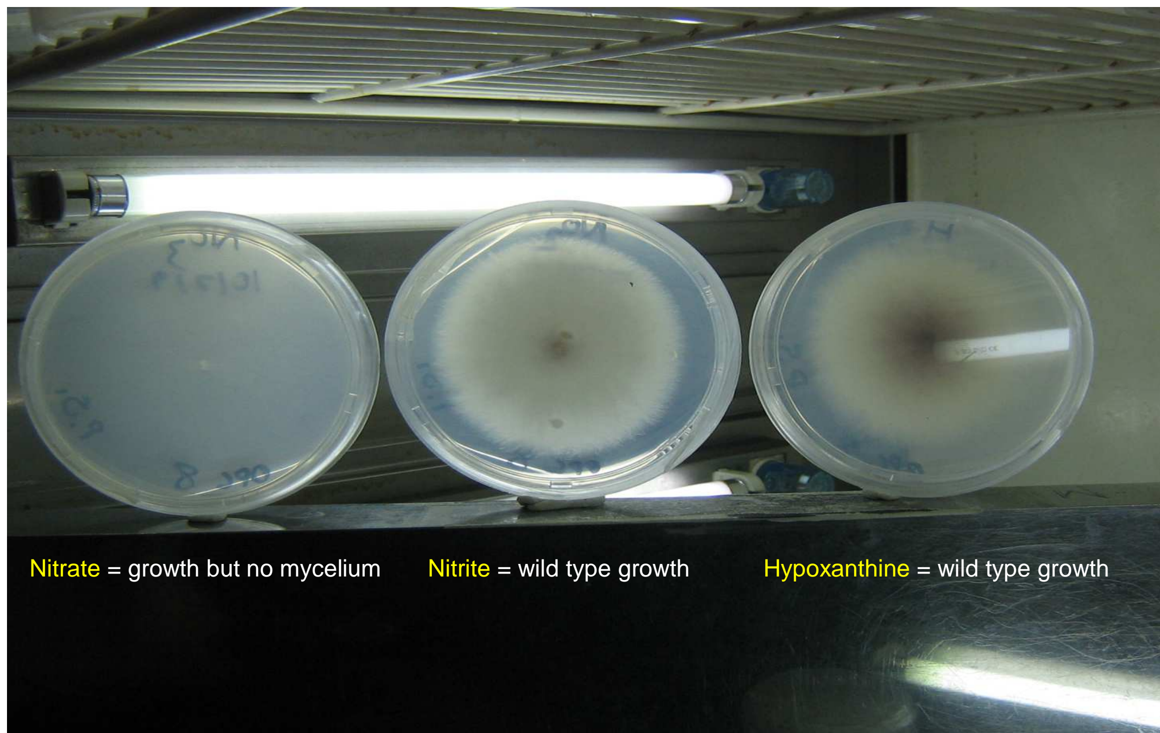

Figure 3.6 Growth of ***nit1*** mutant phenotypes from isolate OPC008 on three different nitrogen sources, nitrate (left), nitrite (middle) and hypoxanthine (right), after 7 days of incubation at 27℃. OPC008 shows the hyphal wild type growth of ***nit1*** mutant on both nitrite medium (middle) and hypoxanthine medium (right) and thin growth on nitrate medium (left).

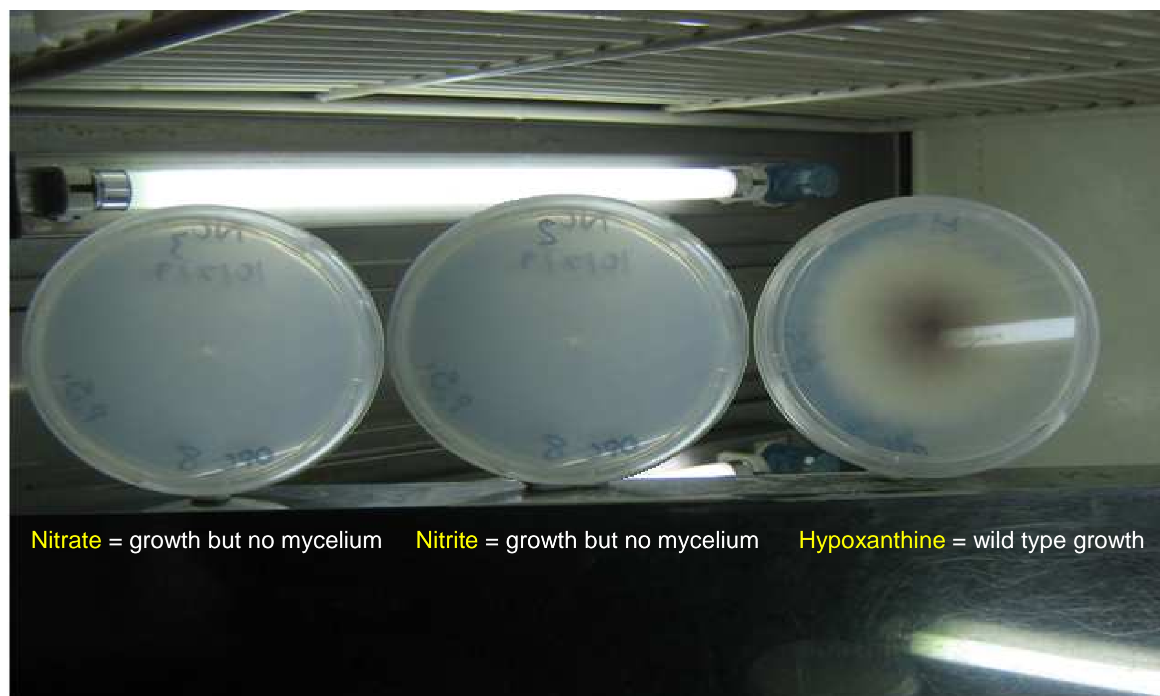

Figure 3.7 Growth of ***nit3*** mutant phenotypes from isolate OPC008 on three different nitrogen sources, nitrate (left), nitrite (middle) and hypoxanthine (right), after 7 days of incubation at 27℃. OPC008 shows the wild type hyphae growth of ***nit3*** mutants on hypoxanthine medium (right) and thin growth on both nitrate medium (left) and nitrite medium (middle).

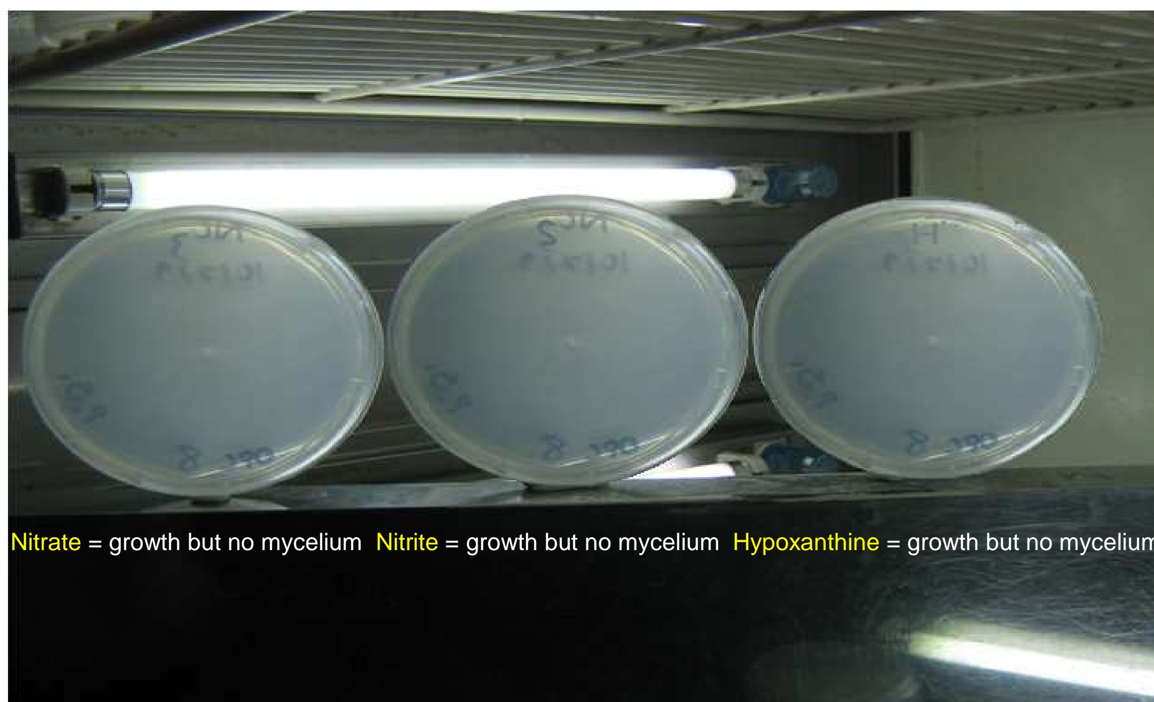

Figure 3.8 **Growth of NitM mutant phenotypes from isolate OPC008 on three different nitrogen sources, nitrate (left), nitrite (middle) and hypoxanthine (right), after 7 days of incubation at 27°C.**

The frequency of *nit1* mutants recovered was considerably higher on PDC than on MMC and RBAM. Consequently, the relative frequency of both the *nit3* and NitM mutants recovered were considerably higher on MMC than PDC or RBAM. *Nit1* mutants were recovered from 33 isolates, while *nit3* mutants were recovered from only 23 isolates, 37 isolates failed to produce NitM mutants, although they were repeatedly exposed to the two chlorate mediums and different concentration of potassium chlorate within the media. The frequency of different *nit* mutants recovered varied amongst different locations (Table 3.7). *Nit1* mutants had the highest occurrence. Twenty one out of 23 *nit* mutants were *nit1* (91.3%) obtained from the Kakamas isolates; 139 out of 189 *nit* mutants were *nit*1 (65.5%) obtained from the Pofadder isolates; 110 were *nit1* mutants (63.9%) out of 202 *nit* mutants obtained from the Prieska isolates; 16 were *nit1* mutants (88.9%) out of 18 *nit* mutants obtained from the KP isolates. Positive controls and testers used in this study were *F. oxysporum* obtained from different parts of the South Africa and/or hosts, and an isolate from Israel, *F. oxysporum* f. sp. *dianthi* (Table 3.8).

Whether isolates were pathogenic or non-pathogenic, *nit* mutants could be easily obtained and their phenotypes shown to have no relationship to their pathogenicity. As a result (Table 3.7), two VCGs were identified among 44 *F. oxysporum* isolates analysed associated with wilt on *H. gordonii* (Figure 3.9). One predominant VCG (Group 1)

contained 39 isolates and a second VCG (Group 2) contained five isolates. No self-compatible *nit* mutants were observed.

Table 3.7 Vegetative Compatibility Groups of *Fusarium oxysporum* phenotypes of *nit* mutants recovered from *H. gordonii*

No.	Isolate	Location	VCGs	Number of nit mutants examined	Classes of complementary nit mutants		
					nit1	*nit 3*	NitM
1	OPC101	Kakamas	1	23	21	2	0
2	OPC001	Prieska	1	3	2	1	0
3	OPC002	Prieska	1	20	18	2	0
4	OPC003	Prieska	2	13	0	0	13
5	OPC004	Prieska	1	5	5	0	0
6	OPC005	Prieska	1	4	4	0	0
7	OPC006	Prieska	1	8	5	3	0
8	OPC007	Prieska	1	3	2	1	0
9	OPC008	Prieska	1	17	7	0	10
10	OPC009	Prieska	1	9	3	6	0
11	OPC010	Prieska	1	16	9	7	0
12	OPC011	Prieska	1	8	8	0	0
13	OPC012	Prieska	1	6	6	0	0
14	OPC013	Prieska	1	7	1	5	1
15	OPC014	Prieska	1	4	0	4	0
16	OPC015	Prieska	1	4	4	0	0
17	OPC016	Prieska	1	5	5	0	0
18	OPC017	Prieska	1	5	5	0	0
19	OPC018	Prieska	1	5	4	0	1
20	OPC019	Prieska	1	4	3	1	0
21	OPC020	Prieska	1	23	8	4	11
22	OPC027	Prieska	1	6	4	2	0
23	OPC028	Prieska	1	8	7	1	0
24	OPC055	Prieska	1	19	19	0	0
25	OPC035	Pofadder	1	16	3	12	1
26	OPC036	Pofadder	1	5	5	0	0
27	OPC037	Pofadder	1	5	1	4	0
28	OPC039	Pofadder	2	2	2	0	0
29	OPC040	Pofadder	2	10	4	6	0
30	OPC041	Pofadder	1	3	3	0	0
31	OPC042	Pofadder	1	1	1	0	0
32	OPC043	Pofadder	2	21	18	0	3
33	OPC044	Pofadder	1	14	11	3	0
34	OPC045	Pofadder	1	15	10	5	0
35	OPC046	Pofadder	1	14	14	0	0
36	OPC047	Pofadder	1	7	6	0	1
37	OPC048	Pofadder	2	7	7	0	0
38	OPC049	Pofadder	1	16	8	8	0
39	OPC050	Pofadder	1	20	19	1	0
40	OPC051	Pofadder	1	11	8	3	0
41	OPC052	Pofadder	1	21	18	3	0
42	OPC053	Pofadder	1	1	1	0	0
43	OPC033	Klein Pella	1	7	7	0	0
44	OPC034	Klein Pella	1	11	9	2	0
Total				**432**	**305**	**86**	**41**

Table 3.8 Phenotypes of *nit* mutants recovered from other hosts and locations around South Africa, Culture Collection of Plant Pathology (CCP) and Israel (CBS) were used as control testers

No.	Isolates	species and or *forma specialis*	VCG code	Number of nit mutants examined	Classes of complementary nit mutants		
					nit1	*nit3*	NitM
T1	OPC223	*F. oxysporum*	NA	3	2	1	0
T2	OPC205	*F. oxysporum*	NA	4	3	1	0
T3	OPC200	*F. oxysporum*	NA	7	5	2	0
T4	OPC201	*F. oxysporum* f. sp. *melonis*	0134	10	7	3	0
T5	OPC202	*F. oxysporum*	NA	5	4	0	1
T6	OPC203	*F. oxysporum*	NA	14	12	1	1
T7	OPC206	*F. oxysporum* f. sp. *asparagi*	1001	14	13	1	0
T8	OPC207	*F. oxysporum* f. sp. *asparagi*	1002	5	4	1	0
T9	OPC204	*F. oxysporum*	NA	11	7	4	0
T10	OPC205	*F. oxysporum*	NA	7	5	2	0
T11	OPC208	*F. oxysporum* f. sp. *asparagi*	1007	6	4	2	0
T12	OPC209	*F. oxysporum* f. sp. *tuberosi*	NA	4	3	1	0
T13	OPC210	*F. oxysporum* f. sp. *cubense*	0120	23	12	11	0
T14	OPC211	*F. oxysporum*	NA	3	2	1	0
T15	OPC212	*F. oxysporum*	NA	14	11	3	0
T16	OPC213	*F. oxysporum* f. sp. *cepae*	0425	12	8	4	0
T17	OPC214	*F. oxysporum*	NA	8	7	1	0
T18	OPC215	*F. oxysporum*	NA	7	5	2	0
T19	OPC224	*F. oxysporum* f. sp. *dianthi*	0202	10	6	3	1
T20	OPC216	*F. oxysporum*	NA	6	4	2	0
T21	OPC217	*F. oxysporum*	NA	4	3	1	0
T22	OPC218	*F. oxysporum*	NA	6	5	1	0
T23	OPC219	*F. oxysporum*	NA	2	1	1	0
T24	OPC220	*F. oxysporum*	NA	8	7	1	0
T25	OPC221	*F. oxysporum f. sp. phaseolus*	0161	3	2	0	1
T26	OPC222	*F. oxysporum*	NA	0	0	0	0
T27	OPC223	*F. oxysporum*	NA	0	0	0	0
Total				196	142	50	4

NA = Not assigned

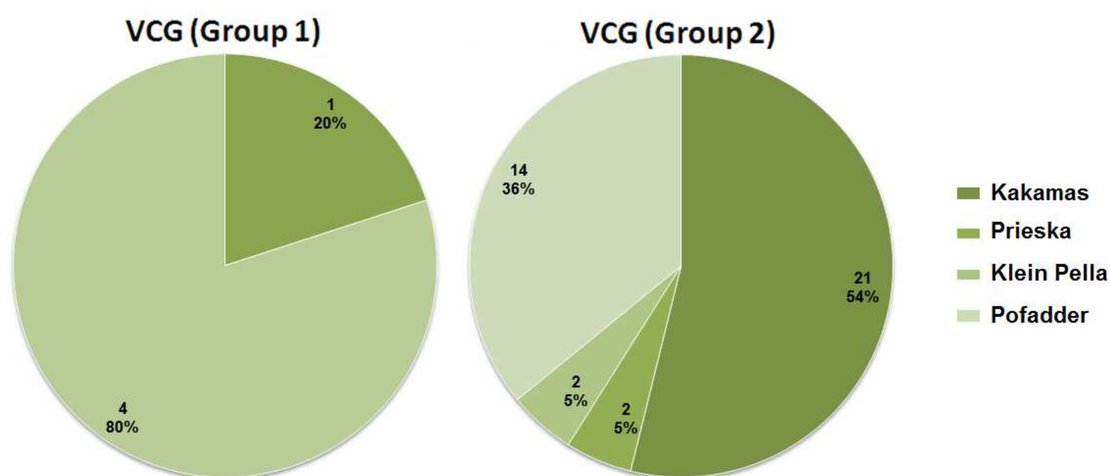

Figure 3.9 Percentage of VCG groups 1 and 2 from various locations in the Northern Cape.

The distribution of the VCGs was complex and comprises isolates from various geographic locations from the Northern Cape Province including pathogenic and no non-pathogenic isolates. Group 1 included 39 isolates (Table 3.7) from all four locations with one isolate from Kakamas, 18 from Pofadder, 23 from Prieska and two from Klein Pella plantations. These showed different levels of pathogenicity on Hoodia plants and included 9 pathogenic isolates, 1 non-pathogenic isolate. Vegetative compatibility Group (Group 2) included 5 isolates: 4 from Pofadder and 1 from Prieska. One non-pathogenic isolate (OPC039) was observed and paired with all nit mutants in VCG (Group 2), from Pofadder. The *nit* mutants of the tested isolates and the tester isolates formed strong heterokaryons. The complementary *nit* mutants chosen as testers of VCG (Group 1) were OPC008-M1 (NitM, parental isolate OPC008) and OPC020-M4 (NitM, parental isolate OPC020); the testers of VCG (Group 2) were OPC003-M13 (NitM, parental isolate OPC003) and OPC043-M3 (NitM, parental isolate OPC043). All the other *nit* mutants generated by other isolates were paired with testers. OPC014 had only the *nit3* phenotype, OPC003 created only the NitM mutant phenotype.

3.3.3.3 Complementation analysis

Physiological complementation between *nit* mutants with different phenotypes was indicated by the dense mycelium growth that developed where the contact zone of the *nit* mutant colonies came in contact with each other and anastomosed to form a heterokaryon (Figure 3.10). The mutants of each individual isolate were first paired among themselves on MM to reveal complementation within the isolate. Complementary *nit* mutants formed dense, wild-type growth on MM as a result of heterokaryosis and were assigned to the same VCG. Vegetatively incompatible isolates were detected by their inability to form a heterokaryon when paired and grown on MM.

Complementation occurred among *nit* mutants with different phenotypes (Table 3.8). The number of NitM mutants obtained from chlorate substrates was evaluated to compare the efficiency of different media in the generation of *nit* mutants useful for VCG analysis. Similar results were obtained in this study compared to studies done by (Correll, 1991) which showed more NitM mutants were produced on MMC. Complementation occurred quickly and heterokaryon hyphal growth was more vigorous in pairings of NitM with *nit1* or *nit3* mutants than in pairings of *nit1* with *nit3* mutants. NitM was more vigorous when pairing with *nit1*, *nit3* and NitM type mutants in all possible combinations and the majority of complementation groups were identified among the NitM mutants.

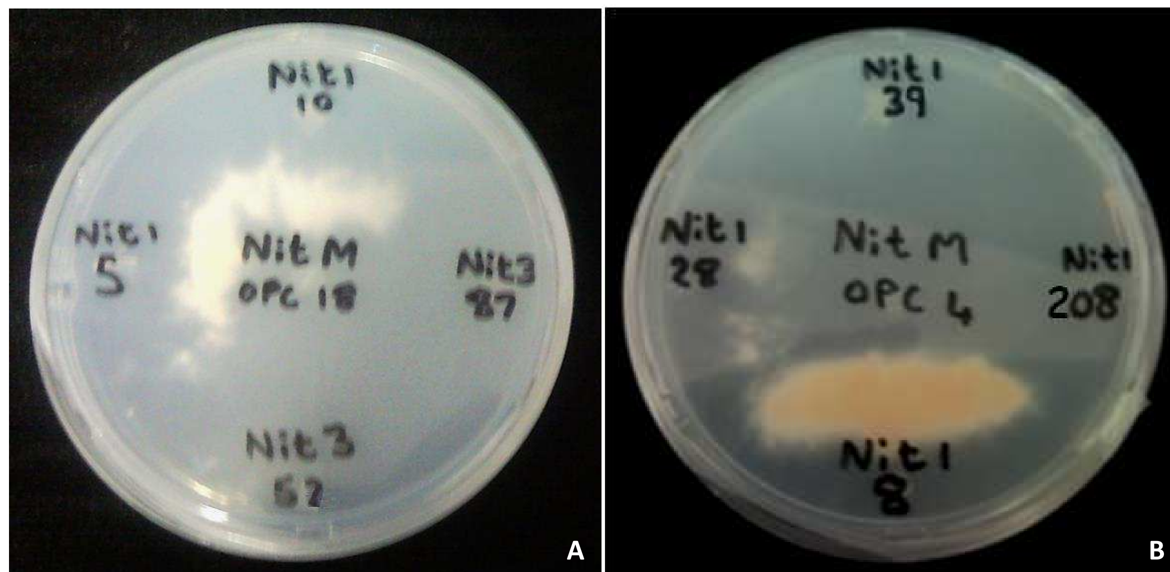

Figure 3.10 A pairing between the complementary and non-complementary mutants among different isolates. **(A)** *nit1* mutants of isolate OPC005-1-1 (1) and (10) of isolate OPC010-1-2 and *nit3* mutants of isolates (87) of isolate OPC224-3-1 and (57) of isolate OPC223-3-1 did not pair with nitM mutant (18) of isolate OPC018-M-1 in plates after 14 days of incubation at 27℃. NitM mutant OPC018-M- 1 of isolate OPC018 failed to form heterokaryons between combinations with isolates OPC224-3-1 and OPC223-3-1. The heavy, white line of growth where the two mutant colonies contact indicates heterokaryon formation. Non complementary mutants, *nit3* (OPC224 and OPC057) out groups selected from CCP OPC223-3-1 and CBS OPC224-3-1 in plates after 14 days of incubation at 27℃. **(B)** *nit1* mutant (8) of isolate OPC008-1-1, (28) of isolate OPC028-1-1, (39) of isolate OPC039-1-8 and (208) of isolate OPC208-1-1 pairing with NitM mutant (4) of isolate OPC004-M-1 in plates after 14 days of incubation at 27℃. NitM mutant OP C004-M-1 of isolate OPC004 failed to form heterokaryons between combinations with isolates OPC039-1-8 and OPC080-1-1. The heavy, pale cream/white line of growth where the OPC008-1-1mutant colonies contacts indicates heterokaryon formation, while a weak and slows complementation occurs with isolate OPC028-1-1. Non complementary mutants, *nit1* (OPC208 and OPC039) out group OPC208-1-1 selected from CCP and non-complementary from isolate OPC039-1-1 shows isolate from the same area to be different in plates after 14 days of incubation at 27℃.

Two complementary *nit* mutants (*nit1* and *nit1*, *nit1* and *nit3*, NitM and NitM) that readily formed vigorous heterokaryons with other mutants were selected as tester isolates when heterokaryons did not form after paring among NitM combination (Table 3.9; Figures 3.10 A and B) they were considered incompatible. In some cases, when *nit1* and *nit3* mutants were paired, subsequent complementation was not evident for 2 to 3 weeks due to slow growth. Normally after 3 weeks the complementation reaction was weak with little aerial mycelium. Parings of *nit1* with *nit3* mutants recovered from the same parental isolate showed successful complementation. Some *nit1* mutants were able to complement one another, as could some NitM mutants. No complementation was observed between any of the *nit3* mutants. From all *nit* mutants generated in this study no heterokaryon self incompatiblity was observed despite different chlorate concentration and different media being used. To be considered as heterokaryon self-incompatible, no complementation would have occurred between the mutants from the same isolates when paired together.

Table 3.9 Complementation reaction between nitrate non-utilizing mutants of *F. oxysporum*

	nit1	*nit3*	NitM
nit1	- or ±	± or -	+
nit3	± or -	-	+
NitM	+	+	+ or -

\+ = Complementation occurs readily;
± = weak and slows complementation occurs;
\- = no complementation occurs.

3.4 Discussion

Fusarium species identified in this study, show a significant amount of variation with respect to morphological and cultural characteristics. The variation explains the ability of *Fusarium* to colonise many diverse hosts. The taxonomy of the genus *Fusarium* is complex and difficulties are experienced in the development of a stable and widely accepted taxonomic system for the genus due to the high degree of variation (Nelson *et al.* 1994). The large number of species described before the 1900's can be accredited partly to variability in many *Fusarium* populations and to scarce criteria for restricting taxa (Nelson *et al.* 1994).

Fusarium wilt on *H. gordonii* was first described as a disease in nurseries (Kakamas and Pofadder) along the Orange River in the Northern Cape in 2004 where *H. gordonii* was cultivated in mass production for sale to pharmaceutical companies interested in the appetite suppressant compound P57 (Philippou *et al.*, 2013). All locations (Kakamas Klein Pella, Pofadder and Prieska) where *F. oxysporum* infected *H. gordonii* were collected for this study are situated within the Northern Cape. In 2004, Kakamas was the first location in the Northern Cape where *Fusarium* wilt on *H. gordonii* was detected. Later, in 2007, *Fusarium* wilt was reported in Pofadder, Northern Cape. Factors that contributed to the *Fusarium* wilt on *H. gordonii* could possibly be related to other agronomic crops displaying *Fusarium* wilt. The reasons include: pathogen has co-evolved with the plant hosts and under ideal conditions an onset of wilt occurs; pathogen was introduced and to survive on the host, it adapted, and/or environmental influence on a host plant and the ability to protect itself from wilt.

Fusarium oxysporum is difficult to remove from the soils and once it has infected a crop, measures have to be taken to control the disease thereafter. Most farmers would implement crop rotation as removing the wilted plants are not an effective method, although some farmers prefer to use chemicals and fungicides as an alternative. Some crops have been bred for *Fusarium* wilt resistance against specific *formae speciales*. Keeping soils well drained is also important in preventing the fungus in getting a footing on crops. Tomato farmers have added lime to soils to increase the pH above 6.5 - 7.0 as *F. oxysporum* spores struggle to develop at higher pH levels. This explains the lower frequencies of wilt on *H. gordonii* observed in the wild. Karroo and Kalahari soils were scattered *H. gordonii* populations grow are known to have above 6.5 pH levels and soils are well drained. *F. oxysporum* specific to *H. gordonii* was present in the soil but inoculum only increased when environmental conditions were right. When compared to the natural environment, a nursery environment is far more favourable. Nurseries use fertilizers that lower pH levels in soil and shading leads to less evaporation of water. Crop rotation during different seasons for protection against wilt is not possible with *H. gordonii* as the plant takes five years to mature.

The nurseries and farms, from which infected *H. gordonii* plants were sampled in the current study are found in the Namaqualand, Kalahari and Upper Karoo in the Northern Cape Province. Following the first report of wilt in Kakamas nursery in 2004, a second report occurred in 2007, in a nursery in Pofadder, 150 km away. Inoculum was introduced from Kakamas as the trial was done to determine if certain chemicals and fungicides could prevent infection in large populations of *H. gordonii* in nurseries. No

other outbreaks of *Fusarium* wilt on *H. gordonii* were reported thereafter. In both instances, Kakamas and Pofadder are the only known locations to have recorded outbreaks, in an area cleared for *H. gordonii* cultivations.

The present study evaluated isolates from all known locations where *H. gordonii* is cultivated in the Northern Cape Province, South Africa. One of the six identified species, *F. oxysporum*, is associated with *Fusarium* wilt on many other agronomic crops worldwide. Other *Fusarium* species in the Northern Cape Province have been observed previously and in this study, this shows the diversity of the *Fusarium* species co-existing in the same environment. *Fusarium* species (*F. culmorum*, *F. dimerum*, *F. equiseti*, *F. nelsonii*, *F. oxysporum* and *F. solani*) are associated with soils and geographic locations where other economical crops and *H. gordonii* grow (Schroers *et al*., 2009; Marasas *et al*., 1998).

There are no previous reports of *F. oxysporum* being associated with *Fusarium* wilt on *H. gordonii* in South Africa, making this the first official report. The fungus was found only in similar temperate regions and the distribution of *Fusarium* species varied when comparing locations. The *H. gordonii* grown in cultivated areas of the Northern Cape Province was hypothesised to provide natural occurring inoculum for *Fusarium* wilt on *H. gordonii*, with warm day temperatures and cooler night temperatures encouraging fungal growth development. The high percentage of *F. oxysporum* isolates at four out of the five locations in the present study may be attributed to optimal conditions created in nurseries by lowering pH of soil with fertiliser and shade net shielding the soil from sun and retaining more water in the soil. *Fusarium* wilt is soil-borne and can survive in the soils for long periods of time. Precautions for future planting in the affected areas should be reconsidered. It can also spread through infected dead plant material, so dead debris should be removed immediately to prevent overwintering of the pathogen. An alternative control method is to improve soil conditions since *F. oxysporum* spreads faster through soils that have high moisture and bad drainage. Other possible control methods include planting of resistant varieties although this is not yet feasible as *H. gordonii* resistant varieties currently do not exist. Using soil and systemic fungicides to eradicate the disease from the soil, flood fallowing and using clean seeds each year needs to be assessed to determine if these strategies could assist with preventing disease spread. This fungus can produce large numbers of macroconidia on plant debris exposed to light and given sufficient moisture. Further complicating control: *F. oxysporum* spores may be transported in two basic ways (1) short distances by means of water splashing and

planting equipment and (2) long distances by means of infected transplants and seeds (Agrios, 2005).

The controlled environment of the greenhouse proved to be very successful in identifying pathogenic isolates. Plants susceptible to wilt disease died and were not able to recover. The control treatment indicated that all isolates collected from *H. gordonii* are not pathogenic to this particular host as was the case with isolate CBS 416.90 which caused wilt disease on 50% of the carnations in treatment #11. Another observation was that isolates OPC003, OPC028 and OPC039 did not cause any wilt disease on *H. annulata,* and OPC039, although being pathogenic and causing wilt in *H. gordonii*. Two isolates, OPC004 and OPC028 did not cause any wilt disease on *H. gordonii,* although isolate OPC004 was found to cause wilt disease on *H. annulata.* Isolates referred to as non-pathogenic should be investigated in the future since using these isolate types would be beneficial to nurseries that have wilt disease. Although only one isolate (OPC101) was highly pathogenic to *H. annulata* with a 100.0% mortality rate, it was interesting to note that it had the same pathogenicity on *H. gordonii.* Non-pathogenic isolates that showed no wilt disease in *H. annulata* were two isolates OPC003.

The use of mutants to produce heterokaryons was required since no other protocol was applicable to the study of *F. oxysporum* populations associated with *H. gordonii*. Chlorate, a nitrate substitute has been used extensively for studying heterokaryosis in Fusarium (Puhalla, 1985). Generally, the growth of chlorate sensitive isolates is limited by the internal functioning and ability to reduce the chlorate to a toxic chlorite by nitrate reductase. Chlorate resistant isolates either do not utilise chlorate or are unable to reduce chlorate to chlorite (Liu and Sundheim, 1995). Nitrate non-utilising mutants are usually unable to reduce chlorate to chlorite due to an alteration at one or more of the loci that control nitrate reductase, as a result making them chlorate resistant. This was advantageous to the study and yielded many *nit* mutants. *Nit* mutants have several advantages over other autotrophic mutations. Chlorate resistant mutants appear spontaneously, with no need for mutagenesis. This bypasses problems associated with mutagenesis, such as unwanted mutations in regions of the genome other than the target gene, such as in genes affecting growth rate or even in genes responsible for vegetative compatibility or avirulence (Correll *et al.*, 1987). The mutants were fast growing, and the frequency of mutation was high.

It was quick and relatively simple to allocate the genotype of a particular mutant into a phenotypic group. Phenotypic tests were performed by placing isolates onto a variety of

nitrogen sources. The three mutant genotypes used in this study were *nit1*, *nit3* and NitM. Chlorate resistant sectors obtained depended on both the isolate and the amount of chlorate in the medium. The three *nit* mutant types were not recovered with equal incidence, with *nit1* mutants usually more than seven times as common as the NitM mutants (Table 3.7). The results showed that only two groups existed and proved the hypothesis to be wrong assuming there would be more VCGs present.

Puhalla (1985) found 16 VCGs among 21 pathogenic isolates of *F. oxysporum* with 6 different *formae specialis*. Molecular and morphological characteristics have been analysed to determine the diversity within pathogenic and non-pathogenic populations of *F. oxysporum* (Appel and Gordon, 1994; Elena and Pappas, 2002). In this study, there were 2 VCGs among 44 isolates of *F. oxysporum*. The study generated 438 *nit* mutants from the 44 wild type isolates to determine which of the two unambiguous groups they fell into. VCG group 1 was relatively diverse as it separated the virulent and non-virulent isolate and after extensive work was done in the pathology tests, it showed that the low virulent pathogens and non-pathogenic isolate OPC039 fell part of the VCG (Group 2).

Isolates recovered from soil as saprophytes are often diverse with respect to VCG. Determining vegetative compatibility between isolates is a good method to determine relatedness of populations, even though vegetative incompatibility does not necessarily determine a large genetic difference. Puhalla (1985) recommended using VCGs and stated that division between isolates was correlated with pathogenicity. Pathogens should therefore rarely take part in recombination and reassortment of the heterokaryon or vegetative incompatibility (vic) loci alleles to produce new VCG phenotypes. Therefore, VCG and pathogenicity are inadvertently correlated rather than related by a cause and effect association. Twenty five years after the original proposal a few conclusions can be drawn. Some pathogenic *F. oxysporum* f. sp. *melonis* isolates showed that there was a strong correlation between pathogenicity and VCG, with the majority of members found in one or a few VCGs (Jacobson and Gordon, 1988; Vakalounakis and Fragkiadakis, 1999). The correlation is weak, or absent, as is the case in other members of these *formae speciales* (*F. oxysporum* f. sp. *asparagi* and *F. oxysporum* f. sp. *lycopersici*) were they have a relatively high amount of VCGs (Elmer and Stephens 1989, Elias and Schneider, 1991).

Isolates obtained from wilted *Hoodia* plants had vegetatively compatible groups that were consistent with pathogenicity to the plant. The finding that a non-pathogenic isolate was vegetatively compatible with a pathogenic isolate is not novel (Appel and Gordon,

1994; Gordon and Okamoto, 1992) and proves that parasexual activity may have taken place which allows diversity and the creation of new races. The identification of pathogenic isolates and non-pathogenic isolate by techniques such as molecular markers still needs to be developed (McFadden *et al.*, 2006). Without sexual reproduction, isolates of *F. oxysporum* which were vegetatively incompatible may have become genetically isolated subpopulations (Jacobson and Gordon, 1988). If this were to occur, biological and ecological differences between VCGs in the various non-pathogenic *F. oxysporum* populations would probably be found (Correll *et al.*, 1986). Alabouvette *et al.* (1985) has demonstrated that non-pathogenic isolates of *F. oxysporum* reduced the disease rate and severity of several vascular wilt diseases induced by various *formae speciales* of *F. oxysporum*. Alabouvette (1999) stated using soils suppressive to *Fusarium* vascular wilt diseases is extremely important to be able to differentiate isolates among the non-pathogenic *F. oxysporum* isolates. Not much is understood concerning the population dynamics and ecological importance of non-pathogenic isolates of *F. oxysporum*, particularly at subspecies level. VCGs are naturally occurring genetic markers and could be used to identify and characterise the different subpopulations of *F. oxysporum* (Correll *et al.*, 1986).

Although no isolates in this study were vegetatively self-incompatible, it is possible that if more isolates were collected from areas where *H. gordonii* is found naturally this phenomenon could have been encountered. Correll (1991) examined an obstruction that forms between two vegetatively self-incompatible mutants in *F. oxysporum* f. sp. *pisi*. Jacobson and Gordon (1988) reported that HSI phenotype in *F. oxysporum* f. sp. *melonis* resulted from an isolate not being able to start or complete heterokaryon formation. The same phenomenon was observed by Correll *et al.* (1987), as heterokaryon self-incompatible phenotype in *F. verticillioides* produced fewer hyphal fusions. The authors hypothesised three possible reasons for vegetatively self-incompatible isolates, namely: (i) they produced dysfunctional enzymes associated with hyphal branching, (ii) they decreased in their amount of fimbriae production, (iii) they lacked the ability to diffuse a substance which signals nearby hyphae and stimulates anastomosis.

The present study is the first time that the pathogen causing *Fusarium*-wilt of *H. gordonii* has been analysed in terms of its population biology. The use of VCGs has proven to be a useful tool in the analysis of fungal populations and is a direct assessment of a combination of multiple genes of adaptive importance of inherited characteristic within populations. The laboratory analysis of VCGs with complementary nitrate non-utilizing

(*nit*) mutants is relatively simple method and requires basic microbiological materials (Correll *et al*., 1987). VCGs are used in determining genotypic diversity, e.g. the occurrence of different genotypes within a population, and for determining if two isolates are identical to one another. However, the use of VCG technique is not an appropriate method for population analyses of pathogenic fungi, since they are not able to differentiate different biological species or for assessing differences that occur above the species level. Although the VCG technique is able to measuring genotypic diversity, it is not able to assess the levels of allele frequencies, e.g. the frequencies of alleles at different *vic* loci. Similarly, although the VCG technique is a powerful tool for determining clonality, it cannot be used for determining relatedness. Finally, the detection of heterokaryosis may be complicated by alleles that prevent formation of a heterokaryon, even if the component isolates are vegetatively compatible, e.g. mutants at heterokaryon self-incompatibility loci. When compared with some other multilocus techniques, such as DNA fingerprint probes, VCGs require less technical laboratory equipment but do not sample as many loci and require more effort to interpret (Leslie, 1993).

Nitrogen responsive genes in *F. oxysporum* (Divon *et al*., 2006) have been reported to be involved in regulating fungal nutrition genes and fitness during the development of the disease. An understanding of the role *vic* loci have in the pathogen's life cycle is important and future studies may lead to the development of 'universally compatible' isolates (Leslie, 1996). Such isolates could be used as delivery mechanism for mycoviruses or other intracellular biocontrol agents into a population that was otherwise so subdivided by VCG as to make biological control by such transmission impossible (Kistler, 1997). For additional direct pathogen control, agents that simulate an incompatible reaction might be used to induce the physiological killing response, providing a novel set of antifungal compounds. If the response is specific, it could possibly eliminate pathogenic isolates while causing no harm to the beneficial non-pathogenic isolates. If the response mechanism is more specific, then it might be possible to identify a specific species or a cluster of species specific to the antifungal agents (Leslie, 1996). Essentially these compounds would activate a 'kill off' response in the target, and may perhaps be environmentally friendly, moderately specific in their action, and appropriate in environments other than plant protection, e.g. medical and veterinary uses. Studies of vegetative compatibility have greatly increased our knowledge of fungal pathogens and a study of the *vic* loci will probably further aid our understanding (Correll, 1991). Although VCGs and molecular techniques have improved our understanding of the pathology, biology and race relationships of *F. oxysporum*

(Correll, 1991 our knowledge and understanding of this complex fungal pathogen is far from complete.

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Chapter 4

Genetic diversity of *Fusarium oxysporum* associated with wilt of *Hoodia gordonii*

4.1 Introduction

Fusarium oxysporum is a widely distributed pathogenic soil borne fungus. This pathogen infects many plant species belonging to different families, specifically targeting hosts by its adapted *formae speciales* (Booth, 1975; Beckmann, 1987; Kistler, 2001). Although more than 150 *formae speciales* have been identified (Hawksworth *et al.*, 1995; O'Donnell and Cigelnik, 1997; Baayen *et al.*, 2000; O'Donnell *et al.*, 2009), only a few of these have been investigated using discrete DNA sequence data to determine the evolutionary origin (O' Donnell *et al.*, 1998). Isolates of *F. oxysporum* f. sp. *cubense*, causing Panama disease of banana and *F. oxysporum* f. sp. *melonis* causing wilt on melons have been shown to have developed from more than one ancestral type (O' Donnell *et al.*, 1998). These results suggest that the genetic origin of each *formae speciales* developed from different types of ancestral isolates within the FOSC. Similarly, isolates within the FOSC, *F. oxysporum* f. sp. *asparagi*, *dianthi*, *gladioli*, and *lini* confirmed the multiple evolutionary origins based on gene sequences (Baayen *et al.*, 2000). They also reported that *F. oxysporum* f. sp. *lilii* and *tulipae* appeared to have developed from the same ancestral type. The FOSC has many *formae speciales* causing unique symptoms on various economically important plants, such as, *Fusarium* wilt, *Fusarium* yellows, stem rot, root rot, Bayoudh disease, dieback and Panama disease. Among the many different diseases caused by this fungus, Fusarium wilt is one of the economically most important diseases (Mai and Abawi, 1987; Correl, 1991). In 2004, wilt disease was first observed on *H. gordonii* in South Africa and the causal agent was identified as *F. oxysporum* (Philippou *et al.*, 2013).

F. oxysporum, as the primary causal agent of *Fusarium* wilt, resides in the section *Elegans* (Wollenweber and Reinking, 1935). Morphological identification of this species is difficult. The most important limitations of morphological identification of *F. oxysporum,* is the inability to differentiate between and amongst the different genetically diverse *formae speciales* which share common morphological features (Taylor *et al.*, 2000). In order to accurately identify this species and to support results based on morphology, different techniques are combined to characterise *F. oxysporum* isolates. These

techniques include pathogenicity tests, vegetative compatibility tests, DNA sequencing and DNA fingerprinting analyses. Of these, multi-gene phylogenetic approaches appear to be the most informative approaches and have resolved many *Fusarium* species (Nirenberg and O'Donnell, 1998; Zeller *et al.*, 2003; O'Donnell *et al.*, 2004), as well as groups within the FOSC (O'Donnell *et al.*, 2009). However, O'Donnell *et al.* (1998) first showed that isolates within a given *formae speciales* did not necessarily originate from the same evolutionary source. Fourie *et al.* (2009) showed that the *F. oxysporum* complex represents at least eight distinct lineages based on the combined analysis of four gene regions the TEF and the mitochondrial small subunit (mtSSU) rRNA genes, the rRNA intergenic spacer (IGS) region and a repeat region encoded in the mitochondrial (mtR) genome. Kawabe *et al.* (2005) confirmed the multiple evolutionary origin of *F. oxysporum* f. sp. *lycopersici* with IGS, MAT and endopolygalacturonase sequences. The worldwide collection of isolates formed three evolutionary clades, each of which contained single or closely related VCGs. Bogale *et al.* (2006) sequenced the partial translation TEF and mtSSU genes of 32 *F. oxysporum* isolates from Ethiopia and separated these isolates into three groups that corresponded with the three major clades of *F. oxysporum* previously defined by O'Donnell *et al.* (1998).

The use of AFLPs developed by Vos *et al.* (1995) to screen the whole genome together with sequencing which targets specific gene regions such as TEF and β-tub region has been used for taxonomic and phylogenetic analysis for various *Fusarium* species (Majer *et al.*, 1996; Baayen *et al.*, 2000; Kiprop *et al.*, 2002; Abdel-Satar *et al.*, 2003; Bogale*et al.*, 2006). AFLP offers two main advantages over other DNA based techniques used in taxonomic and phylogenetic studies of *F. oxysporum*. Firstly, it examines the entire genome rather than considering specific regions as is the case with nucleotide sequence and PCR-RFLP analyses (Majer *et al.*, 1996; Baayen *et al.*, 2000). Secondly, AFLP is highly reproducible unlike RAPD analyses (Vos *et al.*, 1995).

The occurrence of *Fusarium* species, particularly *F. oxysporum* has not been well documented on South African indigenous succulent plants, especially *H. gordonii*. The only detailed reports of a *Fusarium* species associated with disease on *Hoodia* was made by Swart (2008) and with *F. dimerum* as causal agent (Lamprecht *et al.*, 2008; Schroers *et al.*, 2009). The disease caused by *F. dimerum* was associated with black lesions and dry rot in *H. gordonii*. No prior genetic variation information is known for *F. oxysporum* associated with *Fusarium* wilt disease on *H. gordonii.*

The objectives of this study were to assess the phylogenetic relationships and genetic diversity of *F. oxysporum* isolates collected from soil and plant material associated with *Fusarium* wilt on *H. gordonii* using nucleotide sequences and AFLP analyses. It was hypothesised that 1) *F. oxysporum* isolates associated with wilt on *H. gordonii* form part of the FOSC even though multiple evolutionary origins based on gene sequences were suggested, and 2) that a new *formae speciales* within the FOSC had been identified based on DNA analyses from AFLP and gene sequences generated.

4.2 Materials and methods

4.2.1 Collection of fungal isolates

A total of 131 soil and plant tissue samples were collected from five different locations in the Northern Cape Province, South Africa as described in Chapter 3 (section 3.2.1). Locations were selected based on the history and observations of *Fusarium* wilt on *H. gordonii* over seven years (2004 to 2010). Forty four *F. oxysporum* isolates were obtained from these soil and plant tissue samples. Table 4.1 lists all *F. oxysporum* isolates from various substrates associated with wilt on *H. gordonii*, including *F. oxysporum* f. sp. *dianthi* (CBS 416.90) from Israel and a *Fusarium sp*. (NRRL 25221) from genebank database as reference isolates. All samples obtained in this study were collected from soils and plant tissue from nurseries or commercial plantings. The first *F. oxysporum* isolate was collected during 2004 when wilt on *H. gordonii* plants in Kakamas was initially observed (Philippou *et al*., 2013).

4.2.2 Purification of cultures

Single spore subcultures were obtained as described in Chapter 3 (section 3.2.3).

4.2.3 DNA isolation

All isolates morphologically identified as *F. oxysporum* were subcultured onto ten PDA plates per isolate. Isolates were grown on PDA plates incubated in a 12 h light/dark cycle for 7-10 days at 25℃ for optimal growth. After ten days mycelia were scraped from the surface of cultures from PDA plates and transferred to 50 ml sterile tubes. Mycelium material was freeze dried for three days at -60℃ using a Viritis Advantage Freeze Mobile II (New York, USA). Freeze-dried samples were stored at -80℃ until further use.

Table 4.1 ***F. oxysporum*** **isolated from various locations and substrates associated with *H. gordonii***

Isolate ID	Location	Year	Species	Substrate	Health of plant
OPC001	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC002	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC003	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC004	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC005	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC006	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC007	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC008	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC009	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC010	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC011	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC012	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC013	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC014	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC015	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC016	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC017	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC018	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC019	Prieska	2007	*F. oxysporum*	Soil	Wilt
OPC020	Prieska	2009	*F. oxysporum*	Root	Wilt
OPC027	Prieska	2009	*F. oxysporum*	Root	Wilt
OPC028	Prieska	2009	*F. oxysporum*	Root	Wilt
OPC033	Klein Pella	2009	*F. oxysporum*	Root	Wilt
OPC034	Klein Pella	2009	*F. oxysporum*	Root	Wilt
OPC035	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC036	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC037	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC039	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC040	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC041	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC042	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC043	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC044	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC045	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC046	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC047	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC048	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC049	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC050	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC051	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC052	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC053	Pofadder	2007	*F. oxysporum*	Soil	Wilt
OPC055	Prieska	2010	*F. oxysporum*	Stem	Wilt
OPC101	Kakamas	2004	*F. oxysporum*	Stem	Wilt

Before isolation of total genomic DNA, the freeze-dried fungal material was homogenised using Qiagen's TissueLyser (Haan, Germany). A piece of the material was transferred to a 2 ml micro-centrifuge tube with two 5 mm stainless steel ball bearings and fungal tissue

was grounded to a fine powder for 30 s at 30 r/s. Total genomic DNA was extracted from isolates using the CTAB (hexadecyltrimethylammonium bromide) method (Saghai-Maroof *et al.*, 1984). CTAB extraction buffer with a volume of 750 µl (100 mM Tris-Cl (tris (hydroxymethyl) aminomethane), pH 8.0; 20 mM EDTA (ethylenediaminetetra acetate), pH 8.0; 1.4 M NaCl; 2% (w/v) CTAB; 0.2% (v/v) β-mercapthoethanol) was added to approximately 250 µl of grounded fungal tissue and incubated at 65℃ for 1 h. An additional 500 µl of chloroform:isoamylalcohol [24:1 (v/v)] was added and was mixed well followed by centrifugation at 12 000 *g* for five minutes at 5℃. DNA from the aqueous phase was precipitated with 0.66 volumes isopropanol and incubated at room temperature for 20 min followed by centrifugation at 12000 *g* for five minutes. The supernatant was discarded and tubes were drained upside down. The DNA pellet was washed by adding 500 µl ice-cold 70% (v/v) ethanol, followed by incubation for 20 min at room temperature. Samples were centrifuged at 12000 *g* for five minutes at 5℃ and the supernatant discarded. The DNA pellets were air-dried for one hour and re-suspended overnight in 200 µl TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) at 4℃.

RNaseA (0.1 mg/ml) was added and incubated at 37℃ for two hours. DNA was precipitated with 20 µl 7.5 M ammonium acetate and an equal volume chloroform:isoamylalcohol (24:1) and centrifuged at 12000 *g* for five minutes at 5℃. DNA from the aqueous phase was precipitated overnight at 4℃ with 500 µl ice-cold 100% absolute ethanol. Following overnight incubation, tubes were centrifuged at 12000 *g* for 15 min 5℃ and the supernatant discarded. DNA was washed twice with 500 µl ice-cold 70% (v/v) ethanol through centrifugation at 12000 *g* for ten minutes at 5℃. The supernatant was discarded and the DNA air-dried at room temperature and re-suspended in 50 µl TE buffer, pH 8.0.DNA quantity and quality were determined from a 0.8% (w/v) agarose gel with electrophoresis at 80 V in UNTAN (40 mM Tris-Cl; 2 mM EDTA, pH adjusted to pH 7.4 with acetic acid) buffer. DNA was visualised with ethidiumbromide staining under UV light. The concentration of the DNA samples was determined by using a UV spectrophotometer and measuring absorbance at A_{260} and A_{280}. Samples were diluted to a working concentration of 200 ng/µl for subsequent experiments.

4.2.4 Sequencing

F. oxysporum was morphologically identified as the *Fusarium* wilt pathogen on *H. gordonii* in South Africa (Philippou *et al.*, 2013) and occurred at high frequencies across all four sample sites (Chapter 3). The identity of these 44 isolates as

F. oxysporum was further confirmed by using PCR assays. Fragments of three nuclear genes, TEF, β-tub and cmd were sequenced. Amplification and sequencing primers used in this study are listed in Table 4.2.

Table 4.2 Oligonucleotides used in this study

Gene	Primer	Sequence	Size (bp)
Elongation Factor 1-α[a]	EF1	5'-ATGGGTAAGGA(A/G)GACAAGAC-3'	656
	EF2	5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'	
β-tubulin[b]	Bt2a	5'-GGTAACCAAATCGGTGCTGCTTTC-3'	290
	Bt2b	5'-ACCCTCAGTGTAGTGACCCTTGGC-3'	
Calmodulin[c]	CLOX1	5'-CAGCAAAGCATCAGACCACTATAACTC-3'	534
	CLOX2	5'-CTTGTCAGTAACTGGACGTTGGTACT-3'	

Primers described in [a] O'Donnell *et al.* (1998), [b] Glass & Donaldson (1995) and [c] O'Donnell *et al.* (1998)

Genomic DNA was diluted to 1 ng/µl and was amplified using PCR. PCR mixtures contained reaction buffer (1.5 mM $MgCl_2$, 5 µl 10 x Buffer solution, 37.5 µl dH_2O, 0.02 mM of each dNTP, 0.05 U/µl Taq Polymerase (Roche, USA), 100 pmol/µl of each forward and reverse primers TEF (EF1 and EF2) or β-tub (Bt2a and Bt2b) or Cmd (CLOX1 and CLOX2), respectively (Table 4.2) and 1 µl template DNA. DNA products were separated by using agarose gel electrophoresis at 80 V for 45 min and documented using BioRad Gel Doc XR system. Agarose gels consisted of 1% (w/v) agarose in TAE buffer (0.1 M Tris; 0.05 M EDTA; pH 8; 0.1 mM glacial acetic acid) stained with Goldview™ (SBS Genetech) according to the manufacturer's recommendations. PCR products of interest were isolated from agarose gels for further studies and was visualised using a Dark Reader®. These PCR products were purified from agarose gels with the use of the GFX PCR DNA and Gel Band Purification Kit from GE Healthcare UK, Ltd. These purified PCR products were sequenced separately for each primer (EF1, EF2, Bt2a, Bt2b, CLOX1 and CLOX2) with different PCR protocols as required for the three primer sets (Table 4.2). These PCR products were sequenced using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) according to the manufacturer's recommendations. Nucleotide composition was analysed with an Applied Biosystems 3130xl Genetic Analyzer.

The TEF (~656 bp), β-tub (~290 bp) Cmd (~534 bp) gene fragments were PCR amplified using primer pairs EF1 and EF2 (O'Donnell *et al.*, 1998), Bt2a and Bt2b (Glass and Donaldson, 1995) and CLOX1 and CLOX2 (O'Donnell *et al.*, 2000), respectively for each

isolate. PCR mixtures contained reaction buffer (10 mM Tris-HC1 (pH 8.3), 2.5 mM $MgCl_2$, and 50 mM KCl), 2.5 µM of each dNTP, 0.20 µM of each primer, 0.05 U/µl Taq Polymerase (Roche, USA), and approximately 1 ng/µl template DNA. The PCR cycling conditions consisted of an initial denaturation at 94℃ for 4 min followed by 35 cycles of denaturation at 94℃ for 30 sec for EF1 and EF2, as well as Bt2a and Bt2b, and denaturation at 94℃ for 50 sec for CLOX1 and CLOX2 . Annealing for 30 sec at 56℃ for EF1 and EF2 and for 30 sec at 68℃ for Bt2a and Bt2 b compaired to an annealing of 1 min at 61℃ for CLOX1 and CLOX2, then extension at 72℃ for 30 sec. PCRs were terminated after a final extension at 72℃ for 10 m in for (EF1 and EF2) and (Bt2a and Bt2b) and a final extension at 72℃ for 7 min for (CLOX1 and CLOX2). PCR products were purified using the QIAquick PCR purification kit (QIAGEN, GmbH. Germany), and sequenced in both directions using the same primers as for the respective PCRs. For this purpose, the BigDye terminator sequencing kit (Version 3.1, Applied Biosystems. USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used. All amplification and sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems).

4.2.4.1 Phylogenetic analysis

Both forward and reverse strands of the specified genes regions of interest were sequenced to determine the exact nucleotide sequence. TEF, β-tub and Cmd sequences were aligned for each isolate using the program Clustal W 1.83 plug-in to perform multiple alignments (Thompson *et al.*, 1997) in the software Geneious 6.1.4 (Drummond *et al.*, 2011). The alignment was edited manually using the sequence alignment editing program Geneious 6.1.4 (Drummond *et al.*, 2011) and all mutations checked against the electropherograms. Genetic diagrams were constructed with the use of the software Geneious 6.1.4 (Drummond *et al.*, 2011). Sequin version 11.90 application from NCBI (National Centre for Biotechnology Information; www.ncbi.nih.gov) was used to infer amino acid sequences from nucleotide sequences. The amino acid sequences were then compared with relevant amino acid sequences in the NCBI database (GenBank) and Fusarium-ID (http://fusarium.cbio.psu.edu) to determine the presence and positions of introns for positive identification with known isolates within the database. The *F. oxysporum* sequences from this study were aligned with two published TEF, β-tub and Cmd sequences obtained from GenBank. Geneious 6.1.4 (Drummond *et al.*, 2011) software was used to estimate phylogenetic relationships for each of the gene sequence datasets. For this purpose, Tree-alignments was set at 70% similarity with a gap penalty value of 12 and gap expansion value of 3, together with a global alignment with free end

gaps.Cluster analysis using the neighbour joining method based on the unweighted pair-group method using arithmetic averages (UPGMA) was conducted with a nucleotide sequence taxonomy and multivariate analysis system Geneious 6.1.4 (Drummond *et al.*, 2011) software with the data in a nucleotide data matrix to construct a rooted phylogram. UPGMA is a hierarchical algorithm used for clustering isolates into similar groups. Cluster analysis information is sensitive to closely related individuals; therefore the constructed phylogram illustrates the relationship of the *F. oxysporum* isolates to the 2 reference isolates based on the three gene sequence variations. Phylograms were drawn based on the Tamura-nei genetic distance model.

4.2.5 AFLP analyses

A total of 44 isolates were identified, based on both morphology and sequencing of three different genes, as *F. oxysporum* and included in the genetic analysis. These isolates included one isolate from Kakamas, two isolates from Klein Pella, 23 isolates from Prieska, 18 isolates from Pofadder and six reference isolates provided by ARC-PPRI, CBS, Dr Kerry O' Donnell and MRC. The six reference isolates included representatives of one *formae speciales* and two *F. oxysporum* isolates from soil (Table 4.3). AFLP analysis was performed using primer pair combinations *Eco*RI-TG/*Mse*I-TT, *EcoR*I-TC/*Mse*I-AA and *Eco*RI-TC/*Mse*I-AG. AFLPs have been used to distinguish *Fusarium*s species and phylogenetic groups in a number of *Fusarium* species (Baayen *et al.* 2000, Zeller *et al.* 2003, Belabid *et al.* 2004) including *F. oxysporum* (Baayen *et al.* 2000, Belabid *et al.* 2004). Primers and adapters (Table 4.4) were synthesised by Integrated DNA Technologies Inc, Coralville, IA, USA. Oligonucleotides used as adapters for AFLP analysis were purified by the manufactures using polyacrylamide gel electrophoresis (PAGE). Adapters were prepared by mixing equimolar amounts of the single-stranded oligonucleotides, heating for 10 min at 65℃ in a w ater bath and then allowing the mixture to cool down to room temperature. AFLP analysis was performed according to Vos *et al.* (1995) with minor modifications as described by Herselman (2003).

4.2.5.1 Restriction digestion and ligation of genomic DNA

Two restriction enzymes, *Eco*RI (six bp recognition site) and *Mse*I (four bp recognitionsite) were used to digest genomic DNA from the *Fusarium* isolates. The 50 µl reaction mixture consisted of approximately 1 µg of genomic DNA of each fungal isolate was digested with 4 U *Mse*I (New England Biolabs, Ipswich, MA, USA) and 1 x *Mse*I buffer [50 mM NaCl, 10 mM Tris-Cl pH 7.9, 10 mM MgCl2, 1 mM dithiothreitol (DTT)] for

five hours at 37℃. After *Mse*I digestion, restriction fragments were further digested with 5 U *Eco*RI (Roche Diagnostics, Mannheim, Germany) and NaCl to a final concentration of 100 mM, followed by overnight incubation at 37℃ . A 10 µl reaction mixture containing 50 pmol *Mse*I-adapter, 5 pmol *Eco*RI-adapter, 1 U T4 DNA Ligase (USB Corporation, Cleveland, Ohio, USA), 0.4 mM adenosine 5'-triphosphate (ATP) and 1x T4DNA Ligase buffer (66 mM Tris-Cl pH 7.6, 6.6 mM MgCl2,10 mM DTT, 66 µM ATP) was added to the 50 µl restriction digestion mix to ligate the adapters. The ligation reaction mixture was incubated overnight at 16℃.

4.2.5.2 Pre-amplification reactions

Pre-amplification reactions were performed in 20 µl reaction mixtures containing 5 µl template DNA from undiluted restriction/ligation mixtures, 30 ng of each pre-amplification primer [*Mse*I-primer+0 and *Eco*RI-primer+0 (Table4.4)], 1 x Promega polymerase buffer, 2 mM $MgCl_2$, 200 µM 2'-deoxynucleoside 5'-triphosphates (dNTPs) and 0.02 U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA). All PCR-amplifications were performed using a PCR thermal cycler (DNA Engine DYAD™, BIO-RAD, USA) using the following cycling programme: an initial denaturation step at 94°C for five minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for one minute and 72°C for one minute and a final 10 min elongation at 72°C and then held at 10°C. The quality and quantity of pre-amplification

Table 4.3 Reference isolates representing three *F. oxysporum* isolates and two other *Fusarium* species used in this study

Study code	Species name	Host or substrate	Geographic origin
OPC205	*F. oxysporum*	Wheat rhizosphere	Free State, SA
OPC223	*F. oxysporum*	Wheat rhizosphere	Free State, SA
OPC224	*F. oxysporum* f. sp. *dianthi*	Carnation	Bet Dagan, Isreal
OPC225	*F. verticillioides*	Maize	Pretoria, SA
OPC226	*F. graminearum*	Wheat	USA/Hungary/Netherlands/Iran
OPC227	*F. graminearum*	Wheat	USA/Hungary/Netherlands/Iran

Table 4.4 Sequences for adapters and primers used for ligation reactions, preselective and selective amplification of *F. oxysporum* isolates (Baayen *et al.* 2000, Belabid *et al.* 2004)

Primer	Type	Sequence (5'-3')
MseI adapter-F	Adapter	GACGATGAGTCCTGAG
MseI adapter-R	Adapter	TACTCAGGACTCAT
EcoRI adapter-F	Adapter	CTCGTAGACTGCGTACC
EcoRI adapter-R	Adapter	AATTGGTACGCAGTCTAC
MseI primer+0	Pre-selective	GACGATGAGTCCTGAGTAA
EcoRI primer+0	Pre-selective	CTCGTAGACTGCGTACCAATTC
MseI primer+2	Selective	GACGATGAGTCCTGAGTAA**NN**
		NN=AA, AG or TT
EcoRI primer+2	Selective	CTCGTAGACTGCGTACCAATTC**NN**
		NN=TC or TG

reactions were estimated by separation through a 1.5% (w/v) agarose gel in 1 x UNTAN buffer at 80 V for one hour. Based on electrophoresis results, pre-amplification reactions were diluted accordingly (1:10 to 1:50) with 1 x TE buffer (pH 8.0) prior to selective amplification.

4.2.5.3 Selective amplification reactions

The pre-selective amplified DNA was used as the template for the AFLP analysis. The selective PCR reactions were performed using three primer combinations, namely *Eco*RI-AA/*Mse*I-AT, *EcoR*I-CC/*Mse*I-CG and *Eco*RI-TG/*Mse*I-TT. Reactions contained 5 µl pre-amplified DNA, 1x Promega *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 100 µg/ml bovine serum albumin, 30 ng of *Mse*I primer+2 (Table 4.3), 30 ng of *Eco*RI primer+2 (Table 4.4) and 0.75 U Go*Taq*® Flexi DNA polymerase (Promega) in a final volume of 20 µl. The selected primers have been used for fingerprinting to distinguish within the species complex and phylogenetic groups in *Fusarium oxysporum* (Baayen *et al.* 2000; Belabid *et al.* 2004; Bogale *et al.* 2006). The AFLP-PCR amplification programme was: 94℃ for five minutes followed by 94℃ for 30 seconds, 65℃ for 30 seconds, decreasing by 1℃ every cycle and 72℃ for one minute for nine cycles followed by 94℃ for 30 seconds, 56℃ for 30 seconds and 72℃ for one minute for 25 cycles and one cycle of 72℃ for two minutes followed by a 10℃ hold.

4.2.5.4 Visualisation of amplified fragments

The AFLP-PCR products were separated on a 5% (w/v) denaturing polyacrylamide gel [19:1 acrylamide:bis-acrylamide, 7 M urea, 1x TBE Buffer (89 mM Tris-Cl, 89 mM boric acid, 2.0 mM EDTA)]. The amplified reactions were mixed with equal volumes formamide loading dye [98% (v/v) de-ionised formamide, 10 mM EDTA (pH 8.0), 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol]. These reactions were denatured by incubation for five minutes at 94℃. After denatura tion, mixtures were immediately placed on ice prior to loading. Each PAGE gel contained at least 60 individuals, which represented one primer pair combination. Samples were compared to the *F. oxysporum* reference isolates (Table 4.3), which were used as standards in this experiment and fragment sizes were determined by comparison with a 100 bp DNA ladder (Promega). Samples were set up and loaded arbitrarily to enable unbiased scoring. Electrophoresis was performed at constant power of 80 W for approximately two hours.

The separated AFLP-PCR products were visualised by silver staining by following the Silver Sequence™ DNA Sequencing System manual (Promega). After the final wash step, stained gels were left overnight in an upright position to air dry and then photographed. The dried stained PAGE gel was positioned on photographic paper (Ilford Multigrade IV RC) the same size as the gel and exposed to a dim white light for approximately 20 seconds. The fragment sizes were determined by comparison with a 100 bp DNA ladder (Promega). Scorable polymorphic AFLP bands, ranging from 200bp to 800 bp were scored manually as 1 when present and 0 when absent in each individual and used to construct a binary data matrix. Scored data were used to construct a data matrix for statistical analysis. Homologous AFLP-PCR fragments in different individuals were assumed to represent the same allele.

4.2.5.5 Data analyses

4.2.5.5.1 Analysis of genetic variance

Cluster analysis using the sequential agglomerative hierarchical nested cluster analysis (SAHN) clustering method based on the the unweighted pair-group method using arithmetic averages (UPGMA) was conducted with a numerical taxonomy and multivariate analysis system (NTSYSpc) version 2.21n software (Rohlf, 2000) with the data in a binary data matrix to construct a rooted dendrogram. UPGMA is a hierarchical algorithm used for clustering isolates into similar groups. Cluster analysis information is

sensitive to closely related individuals; therefore the constructed dendrogram illustrates the relationship of the *F. oxysporum* isolates to the six reference isolates based on AFLP allele variation. Genetic similarities of isolates were compared by using the DICE coefficient method (Dice, 1945). Cophenetic correlation coefficients were calculated by using COPH and MXCOMP procedures as implemented in NTSYSpc to measure the goodness of fit of the cluster analysis to the similarity based analysis of the binary data matrix. The goodness of fit (r) value indicates a very good fit if $r \geq 0.9$, a good fit with $0.8 \leq r < 0.9$, a poor fit with $0.7 \leq r < 0.8$ and a very poor fit with $r < 0.7$ (Rohlf, 1997).

Allelic polymorphic information content (PIC) was done as it measures the information from data generated by genetic markers (Botstein *et al.*, 1980). PIC for AFLP dominant markers was calculated using the equation:

$$PIC = 1 - [f^2 + (1 - f)^2]$$ (De Riek *et al.*, 2001)

The “f” in the equation, is the frequency of the marker in the data set. Only polymorphic markers were used to display PIC distribution. PIC values of all polymorphic fragments for each primer pair were averaged to give the PIC value for the primer pair. PIC evaluates polymorphism of a marker by characterising the efficiency of each primer for detecting polymorphic loci (Shete *et al.,* 2000).

4.3 Results

4.3.1 Sequencing analyses

The 44 isolates identified as *F. oxysporum* based on morphology (Chapter 3) were confirmed as *F. oxysporum* with PCR sequences from TEF, β-tub, Cmd genes. The reference isolates include isolates from Genbank*, F. oxysporum* f. sp. *dianthi* and *Fusarium* sp. (NRRL 25221) was included in the sequencing analysis, as controls. The results were consistent with the morphological characterisation of all of these isolates as *F. oxysporum*. *F. oxysporum* f. sp. *dianthi* showed to be 99.8% similar to isolate OPC101 using TEF data however this was not the case with the other two gene sequences. Only these *F. oxysporum* isolates were included in the subsequent assays.

Sequence analysis of the aligned sequences of the 44 isolates of *F. oxysporum* used in this study, identified two TEF haplotypes (Figure 4.1 A), one haplotype for β-tub (Figure 4.1 B) and one haplotype for Cmd (Figure 4.1 C). Sequences of TEF contributed most of

the phylogenetic signal (four and two informative characters or synapomorphies, respectively); all mutations were identified in the coding region of the gene. Sequences from β-tub and Cmd from all isolates were identical and none showed any mutations confirming how highly conservative these genes are. Phylograms based on UPGMA analysis of the sequence data from each of the three loci, rooted with a sequence of *Fusarium* sp. (NRRL 25221) by the out-group method, are shown in Figure 4.1.

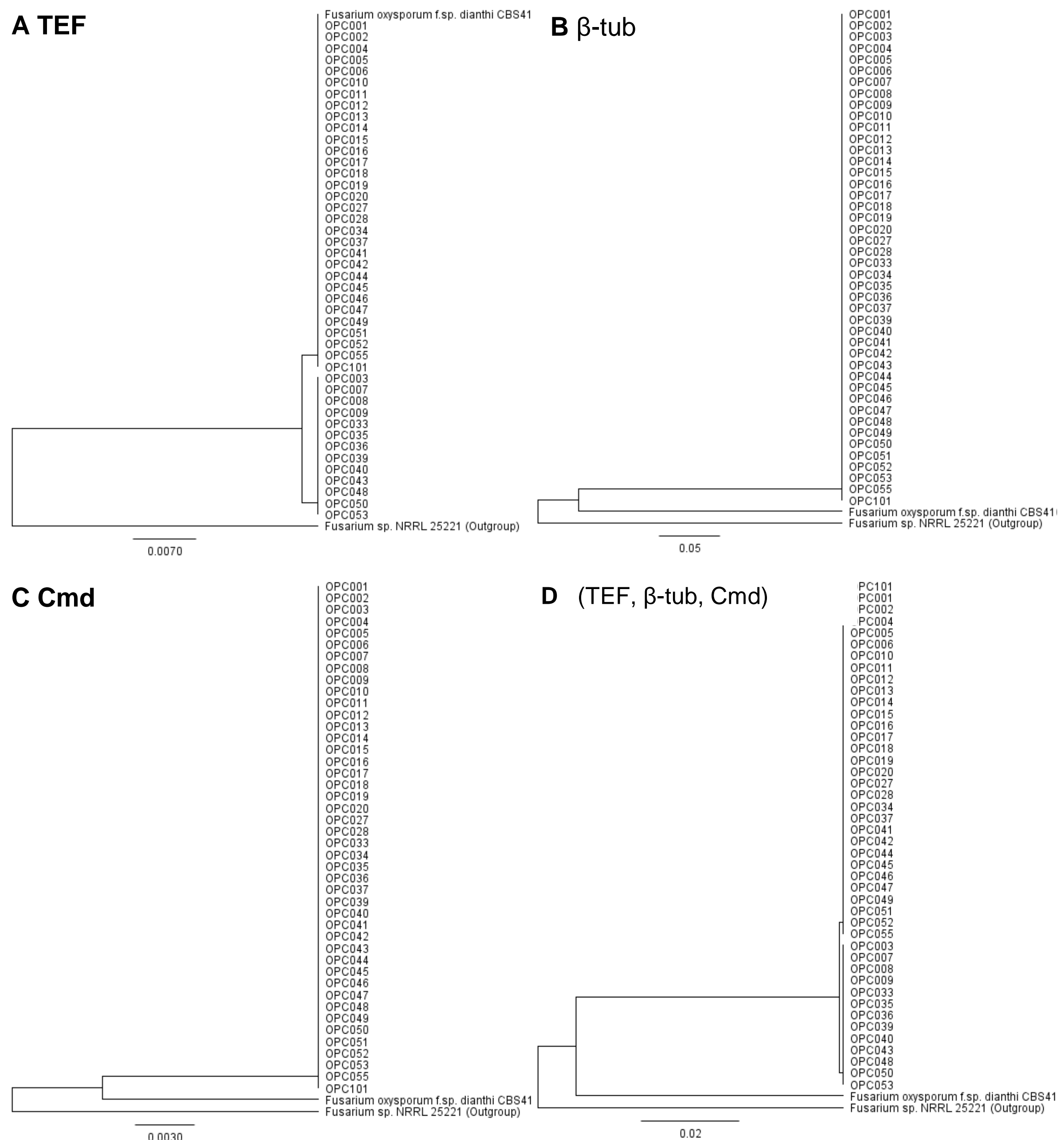

Figure 4.1 Phylograms inferred from UPGMA analysis of sequence data from each of the three loci and combined loci rooted with a sequence of *Fusarium* sp. (NRRL 25221). Numbers by nodes represent the similarity consensus of 70%. Sequences were identified from Genbank using BLAST. Sequences were downloaded then aligned using the Geneious Aligner and a phylogenetic tree built using PhyML. All of these steps were performed within Geneious Basic. **A**, Translation elongation factor 1 alpha (TEF) **B**, Beta tubulin (β-tub) **C**, Calmodulin (Cmd) **D**, Consensus tree inferred from combined analysis of translation elongation factor 1 alpha, beta tubulin and calmodulin gene sequences.

The same underlying phylogeny appears to have been observed in each of the gene genealogies; since two distinct clades in TEF were observed and one group for both β-tub and Cmd. The sequences of the isolates from the two genes, β-tub and Cmd, respectively have shown to be similar to each other. Even though two separate clades can be observed in the TEF phylogram, the isolates in each of these clades has shown 100% similarity. No homoplacy was observed because all three phylogram trees have a consistency index of 1.0.

Results of the combinability test implemented in Geneious 6.1.4 software (Drummond *et al.*, 2011) indicated that the sequences of the three genes could be analysed as a combined data set ($P = 1$ when each of the three data sets was constrained to the 70% similarity consensus of the other partitions). The UPGMA analysis of the combined sequence data from the 44 isolates of *F. oxysporum* (4 synapomorphies and 2 automorphies) yielded a single consensus phylogeny tree (Figure 4.1 D). *F. oxysporum* isolates were resolved as two distinct groups. Group 1 contained 33 isolates from all locations. Group 2 consisted of 11 isolates from three of the locations (Klein Pella, Pofadder and Prieska) found in soil and root samples.

4.3.2 AFLP analyses

Evaluation of the genetic diversity within the identified group of *F. oxysporum* isolates associated with wilt on *H. gordonii* were done using AFLPs as it is highly informative and reproducible. We identified 89 polymorphic AFLP fragments across the 44 *F. oxysporum* isolates. These fragments were between 200 and 800 bp in length. The three primer pair combinations used for selective amplification generated 90 scorable fragments at average per primer pair, of which 28 (31.1%) to 31 (34.4%) were polymorphic. An example of an AFLP fingerprint generated using primer pair combination EcoRI-TC/MseI-AA is given in Figure 4.2.

The AFLP primer combinations *Eco*RI-TG/*Mse*I-TT and *Eco*RI-TC/*Mse*I-AA yielded the highest (31 fragments) number of polymorphic fragments and the smallest number of polymorphic fragments (28) were generated by primer combination *Eco*RI-TG/*Mse*I-AG. The PIC values for two of the three primer combinations were similar, while the third primer combination was slightly higher than the two similar primer combinations.

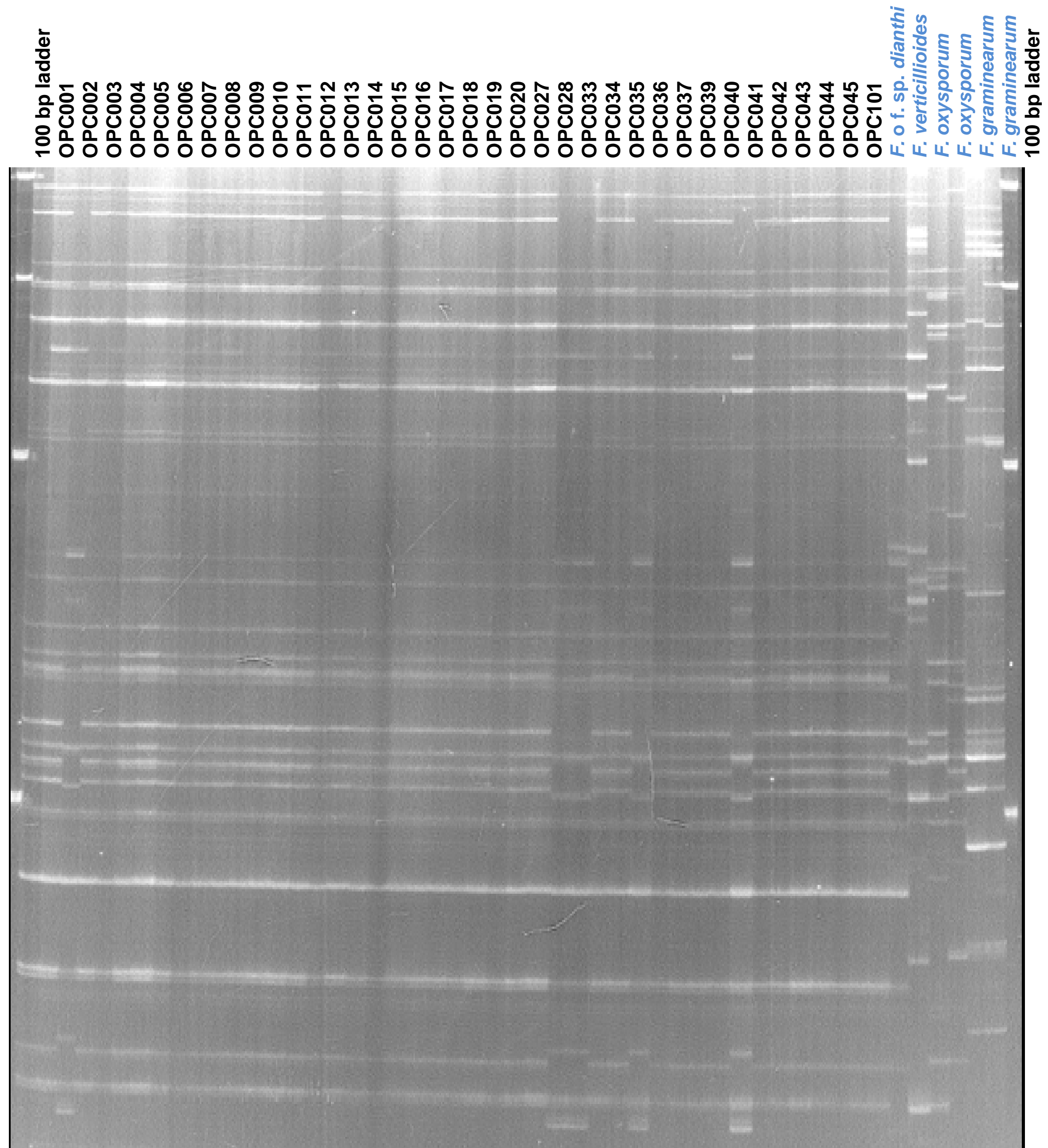

Figure 4.2 AFLP fingerprint generated using primer pair combination *Eco*RI-TC/*Mse*I-AA. OPC codes represent the isolate profiles of the 44 *F. oxysporum* isolates from the four sampled locations. Reference isolates indicated in blue.

These PIC values in spite of the significantly low range in the number of polymorphic fragments indicate that a primer pair producing the highest number of polymorphic fragments is not necessarily the most informative primer combination (Table 4.5).

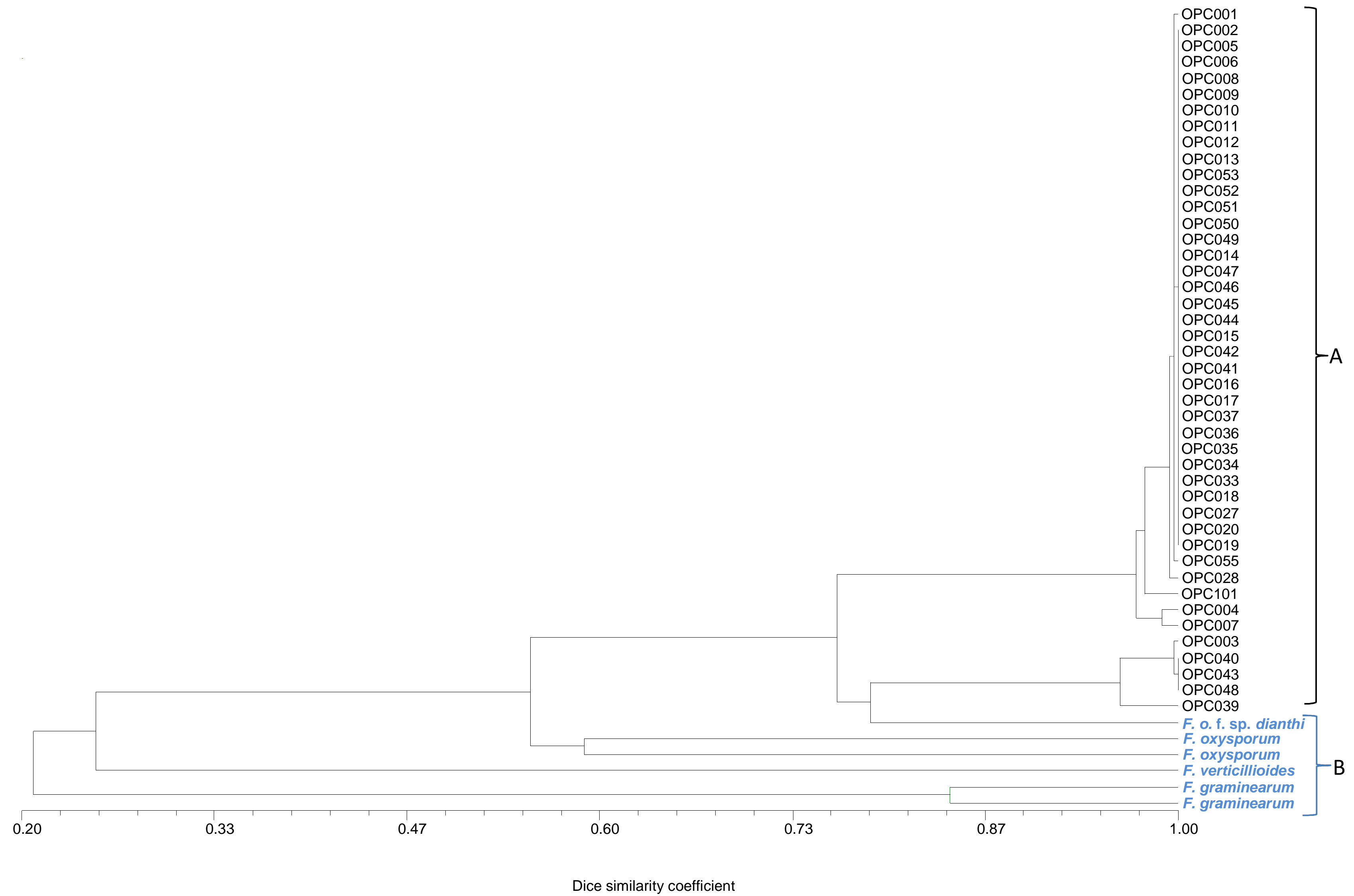

Figure 4.3 Dendrogram generated using NTSYSpc and UPMGA clustering using Dice's similarity coefficient, illustrating clustering of *F. oxysporum* isolates and out-groups.

Table 4.5 Selective primer combinations, scored polymorphic fragments and polymorphic information content values for primers used in this study

Primer combination	Scored polymorphic fragments	Polymorphic information content
PC1 - *Eco*RI-TG/*Mse*I-TT	30	0.31
PC2 - *Eco*RI-TC/*Mse*I-AA	31	0.32
PC3 - *Eco*RI-TG/*Mse*I-AG	28	0.41

Using all 45 *F. oxysporum* isolates associated with *H. gordonii*, six primers were evaluated for the number of polymorphic bands that the primer combinations generated. Out of the three primer combinations (PC), E-TG/M-TT (PC1), E-TC/M-AA (PC2) and E-TG/M-AG (PC3), two were chosen for the study based on literature and one on preliminary evaluations. These three primer combinations generated a total of 89 distinctly polymorphic bands, where the number of polymorphic bands per primer combination [PC1 (30 bands); PC2 (31 bands) and PC3 (28 bands)] seem to be almost equal. Genetic relationships among the isolates based on the datasets obtained from the three AFLP primer combinations were analysed as a combined dataset. The combined dataset resulted in 39 of the *F. oxysporum* isolates, including OPC 101, in cluster A and 5 isolates grouped into cluster B (Figure 4.3) at a similarity of about 76.2%.

Three smaller groups can be observed in cluster A with a similarity of about 97%. The isolates in cluster B further resolved into two groups with a similarity of about 95.5%. Both cluster A and B contain pathogenic and non-pathogenic *F. oxysporum* isolates associated with *Fusarium* wilt on *H. gordonii*. Cluster B includes *F. oxysporum f. sp. dianthi*, one of the out-groups that has shown high similarity with the other *F. oxysporum* isolates when evaluated on morphological characteristics. The other out-group isolates indicated a 55.5% similarity among the *F. oxysporum* isolates, 26.0% similarity with *F. verticillioides* and a 21.0% similarity with *F. graminearum*. Approximately 55.0% similarity which may be somewhat low for use as a cut-off point for AFLP based species recognition in the FOSC.

4.3.2.1 Analysis of genetic variance

The cophenetic correlation, using the goodness of fit (r), which measures the correlation between the similarity represented on the dendrogram as well as the actual degree of similarity, resulted in r = 0.82 for the UPGMA clustering method based on Dice's similarity

coefficient. This value shows that the dendrogram can be used as an indicator of genetic diversity between the *F. oxysporum* isolates.

The dendogram showed that there was no relationship between AFLP clusters and geographic origin. Cluster A can further be divided into three groups. The first group within this cluster includes a significant number of clonal genotypes (75.0%) which consist of 33 different isolates from all four locations. Little genetic variation had been observed within this group. The isolates within this group which are not included in the clonal genotypes, includes two isolates from Prieska and one from Klein Pella. The second group of isolates within cluster A consists only of OPC 101. This isolate was collected from Kakamas and was the isolate that have shown to be the most virulent during the pathogenicity tests conducted (Chapter 3). The third group within cluster A includes two isolates with 96.8% similarity from Prieska. The genetic variation between the clonal genotypes and the other the isolates in cluster A range from 95 to 98% similarity. Thus the genetic compilation between isolates in cluster A can be described as significantly low.

Cluster B also indicated the formation of three smaller groups within this cluster. The level of genetic variation in cluster B tends to be higher than that in cluster A. Cluster B only show a few clonal genotypes (6.8% from total isolates). Group one of cluster B consists of four isolates. Three of these isolates comprise of clonal genotypes. These three isolates are all from Pofadder. The fourth isolate of group one is from Prieska and shows 98% similarity to the clonal genotypes. The second group in this cluster only includes isolate OPC 039 from Pofadder. This isolate show to be 94% similar to the clonal genotyes in cluster B. Group three in cluster B consist of only one isolate, *F. oxysporum* f. sp. *dianthi.* This isolate was included in the study as an out-group. *F. oxysporum* f. sp. *dianthi* had previously been associated with *Fusarium* wilt on carnations. When comparing the two clusters there is a dissimilarity of 23.8% between cluster A and B.

4.4 Discussion

The pathogen, *F. oxysporum* associated with *H. gordonii* wilt has not been previously identified as a causal agent prior to the present study. Morphological identification of *F. oxysporum* was relatively easy, although it is impossible to distinguish *F. oxysporum* isolates of various *formae speciales* within the FOSC. It was only after DNA sequence

comparisons were done to differentiate between various FOSC isolates that these isolates were correctly identified. DNA sequence analyses determined the *F. oxysporum* DNA sequence using similarity search (BLASTN) as a means of diagnosing and was not always clear since, in some cases, information from sequences from Cmd gene found the highest similarity in different species than those obtained using TEF and β-tub gene sequences. Another issue was the low amount of data on FOSC using Cmd gene sequences. However, phylogenetic analyses resolved all unclear morphological differentiation and allowed for accurate identification of the species.

F. oxysporum isolates from MRC, South Africa and Israel have been characterised to determine the level of genetic diversity within and between the isolates associated with *H. gordonii* and were included in the AFLP analyses. The genetic diversity observed in *F. oxysporum* isolates depended on the techniques used and the number of the isolates evaluated. AFLP analysis is a highly reproducible and polymorphic fingerprinting technique, which has been used in numerous genetic studies to evaluate genetic differences across large parts of the pathogen’s genome in a single assay, with no prior sequence information. AFLP analyses of *F. oxysporum* isolates from MRC isolates from South Africa showed a far relationship as compared with the isolate from Israel which indicated a closer relation to isolates from cluster B. Results showed relatively high levels of genetic diversity in *F. oxysporum* within isolates sampled across the Northern Cape Province when compared with other *formae speciales* from FOSC. AFLPs were used in the current study to evaluate and characterise the genetic diversity of *F. oxysporum* isolates associated with *H. gordonii* wilt in South Africa and to associate South African isolates with previously described phylogenetic isolates within FOSC. To date, South African *F. oxysporum* isolates causing wilt on *H. gordonii* have not been evaluated for genetic variation or association with known isolates in the FOSC. No studies have been reported or identified the genetic variation of 44 South Africa *F. oxysporum* isolates measured genetic diversity.

Analyses of AFLP and sequencing (TEF, β-tub and Cmd) data separated the *Hoodia* isolates of *F. oxysporum* into two well supported and concordant clades. The AFLP analyses further grouped these isolates into two clusters. The separation of these clusters was not supported by the presence of TEF, β-tub and Cmd nucleotide sites that were fixed differentially between the two clusters. Isolates residing in these clusters were in no way correlated to geographic origin or source of the isolates. Results of this study also showed

that the *F. oxysporum* isolates from *Hoodia* rhizosphere soil and plant tissue reside in distinct clusters within the so-called clade A and clade B of the *F. oxysporum* isolates tested.

The specific relationships of the two clades inferred from the data in this study are not entirely congruent. Based on data from the present study, isolates representing the FOSC are split into many lineages, one containing clades A and B. However, based on the combined TEF, β-tub and Cmd gene sequences that clade A and B represents the most ancient clade and are more closely related to one another. The three genes sequenced revealed to be highly conservative, this shows that it both clades originated from the same ancestor or that they are slowly evolving into a new clade. This incongruence between findings in the study may be due to differences in the taxon sets used or because of other phenomena such as long branch attraction (Lyons-Weiler and Hoelzer, 1997).

The composition of the two *F. oxysporum* clades emerging from the present study showed that clade A and B included only *F. oxysporum* isolates causing wilt on *H. gordonii* which are suggested to have originated in South Africa. Clade B included five *F. oxysporum* isolates causing wilt on *H. gordonii*, two of which have recently been reported as non-pathogenic in the previous chapter (Chapter 3, Philippou *et al*., 2013). The sequences that showed variation have been submitted and appear in the GenBank database under the *F. oxysporum* isolates causing wilt on *H. gordonii*. The clades A and B also included all of the *F. oxysporum* isolates causing wilt on *H. gordonii* obtained from plant tissues and soils in the present study.

The TEF sequence data separated the 44 *F. oxysporum* isolates into two well supported groups, where group 1 was closely related to group 2. However, the relationship among group 1 and 2 showed that the two groups are closely related remains unclear because the sequence data does not have sufficient information to determine the phylogenetic affinity of Lineage. Although it is difficult to conclude whether this lineage is more closely related to clades A and B, the AFLP results suggest that they all belong to the same lineage. Analyses of TEF sequences in this study showed that a single isolate (OPC 101) was similar to all the isolates in clade A. This isolate grouped within clade A of the clusters that emerged from the AFLP analyses. The TEF data further showed that isolate OPC 101 grouped with the CBS 416.90 (*F. oxysporum* f. sp. *dianthi*). It also suggests that more comprehensive sampling of *Hoodia* soils need to be undertaken to find additional isolates of this type. Analyses of both

β-tub and Cmd in this study proved that the genes were similar in all isolates, supporting the hypothesis that they all originate from the same lineage. The PIC values generated from the AFLP analyses also supports this hypothesis as the PIC values are low which shows that the isolates are very similar and some isolates are clonal.

The two groups emerging from the sequence analyses and those based on the AFLP data were closely concordant. AFLP analyses sampled the entire genome (Vos *et al.*, 1995), whereas gene genealogies analysed specific nuclear genes (Majer *et al.*, 1996), proving that the genes are highly conservative. The AFLP analyses provide independent measures of the evolutionary history of the organism. As a result, phylogenies supported by the two techniques are likely to reliably reflect the evolutionary history of the *F. oxysporum* isolates included in this study.

AFLP analyses revealed two major clusters within the South African isolates of *F. oxysporum* isolates associated with wilt on *H. gordonii*. Separation of these clusters was also supported by the presence of 6 TEF nucleotide sites which were fixed differently between the two clusters. The genetic diversity of isolates within the AFLP cluster A was greater than that in cluster B. However, the genetic differentiation between isolates in the two AFLP based clusters appeared to be small. This suggests that all the isolates of *F. oxysporum* isolates associated with *H. gordonii* represent a single species. Nonetheless, the AFLP data, and sequencing data from (TEF, β-tub and cmd) to a lesser extent suggest that this single species is either in the process of separating into two different species or that the two clusters are in the process of converging into one species. The assistance of sequencing aids tremendously in identifying and placing *F. oxysporum* in the specific and highly variable FOSC as is the point in case with the various isolates from CBS and MRC isolates where Israel isolates were more closely related than the MRC isolates which was more geographically closer. Nevertheless, such an AFLP based tool for recognising species among the morphologically different *Fusarium* spp. was of great value.

The *F. oxysporum* isolates associated with *H. gordonii* treated in this study were obtained from nurseries and farm soils, vascular tissue and root samples of wilted *H. gordonii* plants, soil from wheat roots collected from the Free State Province and one isolate collected from wilted carnations in Israel. However, the grouping observed among these isolates did not correlate in any way with the source (host or substrate) or

geographical origins of the isolates. Although these isolates have been tested for pathogenicity, nothing is known regarding their mycotoxic productions and ecological relevance. However, in *F. oxysporum* for example, several genetic diversity studies have shown that some pathogenic isolates could have arisen from presumably non-pathogenic isolates, and *vice versa*, through mutations involving a few genes (Woo *et al*. 1996, Baayen *et al*. 2000).

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Chapter 5
General conclusions

Fusarium wilt was first reported in 2004 where it destroyed about 95% of *Hoodia* plants in a commercial nursery in Kakamas. In 2007, the reappearance of *Fusarium* wilt was observed in Pofadder this time on *Hoodia* seedlings having the same devastating effect. Farmers do not plant in semi-arid areas unless under irrigation. A novel crop such as *Hoodia*, makes it an ideal choice to be introduced in such a region as no/or very little water is needed. Although, the plant is endangered, growing these plants in cultivated nurseries would assist in future projects to help in the re-introduction of the species into the wild. *Fusarium* wilt is a contributory factor and economic discouragement for *Hoodia* production in South Africa and for pharmaceutical companies investing in the plant by trying to extract the appetite suppressing compound (P57) used in diet supplements.

Fusarium wilt on *H. gordonii* is caused by *F. oxysporum*. This pathogen had been identified and characterised as the most prominent species in all locations where *Hoodia* was commercially cultivated. The main reason to study the host and wilt disease is based on the concept of improved disease management strategies in commercial nurseries and to improve future breeding programmes. Morphological characterisation was used to identify 89 isolates and to determine the causal agent of wilt on *H. gordonii*. Based on morphological identification of *Fusarium* species from all four locations where *Hoodia* was collected from in commercial plantings in the Northern Cape Province, six *Fusarium* species were identified. Only one of the six species, *F. oxysporum*, had previously been associated with wilt on other plant hosts. In this study, *F. oxysporum* was found in high frequencies at all four locations and was therefore identified as the primary causal pathogen of *Fusarium* wilt on *Hoodia*. The reasons for wilt to have occurred was possibly due to an increased source of host plants which were susceptible to the inoculum found in the soil, introduction from an unhealthy wilted plant and/or infected seed material not treated prior to planting. The pathogen was introduced from a familiar environment where plants were separated by at least > 2 to 3 m from each other to clustered nursery environment. The pathogen destroyed most of the plants in the nurseries as *H. gordonii* are sparsely found in the natural environment so occurrences were too low in frequency to make an impact. However, examples from known cultivated crops prove that disease occurrence is greater in all major cultivated areas in comparison with alternative wild types. Therefore, for an effective breeding programme to be implemented, plants should be screened, treated (fungicide) and selected based

on the important traits (resistance to disease, size and level of P57 compound), together with a good plant health management programme.

As part of the evaluation of the genetic identification of the *F. oxysporum* isolates, VCGs showed that two groups were identified, which is of great importance as this proves that only isolates paired with one another were related and shared compatible *nit* genes. Based solely on *nit* genes, compatible genes will be shared with identical gene pairs for anastomosis to occur. Many *nit* mutants were generated and paired amongst each other and known VCGs in the FOSC. Isolates paired with the known isolates from FOSC which showed non compatibility. The use of VCGs showed how closely related the isolates were but beyond the *nit* genotypes, molecular techniques where pursued for further investigation into the genetic makeup of the isolates.

The phylogenetic analysis using specific genes (TEF, β-tub and Cmd) were sequenced and compared to known isolates on the NCBI database (Genbank) to determine how related they were within the FOSC and amongst each other. These three specifically targeted gene regions indicated the potential for identifying the species and determined the relationship amongst the other known isolates from FOSC collected in Genbank. The sequence of the TEF gene done suggests that the isolates used in this study is identical to *F. oxysporum* f. sp. *dianthi*. The combined data of all three genes involved, however suggest that there is genetic differences between the isolates and *F. oxysporum* f. sp. *dianthi*. This proves how conservative the TEF gene is and that evolution in this region is really slow. The Cmd gene showed the highest similarity with different species than those obtained using TEF and β-tub gene sequences. This prompted the study to focus on the whole genome to identify the genetic variation between the isolates compared to other species and within the FOSC.

AFLPs were used to determine the genetic fingerprint of the *F. oxysporum* isolates causing wilt on *H. gordonii*. A total of 54 *F. oxysporum* isolates were analysed for fingerprinting against four reference isolates. The four reference isolates included representatives of the two isolates from the FOSC and two out-groups. AFLP analyses identified two major clusters within the *F. oxysporum* isolates associated with wilt on *H. gordonii*. However, the genetic differentiation between isolates in the two AFLP based clusters appeared insignificant. This suggests that all the isolates of *F. oxysporum* isolates associated with *H. gordonii* represent a single species and possibly a new *formae specialis*. AFLP based tool for recognising species among the morphologically different *Fusarium* spp. assisted greatly with DNA fingerprinting. Knowledge on the

genetic make-up of the pathogenic isolates provided information about the isolates collected, when compared within the FOSC and related species, proving that the isolates obtained in this study were similar in terms of genus level, based on the AFLP profiles and DNA sequences. This knowledge can furthermore be extended with the development of managent strategies, including resistance breeding against the disease. These isolates however were unique in terms of sub species level (*formae specialis*) based on the VCGs and DNA sequences. This aspect should be exploited further with more isolates from the different localities.

The morphological evaluation of the host plant, *H. gordonii* was undertaken to determine variations amongst different wild and cultivated plants. Morphological data obtained amongst the different wild *H. gordonii* plantings showed levels of variation within and amongst the different localities. Total soluble solids showed a correlation with the environment where in the plants grew. Plants under attack by pathogens induce several structural and biochemical defence mechanisms such as hypersensitive responses, pathogenesis-related proteins, secondary metabolites as well as antifungal enzymes. A possible activation of certain responses activated in the field as compared to the commercial greenhouse may explain why disease is more prevalent in the greenhouse plants. The better understanding of the role of physiological compounds that play a role in disease resistance will help in the development of resistance breeding and disease management. Knowledge gained from analyses using morphological characterisation and total soluble solids of this *H. gordonii* population has provided insight on how we can utilise the data to determine the relatedness of various *H. gordonii*. In this study we observed that the locations separated the plants into separate clusters thus proving that the environmental factors play a significant role as well as the variations of traits amongst and within the plant samples collected. The study provided valuable information on identifying favorable conditions for the plant which could assist with the future development of breeding projects and disease management strategies specifically against wilt.

Future research should focus on the information gained from this study. A large sample size from all known locations where *H. gordonii* grows is suggested with more data from the wild, nurseries and other cultivations in terms of temperature, watering/rainfall and shading/fauna species. One aspect that needs further investigation is the possibility of the change of the dominant *Fusarium* wilt causal species with known non-pathogenic species that have been identified in this study. This strategy may be used to inoculate soils with the non-pathogen species to help maintain a higher population size thus

limiting the spread of the pathogen if it is introduced, since breeding for a resistant *H. gordonii* variety might take too long to achieve, since the plant takes about five to seven years to reach maturity. Although two VCGs were identified the possibilities of other closely related isolates do exist and need to be identified in a future study. Another aspect that needs further investigation is the influence of mycotoxins, which play a role in human consumption and on the pathogenicity of *Fusarium* wilt causing pathogens, together with the total soluble solids of plant sap produced by the host plant, which is the pathogens food source. Future studies should consider the pathogenicity of the isolates in correlation to nurseries and wild plants, since it is a known fact that disease frequency increases, when the host plants are grown in cultivated areas as compared to low frequencies in the wild were they are found individually and scattered in sparse areas. Soil samples should be taken from the already identified plants in the wild to determine the frequency and determine if *F. oxysporum*, pathogenic and non-pathogenic isolates, are also found in the wild. Furthermore, the transportation of inoculum by humans and natural means should be investigated to establish the spread of *Fusarium* wilt in nurseries growing *H. gordonii*, along with the development and age of the isolates. Lastly, genetic fingerprinting (with a special permit) should be done to correlate with morphological data, when samples are obtained from the same wild plants in the future.

Summary

Hoodia gordonii has been used by the San people for centuries as an appetite suppressant while they were on long hunting trips. These succulents are globally known as an important component in diet supplements and products which assist in weight-loss. Together with other plants these contribute towards a multimillion US dollar market, as many pharmaceutical and nutritional companies have made significant financial investments in the research and development for people who suffer from weight problems and obesity. In South Africa, *H. gordonii* is classified as being endangered, because so many pharmaceutical companies cashed-in on the dietary characteristics of these succulents. The plant is found in the South Western parts of Southern Africa mainly in the Karoo, Kalahari and Namib deserts. Plants were also grown in cultivated nurseries under favourable conditions for commercial use. However, diseases have dramatically hampered production in nurseries with almost total loss of crop. Although not many diseases have been documented on this plant, *Fusarium* wilt, a devastating fungal disease of *H. gordonii*, caused by *F. oxysporum* had been identified. This disease is not only responsible for economic losses, but also contaminates the soils with spores, which remain dormant until the next season as inoculum. This study concentrated on the morphological characterisation and molecular identification of the *Fusarium* wilt causing pathogen present in four areas (Kakamas, Klein Pella, Pofadder and Prieska) as well as the morphological evaluation of the host. *Fusarium oxysporum* was identified as the causal agent of *Fusarium* wilt on *H.gordonii* plants. The AFLP analysis and DNA sequences resulted in two distinct groups. Those that clustered in the AFLP cluster B were also grouped in a TEF cluster B, however some isolates from AFLP cluster A also grouped together with the TEF cluster B. The low genetic variation revealed by the AFLP analysis indicated that differences amongst the pathogen isolates occur, but the DNA sequences confirmed that these isolates share a common ancestor. DNA sequencing analysis was used to place 44 South African *F. oxysporum* isolates into the phylogenetic groups as described by O'Donnell and associates. South African *F. oxysporum* isolates clustered into two groups. The observed genetic variation amongst individual isolates was lower than the genetic variation between out-group isolates. Two clusters were identified; within each cluster isolates had a relatively high frequency of clones. These clones confirm that these pathogen isolates share similar allele frequencies. Results from sequencing data showed that the isolates fall within the FOSC, however there was no isolates identified that show 100% similarities when compared with all three genes sequences with recorded genetic sequences of *F. oxysporum* isolates in other parts of

the world. Therefore, based on the current taxonomic system, of host specificity the fungus only infects *H. gordonii*, so the study has proved that a possible new formae specialis has been identified. New preventative measures must be applied to the host when planting in nurseries. Knowledge gained from analyses of the genetic fingerprinting, DNA sequencing of these isolates and the mporphological evaluation of the host might assist with the development of effective control strategies, i.e. resistance breeding against *Fusarium* wilt. This will provide an incentive to potential farmers to plant *H. gordonii*, thus improving production of this succulent for pharmaceutical companies and nurseries in South Africa.

Keywords: Soil-borne pathogene, genetic variation, DNA fingerprinting, VCG, pathogenisity, P57 components, morphological characterization, *Fusarium* wilt

Opsomming

Hoodia gordonii is al vir eeue deur die San as eetlus-demper op hul lang jagtogte gebruik. Hierdie vetplant is wêreldwyd bekend as ‘n belangrike komponent in dieet-aanvullings en produkte wat met gewigsverlies help. Saam met ander plante dra dit by tot ‘n multimiljoen Amerikaanse dollar mark, deurdat baie farmaseutiese en voedingsmaatskappye betekenisvolle finansiële beleggings in navorsing en ontwikkeling maak, vir oorgewig- en mense wie aan vetsug ly. In Suid-Afrika word *H. gordonii* as ‘n bedreigde spesie geklassifiseer, omdat so baie farmaseutiese maatskappye probeer muntslaan uit die waardevolle voedingseienskap van hierdie vetplant. Die plant word in die Suidwestelike gedeelte van Suid-Afrika gevind hoofsaaklik in die Karoo, Kalahari en Namib-woestyn. Plante was ook onder gunstige toestande vir kommersiële gebruik, in kwekerye aangeplant. Siektes het die produksie so drasties belemmer dat aanplantings omtrent uitgewis is, alhoewel nie baie siektes vir die gewas aangeteken is nie. *Fusarium* verwelksiekte is ‘n vernietigende swamsiekte wat deur *F. oxysporum* veroorsaak word en op *H. gordonii* geïdentifiseer is. Hierdie siekte is nie net verantwoordelik vir ekonomiese verliese nie, maar besoedel die grond met spore wat in ‘n dormante toestand agterbly en die volgende groeiseisoen as innokulum dien. In hierdie studie is klem op die morfologiese karakterisering en molekulêre identifikasie van die fungus verantwoordelik vir Fusarium verwelksiekte uit vier gebiede (Kakamas, Klein Pella, Pofadder en Prieska) asook die morfologiese evaluasie van die gasheerplant, gelê. Fusarium oxysporum was geïdentifiseer as die patogeen wat wat hoofsaaklik vir Fusarium verwelksiekte op H. gordonii verantwoordelik is. Resultate van die AFLP (geamplifiseerde fragment lengte polimorfisme) analises en DNS volgorde bepalings toon dat die patogeen isolate in twee definitiewe groepe verdeel. Die groepe wat in die AFLP kluster B gegroepeer het is ook in die TEF kluster B aangetref, alhoewel sommige isolate van die AFLP kluster A ook saam in die TEF kluster B voorgekom het. Die lae genetiese variasie wat deur die AFLP analysis aangetoon word, dui daarop dat verskille tussen die isolate wel voorkom, maar ook dat die isolate ‘n gemeenskaplike voorouer deel. Volgorde bepalings-analises is gebruik om 44 Suid-Afrikaanse *F. oxysporum* isolate in philogenetise groepe, soos beskryf deur O’Donnell en medewerkers, te verdeel. Suid-Afrikaanse isolate groepeer in twee filogenetiese groepe. Die waargenome genetiese variasie binne individuele isolate was laer as die genetiese variasie tussen buite-groepe isolate. Twee groepe is geïdentifiseer en binne elke groep was daar ‘n relatiewe hoë frekwensie van klone. Die klone bevestig weer dat hierdie isolate ooreenstemmende alleel frekwensies deel. Resultate van die DNS volgorde bepalings data dui aan dat die isolate binne die FOSC

groepeer. Alhoewel daar 100% ooreenstemming tussen isolate in die drie geen-volgordes voorgekom het, het dit goed vergelyk met aangetekende DNS volgordes van *F. oxysporum* isolate uit ander dele van die wêreld. Gebasseer op die huidige taksonomiese sisteem van die spesifike gasheerplant is gevind dat die swam net *H. gordonii* affekteer, dus het die studie heel moontlik ‘n nuwe formae specialis geïdentifiseer. Nuwe voorsorgmaatreëls moet op die gasheerplant toegepas word wanneer daar in ‘n kwekery geplant word. Die inligting/kennis wat deur die genetiese-analises, DNS volgordebepaling van die isolate en morfologiese evaluasie van die gasheerplant ingewin is mag help met die ontwikkeling van beheer-strategieë vir weerstandsteling teen *Fusarium* verwelksiekte. Dit sal aan potensiële boere die voordeel gee om die verbeterde *H. gordonii* aan te plant en sodoende produksie verhoog vir die farmaseutiese mark en vir kwekerye in Suid-Afrika.

Sleutelwoorde: Grondgedraagte patogene, genetiese diversiteit, DNS-vingerafdrukke, VCG, patogenisiteit, P57 komponent, morfologiese karakterisering, *Fusarium* verwelksiekte

Appendix I:
Composition of mediums for morphological characterisation and vegetative compatability groups of Fusarium oxysporum isolates

Carnation Leaf-Piece Agar (CLA)

2% (w/v) Agar bacteriological grade (BioLab®, Merck®)

CLA is a natural substrate medium that is suitable for the purification and identification of *Fusarium* species. Sporodochia formed on the carnation leaves contain uniform macroconidia, needed for morphological identification. The agar was suspended in distilled water and autoclaved at 121℃. The medium was added to Petri dishes containing carnation leaves under sterile conditions (Fisher *et al.*, 1982). The carnation leaf pieces were prepared from fresh carnation leaves. After harvest the leaves were dried in an oven until brittle and then sterilised by gamma irradiation. These leaves can be stored at room temperature for up to 12 months before use.

Potato Dextrose Agar (PDA)

3.9% (w/v) PDA bacteriological grade (BioLab®)

PDA was prepared by suspending the PDA powder in distilled water and autoclaving the suspension at 121℃. This carbohydrate-rich medium is suitable for determining colony morphology, pigmentation and growth rates of cultures of most *Fusarium* species under standard conditions.

Spezieller Nährstoffarmer Agar (SNA)

0.1% (w/v) Potassium phosphate (KH_2PO_4)
0.1% (w/v) Potassium nitrate (KNO_3)
0.05% (w/v) Magnesium sulphate ($MgSO_4$•$7H_2O$)
0.05% (w/v) Potassium chloride (KCl)
0.02% (w/v) Glucose
0.02% (w/v) Sucrose
2% (w/v) Agar bacteriological grade (BioLab®, Merck®)

SNA was prepared by suspension and autoclaving of the above-mentioned chemicals in distilled water. This weak nutrient agar can be used for the identification, purification and maintenance of *Fusarium* isolates. It is suitable for sporulation of *Fusarium* isolates and promotes the formation of morphologically uniform macroconidia (Nirenberg, 1976).

Van Wyk's Agar (VWA)

1% (w/v) Glycerin
0.05% (w/v) L-Alanine
0.005% (w/v) Rose bengal
0.1% (w/v) Urea
0.1% (w/v) Pentachloronitrobenzene (PCNB)
1.5% (w/v) Agar bacteriological grade (BioLab®, Merck®)

VWA was prepared by suspension and autoclaving at 121℃ of the above-mentioned chemicals in distilled water. This medium is suitable for the selective isolation of *Fusarium* species from plant material, debris and seeds (Van Wyk *et al.*, 1986).

Water Agar (WA)

1.5% (w/v) Agar bacteriological grade (BioLab®, Merck®)

WA was prepared by suspending the agar powder in distilled water and autoclaving the suspension at 121℃. This medium is suitable for ge rminating *Fusarium* spores under standard sterile conditions.

Basal Medium (BM)

3.0% (w/v) Sucrose
0.1% (w/v) KH_2PO_4
0.05% (w/v) $MgSO_4 \cdot 7H_2O$
0.05% (w/v) KCL
0.05% (w/v) $FeSO_4 \cdot 7H_20$
2.0% (w/v) Agar bacteriological grade (BioLab®, Merck®)
0.02% (w/v) Trace Element: (95 ml dH_2O; 5 g Citric acid; 5 g $ZnSO_4 \cdot 7H_2O$; 1 g $Fe\ (NH_4)_2\ (SO_4)_2 \cdot {}_6H_20$; 0.25 g $CuSO_4 \cdot 5H_20$; 0.05 g $MnSO_4 \cdot H_2O$; 0.05 g $H3BO_4$; 0.05 g $NaMoO_4. \cdot 2H_2O$)

BM was used to prepare other media needed to determine VCGs (Correll *et al.*, 1987).

PDA Containing Chlorite (PDC)

3.9% (w/v) PDA (potato dextrose agar) (BioLab®)
0.5% (w/v) agar, Agar bacteriological grade (BioLab®, Merck®)
1.5% - 3.0% (w/v) $KClO_3$

First potassium chlorate ($KClO_3$) was used at the rate of 15 g L^{-1} (1.5%) and if the actively growing sectors were not produced, the amount of $KClO_3$ was gradually increased to 30 g L^{-1} (3%). PDC was used to create mutations in the isolates. (Correll *et al.*, 1987).

Minimal Medium Containing Chlorite (MMC)

3.0% (w/v) Sucrose
0.2% (w/v) $NaNO_3$
1.6% (w/v) L-asparagine
1.5% (w/v) $KClO_3$

This medium was adjusted to the final volume of 1 L by adding BM. First potassium chlorate ($KClO_3$) was used at the rate of 15 g L^{-1} (1.5%) and if the actively growing sectors were not produced, the amount of $KClO_3$ was gradually increased to 30 g L^{-1} (3%) (Correll *et al.*, 1987). MMC was used to create mutations in the isolates. (Correll *et al.*, 1987).

Rose Bengal Amended Medium (RBAM)

3.5% (w/v) Czapek-Dox broth (Difco, Detroit, Mich.)
2.5% (w/v) $KClO_3$
5.0% (w/v) Rose Bengal
1.0% (w/v) of 0.5 mg/ml stock solution ethanol

RBAM was adjusted to pH 7.0 – 7.2 with HCl or NaOH prior to addition of 2.0% (w/v) agar, Agar bacteriological grade (BioLab®, Merck®) (Bell and Crawford, 1967). RBAM was used for selecting nitrate-metabolism mutants from strains with reduced sensitivity to chlorate (Elias and Cotty, 1994).

Minimal Medium Containing Nitrate (NMM)

NMM was made by adding 2 g sodium nitrate ($NaNO_3$) into 1 L of BM before autoclaving.

This medium was used to identify *nit* mutants and to determine VCGs through complementation tests (Correll *et al*., 1987).

Ammonium Medium (AMM)

AMM was made by adding 1 g ammonium tartrate [$(NH_4)_2C_4H_4O_6$] into 1 L of BM before autoclaving.

This medium was used to identify *nit* mutants and to determine VCGs through complementation tests (Correll *et al*., 1987). The AMM is the control. Strains that will not grow on this medium have another nutritional requirement that needs to be diagnosed.

Minimal Medium Containing Hypoxanthine (HMM)

HMM was made by adding 0.2 g hypoxanthine ($C_5H_4N_4O$) into 1 L of BM before autoclaving.

This medium was used to identify *nit* mutants and to determine VCGs through complementation tests (Correll *et al*., 1987). HMM is used to differentiate NitM mutants, as these mutants are unable to utilise hypoxanthine as a sole nitrogen source due to a defective molybdenum co-factor that is shared by nitrate reductase and purine dehydrogenase.

Uric Acid Medium (UAM)

UAM was made by adding 0.2 g uric acid ($C_5H_4N_4O_3$) into 1 L of BM before autoclaving.

This medium was used to identify *nit* mutants and to determine VCGs through complementation tests (Correll *et al*., 1987).