IDENTIFICATION AND CHARACTERISATION OF SOME PHYTOPATHOGENS INFECTING SOUTH AFRICAN INDIGENOUS ORNAMENTAL PLANTS

C.A. Becker

BSc (UKZN); BSc (Hons) (UKZN)

Submitted in partial fulfilment of the requirements for the degree of

Master of Science

In the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal, Pietermaritzburg

South Africa

November 2014

DISSERTATION SUMMARY

South Africa is endowed with a large number of native plant species and many of the country's plants have become important floricultural crops both locally and internationally. Three genera of South African monocotyledonous plants are particularly prominent floricultural crops of high value to the industry, namely *Clivia, Strelitzia* and *Zantedeschia*. A single species from each of these genera have stood out in the industry and become the most well-known representatives of their respective genera. These plants are *Clivia miniata, Strelitzia reginae* and *Zantedeschia aethiopica*. The reasons that each are of value to the industry are as follows: *C. miniata* has large orange flowers and glossy evergreen leaves and is able to grow well in shaded areas and in pots. *Strelitzia reginae* has bold flowers and foliage, which are highly desirable to consumers, and can be grown as a pot plant or in the garden. *Zantedeschia aethiopica* has attractive white flowers and is grown for as a cut flower, pot plant or in the garden.

A number of highly apparent disease symptoms were observed on plants of *C. miniata, S. reginae* and *Z. aethiopica* growing mainly in KwaZulu-Natal, South Africa. Further samples of diseased plant material were also obtained from the Gauteng and Mpumalanga provinces to supplement the samples obtained from plant producers in KwaZulu-Natal. Six apparent disease complexes formed the focus of the studies presented in this dissertation. Three distinct diseases afflicting *C. miniata* and related *Clivia* species were observed and studied, two distinct diseases of *S. reginae* were observed and studied and one disease of *Z. aethiopica* was documented and studied.

The three diseases observed on *C. miniata* were: (i) an anthracnose disease causing symptoms of leaf spotting and necrosis; (ii) a soft rot of the leaves and stems of afflicted plants and (iii) foliar streaking and mottling speculated to be caused by viral infection. The anthracnose disease was attributed to infection of *C. miniata* by a species of *Colletotrichum*. A fungal isolate was obtained from senescing, symptomatic leaf material and identified as a species of *Colletotrichum*, based on its cultural and morphological characteristics. Evaluation of Koch's postulates with the isolate demonstrated its pathogenicity towards *C. miniata* and morphological characterisation of the conidia of the isolate identified it as *C. karstii*. The morphological identification was corroborated by a Maximum Likelihood (ML) phylogenetic analysis of the ITS region of the isolate, which indicated well-supported clustering with other *C. karstii* isolates. This is the first report of *C. karstii* causing an anthracnose disease of *C. miniata* in South Africa.

Two outbreaks of the soft rot disease of *C. miniata* were observed in commercial nurseries where *C. miniata* was being grown, one in Howick, KwaZulu-Natal and the other in White River, Mpumalanga, South Africa. A bacterial isolate from each outbreak was demonstrated to cause identical soft rot symptoms on inoculated plants, in fulfilment of Koch's postulates. Both bacterial isolates exhibited cultural and morphological characteristics similar to members of the bacterial genus *Pseudomonas*. A BLAST search of the 16S rRNA region of the White River isolate in the Ribosomal Database Project database was performed and indicated similarities to a number of *Pseudomonas* species, corroborating the morphological assessments of the isolate. This is the first report of soft rot of *C. miniata* caused by a *Pseudomonas* species in South Africa.

The virus-like disease symptoms studied were observed on *C. caulescens, C. miniata* and numerous interspecific hybrid plants. Transmission electron microscopy (TEM) of sap samples obtained from symptomatic showed the presence of filamentous viral particles in the leaf sap preparations and attempts to purify viral samples from symptomatic leaf material were successfully carried out. Reverse transcription polymerase chain reaction (RT-PCR) was implemented in attempts to amplify viral nucleic acids from leaf samples and purified virus preparations. These attempts were, however, unsuccessful in generating amplicons for phylogenetic analyses. Attempts were made to study possible viral transmission methods, through the mechanical inoculation of *Clivia* plants and *Nicotiana tabacum*. These transmission studies were inconclusive as none of the inoculated plants developed any symptoms. The possibility that these viruses are novel entities was raised by the results of the research carried on this subject.

The two diseases affecting *S. reginae* were: (i) foliar spotting and necrosis and (ii) a flower blight. The foliar disease was attributed to infection by a species of *Pestalotiopsis*, a genus of known phytopathogen fungi. Morphological characterisation of the versicolorous conidia of the *Pestalotiopsis* isolate shown to be pathogenic on *S. reginae* indicated affinities to *P. clavispora.* A neighbour-joining (NJ) phylogenetic analysis of the ITS sequence data for the isolate placed it within a well-supported clade comprised of *Pestalotiopsis* species with versicolorous conidia. The relationships between the *Pestalotiopsis* species within the versicolorous clade was, however, not well resolved by the ITS-based phylogenetic inference and concatenated phylogenetic analysis of the ITS, β-tubulin and *tef1* sequence data for this isolate will be required for accurate taxonomic placement within the genus. This is the first report of a leaf blight of *S. reginae* caused by a *Pestalotiopsis* species in South Africa.

The flower blight observed to afflict *S. reginae* was demonstrated to be caused by a phytopathogenic bacterium, belonging to the bacterial genus *Pantoea*. The morphological and biochemical characteristics of the isolate were consistent with various *Pantoea* species. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) and a BLAST analysis of the 16S rRNA region of the isolate's genome indicated affinities to *P. agglomerans*, a documented phytopathogen. This is the first report of a *Pantoea* species as a phytopathogen of *S. reginae* and also the first report of a floral disease of *S. reginae* in South Africa.

The disease symptoms commonly observed on *Z. aethiopica* in KwaZulu-Natal were a conspicuous foliar blight of infected leaves. The etiology and symptoms of the foliar disease observed in Pietermaritzburg were similar to those previously reported to be caused by the bacterium *Xanthomonas campestris* pv. *zantedeschiae*. Koch's postulates were successfully carried out with the bacterial strain consistently isolated from symptomatic leaf material. PCR amplification of the 16S rRNA region isolate indicated similarities to various species of *Pseudomonas,* chiefly *P. putida*, but not to any *X. campestris* isolates. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) of the peptidic profile of the isolate corroborated the 16S rRNA based assessment of its identity as a *Pseudomonas* sp but did not provide a strong indication of species identity. This is the first report of a foliar blight of *Z. aethiopica* caused by a *Pseudomonas* sp. in South Africa.

Cultures of all the successfully isolated phytopathogens were deposited at the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) with the following accessions numbers: *C. karstii* PPRI 16882, *Pseudomonas* sp. BD 1294 and *Pseudomonas* sp. BD 1295 [*Clivia* pathogens]; *Pestalotiopsis* sp. PPRI 16883 and *Pantoea* sp. BD 1293 [*Strelitzia* pathogens] and *Pseudomonas* sp. BD 1296 [*Zantedeschia* pathogen].

The research carried out and presented in this dissertation highlights a number of new records of plant diseases and their associated phytopathogens for South Africa. It is indicative of the need for further studies of phytopathogens infecting floricultural crops in South Africa in order to update the field and provide management strategies for floricultural practitioners in the country.

DECLARATION

I, CALVIN ALEXANDER BECKER, declare that

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

(iii) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

(iv) This dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

(v) This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

Signed……

CALVIN ALEXANDER BECKER

Signed……

PROFESSOR MARK LAING

SUPERVISOR

ACKNOWLEDGEMENTS

Professor Mark Laing for his mentorship and for providing the opportunity to carry out the research presented in this thesis

My parents for their unwavering support and encouragement to follow my dreams

The following organisations for their financial assistance throughout the course of my studies:

The Agar Hamilton Trust

The National Research Foundation of South Africa

The Barn Owl Trust

The Clivia Society of South Africa

Plant Health Products and the Agricultural Sector Education Training Authority

The staff and students of the Discipline of Plant Pathology

Mr Richard Burgdorf

Dr Sandi Willows-Munro

The staff of the Microscopy and Microanalysis Unit in Pietermaritzburg

TABLE OF CONTENTS

DISSERTATION INTRODUCTION

South Africa has been identified by Conservation International as a 'megadiverse country', and a number of irreplaceable biodiversity hotspots are recognised in the region (Mittermeier *et al.,* 1997; Raimondo *et al.,* 2013). It is estimated that approximately 20 000 species of vascular plants (approximately 5% of the world's total plants) occur naturally within the borders of the country (Manning, 2003; Pooley, 2005; Van Jaarsveld, 2006; Raimondo *et al.,* 2013.) Many indigenous plant species that were originally native to the country were admired and collected by early travellers and botanists during their explorations of the region and then transported back to botanical hubs in the developing world. The descendants of these originally South African plant species have since made significant contributions to the development of the global floricultural industry (Reinten *et al.,* 2011).

Floriculture is a branch of horticulture that deals with the cultivation of plants for their flowers (and other ornamental characteristics that people admire), rather than for human consumption, animal feed or other purposes (Singh, 2006). The floricultural industry that has developed in the modern world is significant and many international organisations devoted to the expansion of the industry exist, including the International Association of Horticultural Producers (AIPH: http://www.aiph.org/site/index_en.cfm) and the Floriculture Research Alliance (FRA: http://floriculturealliance.org/). Floricultural productivity contributes a significant portion to the agricultural sector of the GDP of a number of developing countries, including South Africa (Bester *et al.,* 2009; Netnou-Nkoana & Eloff, 2012). Khachatryan *et al.* (2014) have stated that they 'expect that the demand for floriculture and nursery crops and landscaping services will likely increase in the next several years' in the USA'.

Floricultural practitioners, like all horticultural practitioners, are faced with a number of constraints that can affect their plants, production cycles and potential profits (Mwanga & Cloete, 2003; Sergeant, 2004; Emana & Gebremedhin, 2007). One challenging issue faced by all ornamental plant producers is the need to produce high-quality, unblemished plants and plant products to supply markets and consumers that demand perfection (Hartmann *et al.,* 2011). Plant diseases have the potential to dramatically reduce the profits of ornamental plant producers because: (a) susceptible plant varieties can be killed outright or stunted by pathogen infection; (b) pathogens can create blemishes on and cause other disfigurements of crop products, which can seriously reduce their aesthetic appeal and subsequent marketability; and (c) the costs associated with the requisite levels of disease control to obtain entirely unblemished crop products are often high.

The aims of this research project were threefold: firstly, to investigate the potential role of phytopathogens in prevalent plant diseases observed on indigenous ornamental plant species growing in Pietermaritzburg, KwaZulu-Natal, South Africa; secondly, to attempt to identify any phytopathogenic organisms implicated as being responsible for the plant diseases investigated; and, thirdly, to provide a review of disease literature (pertinent to the ornamental plant species studied) for future researchers in the field. The three plant species chosen to form the basis of these studies were *Clivia miniata* Regel, *Strelitzia reginae* Aiton and *Zantedeschia aethiopica* Spreng. These species were chosen because of their widespread appeal as floricultural crops, the ubiquity of disease symptoms observed on plants wherever they were being grown and the apparent lack of any recent, detailed investigations of their diseases in South Africa.

The specific goals of this project were to isolate and characterise the suspected phytopathogenic organisms responsible for causing (i) leaf spotting and dieback observed on *C. miniata*, (ii) leaf and petiole blight observed on *S. reginae,* (iii) flower blight of *S. reginae,* (iv) necrotic soft rots of *C. miniata*, (v) leaf blight and necrosis observed on *Z. aethiopica* and (vi) virus-like diseases of *C. miniata* and other *Clivia* species. Symptoms were regularly observed on plants of these species wherever they were cultivated, in both home gardens and in commercial settings, in KwaZulu-Natal, South Africa. Some diseased plant material examined for this thesis also came from other South African provinces. All these diseases have been widely observed and reported by many commercial and hobbyist growers of the plants for a number of years. Definitive studies, utilising both morphological and molecular identification criteria to identify pathogenic microbes implicated in the disease complexes observed in this study, have not been carried out previously in South Africa.

REFERENCES

Bester, C., Blomerus, L.M. & Kleynhans, R. (2009) Development of new floriculture crops in South Africa. Acta Horticulturae 813: 67-72.

Emana, B. & Gebremedhin, H. (2007) Constraints and opportunities of horticulture production and marketing in eastern Ethiopia. Drylands Coordination Group Report 46: 35-37.

Hartmann, H.T., Kester, D.E., Davies, F.T. & Geneve, R.L. (2011) Hartmann & Kester's Plant Propagation – Principles and Practices. 8th ed. Prentice Hall, USA. pp. 14-281.

Khachatryan, H., Hodges, A.W. & Steed, S. (2014) Floriculture crops economic outlook for 2014. University of Florida IFAS Extension FE941: 5-6.

Manning, J. (2003) Photographic Guide to the Wildflowers of South Africa. Briza Publications, South Africa. pp. 12-16.

Mittermeier, R.A., Gil, P.R. & Mittermeier, C.G. (1997) Megadiversity: Earth's Biologically Wealthiest Nations. Conservation International, USA.

Mwanga, R.O.M. & Cloete, M. (2003) The role of horticulture: issues, opportunities and constraints. Acta Horticulturae 621: 45-51.

Netnou-Nkoana, N. & Eloff, J. (2012) The South African floricultural industry and the Plant Breeders' Rights Act: a short review. World Patent Information 34: 224-228.

Pooley, E. (2005) A Field Guide to Wildflowers of KwaZulu-Natal and the Eastern Region. The Flora Publications Trust, South Africa. pp. 12-16.

Raimondo, D.C., von Staden, L. & Donaldson, J.S. (2013) Lessons from the conservation assessment of the South African megaflora. Annals of the Missouri Botanical Garden 99: 221-230.

Reinten, E.Y., Coetzee, J.H. & Van Wyk, B.E. (2011) The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77: 934-946.

Sergeant, A. (2004) Horticultural and floricultural exports – constraints, potential and an agenda for support for the Tanzania diagnostic trade integration study. World Bank DTIS. pp. 1-67.

Singh, A.K. (2006) Flower Crops – Cultivation and Management. New India Publishing Agency, India. pp. 1-6.

Van Jaarsveld, E. (2006) The Southern African *Plectranthus* and the Art of Turning Shade to Glade. Fernwood Press, South Africa. pp. 9-10.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The relationship between plants and humans has a long, complex and interesting history. The food, feed, fibre and medicinal products derived from plants for the myriad of uses that these products are used today are invaluable as the basis for foods and industries the world over (Singh, 2006). Although plants are chiefly grown for their contribution towards mankind's everyday survival, significant interest is also vested in growing plants for another purpose, namely the decorative and ornamental value provided by their flowers, fruits and foliage (Save, 2009).

The impact of pathogens on agricultural crop plants is well known and forms the core of plant pathology – the study of the microorganisms and environmental factors that cause disease in plants, the mechanisms by which diseases these factors induce diseases in plants, and the methods of preventing and controlling diseases to reduce they damage that they cause (Agrios, 2005). Ornamental plants are subject to attack by a range of pests and pathogens. Since considerable economic interest is vested in ornamental plant production, the deleterious effects of phytopathogens on ornamental plants translate into direct economic losses for the horticultural industry.

A key step in the control of any plant disease is to identify the organism responsible for causing disease in the affected plants (Moorman, 2006). Once the pathogen has been identified, control measures can be developed appropriately (Moorman, 2006). Research into the identification of pathogens associated with economically important ornamental plants is of importance to the stability of the global industry.

1.2 THE DEVELOPMENT OF THE INTERNATIONAL FLORICULTURAL INDUSTRY AND ITS INFLUENCES ON THE SOUTH AFRICAN INDUSTRY

Plants have been grown by man for their ornamental value for thousands of years. Heywood (2003) stated that 'the introduction of wild species into cultivation for ornament began in several civilizations not long after the first domestications of plants for agriculture'. Flowers have long held special meaning and significance in many cultures and, in many cases, there is such an intricate association of flowers with social beliefs and cultural practices that the symbolism attached to and associated with certain flowers can be traced back throughout history to rites involving life, death, birth, marriages and burials.

Flowers are also grown for many other purposes aside from their decorative and religious value. Spices such as saffron and cloves are derived from floral structures, vegetables such as broccoli, cauliflower and artichokes are the flowering heads of *Brassica oleracea* L and *Cynara cardunculus* L. eaten as vegetables, hop flowers are a crucial ingredient in the making of beer, and many of the active ingredients in essential oils and fragrances were or still are derived from aromatic compounds extracted from plants (Heywood, 2003).

The pursuit of plants for their ornamental value has sometimes led to spectacular investments of time and resources being made to obtain certain types of plants, held to be highly desirable by society at the time. The Dutch phenomenon of tulip mania, which peaked in 1636-1637, saw single tulip bulbs selling for extremely high prices but this peak was soon followed by a dramatic and sudden collapse where the same bulbs could be sold for only a fraction of their peak value (Garber, 1989; Hull, 2009). A similar 'bubble' developed in Victorian England in the 19th century, where orchids became the group of plants that were excessively coveted. This period is termed 'orchidelirium' and saw wealthy orchid fanatics financing expeditions to most parts of the world to collect orchids, which were auctioned in London upon their return and often fetched extravagant prices (Orlean, 2000).

In today's world, the growth of plants for their ornamental value has developed into a major international industry known as floriculture. Floriculture can be defined as the 'cultivation, production and marketing of floral crops' (Singh, 2006). Due to the ubiquity of plants in everyday life, floral crops are very diverse in nature but generally include bedding/garden plants (herbaceous perennials), potted bedding and garden plants (annuals, perennials and herbaceous perennials), annual bedding and garden flats, flowering hanging baskets, potted flowering plants (including orchids, roses, spring-flowering bulbs and African violets), foliage plants, cut flowers, cut cultivated greens and propagative floriculture materials (Anonymous, 2011a).

Wentzel & Pinckaers (2005) estimated that the revenues generated worldwide from the export of plants (namely lives trees, bulbs, cut flowers and ornamental foliage) to be US$ 12.4 billion in 2003. Matthee *et al.* (2006) stated that the international revenue generated from the import and export of floricultural products exceeded US$ 18 billion in 2006. A report released in 2013 by United States Department of Agriculture (USDA) on the US floricultural industry estimated that the total wholesale value of floricultural crops produced in the USA in 2012 was approximately US$ 4.13 billion (Anonymous, 2013).

According to Nell (2007), scientific research into floricultural production since the beginning of the 20th century can be chronologically characterised in the following manner: most research performed in the early 20th century was on optimising production techniques for various new flowering crops, such as carnations, chrysanthemums and snapdragons. In the 1940s, an understanding of photoperiod and its effect on plant growth allowed the commercial flower industry to begin producing year round flower crops. In the 1950s and 1960s, emphasis was placed by researchers on understanding plant nutrition and sources of nutrients, application times and methods for fertilisation to increase production.

A shift in the movement of flower production centres further from their markets began in the early 1960s. This took place because producers found better environmental conditions for production, cheaper labour and increased availability of natural resources further away from urban centres (Nell, 2007). Up until the 1970s, however, floricultural products were still generally produced for the local market or for a country's immediate neighbours due to the perishable nature of most floral crops (Reid *et al.,* 2008).

The subsequent development of air travel now offers plant producers opportunities for the international transport of plant material that would not have been possible beforehand. Frequent and reliable air travel, coupled with improvements in postharvest crop treatments, now means that consumers can receive high quality floricultural crops from almost anywhere and in a timely manner (Nell, 2007). It is air travel that allows South Africa to send cut flowers, live plants, foliage plants and flower bulbs to the main markets for these exported products in Europe, the United States and Asia (Van Rooyen, 2005).

The floriculture industry in South Africa is small but comprises an important segment of the South African agricultural sector (Bester *et al*., 2009). Boshoff & Coetzee (2007) estimated that sales on local floriculture markets grossed R260 million (US$ 37 million) and revenues generated from plant product exports amounted to R371 million (US$ 53 million) in 2005. Netnou-Nkoana & Eloff (2012) ranked 'flower production as one of the most efficient contributors to development and growth in the South African economy' and state that 'the South African floriculture industry has the opportunity to grow into a significant player on the international stage'.

1.3 SOUTH AFRICAN FLORAL DIVERSITY AND ITS IMPACT ON GLOBAL FLORICULTURE

European exposure to South African flora began in the late 1600s and early 1700s, at the time that Linnaeus was developing his ideas and systems for plant classification and nomenclature (Goldblatt, 1978). The impact of these unusual plants was so great that Southern Africa became one of the first areas outside of Europe to be botanically explored and have its plants scientifically classified (Goldblatt, 1978).

Today, South Africa is acknowledged to have a rich botanical wealth and diversity and it has been estimated that approximately 23 000 plant species are native to the country (Van Jaarsveld, 2006). As many as 19 000 of these species are angiosperms, or flowering plants, and hence may be of some floricultural value (Manning, 2003). The diversity exhibited by South African flora is a result of the interplay between the diversity of geographic and climatic factors that influence the country's landmass and South African vegetation is divided into at least eight biomes (Manning, 2003). Each biome represents a distinct community of plants characterised by the dominant biology exhibited by its component plants, which in turn is a direct response to climatic and geographic factors influencing the area which each biome occupies (Rutherford, 1997). The distribution of these biomes can be seen in Figure 1.1, where South Africa's nine provincial capital cities are also indicated.

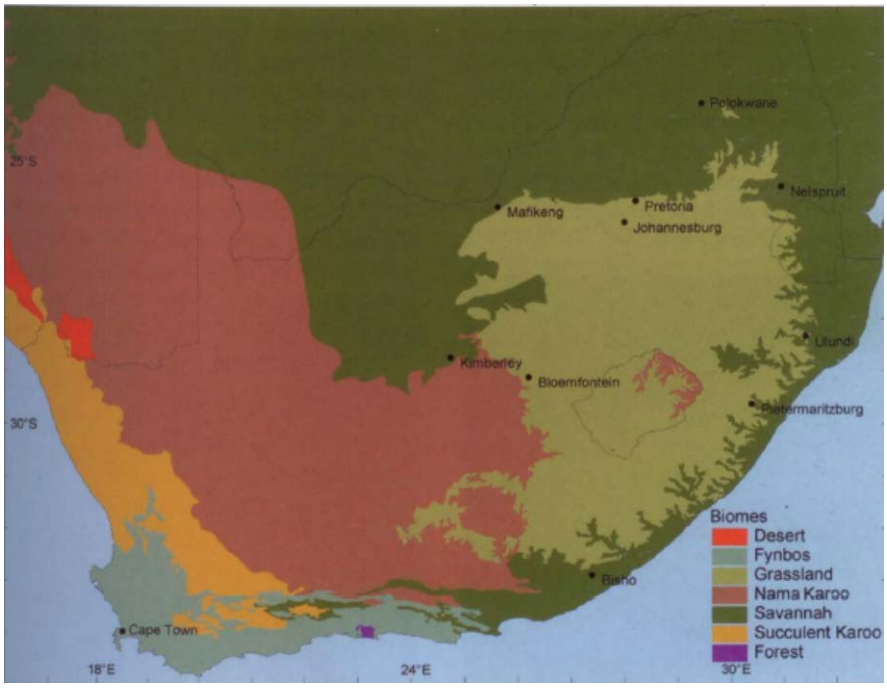


Figure 1.1 Distribution of South African vegetation biomes (Manning, 2003)

Reinten *et al.* (2011) provided a comprehensive review of South African plant species grown for ornamental use worldwide, which is reproduced in Table 1.1 below.

Today, while these established indigenous species make significant contributions to the international cut-flower and bedding plant trades, there is continued interest in the large number of wild species found in South Africa that are yet to be cultivated (Pooley, 2005; Van Jaarsveld, 2006). Reinten *et al.* (2011) noted that South Africa is viewed internationally as 'a hotspot of diversity and an important source of potential new cut flower cultivars'. It is likely that South Africa is also a 'hotspot' for new plant material for all other aspects of floricultural production as mentioned by Anonymous (2011a).

Southern Africa is acknowledged to have a substantially richer flora than both East and West Africa (Linder, 2001). Within South Africa itself there are areas of especially high plant diversity - particularly well known is the fynbos biome in the southwestern Cape but Thuiller *et al.* (2006) identified additional centres of high species diversity in the eastern escarpment of South Africa, where a complex mosaic of forest, savanna and grassland communities occurs. Centres of plant diversity (CPDs) are defined as 'areas of global botanical importance with high diversity and large numbers of endemic or threatened species with social, economic, cultural or scientific importance' (Pooley, 2005).

Pooley (2005) observed that two of these CPDs lie within the province of KwaZulu-Natal in eastern South Africa. These are the Maputaland (also known as Tongaland) Region and the Drakensberg Alpine CPDs, which occur in the extreme northeast and southwest of the province respectively (Cowling & Hilton-Taylor, 2003; Pooley, 2005). Pooley (2005) estimated that more than 11 000 plant species, subspecies and varieties of flowering plants (excluding grasses, trees and large woody plants) occur in the KwaZulu-Natal region covered by her field guide,.

The University of KwaZulu-Natal- Pietermaritzburg campus, where the research for this thesis was conducted, is situated in the city of Pietermaritzburg in the province of KwaZulu-Natal. Aside from serving as KZN's capital, Pietermaritzburg straddles the border between the savannah and grassland biomes at an altitude of 596 m above sea level (Manning, 2003; Richards *et al.,* 2006) (Figure 1.1). The climate is subtropical with an average annual rainfall of 850 mm falling mainly during the hot, humid summers (Annandale *et al.,* 2002).

Table 1.1 Indigenous South African plant species of commercial interest in the international floricultural trade. Uses indicated are: F = fresh flowers, D = dried flowers, Fol = foliage (leaves, stems, fruits or small flowers), Pot = potted flowers (excluding foliage plants) (Reinten *et al.,* 2011)

Species and family	Common and/or commercial name(s)	Use and/or potential use: + = low or none, ++ average, +++ = high
Adromischus species; Crassulaceae	Calico hearts	Pot+++
Agapanthus africanus (L.) Hoffmanns. (=*A. umbellatus* L'Hér.); Agapanthaceae	African lily, blue lily, blue african lily, lily of the Nile	F+++, Pot++
Agapanthus praecox Willd. (=*A. orientalis* (F.M.Leight.) F.M.Leight.); Agapanthaceae	African lily	F+++, Pot+
Agathosma species; Rutaceae	Buchu, anise buchu	Fol+++, Pot++, D++
Albuca species; Hyacinthaceae	Albuca, slime lily	Pot+++
Amaryllis belladonna L.; Amaryllidaceae	Belladonna lily, miniature amaryllis, cape belladonna, jersey lily	F+++, Pot+++
Androcymbium species; Colchicaceae	Cup-and-saucer, men-in-a-boat	Pot++
Anthospermum aethiopicum L.; Rubiaceae	Anthospermum, new look	Fol++
Arctotis x*hybrida*; Asteraceae	African daisy	Pot+++
Arctotis venusta Norl. (=*A. stoechadifolia* P.L.Bergius); Asteraceae	Blue-eyed african daisy	Pot++
Argyroderma species; Aizoaceae	Baby bottoms	Pot+
Aristea species; Iridaceae	Aristea	Pot+++
Aspalathus species; Fabaeae	Cape pea-flowers	Fol+
Asparagus species; Asparagaceae	Asparagus fern	Fol+++
Aulax umbellata (Thunb.) R.Br.; Proteaceae	Featherbush	Fol++
Babiana species; Iridaceae	Babiana	Pot+++
Begonia sutherlandii Hook.f.; Begoniaceae	Begonia	Pot++
Berzelia abrotanoides (L.) Brongn.; Bruniaceae	Abrotan	Fol++, D++
Berzelia galpinii Pillans; Bruniaceae	Baubles, galpinii	F++, Fol++, D++
Berzelia lanuginosa (L.) Brongn.; Bruniaceae	Berzelia, lanuginosa, Cape greens, kol-kol	Fol++, D++
Berzelia squarrosa (Thunb.) Sond.; Bruniaceae	Squarrosa	Fol+, D+
Brunia albiflora E.Phillips; Bruniaceae	Albiflora, white brunia	F+++, Fol+++, D+++
Brunia alopecuroides Thunb.; Bruniaceae	Alopecuroides	Fol+++, D+++
Brunia laevis Thunb.; Bruniaceae	Silver brunia	Fol++, D++
Brunia nodiflora L.; Bruniaceae	Spray brunia, stompie	Fol+++, D+++
Brunia stokoei E.Phillips; Bruniaceae	Rooistompie	Fol+, D+
Brunia alopecuroides Thunb.; Bruniaceae	Strawberry berzelia, white berzelia, red berries	Fol+, D+
Bulbinella latifolia (L.f.) Schult. and Schult.f.; Asphodelaceae	Cat's tail	F++
Bulbinella nutans (Jacq.) Spreng.; Asphodelaceae	Cat's tail	F++
Chaenostoma subspicatum Benth. (=*Sutera subspicatum*); Scrophulariaceae	Sutera	Pot++
Chlorophytum comosum (Thunb.) Jacq.; Anthericaceae (Asparagaceae)	Spider plant, hen-and-chickens	Pot+++
Clivia miniata (Lindl.) Regel; Amaryllidaceae	Clivia, orange lily, bush lily, fire lily, flame lily	Pot+++
Conophytum species; Aizoaceae	Buttons	Pot++
Crassula species; Crassulaceae	Stonecrops	Pot+++
×*Crinodonna* cultivars (*Amaryllis belladonna* L. ×*Crinum*); Amaryllidaceae	Crinodonna	F+++
Crocosmia aurea (Pappe ex Hook.) Planch.; Iridaceae	Crocosmia	F+++, D++
Crocosmia×*crocosmiiflora*; Iridaceae	Montbretia	F+++, D++
Cyanella species; Tecophilaeaceae	Lady's-hand	Pot++
Cyperus papyrus L.; Cyperaceae	Papyrus, Egyptian paper plant	Fol+++
Cyperus textilis Thunb.; Cyperaceae	Mat sedge	Fol++
Cyrtanthus species; Amaryllidaceae	Fire lily	F+++, Pot+++
Daubenya aurea Lindl.; Hyacinthaceae	Pincushion lily	Pot++
Diosma subulata J.C.Wendl.; Rutaceae	Florist buchu	Fol+++
Disa species and cultivars	Disa	F++, Pot++
Eleusine coracana Gaertn.; Poaceae (Gramineae)	Finger millet	D++
Empodium species; Hypoxidaceae	Autumn star	Pot++
Erica species; Ericaceae	Heather	F++, Pot++, D+
Eriocephalus racemosus L.; Asteraceae	White cotton	Fol
Eucomis autumnalis (Mill.) Chitt.; Hyacinthaceae	Pineapple lily	F+++, Pot+++
Eucomis bicolor Baker; Hyacinthaceae	Pineapple lily	F+++, Pot+++
Eucomis comosa (Houtt.) Wehrh.; Hyacinthaceae	Pineapple lily	F+++, Pot+++
Euryops pectinatus Cass.; Asteraceae	Bush daisy	Pot++
Ferraria species; Iridaceae	Spider iris	Pot++

Species and family	Common and/or commercial name(s)	Use and/or potential use: + = low or none, ++ average, +++ = high
Freesia × hybrida (and other species); Iridaceae	Freesia	F+++, Pot+++
Gazania krebsiana Less.; Asteraceae	Gazania	Pot+++
Geissorhiza species; Iridaceae	Satinflower, wine cup	Pot++
Gerbera × hybrida; Asteraceae	Gerbera	F+++, Pot+++
Gerbera jamesonii Bolus ex Adlam; Asteraceae	Gerbera, barberton daisy, transvaal daisy	F++, Pot++
Gethyllis species; Amaryllidaceae	Kukumakranka	Pot++
Gibbaeum species; Aizoaceae	Ostrich toes	Pot++
Gladiolus carneus D.Delaroche; Iridaceae	Painted lady	F++, Pot++
Gladiolus cultivars; Iridaceae	Gladiolus, glad, sword lily	F+++, Pot++
Gladiolus tristis L.; Iridaceae	Ever-flowering gladiolus, marsh afrikaner	F++, Pot++
Gloriosa superba L. (=*G. rothschildiana* O'Brien); Colchicaeae	Flame lily, glory lily	F+++, Pot+++
Gomphocarpus physocarpus E.Mey. (=*Asclepias physocarpa* Schltr.); Apocynaceae	Milkweed, swan plant	Fol++
Haemanthus albiflos Jacq.; Amaryllidaceae	Paintbrush	Pot++
Haemanthus coccineus L.; Amaryllidaceae	Blood flower, April fool	Pot++
Haemanthus humilis Jacq.; Amaryllidaceae	Paintbrush	Pot++
Haworthia species; Xanthorrhoeaceae	Haworthia	Pot+++
Helichrysum eximium Less. (=*Helipterum eximium* DC.); Asteraceae	Strawberry everlasting	F++, D++ (Red data species; cultivated material only)
Hesperantha species; Iridaceae	Hesperantha	Pot+++
Hessea species; Amaryllidaceae	Umbrella lily	Pot++
Hypoxis species; Hypoxidaceae	Star grass	Pot++
Ischyrolepis subverticillata Steud.; Restionaceae	Restios, besemriet	Fol++
Ixia cultivars; Iridaceae	African corn lily, wand flower	F+++, Pot+++
Kalanchoe species; Crassulaceae	Flaming katy	Pot+++
Kniphofia tysonii Baker; Asphodelaceae	Red hot poker, torch lily	F++
Kniphofia uvaria (L.) Oken; Asphodelaceae	Red hot poker, torch lily	F++
Lachenalia aloides (L.f.) Engl.; Hyacinthaceae	Cape cowslip, lachenalia	Pot+++
Lanaria lanata (L.) T.Durand & Schinz; Lanariaceae	Lambtails	Fol+
Lapeirousia species; Iridaceae	Cabong, lapeirousia, painted petals	Pot++
Ledebouria species; Hyacinthaceae	African squill	Pot++
Leonotis leonurus (L.) R.Br.; Lamiaceae	Lion's ear	F+++, D++, Fol++
Leonotis nepetifolia Schimp. ex Benth.; Lamiaceae	Wild dagga, lion's tail	Fol++
Leucadendron adscendens R.Br.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron argenteum (L.) R.Br.; Proteaceae	Silver tree	Fol++, D++
Leucadendron conicum (Lam.) I.Williams; Proteaceae	Cone bush	Fol+++, D++
Leucadendron coniferum (L.) Meisn.; (=*L.sabulosum* T.M.Salter); Proteaceae	Cone bush	Fol+++, D++
Leucadendron comosum (Thunb.)R.Br.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron daphnoides (Thunb.) Meisn.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron decorum R.Br.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron discolor E.Phillips and Hutch.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron floridum R.Br.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron galpinii E.Phillips and Hutch.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron laureolum (Lam.) Fourc.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron laxum I.Williams; Proteaceae	Cone bush, smart rose	Fol+++, D++
Leucadendron linifolium (Jacq.) R.Br.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron macowanii E.Phillips; Proteaceae	Acacia-leaf cone bush	Fol+++, D++
Leucadendron muirii E.Phillips; Proteaceae	Cone bush	Fol+++, D++
Leucadendron nervosum E.Phillips and Hutch.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron orientale I.Williams; Proteaceae	Cone bush	Fol+++, D++
Leucadendron platyspermum R.Br.; Proteaceae	Cone bush	Fol+++, D++
Leucadendron rubrum Burm.f. (=*L. plumosum* R.Br.); Proteaceae	Cone bush	Fol+++, D++
Leucadendron salicifolium (Salisb.) I. Williams; Proteaceae	Cone bush	Fol+++, D++
Leucadendron salignum P.J.Bergius; Proteaceae	Conebush	Fol+++, D++
Leucadendron stelligerum I. Williams; Proteaceae	Cone bush	Fol+++, D++
Leucadendron tinctum I. Williams; Proteaceae	Cone bush	Fol+++, D++
Leucadendron xanthoconus (Kuntze) K.Schum; Proteaceae	Cone bush	Fol+++, D++
Leucospermum species (selected); Proteaceae	Pincushions	F+++, Pot++
Leucospermum catherinae Compton; Proteaceae	Pincushion	F+++, D++
Leucospermum conocarpodendron H.Buek; Proteaceae	Pincushion	F++, D++

Species and family	Common and/or commercial name(s)	Use and/or potential use: + = low or none, ++ average, +++ = high
Leucospermum cordifolium (Knight) Fourc.; Proteaceae	Pincushion	F+++, D++, Pot++
Leucospermum cuneiforme (Burm.f.) Rourke; Proteaceae	Pincushion	F++, D++
Leucospermum erubescens Rourke; Proteaceae	Pincushion	F+++, D++
Leucospermum glabrum R.Br.; Proteaceae	Pincushion	F++, D++
Leucospermum lineare R.Br.; Proteaceae	Pincushion	F++, D++
Leucospermum patersonii E.Phillips; Proteaceae	Pincushion	F++, D++
Leucospermum reflexum H.Buek ex Meisn.; Proteaceae	Pincushion	F+++, D++
Leucospermum rodolentum (Salisb. ex Knight) Rourke; Proteaceae	Pincushion	F++, D++
Leucospermum saxosum S.Moore; Proteaceae	Pincushion	F++, D++
Leucospermum tottum R.Br.; Proteaceae	Pincushion	F++, D++
Leucospermum truncatulum (Salisb. ex Knight) Rourke; Proteaceae	Pincushion	Fol+++, D++
Leucospermum vestitum (Lam.) Rourke; Proteaceae	Pincushion	F++, D++
Limonium peregrinum (P.J.Bergius) R.A.Dyer; (=*L. roseum* Kuntze); Plumbaginaceae	Statice	F+++, D+++
Lithops species; Aizoaceae	Flowering stones	Pot++
Lobelia erinus L.; Campanulaceae	Edging lobelia, trailing lobelia	Pot+++
Massonia species; Hyacinthaceae	Hedgehog lily	Pot+
Metalasia muricata R.Br.; Asteraceae	Blombos	F+++
Mimetes cucullatus (L.) R.Br.; Proteaceae	Common pagoda, *rooi stompie*	Fol++, D++
Mimetes hirtus (L.) Salisb. Ex Knight; Proteaceae	Marsh pagoda, hairy mimetes	Fol++, D++
Moraea species; Iridaceae	Moraea, peacock iris	Pot++
Nebelia paleacea Sweet; Bruniaceae	Nebelia, *bergstompie*	Fol++
Nerine bowdenii W.Watson; Amaryllidaceae	Guernsey lily, spider lily, nerine	F+++
Nerine sarniensis Herb.; Amaryllidaceae	Guernsey lily, spider lily	F+++
Nymphaea nouchali Burm.f.; Nymphaeaceae	Water lily	F+
Ornithogalum dubium Houtt.; Hyacinthaceae	Orange star flower	F++, Pot++
Ornithogalum saundersiae Baker; Hyacinthaceae	Chincherinchee, star-of-Bethlehem	F++, D++
Ornithogalum thyrsoides Jacq.; Hyacinthaceae	Chincherinchee, star-of-Bethlehem	F+++, D++
Ornithoglossum species; Colchicaceae	Snake lily	Pot+
Paranomus species; Proteaceae	Scepter	F++
Pelargonium cordatum L'Hér.; Geraniaceae	Geranium, pelargonium, storksbill	Pot++
Pelargonium×*domesticum* Geraniaceae	Regal pelargonium, regals	Pot+++
Pelargonium graveolens L'Hér.; Geraniaceae	Geranium, pelargonium, storksbill	Pot++
Pelargonium peltatum (L.) L'Hér.; Geraniaceae	Ivy geranium, ivy-leaved geranium, hanging geranium	Pot+++
Pelargonium zonale (L.) L'Hér.; Geraniaceae	Geranium, zonal pelargonium, storksbill	Pot+++
Phaenocoma prolifera D.Don; Asteraceae	Everlasting	F++, D+++
Phylica ericoides L.; Rhamnaceae	Cape myrtle, white phylica	F++, D+++,
Phylica lasiocarpa Sond.; Rhamnaceae	Snowtops	Fol++, D++
Phylica plumosa L. (=*P. pubescens* Aiton); Rhamnaceae	Green phylica	F+++, D+++, Pot++
Polyxena species; Hyacinthaceae	Cape hyacinth	Pot+
Protea aristata E.Phillips; Proteaceae	Ladismith protea	F+ (unpleasant odor)
Protea compacta R.Br.; Proteaceae	Bot river protea	F+++
Protea cynaroides (L.) L.; Proteaceae	King protea, giant protea	F+++, D++, Pot++
Protea effusa E.Mey. ex Meins.; Proteaceae		F++, D++
Protea eximia (Salisb. Ex Knight) Fourc.; Proteaceae		F+++, D++, Pot++
Protea grandiceps Tratt.; Proteaceae		F++, D++
Protea laurifolia Thunb.; Proteaceae		F+++, D+++
Protea lacticolor Salisb.; Proteaceae		F++, D++
Protea longifolia Andrews; Proteaceae		F++, D++
Protea lorifolia (Salisb. Ex Knight) Fourc.; Proteaceae		F++, D++
Protea magnifica Link; Proteaceae	Queen protea	F+++, D++
Protea mundii Klotzsch; Proteaceae		F++, D++
Protea nana (P.J.Bergius) Thunb.; Proteaceae		Fol++, D++
Protea neriifolia R.Br.; Proteaceae	Bearded protea, oleander leaf protea	F+++, D++
Protea obtusifolia H.Buek ex Meins.; Proteaceae		F+++, D++
Protea pityphylla E.Phillips; Proteaceae		F++, D++
Protea repens (L.) L.; Proteaceae	Sugarbush, sugar protea	F+++, D++
Protea scolymocephala (L.) Reichard; Proteaceae		F++, D++
Protea speciosa (L.) L.; Proteaceae		F++, D++
Protea susannae E.Phillips; Proteaceae		F+, D++ (leaves with unpleasant odor)

Species and family	Common and/or commercial name(s)	Use and/or potential use: + = low or none, ++ average, +++ = high
Pteronia paniculata Thunb.; Asteraceae	Gum bush, *gombossie*	Fol+
Retzia capensis Thunb.; Stilbaceae	Honeyflower, *heuningblom*	Fol+(Red data species; cultivated material only)
Rhodocoma species; Restionaceae	Restios	Fol++
Rhodohypoxis baurii (Baker) Nel; Hypoxidaceae	Red star, rosy posy, spring starflower	Pot+++
Romulea species; Iridaceae	Romulea	Pot+++
Rumohra adiantiformis (G.Forst.) Ching; Dryopteridaceae	Leather fern, leatherleaf fern, baker fern, iron fern, seven week fern	Fol+++
Sandersonia aurantiaca Hook.; Colchicaceae	Christmas bells, chinese lantern lily	F+++, Pot+++
Sansevieria trifasciata Hort. ex Prain; Dracaenaceae or Asparagaceae	Mother-in-law's tongue, bowstring hemp	Fol+++, Pot+++
Serruria florida (Thunb.) Salisb. Ex Knight; Proteaceae	Blushing bride, spiderheads	F+++, D+++, Pot++
Serruria rosea E.Phillips; Proteaceae	Spiderheads	F+++, D+++, Pot++
Sorghum bicolor (L.) Moench; Poaceae	Sorghum, great millet, broomcorn	Fol+
Sorghum nigrum Roem. and Schult.; Poaceae	Black millet, black sorghum, black witches' broom	Fol++
Sparaxis tricolor (Schneev.) Ker Gawl; Iridaceae	Sparaxis, harlequin flower	F+++, Pot++
Spiloxene species; Hypoxidaceae	Cape star	Pot+++
Staavia radiata Dahl; Bruniaceae	Glass eyes	Fol+++
Stoebe plumosa Thunb.; Asteraceae	Stoebe	Fol++, D++
Stoebe vulgaris Levyns; Asteraceae	Bankrupt bush	Fol++, D++
Strelitzia reginae Banks; Strelitziaceae	Bird-of-paradise, crane flower	F+++, Fol+++
Streptocarpus ×hybridus; Gesneriaceae	Cape primrose, florist streptocarpus	Pot+++
Strumaria species; Amaryllidaceae	Cape snowflake	Pot++
Syncarpha vestita (L.) B.Nord.	White everlasting	F+++, D+++
Syringodea species; Iridaceae	Cape crocus	Pot+
Thamnochortus insignis Mast.; Restionaceae	Shell reed	Fol++, D++
Thunbergia alata Sims; Acanthaceae	Black-eyed Susan vine	Pot++
Trichocephalus stipularis (L.) Brongn. (=*Phylica stipularis* L.); Rhamnaceae	Hairy heads	Fol++, D++
Tritonia crocata Ker Gawl.; Iridaceae	Tritonia, flame freesia, garden montbretia	F++, Pot++
Tritonia cultivars; Iridaceae	Blazing star, garden montbretia	F+++, Pot+++
Tulbaghia simmleri Beauverd; Alliaceae	Broad-leaved wild garlic	F+++, Pot+++
Tulbaghia violacea Harv.; Alliaceae	Wild garlic	F++, Pot++
Tylecodon species; Crassulaceae	Miniature baobab	Pot++
Veltheimia species; Hyacinthaceae	Sand lily	Pot+++
Venidium fastuosum Stapf; Asteraceae	Cape daisy, monarch-of-the-veld	F++
Wachendorfia species; Haemodoraceae	Butterfly lily	F++
Walleria species; Tecophilaeaceae	Potato lily	Pot+
Watsonia cultivars; Iridaceae	Watsonia, bugle lily	F+++
Wurmbea species; Colchichaceae	Spider lily	Pot++
Zantedeschia aethiopica (L.) Spreng.; Araceae	Calla lily, arum lily	F+++, Pot++
Zantedeschia albomaculata (Hook.) Baill; (=*Z. melanoleuca* (Hook.f.) Engl.); Araceae	Calla lily, arum lily	F++, Pot++
Zantedeschia elliotiana (W.Watson) Engl.; Araceae	Calla lily, arum lily	F++, Pot++
Zantedeschia jucunda Letty; Araceae	Calla lily, arum lily	F++, Pot++
Zantedeschia pentlandii (R.Whyte ex W.Watson) Wittm.; Araceae	Calla lily, arum lily	F+++, Pot+++
Zantedeschia rehmannii Engl.; Araceae	Calla lily, arum lily	F+++, Pot+++

1.4 PROPAGATION OF ORNAMENTAL PLANTS

Taxonomic nomenclature is important when it comes to the naming of ornamental plants as many present-day ornamental varieties only exist in cultivation and have been derived from natural variants of wild plant species, through mutagenesis or through complex intra- and interspecific hybridisation carried out by plant breeders (Jain, 2006; Hartmann *et al.,* 2011). These plants are termed cultivars and are distinct from plant species in that plant species are capable of more or less maintaining their distinctive characteristics through subsequent generations due to their natural genetic characteristics that delineate them. Cultivars would likely change dramatically or disappear entirely if not maintained by genetic selection by plant breeders and propagators (Hartmann *et al.,* 2011).

Plants can be propagated in numerous ways, but the basic distinction that can be made between the various methods is whether the new plants are produced in a sexual nature by the parent material (seed) or whether the new plants are produced in an asexual manner (vegetative propagation). Since many plants naturally exhibit numerous modes of propagation to maximise their survival, it holds true that many common ornamental plants can be propagated both from seed and vegetatively. The use of either form depends on the type of plant being propagated and on the specific situation for which the plants are grown (Hartmann *et al.,* 2011).

1.4.1 Seed propagation

Seed propagation is the most common method used for the production of ornamental plants (Hartmann *et al.,* 2011). Almost all plant species reproduce via the production of seed and it follows that harvesting and sowing of seed is possibly the easiest method of raising ornamentals. In some cases, plants can only be propagated from seed and vegetative methods do not work. Seeds also provide advantages towards the producer that working with living plant material does not confer (Lambardi *et al.,* 2006). These advantages are namely that seeds are easier to store, transport and handle than live material, seed sowing can be mechanised in a way that vegetative propagation cannot, and seeds are more resistant to damage caused by diseases and pests than living material is (Lambardi *et al.,* 2006).

The cultivars produced by ornamental plant breeders often exhibit genotypic stabilisation that results in phenotypic stabilisation amongst the progeny plants grown from seed (Hartmann *et al.,* 2011). This is particularly true for annual and biennial ornamentals, whose short life cycles allow for rapid selection and fixation of desirable traits in the population (Callaway & Callaway, 2000). Genetic variation due to the inherent processes that occur during the production of gametes is limited in these instances since the parent plants exhibit little

variation themselves. However, sexual reproduction inherently generates variation amongst the offspring produced if the parents exhibit variation themselves (Snustad & Simmons, 2006).

Plant groups such as woody perennials and ornamental trees are also commonly propagated from seed. However, since these types of plants possess much longer lifecycles than annuals or biennials, it is harder for breeders to work towards genetic fixation of desirable traits (Hartmann *et al.,* 2011). As such, these traits may not be consistently passed onto the next generation of seedlings unless appropriate measures are followed (Hartmann *et al.,* 2011).

1.4.2 Vegetative propagation

In some instances, ornamental cultivars do not breed true from seed because their genetics are not stabilised or they do not constitute inbred lines and so the use of seed is nullified for their propagation. Plants, however, possess an important feature that allows them to regenerate from severed parts. Snustad & Simmons (2006) term this feature 'totipotency' and define it further as 'the ability of a single cell to produce all the differentiated cells of the mature plant'.

This is in effect asexual reproduction on the plant's part and vegetative propagation of plants results in the production of new, genetically identical individuals from the original parent material. These new plants, being clones, will display identical characteristics to the parent plant material. If the cultivar in question cannot be reproduced faithfully from seed, then clonal propagation is the only method that can be used to grow new plants from the initial parent that displays good characteristics to the plant breeder.

Clonal regeneration is an important method of propagation for many ornamentals and cuttings are the chief type of regeneration propagule used across the scope ornamentals (Smith, 2007; Hartmann *et al.,* 2011). Plant tissue culture is now also a widely used and successful form of vegetative propagation and Moyo *et al.* (2011) stated that 'plant tissue culture and its related biotechnology techniques have made significant contributions in the growth of the floriculture industry through mass micropropagation of healthy plantlets and the facilitation of molecular breeding applications'.

Synthetic or artificial seeds are a relatively recent addition to the array of vegetative propagation methods used for ornamentals and combine the advantages of clonal propagation with those of seed propagation mentioned previously (Lambardi *et al.,* 2006).

1.5 THE PRESENCE, IMPACT AND CONTROL OF PLANT DISEASES IN THE FLORICULTURAL INDUSTRY

The horticultural industry is rigorous in its maintenance of quality standards and any irregularities in the quality of the product reduce its value significantly. Townsley-Brascamp & Marr (1995) analysed ornamental plant attributes that affect consumer appreciation and found that apparent plant health had the greatest effect on whether the consumers purchased a plant or not. Consumers judged plant health by the absence of pests and diseases, and the bushiness of the plants. The authors stress the importance of maintaining healthy plants and the need for research into pests and diseases of ornamentals.

Hence, plant pathogens are important during the growth of ornamental plants because of the damaging effects they can have on the health of the plants and subsequent losses of product quality. Bacteria, fungi and viruses are the three chief groups of phytopathogens responsible for diseases in ornamental plants (Hartmann *et al.,* 2011). Viroids, phytoplasmas and nematodes are also responsible for diseases of ornamental crops (Harden, 1992).

Table 1.2 below provides some estimates of the economic value of ornamental crop losses experienced in Georgia, USA in 2006 due to the presence of pathogens (Martinez, 2007). The losses incurred by the industry equate to approximately 10 % of Martinez's (2007) estimate of the entire value of the industry for that year. It is clear that pathogens cause significant economic losses to the industry and as Hartmann *et al.* (2011) noted, plant pathogenic bacteria, fungi and viruses were primarily responsible for these losses.

Table 1.2 Economic losses for ornamental production experienced in Georgia, USA in 2006 (Martinez, 2007)

Disease (Ornamental production)	**% Reduction in Crop Value**	**Damage ($ Millions)**	**Cost of Control ($ Millions)**	**Total ($ Millions)**
Bacterial Diseases (Fire Blight, Leaf Spots)	0.4	2.42	0.8	3.22
Fungal Leaf Spots, Stem Cankers, Needle Blights	1.5	9.09	6.5	15.59
Root and Crown Rots	3.0	18.19	8.2	26.39
Powdery Mildew	0.5	3.03	1.8	4.83
Botrytis Blight	0.2	1.21	1.2	2.41
Virus (Tswv, Insv, Hosta Virus X)	0.2	1.21	0.1	1.31
Minor Diseases (Rust, Downy Mildew, Nematode)	1.0	6.06	2.6	8.66
Total (Ornamental Production)	**6.8**	**41.22**	**21.2**	**62.42**

Damping off is probably the most common disease encountered when ornamentals are grown from seed (Hartmann *et al.,* 2011). This syndrome is caused by a number of soil-borne fungi, namely species of *Fusarium* Link*, Phytophthora* de Barry*, Pythium* Nees and *Rhizoctonia* DC. (Agrios, 2005). The first three genera are readily and rapidly disseminated by surface water and so suppressing them in the water used at all stages of growing the plants is critical to their control (Hartmann *et al.,* 2011).

Daughtrey (2007) stated that fungi are likely to cause disease during all stages of ornamental propagation. In addition to the four fungal genera that cause damping off, other problematic fungi are species of *Botrytis* P. Micheli*, Cylindrocladium* Morgan*, Sclerotinia* Fuckel and *Thielaviopsis* Went (Daughtrey, 2007).

Sobiczweski (2008) observed that although only approximately 2 % of known bacterial species are phytopathogenic, the presence of these organisms in plant production systems presents an important factor limiting the growth and cropping of infected plants and economic losses can be significant. Newman (2008) noted that commonly encountered bacterial greenhouse diseases include bacterial wilts (caused by *Pseudomonas* Migula sp. and *Ralstonia solanacearum* Yabuuchi *et al.*), bacterial blight (caused by *Xanthomonas* Dowson sp.), soft rot (caused by *Erwinia* Winslow *et al.* and *Pectobacterium* Waldee sp.) and crown gall (caused by *Agrobacterium tumefaciens* Smith & Townsend).

Viruses infecting ornamental crops are diverse and include members of many genera. Commonly occurring genera include cucumoviruses, potyviruses, tobamoviruses and tospoviruses (Daughtrey & Benson, 2005; Tomioka *et al.,* 2012). The tospoviruses Impatiens necrotic spot virus (INSV) and Tomato spotted wilt virus (TSWV) are particularly widespread amongst ornamental plant genera. McDonough *et al.* (1999) provided the following list of ornamentals (Table 1.3) that are known to be infected by one or both of these viruses.

Newman (2008) summarised methods to accomplish effective disease control in floricultural crops and defined three main areas on which producers should focus, namely: i) sanitation, ii) clean stock and iii) seed treatment. These three principles should be integrated for the best chances of controlling important diseases. Hartmann *et al.* (2011) emphasised that plant pathogen management begins prior to propagation taking place, through the proper manipulation of stock and container grown plants from which seeds or vegetative propagules are obtained.

The presence of pathogens often results in the production of plants of inferior quality, leading to profit losses because later finishing and selling phases of the crop will be delayed or

nullified if the plant dies (Hartmann *et al.*, 2011). Stock plants and propagative material should be kept as disease-free and clean as possible before propagation and cultivation media and beds should be treated to exclude and suppress pathogens wherever possible.

Table 1.3 Host plants for tospoviruses TSWV and INSV listed by greenhouse crop type (McDonough *et al.*, 1999)

Bedding Plants	TSWV	INSV
Begonia	+	+
Blue daisy	+	
Browallia		+
Caladium		+
Celosia		+
Coleus		+
Dahlia	+	+
Dusty miller		+
Eggplant	+	+
Fuschia	+	
Gazania	+	+
Geranium	+	+
Gomphrena		+
Impatiens	+	+
Lobelia	+	+
Maltese cross		+
Marigold		+
Moss rose	+	+
Nasturtium		+
New Guinea Impatiens		+
Petunia	+	+
Phlox		+
Salvia	+	+
Sea lavender	+	
Star of Bethlehem	+	
Stock		+
Strawflower	+	+
Swan River daisy		
Verbena		+
Zinnia		+
Foliage plants		
Arrowhead vine		+
Bird's Nest fern		
Chinese evergreen	+	+
Cordyline	+	+
Dieffenbachia	+	
Dracaena	+	+
Japanese aralia	+	
Kalanchoe	+	+
Maranta	+	+
Oleander	+	
Pedilanthus		+
Piggyback plant		+
Pothos		+
Rubber tree	+	+
Schefflera		+
Swedish Ivy		+
Tradescantia		+
Weeping fig	+	
Zebra plant		+
Non-Ornamentals		
Broadbean	+	
Celery	+	
Endive	+	
Garden bean	+	
Lettuce	+	
Pepper	+	+
Spinach	+	+
Tarragon		+
Tomato	+	+
African violet	+	+
Alstromeria	+	+
Amazon lily		+
Amaryllis	+	+
Anemone		+
Anthurium	+	+
Ardisia	+	+
Asiatic lily	+	+
Bromelia	+	
Calceolaria		+
Calla lily	+	
Chrysanthemum	+	+
Clivia	+	
Cyclamen	+	+
Eucharis	+	
Exacum		+
Florist's cineraria	+	
Gardenia	+	+
Gerbera	+	+
Gladiola	+	+
Gloxinia	+	+
Hoya		+
Hydrangea	+	+
Lantana	+	
Lipstick plant		+
Lisianthus	+	+
Mother of thousands		+
Oncidium	+	
Oxalis		+
Peace lily	+	
Peperomia	+	+
Phalaenopsis	+	
Primula	+	+
Rain daisy	+	
Ranunculus	+	+
Rhododendron	+	
Ruscus		+
Schizanthus	+	+
Snapdragon		+
Statice	+	
Stephanotis	+	+
Streptocarpus	+	+
Thanksgiving cactus		+
Perennials		
Ajuga		+
Aster	+	
Barberry		+
Bee balm		+
Bishop's weed		+
Black-eyed susan		+
Campanula		+
Catnip		+
Columnea	+	+
Delphinium		+
English daisy		+
Forget-me-not		+
Foxglove		+
Gaillardia	+	
Gentian		+
Hosta		+
Osteospermum	+	
Pentstemon		+
Peony		+
Physostegia		+
Polemonium		+
Poppy		+
Red Valerian	+	+
Sedum		+
Shasta daisy		+
Turtlehead		+
Veronica		+
Vinca	+	+
Weeds		
Bittercress		+
Chickweed		+
Dandelion	+	
Field bindweed	+	
Galinsoga		+
Horseweed	+	
Jewelweed		+
Lamb's quarters	+	

1.6. TAXONOMY, CULTIVATION AND PROPAGATION OF *CLIVIA* Lindl., *STRELITZIA* Ait. AND *ZANTEDESCHIA* L.

1.6.1 *Clivia* (Bush lily)

The genus *Clivia* was named after Lady Clive, the Duchess of Northumberland, by John Lindley after she managed to get the first example of the type species (*Clivia nobilis* Lindl.) to flower in London in 1828 (Pooley, 2005). The genus belongs to the family Amaryllidaceae and consists of 6 species of evergreen, rhizomatous perennials that produce dark green, strap-shaped leaves and umbels of orange or rarely yellow flowers (Aubrey, 2001a; Conrad & Snijman, 2011). An important distinction made amongst *Clivia* species is between that of the pendulous flowered species [*C. caulescens* (Dyer)*, C. gardenia* (Hook.)*, C. nobilis* (Lindl.)*, C. mirabilis* (Rourke) and *C. robusta* (Murray, Ran, De Lange, Hammet, Truter & Swanevelder)] and the only open flowered species, *C. miniata* (Regel) (Winter, 2006).

Members of the genus generally inhabit areas of the forest and thicket biomes in the provinces of eastern South Africa, namely KwaZulu Natal, the Eastern Cape, Mpumulanga and Limpopo, and Swaziland that receive summer rainfall (Aubrey, 2001a). Figure 1.2 (Dixon, 2011) illustrates the geographic distribution of each species in South Africa. *C. mirabilis* is very unusual in that is occurs in the Northern Cape in an area with a semi-arid, Mediterranean climate that receives only winter rainfall, 800 km away from the closest natural populations of any other clivia species (Rourke, 2002).

There is significant phenotypic variation both between and within each clivia species for a number of traits related to their flowers, leaves and plant morphology. In addition, natural hybrids amongst the various species are known to occur where some of the species overlap in distribution (Dixon, 2011). The vast array of forms which clivia species and their hybrids exhibit allows ample scope for breeding programmes to develop desirable species and interspecific hybrid cultivars for use as pot plants worldwide (Reinten *et al.,* 2011). Some examples of the variation present in the genus are illustrated in Figure 1.3 below.

Clivias are propagated from seed or vegetatively from offsets. Seeds arise from sexual reproduction and so growing plants from seed is the standard method used for producing plants for breeding programmes and for raising plants that are not named cultivars, where variation amongst the progeny is desired. The removal of suckers from around the base of mature plants is commonly used to increase numbers of specific, named clivia cultivars. Since each sucker is a clone of the parent plant, this ensures that each new plant is identical to its parent. Vegetative propagation is desirable to increase the best plants obtained as the outcome of a breeding program. Clivias are difficult to propagate by tissue culture, however.

Clivias are relatively easy to cultivate but will not tolerate frost or prolonged cold periods and as such most clivia plants grown for the floricultural markets of Europe and North America are produced for the flowering pot plant trade (Duncan, 1999). Since clivias in most markets are grown primarily for the appeal of their flowers, breeders work tirelessly to develop better cultivars with better flowering characteristics in terms of flower colour, size, shape, petal broadness and arrangement as well as towards a balanced number of flowers per umbel to best display the blooms (Aubrey, 2001a). The architecture of the plant and the broadness and colour of the leaves in terms of variegation can also be highly valued, as is particularly evident in China, Japan and Korea where clivia are grown as permanent house plants and hence admired for more than just their flowers (Duncan, 1999).

Since the initial discovery of clivia in South Africa in the 1800s, there has always been substantial interest in the cultivation and breeding of the genus. Centres of breeding included England in the 19th and 20th centuries, Belgium during the same time period and still today, and China, Japan and Korea during the last 30 years (Duncan, 1999; Dixon, 2011). Interest in growing clivias has seen a renewed upsurge, both in South Africa and abroad, since the formation of the Clivia Society in 1992 (Dixon, 2011). Although the Clivia Society is located in South Africa, it provides a ‘conduit for the enthusiasm and energy of clivia devotees worldwide’ and the society exists ‘to facilitate the common interests of clivia clubs worldwide through, amongst others, the publication of a yearbook and quarterly newsletters, the registration of named cultivars, development of judging criteria and fostering research on Clivia’ (Anonymous, 2011b).

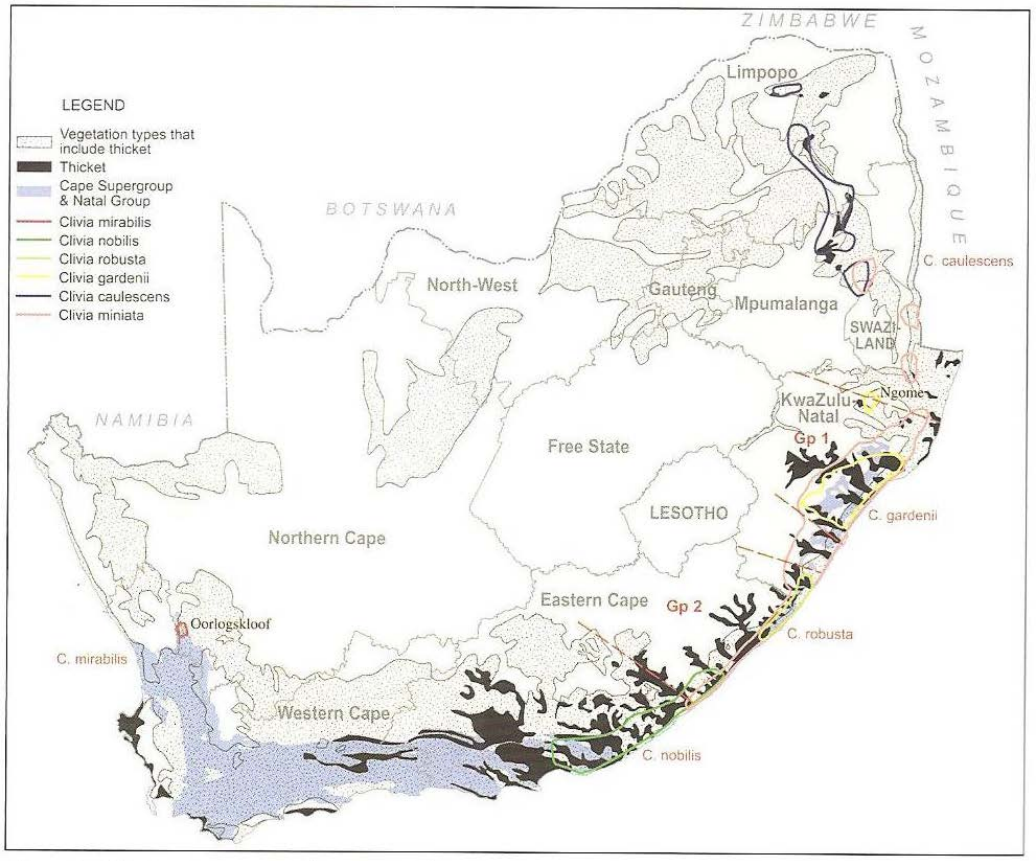


Figure 1.2 Distribution map of the genus *Clivia*, showing its relationships with Southern African subtropical thicket vegetation and the sandstones of the Cape Supergroup and Natal Group. The range limits of Groups 1 and 2, two yellow-flowered forms of *C. miniata* are also shown. (Dixon, 2011).

Figure 1.3 Representatives of the genus *Clivia* – *C. miniata, C. nobilis* and a *C. miniata* cultivar (clockwise from top left) (Aubrey, 2001a; Winter, 2006)

1.6.2 *Strelitzia* (Bird of paradise flower)

The genus *Strelitzia* consists of five species of banana-like plants, occurring naturally in the forest and thicket biomes in the eastern regions of Southern Africa, specifically in South Africa and Zimbabwe (Pooley, 2005; Cron *et al.,* 2012). The genus is the type of its family, the Strelitziaceae, and was named in honour of Queen Charlotte from the house of Mecklenburg-Strelitz and wife to George III of England (Pooley, 2005). The five *Strelitzia* species are *Strelitzia alba* (Skeels)*, S. caudata* (Dyer)*, S. juncea* (Link)*, S. nicolai* (Regel & Koern.) and *S. reginae* (Ait.) (Hensley *et al.,* 2008; Xaba; 2011; Vieira *et al.,* 2012). All species are found in eastern South Africa, in a broadly similar geographic area to the various *Clivia* species, with outlying populations of *S. caudata* in the eastern highlands of Zimbabwe (Cron *et al.,* 2012). The distribution of the various species is outlined in Figure 1.4 below.

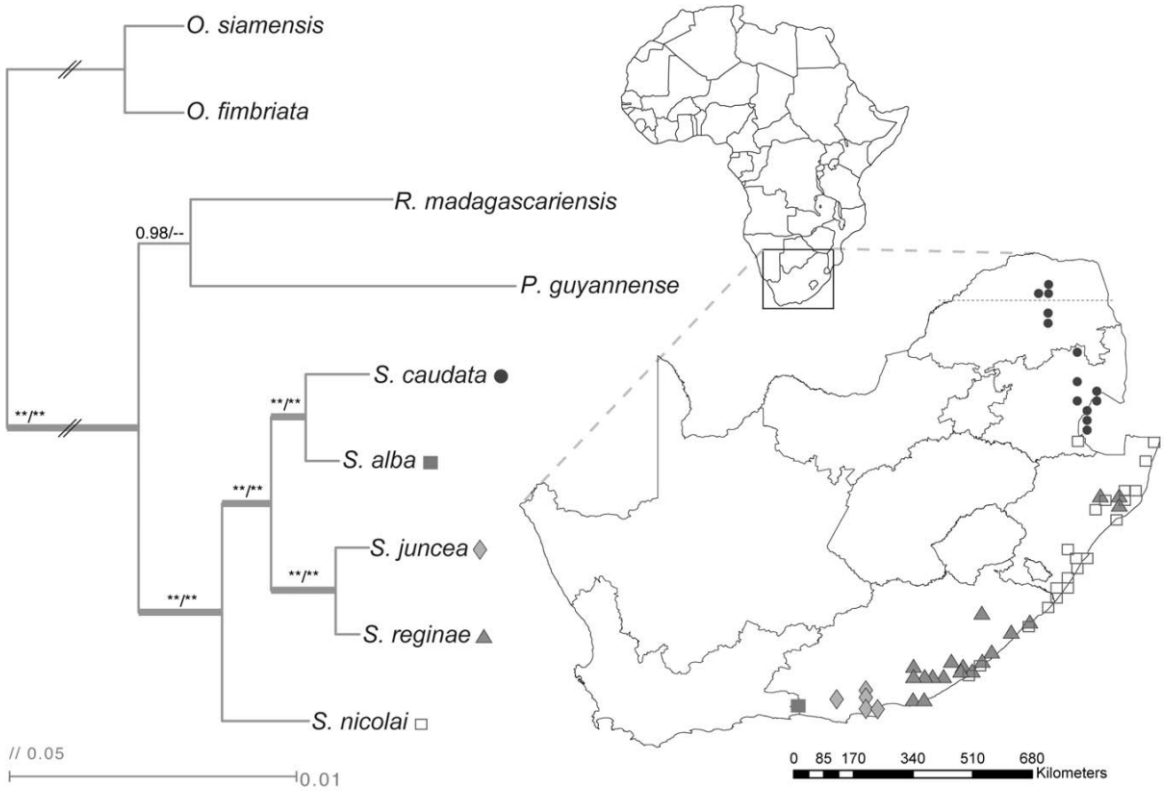


Figure 1.4 Phylogenetic analysis of combined molecular and morphological data for *Strelitzia* and distribution of the five species in South Africa (Cron *et al.,* 2012)

The most widely known species is *S. reginae*, which is grown as a cut flower and pot plant worldwide and used extensively for outdoor landscaping purposes in areas with similar climates to its temperate native range (Hensley *et al.,* 2008; Hoffman *et al.,* 2011; Xaba, 2011). Vieira *et al.* (2012) commented that *S. reginae* is admired for cut flower production because its orange and blue flowers are brightly coloured, large, develop on a long, sturdy stem and exhibit good postharvest durability and longevity. A typical flower of *S. reginae* is

illustrated in Figure 1.5a below. Plants produce the most flowers when cultivated in full sun and in well-drained soil and can form clumps large enough to hold dozens of flower spikes at a time (Hensley *et al.,* 2008).

Although highly valued for its flowers, *S. reginae* does possess some traits which present difficulties in cultivation. North *et al.* (2010) noted that *S. reginae* is 'one of the very few important cut flower plants for which no uniform cultivars are available'. However, Kirstenbosch Botanical Gardens in South Africa have maintained a breeding programme since the late 1990s, with the most notable release being *S. reginae* 'Mandela's Gold'- a yellow-flowered variety that breeds true to type from seed and is illustrated in Figure 1.5b (Xaba, 2011).

Figure 1.5 (a) Bird-of-paradise (*Strelitzia reginae*) displaying showy orange and blue flowers that are highly valued as cut flowers worldwide (photograph taken by author), (b) *Strelitzia reginae* 'Mandela's Gold' – a yellow-flowered cultivar that breeds true to type (Notten, 2002)

The general lack of cultivar development in *S. reginae* is attributed to the fact that conventional methods of propagation are slow. Strelitzias are generally propagated from seed or vegetatively via division of plants (Criley, 1999; Vieira *et al.,* 2012) but seed-grown plants take 3-5 years to first flowering and divisions take 1-2 years to re-establish (Hensley *et al.,* 2008). North *et al.* (2010) cited a need for the development of tissue culture techniques

that can reliably and quickly generate large numbers of plants to allow expansion of production, which is not yet accessible to Strelitzia growers.

1.6.3 *Zantedeschia* (Calla lily)

Zantedeschia is a member of the family Araceae and the genus is comprised of seven species, all endemic to South Africa (Welsh & Clemens, 1992; Pooley, 2005). The genus was named in honour of an Italian physician and botanist, Francesco Zantdeschi, and plants were first introduced to horticulture in Europe in the 1600s (Pooley, 2005). The members of the genus are informally separated into two groups, namely the Group 1 species, *Z. aethiopica* (L.), and the five Group 2 species, namely *Z. albomaculata* (Hook.), *Z. elliottiana* (Watson), *Z. jucunda* (Letty), *Z. pentlandii* (Watson) and *Z. rehmanii* (Engl.) (Griffin & Grobicki, 2000; Kuehny, 2000). The seventh species, *Zantedeschia odorata* (Perry), shares characteristics of both groups (Griffin & Grobicki, 2000).

Zantedeschia aethiopica is classified separately to the rest of the genus due to difference in its rooting structures and the fact that it is evergreen under suitable growing conditions (Kuehny, 2000; Ghimire *et al.,* 2012). *Zantedeschia aethiopica* grows from a long, branched rhizome and can flower year round if it remains evergreen (Kuehny, 2000). It grows naturally in marshy areas and produces white spathes that are sturdy in nature and are hence attractive for cut flowers due to their long shelf lives (Lazzereschi *et al.,* 2011; Ghimire *et al.,* 2012). It is illustrated in Figure 1.6 below.

Figure 1.6 *Zantedeschia aethiopica* flower and plant (Aubrey, 2001b).

The group 2 species are deciduous geophytes that grow from perennial tubers/compact stems and their growth is characterised by a cycle of alternate periods of growth and rest (Welsh & Clemens, 1992; Naor *et al.,* 2006). Many hybrid cultivars have been developed

from these deciduous *Zantedeschia* species and they are popularly grown as outdoor garden plants, cut flowers and pot plants worldwide (Kuehny, 2000; Sandler-Ziv *et al.,* 2011; Ghimire *et al.,* 2012). Chief countries of coloured *Zantedeschia* production are Holland, New Zealand and the US (Sandler-Ziv *et al.,* 2011). Table 1.4 below is reproduced from Kuehny (2000) and lists the variety of commercially grown *Zantedeschia* species and hybrids available in international markets. Figure 1.7 shows four other *Zantedeschia* species that have contributed to the breeding of selections and cultivars from these Group 2 species and show some of the variation in the genus with which breeders can work to develop new and better cultivars.

Figure 1.7 *Zantedeschia* species that have contributed to the floricultural development of the genus - *Z. albomaculata, Z. elliotiana, Z. rehmannii* and *Z. pentlandii* (clockwise from top left) (Singh, 2012).

Table 1.4 *Zantedeschia* species and cultivars used for commercial production (Kuehny, 2000)

Species and cultivar	Flower color
Z. aethiopica (L.) Sprengel	Green at base outside, otherwise white
Childsiana	White
Devoniensis	Large white
Gigantea	Sulphur-yellow with small purple-black blotch
Green Goddess	Green
Little Gem	Small white
Perle von Stuttgart	Small white
White Giant	Large white
White Superior	Large creamy white
White Swan	Ivory-white
Z. albomaculata (Hook.) Baill.	Dark purple inside base, elsewhere white, cream, straw-colored, greenish yellow, or rare
Aurata	Yellow
Best Gold	Dark yellow
Black Eyed Beauty	Cream/white
Bridal Blush	Naples yellow
Cameo	Salmon to pale apricot
Candy	Dark pink
Carmine Red	Violet-purple
Crowbrough	White
Little Jimmy	Cream white
Z. albomaculata (Hook.) Baill. subsp. *albomacualta*	White, ivory or cream, rarely pale yellow or coral-pink
Z. albomaculata (Hook.) Baill. subsp. *macrocarpa* (Engl.) Letty	Cream or straw-colored
Z. albomaculata (Hook.) Baill. ×*elliotiana* (Wats.) Engl.	Yellow, with crimson blotch at the base
Hybrid Yellow or Golden Star	Bright yellow
Z. elliotiana (Wats.) Engl.	Bright golden-yellow
Galaxy	Dark Indian-lake
Golden Sun	Canary yellow
Harvest Moon	Lemon-yellow
Heart Glow	Fiery red
Z. elliottiana (Wats.) Engl. ×*adlami* Leichtlin	Sulphur-yellow, black blotch
Z. elliottiana (Wats.) Engl. ×*aethiopica* (L.) Sprengel Elliotiopica	
Z. elliotiana (Wats.) Engl. ×*albomaculata* (Hook.) Baill. subsp. *albomaculata* Taylori	Yellow with dark blotch
Z. elliottiana (Wats.) Engl. ×*pentlandii* (Wats.) Wittm.	
After Glow	Rounded, peach-orange, red in throat
Aztec Gold	Rounded, gold maturing to burnt orange
Black Magic	Rounded, clear-yellow, throat black
Crystal Glow	Pale pink
Golden Affair	Oval, clear yellow
Majestic Red	Pointed, rich deep red
Mauve Mist	Pointed, clear mauve
Pacific Pink	Oval pointed, slightly fluted, bright pink
Pastel Magic	Pointed with waved edge, clear lemon
Pink Persuation	Oval to pointed, rich pink
Pixie	Pointed, apricot
Regel Charm	Large pointed, blushed orange-red at maturity
Vanity Fair	Pointed, pale yellow with pink flush
Velvet Cream	Cream
Z. elliottiana (Wats.) Engl. ×*rehmanni* Engl. Flame	Yellow blushing red
Z. jucunda Letty	Bright golden-yellow with a dark purple area inside at base
Lavender Petite	Fuchsia-purple
Maroon Dainty	Violet-purple
Mrs. Roosevelt	Creamy yellow
Z. rehmannii Engl.	White to pink or dark purple
Carminea	Large, dark carmine
Crystal Blush	White dark purple (at pollen shed)
Gem Lavender	Lavender purple
Gem Rose	Deep rose
Little Suzy	Pink
Soft Glow	Barium-yellow
Tony	Aureolin
Z. rehmannii Engl. ×*albomaculata* (Hook.) Baill. subsp. *albomaculata*	Intense pink color
Z. rehmannii Engl. ×*elliotiana* (Wats.) Engl.	Brilliant red
Z. rehmannii Engl. ×*elliotiana* (Wats.) Engl. Ragionieri	White tinged pink
Z. rehmannii Engl. ×*pentlandii* (Wats.) Wittm.	Rounded, mauve-pink
Z. rehmannii Engl. ×*pentlandii* (Wats.) Wittm. Marguerita	Salmon-pink
Z. sp. Rubylike Rose	Deep rose
Z. sp. Rubylike Pink Ice	Cool pink (to lavender outdoors)
Z. sp. Garnet Glow	Bright hot pink
Z. sp.Treasure	Bronze orange
Z. sp. Mango	Orange red with bronze overlay
Z. sp. Super Gem	Deep rose (to rose-lavender outdoors)

Zantedeschia species can be easily propagated from seed or from division of offsets from the rhizomes/tubers of mature plants (Sandler-Ziv *et al.,* 2011). Seed-grown plants may exhibit variation amongst individuals and this method can be useful for the development of better cultivars in breeding programmes or in circumstances where large numbers of plants are required and variation is amongst the progeny is not detrimental to the production process.

Hybrid *Zantedeschia* cultivars will not reproduce faithfully from seed and so methods of vegetative propagation are used for commercial multiplication of these varieties (Welsh & Clemens, 1992; Lazzereschi *et al.,* 2011). Tubers of cultivars can be divided, as for the *Zantedeschia* species, but the production of plantlets *in vitro* through the use of tissue culture techniques is the most common method employed these days (Welsh & Clemens, 1992; Sandler-Ziv *et al.,* 2011).

1.7 SUMMARIES OF DISEASE LITERATURE AVAILABLE FOR *CLIVIA*, *STRELITZIA* AND *ZANTEDESCHIA*

Clivia and *Strelitzia* species, hybrids and cultivars are affected by a variety of diseases caused by a number of phytopathogenic organisms. Tables 1.5-1.12 below present a summary of the known pathogens that affect the genera.

1.7.1 *Clivia* pathogens

Available literature cites a number of diseases of *Clivia* species and hybrids in South Africa and internationally. Fungi seem to be the most numerous pathogens reported, whilst bacterial and viral diseases seem to be more limited in number, based on the published literature.

Table 1.5 Known bacterial pathogens of *Clivia*

Genus	Species	Common name of disease and/or host organs affected	*Clivia* species	Reference
Erwinia	*cypripedii*	brown rot	Clivia spp.	Han & Choi (1994)
Pectobacterium	*carotovorum* subsp. *carotovorum*	soft rot	*Clivia miniata*	Choi & Lee (2000); Laing (2009)
Pseudomonas	*syringae* pv. *syringae*		*Clivia miniata*	Khan & Rudolph (1997)

Table 1.6 Known fungal pathogens of *Clivia*

Genus	Species	Common name of disease and/or host organs affected	*Clivia* species	Reference
Alternaria	*tenuissima*		*Clivia* spp.	Swart (2005)
Blennoria	sp.		*Clivia* spp.	Oprea & Neamtu (2001)
Colletotrichum	*boninense*	leaf spot	*Clivia* spp.	Moriwaki *et al.* (2003); Hyde *et al*. (2009); Silva-Rojas & Avila-Quezada (2011)
	cliviae	leaf spot	*C. miniata*	Hyde *et al.* (2009); Yang *et al.* (2009)
	gloeosporiodes	anthracnose	*C. miniata*	Whitlock (1986); Crous *et al.* (2000)
	karstii	anthracnose	*C. miniata*	Damm *et al.* (2012b)
	trichellum	anthracnose	*C. miniata*	Kim *et al.* (2001)
	sp.		*Clivia* spp.	Swart (2005)
Fusicoccum	*luteum*		*C. miniata*	Braithwaite *et al.* (2006)
Fusarium	*oxysporum*		*C. miniata*	Swart (2004)
	sp.	damping off	*Clivia* spp.	Stroh (2002)
Phoma	sp.		*C. miniata*	Swart (2004)
Phytophthora	sp.	damping off	*Clivia* spp.	Stroh (2002)
Pythium	sp.	damping off	*Clivia* spp.	Stroh (2002)
Stagnospora	*curtisii*	leaf and stalk spot	*Clivia* spp.	Hill, (1982); Stroh (2002); Punithalingam & Spooner (2005)
Strelitziana	*cliviae*		*C. miniata*	Crous & Groenewald (2012); Crous *et al.* (2012a)
Macrophoma	*agapanthii*	leaf dieback	*Clivia* spp.	Stroh (2002)
	sp.	leaf spot	*C. miniata*	Zhang (1990)
Rhizoctonia	*solani*	damping off	*Clivia* spp.	Stroh (2002)
Sclerotium	*rolfsii*	collar rot	*Clivia* spp.	Stroh (2002)

Table 1.7 Known viral pathogens of *Clivia*

Genus	Species	Symptoms of infected plants	*Clivia* species	Country reported	Reference
Potexvirus	Undetermined		*Clivia* hybrids	New Zealand	Hammet (2004)
Potyvirus	Narcissus late season yellows virus (NLSYV)	streaking and mottling of leaves	*C. gardenii, C. miniata* 'Belgium hybrid', *C. miniata* var. *citrina*	Australia	Adcock (2007)
Tospovirus	Iris yellow spot virus (IYSV)		*Clivia* spp.	Japan	Jones (2005); Gent *et al.* (2006)
	Tomato spotted wilt virus (TSWV)		*Clivia* spp.	Holland, USA	Verhoeven & Roenhorst (1994); McDonough *et al.* (1999)

1.7.2 *Strelitzia* pathogens

Bacterial and fungal diseases have been reported on *Strelitzia* in South Africa and internationally. Fungal diseases seem to be slightly more numerous than bacterial diseases, whilst there are no reports of any viral pathogens on *Strelitzia* published in available literature.

Table 1.8 Known bacterial pathogens of *Strelitzia*

Genus	Species	Common name of disease and/or host organs affected	*Strelitzia* species	Reference
Acidovorax	*avenae* subsp. *avenae*	bacterial leaf stripe	*S.nicolai*	Wehlburg (1971); Seijo & Peres (2011)
	avenae subsp. *avenae*	bacterial leaf stripe	*S. reginae*	Seijo & Peres (2011)
Burkholderia	*gladioli*	leaf spot and blight	*S. reginae*	Cirvilleri *et al.* (2006a)
Pectobacterium	*carotovorum* subsp. *caratovorum*	brown rot	*S. reginae*	Ogorodnik *et al.* (1996); Abd-El-Khair & Nofal (2001)
Pseudomonas	*syringae* pv. *delphinii*	necrosis and soft rot of flowers	*S. reginae*	Abd-El-Khair & Nofal (2001)
	syringae pv. *syringae*		*S. reginae*	Cirvilleri *et al.* (2005); Cirvilleri *et al.* (2006b).
	sp.	bacterial leaf stripe	*S. reginae*	Wehlburg (1970); Miller (1976)
Ralstonia	*solanacearum*	bacterial wilt	*S. reginae*	Liu *et al.* (2009); Rodrigues *et al.* (2011); Chandrashekara *et al.* (2012)
Xanthomonas	*campestris*		*S. nicolai*	Chase & Jones (1987)
	campestris		*S. reginae*	Chase & Jones (1987); Miller & Chase (1987); Graham *et al*. (1990)

Table 1.9 Known fungal pathogens of *Strelitzia*

Genus	Species	Common name of disease and/or host organs affected	*Strelitzia* species	Reference
Armillaria	*mellea*		*S. reginae*	Davino (1984); Coetzee *et al.* (2001)
Botrytis	*cinerea*	grey mould of flowers	*S. reginae*	Halevy *et al.* (1978); Criley & Paull (1993); McKenzie & Dingley (1996); Finger *et al*. (2003); Braithwaite *et al*. (2006)
Calonectria	*ilicicola*	brown leaf spot	*S. reginae*	Hirooka *et al.* (2008)
Colletotrichum	*gloeosporiodes*	anthracnose	*S. reginae*	Korade *et al.* (2001); Lin *et al.* (2006)
Cylindrocarpon	*destructans*	root rot and leaf wilt	*S. reginae*	Grasso & Cutuli (1972)
Cylindrocladium	*clavatum*		*S. reginae*	Barnard *et al.* (1989)
	pteridis		*S. reginae*	Crous & Wingfield (1994) ; Kubota (2001)
Fusarium	*culmorum*	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	moniliforme	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	oxysporum	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	roseum	flowers	*S. reginae*	Garibaldi (1964)
	solani	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	sporotrichiodes		*S. reginae*	Braithewaite *et al.* (2006)
Microsphaeropsis	sp.	leaf and peduncle spot	*S. reginae*	Agosteo & Pennisi (1996)
Penicillium	sp.		*S. reginae*	Finger *et al.* (2003)
Pestalotiopsis	*versicolor*	leaf spot and blight	*S. nicolai*	Braithewaite *et al.* (2006)
Phoma	*nigricans*		*Strelitzia* sp.	Braithewaite *et al.* (2006)
Phyllacora	*strelitziae*	tar spot	*S. alba*	Doidge (1942); Crous *et al.* (2000)
Phytophthora	*nicotianae*	leaf blight and root and foot rot	*S. reginae*	Luongo *et al.* (2010)
Sclerotium	*rolfsii*	southern blight	*S. reginae*	Polizzi *et al.* (2007)
Strelitziana	*africana*		*Strelitzia* sp.	Arzanlou & Crous (2006); Crous & Groenewald (2012)
Thanatephorus	*cucumeris*		*S. nicolai*	Braithewaite *et al.* (2006)
Toxicocladosporium	*strelitziae*		*S. reginae*	Crous *et al.* (2012b)
Ulocladium	*chartarum*	leaf spot	*S. reginae*	Babelegoto *et al*. (1987); Vannini & Vettraino (2000)

1.7.3 *Zantedeschia* pathogens

Zantedeschia species and hybrids are subject to many diseases, as has been reported in literature from South Africa and abroad. A number of bacterial species have been reported to cause soft rot of plants and tubers and many fungi have been shown to cause spotting and disfigurement of *Zantedeschia* leaves and flowers as well as storage rots of tubers and vascular wilts. Viral pathogens are also prevalent in the genus, with most of the viral species belonging to the Potyviridae.

Table 1.10 Known bacterial pathogens of *Zantedeschia*

Genus	Species	Common name of disease	*Zantedeschia* species	Reference
Chryseobacterium	*indologenes*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Chryseobacterium	sp.		*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Erwinia	*chrysanthemi*	soft rot of tubers	*Zantedeschia* hybrids	Lee & Chen (2002); Mikicinski *et al*. (2010a)
Paenibacillus	*polymyxa*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al.* (2010a)
Pectobacterium	*carotovorum* subsp. *atrosepticum*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Pectobacterium	*carotovorum* subsp. *carotovorum*	soft rot of tubers	*Zantedeschia* hybrids	Wright (1998); Lee & Chen (2002); Snijder & van Tuyl (2002); Snijder *et al*. (2004); Cho *et al*. (2005); Janse (2006); Mikicinski *et al*. (2010a)
Pseudomonas	*marginalis*	soft rot of tubers	*Zantedeschia* hybrids	Krejzar *et al*. (2008); Mikicinski *et al*. (2010a); Mikicinski *et al*. (2010b)
Pseudomonas	*putida*	soft rot of tubers	*Zantedeschia* hybrids	Krejzar *et al*. (2008); Mikicinski *et al*. (2010a)
Pseudomonas	*veronii*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Xanthomonas	*campestris* pv. *zantedeschiae*	bacterial leaf spot and blight	*Z. aethiopica*	Joubert & Truter (1972); Lee *et al.* (2005); Coutinho & Goszczynska (2009)

Table 1.11 Known fungal pathogens of *Zantedeschia*

Genus	Species	Common name of disease	*Zantedeschia* species	Reference
Ascochyta	sp.	leaf and flower spot	*Z. aethiopica*	Crous *et al.* (2000)
Cercospora	*richardiicola*	leaf spot	*Z. aethiopica*	Chupp & Doidge (1948); Crous *et al*. (2000); Vieira & Barreto (2004)
Fusarium	*oxysporum*	vascular wilt	*Zantedeschia* hybrids	Ciampi *et al.* (2009)
Fusarium	*solani*	vascular wilt	*Zantedeschia* hybrids	Ciampi *et al.* (2009)
Leveillula	*taurica*	powdery mildew and leaf spot	*Z. aethiopica*	Doidge *et al.* (1953); Crous & Braun (1996); Crous *et al.* (2000)
Pellicularia	*filamentosa*	crown rot	*Zantedeschia* hybrids	Kuehny (2000)
Phoma	*zantedeschiae*	flower stalk spot	*Z. aethiopica*	Dippenaar (1931); Crous *et al.* (2000)
Phyllosticta	*richardiae*	leaf and flower spot	*Z. aethiopica*	Cejp (1971); Crous *et al.* (2000)
Pythium	*ultimum*	storage rot of tubers	*Zantedeschia* hybrids	Kuehny (2000)
Ramularia	*richardiae*	powdery mildew and leaf spot	*Z. albomaculata*	Crous *et al.* (2000)
Septoria	*aracearum*	leaf spot	*Z. aethiopica*	Doidge *et al.* (1953); Crous *et al.* (2000)

Table 1.12 Known viral pathogens of *Zantedeschia*

Genus	Species	*Zantedeschia* species	Reference
Alfamovirus	Alfalfa mosaic virus (AMV)	*Zantedeschia* spp.	Huang *et al.* (2007)
Cucumovirus	Cucumber mosaic virus (CMV)	*Zantedeschia* spp.	Huang *et al.* (2007)
Potexvirus	Potato virus X (PVX)	*Zantedeschia* spp.	Huang *et al.* (2007)
Potyvirus	Bean yellow mosaic virus (BYMV)	*Zantedeschia* spp.	Huang *et al.* (2007)
	Calla lily latent virus (CLLV)	*Zantedeschia* spp.	Chen *et al.* (2006)
	Dasheen mosaic virus (DsMV)	*Zantedeschia* spp.	Elliot *et al.* (1997); Huang *et al.* (2007); Babu *et al.* (2011)
	Konjac mosaic virus (KoMV)	*Zantedeschia* spp.	Huang *et al.* (2007)
	Turnip mosaic virus (TuMV)	*Zantedeschia* spp.	Huang *et al.* (2007)
	Zantedeschia mild mosaic virus (ZaMMV)	*Zantedeschia* spp.	Huang & Chang (2005); Huang *et al.* (2007)
	Zantedeschia mosaic virus (ZaMV)	*Zantedeschia* spp.	Kwon *et al.* (2002); Huang *et al.* (2007)
Nepovirus	Arabis mosaic virus (ArMV)	*Zantedeschia* spp.	Huang *et al.* (2007)
Tobravirus	Tobacco rattle virus (TRV)	*Zantedeschia* spp.	Huang *et al.* (2007)
Tospovirus	Calla lily chlorotic spot virus (CCSV)	*Zantedeschia* spp.	Chen *et al.* (2005)
	Impatiens necrotic spot virus (INSV)	*Zantedeschia* spp.	Huang *et al.* (2007)
	Tomato spotted wilt virus (TSWV)	*Zantedeschia* spp.	Huang *et al.* (2007)

1.8 PLANT PATHOGEN IDENTIFICATION PROTOCOLS

Agrios (2005) stated 'when a pathogen is found on a diseased plant, the pathogen is identified by reference to special manuals; if the pathogen is known to cause such a disease and the diagnostician is confident that no other causal agents are involved, then the diagnosis of the disease may be considered complete'. If, however, the pathogen found seems to be the cause of the disease but no previous reports exist to support this, Agrios (2005) presents a four-step layout for the overall procedures that need to be followed to successfully and confidently identify the phytopathogenic organism. These steps are collectively known as Koch's postulates and are stated by Agrios (2005) as follows:

1. The pathogen must be found associated with the disease in all the diseased plants examined.

2. The pathogen must be isolated and grown in pure culture on nutrient media and its characteristics described (non-obligate parasites), or it must be grown on a susceptible host plant (obligate pathogens) and its appearance and effects recorded.

3. The pathogen from pure cultures must be inoculated onto healthy plants of the same species or variety on which the disease appears, and it must produce the same disease on the inoculated plants.

4. The pathogen must be isolated in pure culture again and its characteristics must be exactly like those observed in Step 2.

If all the above steps have been followed and proved true, then the isolated pathogen is identified as the organism responsible for the disease.

Agrios (2005) elaborated further that Koch's postulates are applicable to pathogen groups such as fungi, bacteria, nematodes, some viruses, some viriods and spiroplasmas (although this is not always easy to carry out) but that they cannot be carried out with other pathogens (some viruses, phytoplasmas, phloem-inhabiting bacteria, obligate fungi and protozoa) because their culture or purification is not always possible. It is additionally often not possible to reintroduce these pathogens into plants to reproduce disease and thus the acceptance of these organisms as the pathogens of the diseases with which they are associated is tentative. Advances and improvement in techniques for the isolation, culture and inoculation of pathogens may aid in carrying out these postulates for all pathogens (Agrios, 2005).

Since plants are subject to diseases caused by a wide range of pathogens, the techniques employed for identification of different groups of these pathogens may differ. Techniques and methods for the identification of bacterial, fungal and viral pathogens are discussed below:

1.8.1 Identification of phytopathogenic bacteria

Diagnosis and identification of phytopathogenic bacteria is based primarily on disease symptoms, the presence of large bacterial populations in the infected area and the absence of other pathogens (Agrios, 2005). Agrios (2005) stated that 'the easiest and most accurate way to prove that an observed bacterium is the pathogen is through the isolation and growth of pure bacterial cultures and the use of a single pure colony for reinoculation of susceptible host plants to carry out Koch's postulates'. Once the pathogenicity of a bacterial isolate has been confirmed, characterisation can be carried out through a number of microbiological techniques. Goszczynska *et al.* (2000) provide the following schematic (Figure 1.8) for the identification of phytopathogenic bacteria.

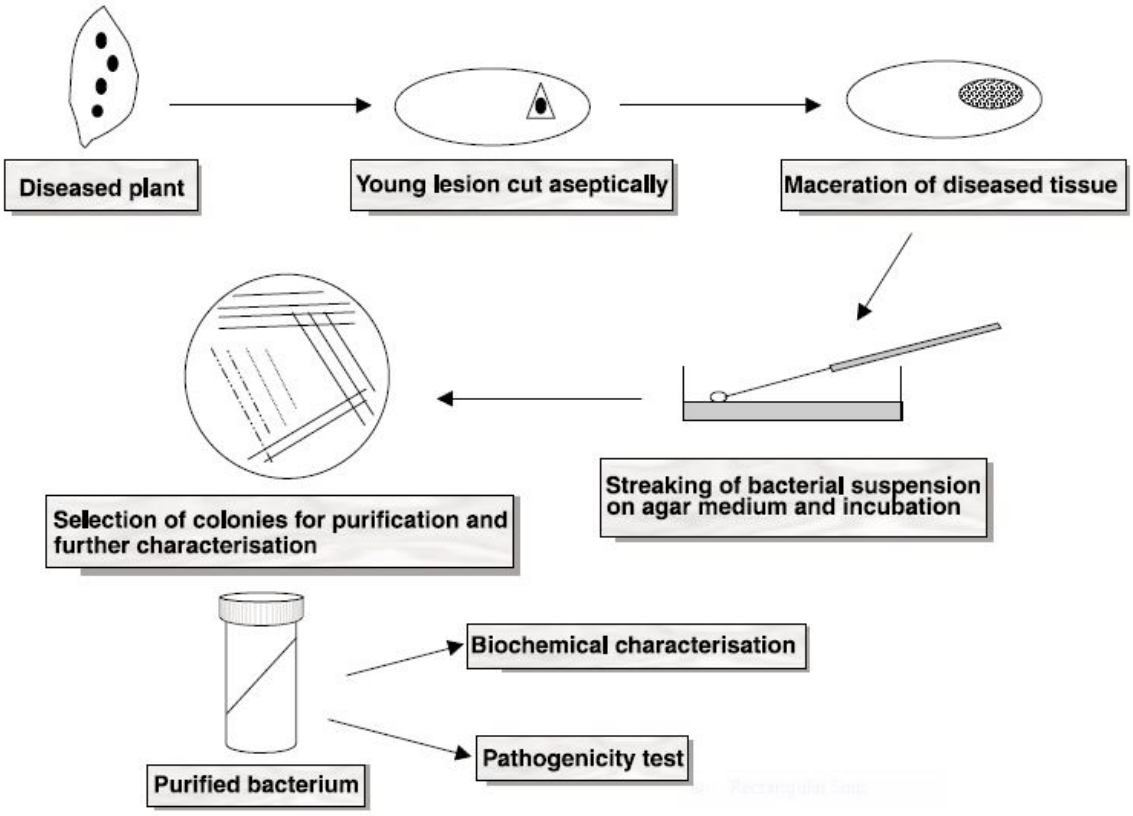


Figure 1.8 Steps taken to isolate and identify phytopathogenic bacteria (Goszczynska *et al.,* 2000)

Techniques used for the characterisation of bacteria in general include: plating of samples on selective or semi-selective media (making preliminary identification of suspected pathogens easier), biologs (profiles of substrates utilized by the bacteria for food) and analysis of fatty acid profiles (Goszczynska *et al.,* 2000; Agrios, 2005). These methods all generate specific profiles for the isolate that can be compared to those of known organisms published in available literature to determine any similarities between the unknown isolate and known bacteria.

Another avenue for bacterial identification is the use of immunodiagnostic or serological techniques such as immunomagnetic separation (IMS), immunofluorescence colony (IFC) staining and forms of enzyme-linked immunosorbent assay (ELISA) (Perombelon & Van der Wolf, 2002). These techniques rely on the availability of mono- and polyclonal antibodies specific to certain groups of bacteria. The antibodies used can be specific to bacterial genera, species or even subspecies and strains and allow for rapid and accurate detection of target organisms, often even from samples where bacterial numbers are very low (Goszczynska & Aveling, 2009). Limitations of these sorts of techniques come into play when

antibodies are not available for detection of target groups or when antibodies display cross-reactivity towards undesired groups of bacteria (Goszczynska & Aveling, 2009).

Matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS) is a technique that generates a spectrum of the constantly expressed, highly abundant ribosomal proteins of bacterial samples (Wybo *et al.,* 2011). Wybo *et al.* (2011) stated that MALDI-TOF MS has the capacity to discriminate between closely related species of bacteria based on their ionised ribosomal protein spectra. Bizzini & Greub (2010) described this technique as 'a rapid and accurate method for the identification of bacteria'.

The development of molecular techniques for bacterial identification has had a major impact on all aspects of microbiology, including the identification and classification of phytopathogenic bacteria (Venter, 2009). Polymerase chain reaction (PCR) is now a routinely used technique to amplify bacterial DNA sequences and Palacio-Bielsa *et al.* (2009) provided a comprehensive review of the PCR techniques and primers published for the identification of plant pathogenic bacteria. Their review strengthens the view of Mumford *et al.* (2006) that 'there are only a few pathogens for which a reliable PCR-based diagnostic system does not exist'.

Palacio-Bielsa *et al.* (2009) and Venter (2009) cited a number of advantages that PCR-based techniques offer for bacterial identification, namely that they are particularly useful for the detection of fastidious and unculturable bacteria (and organisms in general), there is increased specificity and sensitivity over traditional techniques based on culturing diagnostics, and samples can be analysed much more rapidly, allowing for more efficient diagnosis of bacterial diseases and recommendations for their control.

1.8.2 Phytopathogenic fungal identification

Agrios (2005) proposed that a general procedure that should be carried out for the identification of phytopathogenic fungi is as follows: initially one should study the morphology of fungal mycelium, fruiting structures and spores under the microscope. One should then attempt to match this up with appropriate mycological or plant pathology literature to see whether the fungus has been reported to be pathogenic, especially on the host plant on which it was found. If the observed plant symptoms correspond to those in literature, then diagnosis can be considered complete in most cases.

If the fungus/fungi observed are not known to cause plant disease, Agrios (2005) suggested that the fungus may be a saprophyte, or a previously unreported plant pathogen and one should continue through the implementation of Koch's postulates for proof of disease

causation. Fungi can be isolated from diseased plants in a similar manner to bacteria and the procedure outlined in Figure 1.8 is applicable for fungal isolation too.

Additionally, the use of selective fungal media is commonly followed in the isolation and identification of fungi. For example, Moralejo *et al.* (2009) used the selective medium PARBPH to isolate *Phytophthora* sp. from multiple plant tissues and potting media of plants showing showing infection whilst Hadar *et al.* (1989) used PPSM as a standard *Fusarium*-selective medium. In a similar manner to bacteria, the use of media containing compounds known to selectively inhibit the growth of certain groups of fungi and encourage the growth of others can be used to make the preliminary identification of pathogenic fungi easier.

Kabashima *et al.* (2007) noted that ELISA test kits are available for the detection of *Pythium, Phytophthora* and *Rhizoctonia* species and that these kits allow confirmation of the presence of these pathogens in samples in as little as 10 minutes. However, they also mention that incidences of cross-reactivity leading to false positives have been observed with these kits and caution should be exercised in interpreting positive test results without the use of more specific follow-up tests.

Molecular methods can also be used in the diagnosis and identification of fungal diseases. Ghignone & Migheli (2005) cited the nucleic acid based technique of PCR as most suited for early detection of phytopathogenic fungi due to its high sensitivity and potential for automation. The authors also presented a database of PCR primers for phytopathogenic fungi (http://www.sppadbase.com) where researchers can quickly search for primer pairs specific to a target genus or species, the template DNA targeted by the primers, the PCR techniques used and references to the literature in which the techniques were presented. Capote *et al.* (2012) stated that further molecular techniques such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) are commonly used by researchers for fungal identification.

1.8.3 Plant virus diagnosis and identification

Agrios (2005) stated that viral diseases are diagnosed and viruses identified primarily through a combination of five methods:

i) Virus transmission tests to specific host plants (via sap inoculation, grafting or vectors)

ii) Serodiagnostic tests– primarily forms of ELISA only applicable to viruses for which specific antisera are available

iii) Electron microscopy – negative staining of leaf dips or purified virus samples or immune-specific electron microscopy (again only if specific antisera are available)

iv) Microscopic examination of infected cells for inclusions bodies induced by viral presence in the cells – usually diagnostic of the group to which the virus belongs

v) Molecular techniques

Jan *et al.* (2012) elaborated on the various molecular techniques used for plant virus diagnosis and stated that procedures such as reverse transcriptase PCR (for RNA viruses) and various forms of PCR (for DNA viruses) as well as nucleic acid hybridisation are all used for virus identification. Jan *et al.* (2012) further observed that the use of micro-arrays and next generation sequencing (pyrosequencing) are at the forefront of viral detection methods.

The techniques employed for identification of each group of pathogens should be carried out in conjunction with each other, such that the results from each type of analysis support those of the others. The better the concordance between the results of each test, the more confident one can be with the identification of the pathogen one has isolated.

1.9 REFERENCES

Abd-El-Khair, H. & Nofal, M.A. (2001) Flower bacterial soft rot of bird of paradise (*Strelitzia reginae*, Banks) in Egypt and its control. Arab Universities Journal of Agricultural Sciences 9: 397-410.

Adcock, K. (2007) Potyvirus infection in Clivias. Clivia News 16: 11-12.

Agosteo, G.E. & Pennisi, A.M. (1996) Infections of *Microsphaeropsis* sp. on *Strelitzia reginae*. Informatore Fitopatologico 46: 20-22.

Agrios, G.N. (2005) Plant Pathology. 5th edition. Academic Press, USA. pp 37-41.

Anonymous (2011a) Floriculture Crops: 2010 Summary. United States Department of Agriculture and National Agricultural Statistics Service publication. ISSN: 1949-0917. pp. 1-72.

Anonymous (2011b) History of Clivia. http://www.cliviasociety.org/history_of_clivia.html. Accessed 07/06/2013.

Anonymous (2013) Floriculture Crops: 2012 Summary. United States Department of Agriculture and National Agricultural Statistics Service publication. ISSN: 1949-0917. pp. 1-59.

Annandale, J.G., Jovanovic, N.Z., Benade, N. & Allen, R.G. (2002) Software for missing data error analysis of Penman-Monteith reference evapotranspiration. Irrigation Science 21: 57-67.

Arzanlou, M. & Crous, P.W. (2006) *Strelitziana* M. Arzanlou & Crous, gen. nov. *Fungal Planet* 8: 1-2.

Aubrey, A. (2001a) *Clivia miniata* [Lindl.] Regel. http://www.plantzafrica.com/plantcd/cliviaminiata.htm. Accessed: 07/06/2013.

Aubrey, A. (2001b) *Zantedeschia aethiopica* (L.) Spreng. http://www.plantzafrica.com/plantwxyz/zantedeschaeth.htm. Accessed: 10/07/2013.

Babelegoto, N.M., Frisullo, S. & Varvaro, L. (1987) Alterazioni sulle parti epigee di *Strelitzia reginae* Banks causate da *Ulocladium chartarum* (Preuss) Simmons. La Difesa Delle Piante 10: 289-292.

Babu, B., Hegde, V., Makeshkumar, T. & Jeeva, M.L. (2011) Development of non-radioactive probes for specific detection of Dasheen mosaic virus infecting *Amorphophallus paeoniifolius*. Current Science 100: 1220-1225.

Barnard, E.L., El-Gholl, N.E., Schubert, T.S., Leahy, R.M. & Schoulties, C.L. (1989) *Cylindrocladium clavatum* in Florida. Plant Disease 73: 273.

Bester, C., Blomerus, L.M. & Kleynhans, R. (2009) Development of new floriculture crops in South Africa. Acta Horiculturae. 813: 67-72.

Bizzini, A., & Greub, G. (2010) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clinical Microbiology and Infection 16: 1614–1619.

Boshoff, H. & Coetzee, J.C. (2007) Implementation viability of the Goxe BEE floriculture project. Futures Trust and Natural Botanicals 2: 8-14.

Braithwaite, M., Hill, C.F., Ganev, S., Pay, J.M., Pearson, H.G. & Alexander, B.J.R. (2006) A survey of subtropical nursery plants for fungal diseases in Northland. New Zealand Plant Protection 59: 132-136.

Callaway, D. & Callaway, M.B. (2000) Breeding Ornamental Plants. Timber Press, USA. pp. 42-45.

Capote, N., Pastrana, A.M., Aguado, A. & Sanchez-Torres, P. (2012) Molecular tools for detection of plant pathogenic fungi and fungicide resistance. In: Plant Pathology. C.J. Cumagun (ed.). Intech, Spain. pp. 151-202.

Cejp, K. (1971) Some members of the Sphaeropsidales from South Africa. Bothalia 10: 341-346.

Chandrashekara, K.N., Prasannakumar, M.K., Deepa, M., Vani, A. & Khan, A.N. (2012) Prevalence of races and biotypes of *Ralstonia solanacearum* in India. Journal of Plant Protection Research 52: 53-58.

Chase, A.R. & Jones, J.B. (1987) Leaf spot and blight of *Strelitzia reginae* (bird-of-paradise) caused by *Xanthomonas campestris*. Plant Disease 71: 845-847.

Chen, C.C., Chen, T.C., Lin, Y.H., Yeh, S.D. & Hsu, H.T. (2005) A chlorotic spot disease on calla lilies (*Zantedeschia* spp.) is caused by a tospovirus serologically but distantly related to watermelon silver mottle virus. Plant Disease 89: 440-445.

Chen, C.C., Hsu, H.T., Chiang, F.L. & Chang, C.A. (2006) Serological and molecular properties of five potyviruses infecting calla lily. Acta Horticulturae 722: 259-269.

Cho, H.R., Lim, J.H., Yun, K.J., Snijder, R.C., Goo, D.H., Rhee, H.K. & Kim, K.S. (2005) Virulence variation in 20 isolates of *Erwinia carotovora* subsp. *carotovora* on *Zantedeschia* cultivars in Korea. Acta Horticulturae 673: 653-659.

Choi, J.E. & Lee, E.J. (2000) New bacterial soft rot of ornamental foliage plants by *Erwinia carotovora* subsp. *carotovora* in Korea. Plant Disease Research 6: 19-22.

Chupp, C. & Doidge, E.M. (1948) *Cercospora* species recorded for Southern Afica. Bothalia 4: 881-893.

Ciampi, L.P., Nissen, J.M., Venegas, E.G., Fuentes, R.P., Costa, M.L., Schobitz, R.T., Alvarez, E.D. & Alvarado, P.A. (2009) Identification of two species of *Fusarium* Link. that cause wilting of coloured callas (*Zantedeschia aethiopica* (L.) Spreng.) cultivated under greenhouse conditions in Chile. Chilean Journal of Agricultural Research 69: 516-525.

Cirvilleri, G., Bonaccorsi, A., Scuderi, G. & Scortichini, M. (2005) Potential biological control activity and genetic diversity of *Pseudomonas syringae* pv. *syringae* strains. Journal of Phytopathology 153: 654-666.

Cirvilleri, G., Bonaccorsi, A., Vitale, A., Castello, I. & Polizzi, G. (2006a) First report of leaf spot and blight of *Strelitzia reginae* caused by *Burkholderia gladioli* in Italy. Plant Disease 90: 1553.

Cirvilleri, G., Scuderi, G., Bonaccorsi, A. & Scortichini, M. (2006b) Molecular characterisation of *Pseudomonas syringae* pv. *syringae* strains from different host plants using fluorescent amplified fragment length polymorphism. Journal of Plant Pathology 88: 329-333.

Coetzee, M.P.A., Wingfield, B.D., Harrington, T.C., Stiemel, J., Coutinho, T.A. & Wingfield, M.J. (2001) The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. Molecular Ecology 10: 387-396.

Conrad, F. & Snijman, D. (2011). Systematics and phylogeography of *Clivia*. Clivia 13: 9-25.

Coutinho, T.A. & Goszczynska, T. (2009) Leaf spot of arum lilies. In: Bacterial Diseases of Plants in South Africa. T.A. Coutinho, T. Goszczynska, C. Lennox and S.N. Venter (eds.). Briza Publications, South Africa. pp. 85-86.

Cowling, R.M. & Hilton-Taylor, C. (2003) Phytogeography, flora and endemism. In: Vegetation of Southern Africa. R.M. Cowling, D.M. Richardson & S.M. Pierce (eds.). Cambridge University Press, UK. pp. 43-61.

Criley, R.A. & Paull, R.E. (1993) Review: Postharvest handling of bold tropical cut flowers – *Anthurium, Alpinia purpurata, Heliconia* and *Strelitzia.* Acta Horticulturae 337: 201-212.

Criley, R.A. (1999) Landscaping with heliconias, gingers and their relatives. Acta Horticulturae 486: 247-253.

Cron, G.V., Pirone, C., Bartlett, M., Kress, W.J. & Specht, C. (2012) Phylogenetic relationships and evolution in the Strelitziaceae (Zingiberales). Systematic Botany 37: 606-619.

Crous, P.W. & Braun, U. (1996) Cercosporoid fungi from South Africa. Mycotaxon LVII: 233-321.

Crous, P.W. & Groenewald, J.Z. (2012) *Strelitziana cliviae* Crous, *sp. nov.* Fungal Planet description sheet 136. Persoonia 29: 164-165.

Crous, P.W. & Wingfield, M.J. (1994) A monograph of *Cylindrocladium*, including anamorphs of *Calonectria*. Mycotaxon LI: 341-435.

Crous, P.W., Shivas, R.G., Wingfield, M.J., Summerell, B.A., Rossman, A.Y., Alves, J. L., Adams, G.C., Barreto, R.W., Bell, A., Coutinho, M.L., Flory, S.L., Gates, G., Grice, K.R., Hardy, G.E.St.J., Kleczewski, N.M., Lombard, L., Longa, C.M.O., Louis-Seize, G., Macedo, F., Mahoney, D.P., Maresi, G., Martin-Sanchez, P.M., Marvanová, L., Minnis, A.M., Morgado, L.N. & Noordeloos, M. E. (2012a) Fungal Planet description sheets: 128-153. Persoonia 29:146-201.

Crous, P.W., Summerell, B.A., Shivas, R.G., Burgess, T.I., Decock, C.A., Dreyer, L.L., Granke, L.L., Guest, D.I., Hardy, G.E.S.J., Hausbeck, M.K., Hüberli, D., Jung, T., Koukol, O., Lennox, C.L., Liew, E.C.Y., Lombard, L., McTaggart, A.R., Pryke, J.S., Roets, F., Saude, C., Shuttleworth, L.A., Stukely, M.J.C., Vánky, K., Webster, B.J., Windstam, S.T. & Groenewald, J.Z. (2012b) Fungal Planet description sheets: 107-127. Persoonia 28: 138-182.

Crous, P.W., Phillips, A.J.L. & Baxter, A.P. (2000) Phytopathogenic Fungi from South Africa. University of Stellenbosch, Department of Plant Pathology Press, South Africa. pp. 7-50.

Daughtrey, M. (2007) Propagation pathology: the basics. Combined Proceedings International Plant Propagators' Society 57: 293-295.

Daughtrey, M.L. & Benson, D.M. (2005) Principles of plant health management for ornamental plants. Annual Review of Phytopathology 43: 141-169.

Davino, M. (1984) Infections of *Armillaria mellea* on *Strelitzia* plants. Colture Protette 13: 63-65.

Dippenaar, B.J. (1931) Descriptions of some new species of South African fungi and of species not previously recorded from South Africa. South African Journal of Science 28: 284-289.

Dixon, R. (2011) Relationships in *Clivia*. Clivia 13: 63-81.

Doidge, E.M. (1942) Revised descriptions of *Phyllachora* and related fungi. Bothalia 4: 421-463.

Doidge, E.M., Bottomley, A.M., Van der Plank, J.E. & Pauer, G.D. (1953) A revised list of plant diseases in South Africa. Department of Agriculture Science Bulletin 346: 1-122.

Duncan, G.D. (1999) Grow *Clivia*. The National Botanical Institute, South Africa. pp. 2-5.

Elliot, M.S., Zettler, F.W. & Brown, L.G. (1997) Dasheen mosaic potyvirus of edible and ornamental aroids. Plant Pathology Circular 384: 1-4.

Finger, F.L., de Moraes, P.J., Barbosa, J.G. & Grossi, J.A.S. (2003) Vase life of bird-of-paradise flowers influenced by pulsing and term of cold storage. Acta Horticulturae 628: 863-867.

Garber, P.M. (1989) Tulipmania. Journal of Political Economy 97: 535-560.

Garibaldi, A. (1964) *Fusarium roseum* infection on flowers of *Strelitzia reginae*. Rivista della Ortoflorofrutticoltura Italiana 48: 531-534.

Gent, D.H., du Toit, L.J., Fichtner, S.F., Mohan, S.K., Pappu, H.R. & Schwartz, H.F. (2006) Iris yellow spot virus: an emerging threat to onion bulb and seed production. Plant Disease 90: 1468-1480.

Ghignone, S. & Migheli, Q. (2005) The database of PCR primers for phytopathogenic fungi. European Journal of Plant Pathology 113: 107-109.

Ghimire, B.K., Yu, C.Y., Kim, H.J. & Chung, I.M. (2012) Karyotype and nucleic acid content in *Zantedeschia aethiopica* Spr. and *Zantedeschia elliottiana* Engl. African Journal of Biotechnology 11: 11604-11609.

Goldblatt, P. (1978) An analysis of the flora of Southern Africa: it's characteristics, relationships and origins. Annals of the Missouri Botanical Garden 65: 369-436.

Goszczynska, T., Serfontein, J.J. & Serfontein, S. (2000) Introduction to Practical Phytobacteriology. SAFRINET, South Africa. pp. 1-9.

Goszczynska, T. & Aveling, T.A. (2009) Bacterial seed-borne diseases in South Africa. In: Bacterial Diseases of Plants in South Africa. T.A. Coutinho, T. Goszczynska, C. Lennox and S.N. Venter (eds.). Briza Publications, South Africa. pp. 12-16.

Graham, J.H., Hartung, J.S., Stall, R.E. & Chase, A.R. (1990) Pathological, restriction-fragment length polymorphism and fatty acid profile relationships between *Xanthomonas campestris* from citrus and noncitrus hosts. Phytopathology 80: 829-836.

Grasso, S. & Cutuli, G. (1972) A decline of *Strelitzia reginae* caused by *Cylindrocarpon destructans*. Rivista di Patologia Vegetale IV 8: 207-214.

Griffin, N.J. & Grobicki, A.M. (2000) Community income generation through cultivation of high value plants in degraded urban wetlands. WISA presentation: 1-15.

Hadar, E., Katan, J. & Katan, T. (1989) The use of nitrate-nonutilising mutants and a selective medium for studies of pathogenic strains of *Fusarium oxysporum.* Plant Disease 73: 800-803.

Halevy, A.H., Kofranek, A.M. & Besemer, S.T. (1978) Postharvest handling methods for bird-of-paradise flowers (*Strelitzia reginae* Ait.). Journal of the American Society for Horticultural Science 103: 165-169.

Hammet, K. (2004) Virus symptoms and particles – correspondence from the Clivia enthusiasts email group. Clivia News 13: 19-22.

Han, K.S. & Choi, J.E. (1994) Bacterial brown rot of Scarlet Kafir Lily (*Clivia* spp.) caused by *Erwinia cypripedii*. The Plant Pathology Journal 4: 333-335.

Harden, J. (1992) Plant protection – management of pest control techniques. Combined Proceedings International Plant Propagators' Society 42: 99-102.

Hartmann, H.T., Kester, D.E., Davies, F.T. & Geneve, R.L. (2011) Hartmann & Kester's Plant Propagation – Principles and Practices. 8th ed. Prentice Hall, USA. pp. 14-281.

Hensley, D., Deputy, J., Yogi, J. & Leonhardt, K. (2008) Bird-of-Paradise. CTAHR-University of Hawaii Ornamentals and Flowers 27: 1-2.

Heywood, V. (2003) Conservation and sustainable use of wild species as sources of new ornamentals. Acta Horticulturae 598: 43-53.

Hill, C.F. (1982) New plant disease records in New Zealand, 1974-1982. New Zealand Journal of Botany 20: 355-359.

Hirooka, Y., Takeuchi, J., Horie, H. & Natsuaki, K.T. (2008) Cylindrocladium brown leaf spot on *Howea belmoreana* caused by *Calonectria ilicicola* (anamorph: *Cylindrocladium parasiticum*) in Japan. Journal of General Plant Pathology 74: 66-70.

Hoffmann, F., Daniel, F., Fortier, A. & Hoffmann-Tsay, S.S. (2011) Efficient avian pollination of *Strelitzia reginae* outside of South Africa. South African Journal of Botany 77: 503-505.

Huang, C.H. & Chang, Y.C. (2005) Identification and molecular characterisation of Zantedeschia mild mosaic virus, a new calla lily-infecting potyvirus. Archives of Virology 150: 1221-1230.

Huang, C.H., Hu, W.C., Yang, T.C. & Chang, Y.C. (2007) Zantedeschia mild mosaic virus, a new widespread virus in calla lily, detected by ELISA, dot-blot hybridisation and IC-RT-PCR. Plant Pathology 56: 183-189.

Hull, R. (2009) Comparitive Plant Virology. 2nd edition. Elsevier Academic Press, USA. pp 4-9.

Hyde, K.D., Cai, L., Cannon, P.F., Crouch, J.A., Crous, P.W., Damm, U., Goodwin, P.H., Chen, H., Johnston, P.R., Jones, E.B.G.,, Liu, Z.Y., McKenzie, E.H.C., Moriwaki, J., Noireung, P., Pennycook, S.R., Pfenning, L.H., Prihastuti, H., Sato, T., Shivas, R.G., Tan, Y.P., Taylor, P.W.J., Weir, B.S., Yang, Y.L. and Zhang, J.Z. (2009) *Colletotrichum* – names in current use. Fungal Diversity 39: 147-182.

Jain. S.M. (2006) Mutation-assisted breeding for improving ornamental plants. Acta Horticulturae 714: 85-98.

Jan, A.T., Azam, M., Warsi, M.K., Ali, A. & Haq, Q. (2012) Technical advancement in plant virus diagnosis – an appraisal. Archives of Phytopathology and Plant Protection 45: 909-921.

Janse, J.D. (2006) Phytobacteriology: Principles and Practice. CAB International, UK. pp. 105-107.

Jones, D.R. (2005) Plant viruses transmitted by thrips. European Journal of Plant Pathology 113: 119-157.

Joubert, J.J. & Truter, S.J. (1972) A variety of *Xanthomonas campestris* pathogenic to *Zantedeschia aethiopica*. New Zealand Journal of Crop and Horticultural Science 78: 212-217.

Kabashima, J.N., MacDonald, J.D., Dreistadt, S.H. & Ullman, D.E. (2007) Easy on-site tests for fungi and viruses in nurseries and greenhouses. University of California Publication 8002: 1-7.

Khan, I.D. & Rudolph, K. (1997) Pathochemical investigations on a phytotoxin secreted by *Pseudomonas syringae* pv. *syringae* in bush bean leaves (*Phaseolus vulgaris* L.). Developments in Plant Pathology 9: 261-264.

Kim, W.G., Hong, S.K. & Cho, W.D. (2001) Occurrence of anthracnose on English Ivy caused by *Colletotrichum trichellum* in Korea. Mycobiology 29: 107-109.

Korade, B.B., Pawar, D.R. & Joshi, M.S. (2001) Anthracnose disease of spider lily. Journal of Maharashtra Agricultural Universities 26: 70-71.

Krezjar, V., Mertelik, J., Pankova, I., Kloudova, K. & Kudela, V. (2008) *Pseudomonas marginalis* associated with soft rot of *Zantedeschia* spp. Plant Protection Science 44: 85-90.

Kubota, M. (2001) Occurrence of leaf spot on *Strelitzia reginae* caused by *Cylindrocladium pteridis*. Annual Report of the Kanto-Tosan Plant Protection Society 48: 89-91.

Kuehny, J.S. (2000) Calla history and culture. Hortechnology 10: 267-274.

Kwon, S.B., Ha, J.H., Yoon, J.Y. & Ryu, K.H. (2002) Zantedeschia mosaic virus causing leaf mosaic symptom in calla lily is a new potyvirus. Archives of Virology 147: 2281-2289.

Laing, M. (2009) Soft rot of agapanthus, bulbine, clivia and wild ginger. In: Bacterial Diseases of Plants in South Africa. T.A. Coutinho, T. Goszczynska, C. Lennox and S.N. Venter (eds.). Briza Publications, South Africa. pp. 163-164.

Lambardi, M., Benelli, C., Ozudogru, E.A. & Ozden-Tokatli, Y. (2006) Synthetic seed technology in ornamental plants. In: Floriculture, Ornamental and Plant Biotechnology Volume 2. Global Science Books, UK. pp. 347-354.

Lazzereschi, S., Grassotti, A., Cacini, S. & Nesi, B. (2011) *Zantedeschia aethiopica*: Effects of basal thermal treatments on flowering time, yield and quality. Acta Horticulturae 893: 953-960.

Lee Y.A. & Chen K.P. (2002) First report of bacterial soft rot of white flowered calla lily caused by *Erwinia chrysanthemi* in Taiwan. Plant Disease 86: 1273.

Lee, Y.A., Chen, K.P & Chang, Y.C. (2005) First report of bacterial leaf blight of white-flowered calla lily caused by *Xanthomonas campestris* pv. *zantedeschiae* in Taiwan. Plant Pathology 54: 239.

Lin, Q., Lin, G., Liu, F., Zhang, N. & Huang, W. (2006) Biological characteristics of anthracnose of *Strelitzia reginae* and control efficiencies of fungicides against the pathogen. Fujian Journal of Agricultural Sciences 21: 14-19.

Linder, H.P. (2001) Plant diversity and endemism in sub-Saharan tropical Africa. Journal of Biogeography 28: 169-182.

Liu, Y., Kanda, A., Yano, K., Kiba, A., Hikichi, Y., Aino, M., Kawaguchi, A., Mizoguchi, S., Nakaho, K., Shiomi, H., Takikawa, Y. & Ohnishi, K. (2009) Molecular typing of Japanese strains of *Ralstonia solanacearum* in relation to the ability to induce a hypersensitive reaction in tobacco. Journal of General Plant Pathology 75: 369-380.

Luongo, L., Galli, M., Riccioni, L. & Belisario, A. (2010) First report of leaf blight and root and foot rot of a *Strelitzia* caused by *Phytophthora nicotianae* in Italy. Plant Disease 94: 134.

Manning, J. (2003). Photographic Guide to the Wildflowers of South Africa. Briza Publications, South Africa. pp. 12-16.

Marolejo, E., Perez-Sierra, A.M., Alvarez, L.A., Belbahri, L., Lefort, F. & Descals, E. (2009) Multiple alien *Phytophthora* taxa discovered on diseased ornamental plants in Spain. Plant Pathology 58: 100-110.

Martinez, A. (2007) Georgia plant disease loss estimates. University of Georgia Cooperative Extension Special Bulletin 41: 1-24.

Matthee, M., Naude, W. & Viviers, W. (2006) Challenges for the floriculture industry in a developing country: a South African perspective, Development Southern Africa, 23, 511-528.

McDonough, M.J., Gerace, D. & Ascerno, M.E. (1999) Western flower thrips feeding scars and tospovirus lesions on petunia indicator plants. http://purl.umn.edu/51809. Accessed 05/07/2013.

McKenzie, E.H.C. & Dingley, J.M. (1996) New plant disease records in New Zealand: miscellaneous fungal pathogens III. New Zealand Journal of Botany 34: 263-272.

Mikicinski, A., Sobiczewski, P., Pulawska, J. & Treder, J. (2010a) Involvement of *Paenibacillus polymyxa* in the etiology of bacterial soft rot of calla lily. Journal of Plant Pathology 92: 375-380.

Mikicinski, A., Sobiczewski, P., Sulikowska, M., Pulawska, J. & Treder, J. (2010b) Pectolytic bacteria associated with soft rot of calla lily (*Zantedeschia* spp.) tubers. Journal of Phytopathology 158: 201-209.

Miller, J.W. (1976) Bacterial leaf stripe of Bird-of-Paradise. Plant Pathology Circular 169: 1-2.

Miller, J.W. & Chase, A.R. (1987) Leaf spot and blight of bird-of-paradise caused by *Xanthomonas campestris*. Plant Pathology Circular 300: 1-2.

Moorman, G.W. (2006) Disease and Arthropod Management in Greenhouse Floriculture. Pennsylvania State University Publications Distribution Centre, USA. pp. 2-3.

Moriwaki, J., Sato, T. & Tsukiboshi, T. (2003) Morphological and molecular characterisation of *Colletotrichum boninense* sp. nov. from Japan. Mycoscience 44: 47-53.

Moyo, M., Bairu, M.W., Amoo, S.O. & Van Staden, J. (2011) Plant biotechnology in South Africa: micropropagation research endeavours, prospects and challenges. South African Journal of Botany 77: 996-1011.

Mumford, R., Boonham, N., Tomlinson, J. & Barker, I. (2006) Advances in molecular phytodiagnostics – new solutions for old problems. European Journal of Plant Pathology 116: 1-19.

Naor, V., Kigel, J. & Ziv, M. (2006) Control of bud sprouting and elongation in coloured *Zantedeschia* tubers by low-temperature storage. Hortscience 41: 685-687.

Newman, S.E. (2008) Common diseases in commercial floriculture greenhouses. Colorado State University Extension Greenhouse IMP Series 3: 1-8.

Nell, T.A. (2007) Floriculture research in the 21st century: it's more than just science. Acta Horticulturae 755: 27-32.

Netnou-Nkoana, N. & Eloff, J. (2012) The South African floricultural industry and the Plant Breeders' Rights Act: a short review. World Patent Information 34: 224-228.

North, J.J., Ndakidemi, P.A. & Laubscher, C.P. (2010) The potential of developing an *in vitro* method for propagating Strelitziaceae. African Journal of Biotechnology 9: 7583-7588.

Notten, A. (2002) *Strelitzia reginae* Aiton 'Mandela's Gold. http://www.plantzafrica.com/plantqrs/strelitregmandelagold.htm. Accessed: 10/07/2013.

Ogorodnik, L.E., Gvozdyak, R.I., Kabashnaya, L.V. & Yakovleva, L.M. (1996) *Erwinia carotovora* subsp. *carotovora* – a causal agent of brown rot of *Strelitzia*. Mikrobiologichnii Zhurnal 58: 7-10.

Oprea, M. & Neamtu, M.S. (2001) Foliage diseases of ornamental plants induced by pathogenic fungi. Analele Institutului de Cercetări pentru Cereale Protecţia Plantelor 31: 25-28.

Orlean, S. (2000) The Orchid Thief: A True Story of Beauty and Obsession. Vintage Publishing, UK. pp. 8-12.

Orlikowski, L. (1977) Certain fungal diseases of *Strelitzia reginae* Banks. Prace Instytutu Sadownictwa w Skierniewicach, B 2: 163-167.

Palacio-Bielsa, A., Cambra, M.A. & Lopez, M.M. (2009) PCR detection and identification of plant-pathogenic bacteria: updated review of protocols (1989-2007). Journal of Plant Pathology 91: 249-297.

Perombelon, M.C.M. & Van der Wolf, J.M. (2002) Methods for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium carotovorum* subsp. *atrosepticum*) on potatoes: a laboratory manual. Scottish Crop Research Institute Occasional Publication 10: 1-82.

Polizzi, G., Aiello, D., Castello, I., Vitale, A. & Parlavecchio, G. (2007) First report of southern blight on firewheel tree, bay laurel, bird of paradise, Mediterranean fan palm and liverwort caused by *Sclerotium rolfsii* in Italy. Plant Disease 91: 1199.

Pooley, E. (2005) A Field Guide to Wildflowers of KwaZulu-Natal and the Eastern Region. The Flora Publications Trust, South Africa. pp. 12-16, 36-38, 46, 340.

Punithalingam, E. & Spooner, B.M. (2005) New taxa and new records of coelomycetes from *Agapanthus* in the U.K. Kew Bulletin 60: 149-158.

Reid, N., Smith, B.W. & Gatrell, J.D. (2008) Importing change: Canadian competition and the U.S. floriculture industry. The Industrial Geographer 6: 3-19.

Reinten, E.Y., Coetzee, J.H. & Van Wyk, B.E. (2011) The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77: 934-946.

Richards, N.P., Botha, G.A., Schoeman, P., Clarke, B.M., Kota, M.W. & Ngcobo, F.N. (2006) Engineering geological mapping in Pietermaritzburg, South Africa: constraints on development. The Geological Society of London IAEG Paper 407: 1-11.

Rodrigues, L.M., Destefano, S.A., Diniz, M.C., Comparoni, R. & Neto, J. (2011) Pathogenicity of Brazilian strains of *Ralstonia solanacearum* in *Strelitzia reginae* seedlings. Tropical Plant Pathology 36: 409-413.

Rourke, J. (2002) *Clivia mirabilis* (Amaryllidaceae: Haemantheae), a new species from Northern Cape, South Africa. Bothalia 32: 1-7.

Rutherford, M.C. (1997) Categorization of biomes. In R.M. Cowling, D.M. Richardson and S.M. Pierce (eds) Vegetation of South Africa. Cambridge University Press, UK. pp. 91-98.

Sandler-Ziv, D., Gilad, Z., Achiam, M., Luria, G. & Kamenetsky, R. (2011) Effective system for propagation of coloured calla lily (*Zantedeschia*) by cutting and intensive growth. Acta Horticulturae 866: 233-238.

Save, R. (2009) What is stress and how to deal with it in ornamental plants? Acta Horticulturae 813: 241-254.

Seijo, T.E. & Peres, N.A. (2011) First report of *Acidovorax avenae* subsp. *avenae* causing bacterial leaf stripe of *Strelitzia nicolai*. Plant Disease 95: 1474.

Silva-Rojas, H.V. & Avila-Quezada, G.D. (2011) Phylogenetic and morphological identification of *Colletotrichum boninense*: a novel causal agent of anthracnose in avocado. Plant Pathology 60: 899-908.

Singh, A.K. (2006) Flower Crops – Cultivation and Management. New India Publishing Agency, India. pp. 1-6.

Singh, Y. (2012) *Zantedeschia* Spreng. http://www.plantzafrica.com/plantwxyz/zantedeschia.htm. Accessed: 10/07/2013.

Smith, M. (2007) The Plant Propagator's Bible – A Step-by-Step Guide to Propagating Every Plant in Your Garden. Quarto Publishing plc, UK. pp. 6-7.

Snijder, R.C., Cho, H., Hendriks, M.M., Lindhout, P. & Van Tuyl, J.M. (2004) Genetic variation in *Zantedeschia* spp. (Araceae) for resistance to soft rot caused by *Erwinia carotovora* subsp. *carotovora*. Euphytica 135: 119-128.

Snijder, R.C. & Van Tuyl, J.M. (2002) Evaluation of tests to determine resistance of *Zantedeschia* spp. (Araceae) to soft rot caused by *Erwinia carotovora* subsp. *carotovora.* European Journal of Plant Pathology 108: 565-571.

Snustad, D.P. & Simmons, M.J. (2006) Principles of Genetics. 4th edition. John Wiley & Sons Inc., USA. pp. 509-510.

Sobiczewski, P. (2008) Bacterial diseases of plants: epidemiology, diagnostics and control. Zemdirbyste-Agriculture 95: 151-157.

Stroh, H. (2002) Pests and disease affecting *Clivia* in South Africa. Clivia 4: 69-80.

Swart, W.J. (2004) Diagnosing plant disease: What the grower needs to know. Clivia Society Yearbook 6: 61-66.

Swart, W. (2005) Fungal pathogens associated with *Clivia*. Clivia Society Yearbook 7: 91-96.

Thuiller, W., Midgley, G.F., Rouget, M. & Cowling, R.M. (2006) Predicting patterns of plant species richness in megadiverse South Africa. Ecography 29: 733-744.

Tomioka, K., Sato, T., Hanada, K., Yamasaki, F., Nagai, T., Sawada, H., Takeya, M. & Aoki, T. (2012) Plant viruses and viroids released from the NIAS Genebank Project, Japan. Microbiology and Culture Collections 28: 35-40.

Townsley-Brascamp, W. & Marr, N.E. (1995) Evaluation and analysis of consumer preferences for outdoor ornamental plants. Acta Horticulturae 391: 199-206.

Van Jaarsveld, E. (2006) The Southern African *Plectranthus* and the Art of Turning Shade to Glade. Fernwood Press, South Africa. pp. 9-10.

Vannini, A. & Verttraino, A.M. (2000) *Ulocladium chartarum* as the causal agent of a leaf necrosis on *Quercus pubescens*. Forest Pathology 30: 297-303.

Van Rooyen, C. (2005) SA verspeel kanse met blomme [South Africa misses opportunities with flowers]. Landbouweekblad 82: 8-15.

Venter, S.N. (2009) Molecular diagnosis and identification of plant pathogenic bacteria. In: Bacterial Diseases of Plants in South Africa. T.A. Coutinho, T. Goszczynska, C. Lennox and S.N. Venter (eds.). Briza Publications, South Africa. pp. 7-10.

Verhoeven, J. Th. J. & Roenhorst, J.W. (1994) Tomato spotted wilt virus: ecological aspects in ornamental crops in the Netherlands from 1989 up to 1991. Acta Horticulturae 377: 175-182.

Vieira, B.S. & Barreto, R.W. (2004) First report of *Cercospora richardiaecola* causing leaf spots on *Zantedeschia aethiopica* in Brazil. Plant Pathology 53: 813.

Vieira, M.R., Lima, G.P., de Medeiros, D.C., de Souza, A.V. & de Oliveira, E.C. (2012) Genus: *Strelitzia*. Journal of Horticulture and Forestry 4: 178-180.

Wager, V.A. (1972) Records of Diseases of Ornamental Plants Not Previously Recorded in South Africa. Department of Agricultural Technical Services Technical Communication.
Wehlburg, C. (1970) Bacterial leaf stripe of *Strelitzia reginae* Banks. Phytopathology 60: 589.

Wehlburg, C. (1971) Bacterial leaf stripe of *Strelitzia reginae* caused by *Pseudomonas* sp. Plant Disease Reporter 55: 447-448.

Welsh, T.E. & Clemens, J. (1992) Protected cropping of *Zantedeschia* tubers and cutflowers in New Zealand. Acta Horticulturae 319: 335-340.

Wentzel, R. & Pinckaers, M. (2005) USDA Foreign Agricultural Service Global Agriculture Information Network (GAIN) report NL5002. pp. 3-4.

Whitlock, V.H. (1986) *Colletotrichum gloeosporioides* on *Clivia* in South Africa. Plant Disease 70: 800.

Winter, J. (2006) *Clivia nobilis* (Lind.) http://www.plantzafrica.com/plantcd/clivianobilis.htm. Accessed: 07/06/2013.

Wright, P.J. (1998) A soft rot of calla (*Zantedeschia* spp.) caused by *Erwinia carotovora* subspecies *carotovora*. New Zealand Journal of Crop and Horticultural Science 24: 331-334.

Wybo, I., De Bel, A., Soetens, O., Echahidi, F., Vandoorslaer, K., Van Cauwenbergh, M. & Pierard, D. (2011) Differentiation of *cfiA*-Negative and *cfiA*-Positive *Bacteroides fragilis* Isolates by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. Journal of Clinical Microbiology 49: 1961-1964.

Xaba, P. (2011) *Strelitzia reginae* Aiton. http://www.plantzafrica.com/plantqrs/strelitziareginae.htm. Accessed: 07/06/2013.

Yang, Y.L., Liu, Z.Y., Cai, L. Hyde, K.D., Yu, Z.N. & McKenzie, E.H.C. (2009) *Colletotrichum* anthracnose of *Amaryllidaceae*. Fungal Diversity 39: 123-146.

Zhang, N.T. (1990) Report of a national survey on diseases of urban ornamental plants. Forest Pest and Disease 1: 44-46.

CHAPTER 2

ANTHRACNOSE OF *CLIVIA MINIATA* (REGEL) CAUSED BY *COLLETOTRICHUM KARSTII* (YANG *ET AL.*) IN KWAZULU-NATAL, SOUTH AFRICA

2.1 ABSTRACT

Clivia miniata is an important floricultural crop that is extensively grown in South Africa and abroad. It is valued for its large flowers, glossy evergreen leaves and ability to grow in shaded areas. Symptoms of anthracnose, including leaf spotting and necrosis, have been reported by growers of *C. miniata* in KwaZulu-Natal, South Africa. A fungal isolate was obtained from senescing, symptomatic leaf material. It was identified as a species of *Colletotrichum*, based on its cultural and morphological characteristics. Evaluation of Koch's postulates with the *Colletotrichum* isolate confirmed its pathogenicity towards *C. miniata* and confirmed that the symptoms initially observed by growers were due to infection by a *Colletotrichum* species. The conidial characteristics exhibited by this *Colletotrichum* isolate are consistent with those reported for *C. karstii*, previously reported from diseased *C. miniata* in Japan. A portion of the nuclear-encoded ribosomal RNA gene region of the isolate, spanning the 18S ribosomal RNA gene (partial sequence), the internal transcribed spacer 1, 5.8S ribosomal RNA gene, the internal transcribed spacer 2 and the 28S ribosomal RNA gene (partial sequence), was amplified with the primers ITS1-F and ITS4. A Maximum Likelihood (ML) phylogenetic analysis of the ITS region corroborated the morphological characterisation of the isolate and indicated a clustering of the isolate with various isolates of *C. karstii* within the *Colletotrichum* genus. This is the first report of *C. karstii* causing an anthracnose disease of *C. miniata* in South Africa. A representative culture of the isolate has been deposited at the fungal culture collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) under the accession number *C. karstii* PPRI 16882.

2.2 INTRODUCTION

Clivia miniata (Regel) is a slow-growing, shade-loving, evergreen plant that is native to South Africa. Since its discovery in the 1800s, the plant and its hybrids with other species in the genus have become important floricultural crops worldwide (Aubrey, 2001; Pooley, 2005) and are now widely grown throughout the world (Reinten *et al.,* 2011). In mild climates they can be grown in the garden but in countries with a harsher climate, they are either grown as house plants (China, Japan and Korea) or are discarded once they have finished flowering (Europe and the US) (Duncan, 1999; Reinten *et al.,* 2011).

Studies on *Clivia* diseases are of importance to the global industry that has developed around *Clivia* because any reduction in the aesthetic appeal of plants can cause a concomitant reduction in the economic value of the plant. This is particularly the case for foliar diseases of *Clivia* because the diseased and unappealing leaves remain on the plant and in the buyer's sight for a long time. A number of fungal, bacterial and viral diseases of *C. miniata* have been observed wherever it is grown. Fungi are the most commonly reported group of *Clivia* pathogens and Table 2.1 below provides a summary of the literature pertaining to fungal diseases of the genus *Clivia*.

Table 2.1 Literature reporting fungi responsible for causing disease in the genus *Clivia*.

Genus	Species	Common name of disease and/or host organs affected	*Clivia* species	Reference
Alternaria	*tenuissima*		*Clivia* spp.	Swart (2005)
Blennoria	sp.		*Clivia* spp.	Oprea & Neamtu (2001)
Colletotrichum	*boninense*	leaf spot	*Clivia* spp.	Moriwaki *et al.* (2003); Hyde *et al*. (2009); Silva-Rojas & Avila-Quezada (2011)
	cliviae	leaf spot	*C. miniata*	Hyde *et al.* (2009); Yang *et al.* (2009)
	gloeosporiodes	anthracnose	*C. miniata*	Whitlock (1986); Crous *et al.* (2000)
	karstii	anthracnose	*C. miniata*	Damm *et al.* (2012b)
	trichellum	anthracnose	*C. miniata*	Kim *et al.* (2001)
	sp.		*Clivia* spp.	Swart (2005)
Fusicoccum	*luteum*		*C. miniata*	Braithwaite *et al.* (2006)
Fusarium	*oxysporum*		*C. miniata*	Swart (2004)
	sp.	damping off	*Clivia* spp.	Stroh (2002)
Phoma	sp.		*C. miniata*	Swart (2004)
Phytophthora	sp.	damping off	*Clivia* spp.	Stroh (2002)
Pythium	sp.	damping off	*Clivia* spp.	Stroh (2002)
Stagnospora	*curtisii*	leaf and stalk spot	*Clivia* spp.	Hill, (1982); Stroh (2002); Punithalingam & Spooner (2005)
Strelitziana	*cliviae*		*C. miniata*	Crous & Groenewald (2012); Crous *et al.* (2012a)
Macrophoma	*agapanthii*	leaf dieback	*Clivia* spp.	Stroh (2002)
	sp.	leaf spot	*C. miniata*	Zhang (1990)
Rhizoctonia	*solani*	damping off	*Clivia* spp.	Stroh (2002)
Sclerotium	*rolfsii*	collar rot	*Clivia* spp.	Stroh (2002)

Anthracnose, leaf spots and dieback appear to be the most commonly reported symptoms, according to available reports. Similar symptoms have been observed on *C. miniata* in KwaZulu-Natal (KZN), South Africa for a number of years but a cause for this has not yet been established. The symptoms observed on infected plants seem similar to those reported in Table 2.1 to be caused by various *Colletotrichum* (Corda) species. Table 2.2 below summarises reports of similar leaf spot and dieback symptoms that have been assigned to infection by various *Colletotrichum* species. .

Table 2.2 Records of *Colletotrichum* diseases of *Clivia*

Species	Country	Reference
C. boninense	Japan	Moriwaki *et al.* (2003)
C. cliviae	Japan	Yang *et al.* (2009)
C. gloeosporiodes	South Africa	Whitlock (1986)
C. karstii	Japan	Damm *et al.* (2012)
C. trichellum	Iran	Mirabolfathy (1989)

Colletotrichum species tend to primarily cause anthracnose symptoms, such as necrotic, sunken lesions on infected plant leaves, stems, flowers and fruit, on a wide range of plants in tropical and subtropical areas of the world (Agrios, 2005; Cannon *et al.,* 2012). They can also cause seedling blights, and crown and stem rots, and the genus has been ranked as the world's eighth most important group of phytopathogenic fungi (Dean *et al.,* 2012). As Table 2.2 indicates, anthracnose diseases of *Clivia* have been reported before, with the only record from South Africa being made from plants in Johannesburg, Gauteng, South Africa (Whitlock, 1986).

The aim of this study was to identify the phytopathogen responsible for causing a brown leaf spotting and dieback experienced by many commercial and hobbyist *Clivia* growers in KwaZulu-Natal, South Africa.

2.3 MATERIALS AND METHODS

2.3.1 Examination of symptomatic plant material and induction of fungal fruiting bodies on symptomatic tissues

Leaves from mature plants of *C. miniata* that displayed symptoms of the foliar blight were collected in Howick, KZN and placed in a sporulation chamber. High humidity (> 90%) was ensured by misting the leaves daily with distilled water and the temperature was maintained at 20-25°C. The leaves were observed daily for any changes over an 18 day period. Acervuli that developed were examined with a Zeiss Stemi 2000-C stereomicroscope and photographs were taken with a Zeiss Axiocam ERC5s camera and Zeiss Zen 2012 (blue edition) imaging software.

2.3.2 Isolation and morphological identification of the fungus responsible for fruiting body production

The fungus associated with the fruiting bodies was isolated by aseptically transferring a spore mass into 5 ml of sterile, distilled water and mixing gently. A 25 µl sample of the spore suspension was transferred into the centre of a 90 mm malt extract agar (MEA) plate, amended with chloramphenicol at a concentration of 10 mg μl^{-1} to exclude bacterial contamination. The spore suspension was spread over the surface of the plate with a flame-sterilised hockey stick. The plate was incubated upside-down in the dark at 25 ± 2°C for 24 hours and single, germinated spores were aspetically transferred to fresh 90 mm potato dextrose agar (PDA) plates. The growing edges of fungal colonies that had developed after 48 hrs incubation at 25 ± 2°C were transferred to fresh PDA plates to establish pure cultures. Wet mounts of the spores produced by the fungal isolates were examined throughout the

isolation process in order to facilitate morphological identification of the fungal isolate. These were viewed using a Zeiss Axio Scope.A1 light microscope and the spores classified using the keys developed by Baxter *et al.* (1994) and Williams-Woodward (2001).

2.3.3 Pathogenicity test

A conidial suspension of the isolate was prepared by irrigating a 10-day old, sporulating MEA culture with 10 ml sterile, distilled water. A flame-sterilised hockey stick was used to agitate the conidia into suspension. The concentration of the suspension was adjusted to approximately 1 x 10^4 conidia ml^{-1}. The prepared inoculum was sprayed onto the leaves of five *C. miniata* plants using a 1.5 L Pressure Sprayer. The plants were sprayed with the suspension to the point of run-off such that both sides of the leaves were thoroughly covered with the suspension. The plants were covered with polyethylene bags for 24 h following inoculation. Three further plants were inoculated with distilled water instead of a spore suspension, but treated identically otherwise. Plants were observed daily for any changes or symptom development.

2.3.4 Re-isolation of fungi from symptomatic *Clivia* leaves

Approximately 1 cm^2 portions of leaf tissue surrounding the lesions that developed on the inoculated plants were surface sterilised in a 0.35% sodium hypochlorite solution for one minute. The plant material was then rinsed in distilled water, plated on PDA plates and incubated at 25 ± 2°C until fungal growth from the lesions was evident. The tips of the fungal hyphae that grew from the lesions were aseptically transferred to fresh PDA plates and incubated at 25 ± 2°C.

2.3.5 Morphological characterisation of the isolate

2.3.5.1 Spore morphology

A single spore was obtained from a culture of the re-isolated fungus, following the method outlined by Choi *et al.* (1999), and transferred onto a plate of PDA. The fungal culture that grew from the spore was incubated under 24 h UV fluorescence at 25 ± 2°C for seven days, until sporulation was evident. A wet mount of the spores of the isolate was examined using an Olympus AX70 light microscope and images taken using a Nikon DS-Ri1 camera and NIS-elements BR 4.10.00 imaging system. The dimensions of 50 spores were measured at 1000x magnification using the measure function of the imaging software.

A representative culture of the isolate has been deposited at the fungal culture collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) with the accession number PPRI 16882.

2.3.5.2 Culture morphology and growth rate determination

The growth rate of the isolate was assessed by using the flame-sterilised back end of a glass pipette to punch six 6 mm diameter plugs from the growing edge of a five day old culture. These were aspetically transferred to the centre of new 90 mm PDA plates, which were incubated at 25 ± 2°C in the dark for 7 days. The colony diameter (mm) was measured in two perpendicular directions every second day and the mean daily mycelial growth rate (mm day^{-1}) was estimated (Cai *et al.,* 2009; Lima *et al.,* 2013).

2.3.6 Molecular characterisation of the isolate

2.3.6.1 Genomic DNA extraction and amplification and sequencing of the internal transcribed spacer (ITS) rDNA region

Total genomic DNA was extracted from a five day old culture of the isolate grown on PDA using a Qiagen DNeasy Plant Mini Kit. Samples were prepared for DNA extraction by aseptically scraping approximately 100 mg of fresh mycelium off the plate, suspending it in 400 µl DNA extraction buffer provided with the DNeasy kit and adding five sterile plastic beads to the tube before homogenising the mycelium with a Biospec Minibeadbeater for 30 s. The protocol followed thereafter to extract total DNA was as per the manufacturer's instructions.

A portion of the nuclear-encoded ribosomal RNA gene region of the isolate, spanning the 18S ribosomal RNA gene (partial sequence), internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2 and 28S ribosomal RNA gene (partial sequence), was amplified using the primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). A 20 µl PCR reaction was made up with 1 µl of extracted DNA, 10 µl 2X KAPA2G Fast HS ReadyMix, 1.5 µl of a 10 µM solution of each of the forward (ITS1-F) and reverse (ITS4) primers and 6 µl sterile nuclease-free water. The thermal cycling protocol followed that outlined by Weir *et al.* (2012): 4 min at 95°C, then 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 45 s, and lastly 7 min at 72°C. Two PCR reactions were carried out using a G-Storm Goldblock GS1 thermocycler. A negative control containing sterile nuclease-free water in place of template DNA was concurrently run with the samples.

The presence of PCR products was analysed on a 1.5% agarose gel, with SYBR® Safe DNA gel stain added to visualise any DNA present. The size of any PCR products was estimated through comparison with a concurrently run sample of the GeneRuler 100 bp DNA Ladder. The gel was run at 100 V for 50 min and PCR products were viewed using a Syngene G:Box and captured using GeneSnap image acquisition software.

A single PCR reaction showing a band of the correct size [± 615 bp - Weir *et al.* (2012)] (Figure 2.6) was selected for DNA sequencing at the Central Analytical Facility (CAF) at the University of Stellenbosch, South Africa (http://academic.sun.ac.za/saf/about.html). The DNA product was subject to a post-PCR clean up process by the CAF and then sequenced in both directions, using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems).

2.3.6.2 Sequence alignment and phylogenetic analysis

The DNA sequence files supplied by the CAF were edited with BioEdit Sequence Alignment Editor V7.0.9.0 (Hall, 1999). The forward and reverse sequence files were each manually trimmed to include the region bounded by the forward and reverse ITS primers. The two regions from each sequencing direction were aligned using the CLUSTALW (Thompson *et al.,* 1994) add-on in BioEdit and congruency between the forward and reverse sequence data was verified by eye. The forward sequence was subsequently used for all further analyses.

A preliminary genus-level identification of the fungal isolate was investigated using the Basic Local Alignment Search Tool (BLAST) search option in the NCBI GenBank nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Appendix 2.7.1). Verification of the preliminary morphological identification of the isolate as a species of *Colletotrichum* by this search indicated that the ITS sequence should be compared to ITS sequence data housed in the CBS-KNAW Fungal Biodiversity Centre Q-Bank *Colletotrichum* sequence database (http://www.cbs.knaw.nl/colletotrichum), as recommended by Cannon *et al.* (2012). A pairwise sequence alignment, run with the default parameters selected by the software, indicated the 50 closest matches to the ITS region of this isolate (Appendix 2.7.2).

ITS sequence data for phylogenetic analysis, as presented in Table 2.3, was downloaded from GenBank, to represent: members of all the clades proposed by Cannon *et al.* (2012) for the *Colletotrichum* species they studied, the top five matching *C. karstii* isolates indicated by the Q-Bank alignment, from any *Colletotrichum* species reported from *Clivia* (as reported in Table 2.2) and any *Colletotrichum* species recorded from South Africa (Q-Bank Colletrichum database search, keywords: South Africa).

The ITS dataset was assembled using the sequence data explorer of MEGA version 5.2.2 (Tamura *et al.,* 2011) and aligned with MUSCLE (Edgar, 2004). The alignment was optimised by eye before the data set was used to infer the phylogeny of this isolate. The final aligned dataset comprised 442 characters (162 of which were variable and 126 were parsimony informative) from 77 strains, representing 63 *Colletotrichum* species and *Monilochaetes infuscans* CBS 869.96 as the outgroup. Taxon information is presented in Table 2.3 below.

The phylogeny of the isolate was inferred with a maximum likelihood (ML) analysis using Garli 2.0 (Zwickl, 2006). jModelTest 2.1.1 (Guindon & Gascuel, 2003; Darriba *et al.,* 2012) was used, with the Akaike Information Criterion (AIC) selected, to estimate the model of best fit for analysis of the dataset as TIM1+I+G (-ln 4676.5012). The ML analysis of the dataset was performed using the TIM1+I+G model. The most likely tree was generated with an ML analysis and no bootstrapping, followed by another ML analysis with 100 bootstrap replicates to assess nodal support for the most likely tree. These figures are shown in the appendices. FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) was used to view the trees and annotate the most likely phylogenetic tree, presented in Figure 2.7 below.

Table 2.3 Strain accession details of *Colletotrichum* ITS sequences obtained from GenBank

Species	Clade	Culture Acc. No.	Host	Location	GenBank Acc. No.	Reference
Colletotrichum cf. *karstii*	boninense	PPRI 16882	*Clivia miniata*	South Africa	n/a	This study
C. acutatum	acutatum	CBS 112996	*Carica papaya*	Australia	JQ005776	Cannon *et al.* (2012)
C. agaves		CBS 118190	*Agave striata*	Mexico	DQ286221	Sharma *et al*. (2013)
C. annellatum	boninense	CBS 129826	*Hevea brasiliensis*	Colombia	JQ005222	Cannon *et al*. (2012)
C. anthrisci	dematium	CBS 125334	*Anthriscus sylvestris*	Netherlands	GU227845	Cannon *et al*. (2012)
C. asianum	gloeosporioides	ICMP 18580	*Coffea arabica*	Thailand	FJ972612	Cannon *et al*. (2012)
C. australe	acutatum	CBS 116478	*Trachycarpus fortunei*	South Africa	JQ948455	Cannon *et al.* (2012)
C. boninense	boninense	CBS 123755	*Crinum asiaticum*	Japan	JQ005153	Cannon *et al.* (2012)
C. brevisporum		LC0600	Unidentified plant	India	KC790943	Sharma *et al*. (2013)
C. cereale	graminicola	KS20-BIG	*Bromus inermus*	USA	JQ005774	Cannon *et al*. (2012)
C. chlorophyti		IMI 103806	*Chlorophytum* sp.	India	GU227894	Sharma *et al*. (2013)
C. circinans	dematium	CBS 221.81	*Allium cepa*	Serbia	GU227855	Cannon *et al*. (2012)
C. cliviae		CBS 125375	*Clivia miniata*	China	JX519223	Cannon *et al*. (2012)
C. cliviae		CSSK4	*Clivia miniata*	China	GQ485607	Yang *et al.* (2009)
C. coccodes		CBS 369.75	*Solanum tuberosum*	Netherlands	HM171679	Cannon *et al*. (2012)
C. constrictum	boninense	CBS 128504	*Citrus limon*	New Zealand	JQ005238	Damm *et al*. (2012)
C. curcumae	truncatum	IMI 288937	*Curcuma longa*	India	GU227893	Cannon *et al*. (2012)
C. cymbidiicola	boninense	IMI 347923	*Cymbidium* sp.	Australia	JQ005166	Cannon *et al*. (2012)
C. dacrycarpi	boninense	CBS 130241	*Dacrycarpus dacrydioides*	New Zealand	JQ005236	Damm *et al*. (2012)
C. dematium	dematium	CBS 115524	*Vitis vinifera*	South Africa	GU227826	Damm *et al.* (2009)
C. dematium	dematium	CBS 125.25	*Eryngium campestre*	France	GU227819	Damm *et al.* (2009)
C. destructivum	destructivum	CBS 149.34	*Trifolium* sp.	Netherlands	AJ301942	Cannon *et al*. (2012)
C. dracaenophilum		CBS 118199	*Draceana* sp.	China	DQ286209	Cannon *et al*. (2012)
C. fructi	dematium	CBS 346.37	*Malus sylvestris*	USA	GU227844	Cannon *et al*. (2012)
C. fuscum	destructivum	CBS 130.57	*Digitalis lanata*	Unknown	JQ005762	Cannon *et al*. (2012)
C. gigasporum		MUCL 44947	*Centella asiatica*	Madagascar	AM982797	Sharma *et al*. (2013)
C. gloeosporioides	gloeosporioides	ICMP 18694	*Mangifera indica*	South Africa	JX010155	Weir *et al*. (2012)
C. gloeosporioides	gloeosporioides	IMI 356878	*Citrus sinensis*	Italy	EU371022	Cannon *et al*. (2012)
C. godetiae	acutatum	CBS 133.44	*Clarkia* hybrid	Denmark	JQ948402	Cannon *et al.* (2012)
C. graminicola	graminicola	M1.001	*Zea mays*	USA	JQ005767	Cannon *et al*. (2012)
C. higginsianum	destructivum	IMI 349063	*Arabidopsis thaliana*	Trinidad and Tobago	JQ005760	Cannon *et al*. (2012)
C. jacksonii	graminicola	MAFF 305460	*Echinocloa esculenta*	Japan	EU554108	Cannon *et al*. (2012)
C. jasminigenum	truncatum	CGMCC LLTX-01	*Jasminum sambac*	Vietnam	HM131513	Sharma *et al*. (2013)
C. kahawae subsp. *ciggaro*	gloeosporioides	CBS 112984	*Dryandra* sp.	South Africa	JX010237	Weir *et al*. (2012)
C. kahawae subsp. *ciggaro*	gloeosporioides	ICMP 18539	*Olea europaea*	Australia	JX010230	Cannon *et al*. (2012)
C. karstii	boninense	CBS 127597	*Diospyros australis*	Australia	JQ005204	Damm *et al.* (2012)
C. karstii	boninense	MAFF 306204	*Clivia miniata*	Japan	JQ005196	Damm *et al.* (2012)
C. karstii	boninense	CBS 127595	*Musa banksii*	Australia	JQ005178	Damm *et al.* (2012)
C. karstii	boninense	CBS 127591	*Sapium integerrimium*	Australia	JQ005186	Damm *et al.* (2012)
C. karstii	boninense	CBS 127552	*Eugenia uniflora*	Brazil	JQ005217	Damm *et al.* (2012)
C. karstii	boninense	CBS 127536	*Eucalyptus grandis*	South Africa	JQ005201	Damm *et al.* (2012)
C. lilii	spaethianum	CBS 109214	*Lilium* sp.	Japan	GU227810	Cannon *et al*. (2012)
C. lindemuthianum	orbiculare	CBS 144.31	*Phaseolus vulgaris*	Germany	JQ005779	Cannon *et al*. (2012)
C. lineola	dematium	CBS 125337	Apiaceae	Czech Republic	GU227829	Cannon *et al*. (2012)
C. linicola	destructivum	CBS 172.51	*Linum usitatissimum*	Netherlands	JQ005765	Cannon *et al*. (2012)
C. liriopes	spaethianum	CBS 119444	*Lirope muscari*	Mexico	GU227804	Cannon *et al*. (2012)
C. lupini	acutatum	CBS 109225	*Lupinus albus*	Ukraine	JQ948155	Cannon *et al.* (2012)
C. malvarum	orbiculare	CBS 521.97	*Lavatera trimestris*	UK	KF178480	Damm *et al.* (2013)
C. nymphaeae	acutatum	CBS 515.78	*Nymphaea alba*	Netherlands	JQ948197	Cannon *et al.* (2012)
C. orbiculare	orbiculare	CBS 514.97	*Cucumis sativus*	UK	JQ005778	Cannon *et al*. (2012)
C. orchidophilum		CBS 632.80	*Dendrobium* sp.	USA	JQ948151	Cannon *et al*. (2012)
C. paspali	graminicola	MAFF 305403	*Paspalum notatum*	Japan	KC790948	Sharma *et al.* (2013)
C. phaseolorum	dematium	CBS 157.36	*Phaseolus radiatus*	Japan	GU227896	Cannon *et al*. (2012)
C. phormii	acutatum	CBS 118194	*Phormium* sp.	Germany	JQ948446	Cannon *et al.* (2012)
C. phyllanthi	boninense	CBS 175.67	*Phyllanthus acidus*	India	JQ005221	Cannon *et al*. (2012)
C. pseudoacutatum		CBS 436.77	*Phormium* sp.	Germany	JQ948480	Cannon *et al*. (2012)
C. rusci		CBS 119206	*Ruscus* sp.	Italy	GU227818	Cannon *et al*. (2012)
C. sansevieriae		MAFF239721	*Sansevieria trifasciata*	Japan	AB212991	Cannon *et al*. (2012)
C. siamense	gloeosporioides	ICMP 18739	*Carica papaya*	South Africa	JX010161	Weir *et al.* (2012)
C. siamense	gloeosporioides	ICMP 18570	*Persea americana*	South Africa	JX010248	Weir *et al.* (2012)
C. siamense	gloeosporioides	ICMP 18578	*Coffea arabica*	Thailand	JX010171	Cannon *et al*. (2012)
C. spaethianum	spaethianum	CBS 167.49	*Hosta sieboldiana*	Germany	GU227807	Cannon *et al*. (2012)
C. spinaciae	dematium	CBS 128.57	*Spinacia oleracea*	Netherlands	GU227847	Cannon *et al*. (2012)
C. spinosum	orbiculare	CBS 515.97	*Xanthium spinosum*	Australia	KF178474	Damm *et al.* (2013)
C. sublineola	graminicola	CBS 131301	*Sorghum bicolor*	Burkina Faso	JQ005771	Cannon *et al*. (2012)
C. tabacum	destructivum	CBS 161.53	*Nicotiana tabacum*	Zambia	JQ005763	Cannon *et al*. (2012)
C. thailandicum		BCC38879	*Hibiscus rosa-sinensis*	Thailand	JN050242	Sharma *et al*. (2013)
C. thailandicum		LC0596	*Alocasia* sp.	Thailand	KC790941	Sharma *et al*. (2013)
C. theobromicola	gloeosporioides	ICMP 18649	*Theobroma cacao*	Panama	JX010294	Cannon *et al*. (2012)
C. tofieldiae	spaethianum	CBS 495.85	*Tofieldia calyculata*	Switzerland	GU227801	Cannon *et al*. (2012)
C. trichellum		CBS 217.64	*Hedera helix*	UK	GU227812	Cannon *et al*. (2012)
C. trifolii	orbiculare	CBS 158.83	*Trifolium* sp.	USA	KF178478	Damm *et al.* (2013)
C. tropicicola		BCC38877	*Citrus maxima*	Thailand	JN050240	Noireung *et al.* (2012)
C. truncatum	truncatum	CBS 151.35	*Phaseolus lunatus*	USA	GU227862	Cannon *et al*. (2012)
C. verruculosum	spaethianum	IMI 45525	*Crotalaria juncea*	Zimbabwe	GU227806	Cannon *et al*. (2012)
C. yunnanense		CBS 132135	*Buxus* sp.	China	EF369490	Cannon *et al*. (2012)
Monilochaetes infuscans		CBS 869.96	*Ipomoea batatas*	South Africa	GU180626	Cannon *et al*. (2012)

2.4 RESULTS

This *Clivia* disease first became apparent as small tan- to dark-brown spots/lesions on the leaves, particularly on new growth in spring, on plants of all ages. In some cases, a large necrotic zone develops that is surrounded by a prominent yellow halo. Infected leaves turn chlorotic and die off, leaving a weakened and blemished plant, which may die later.

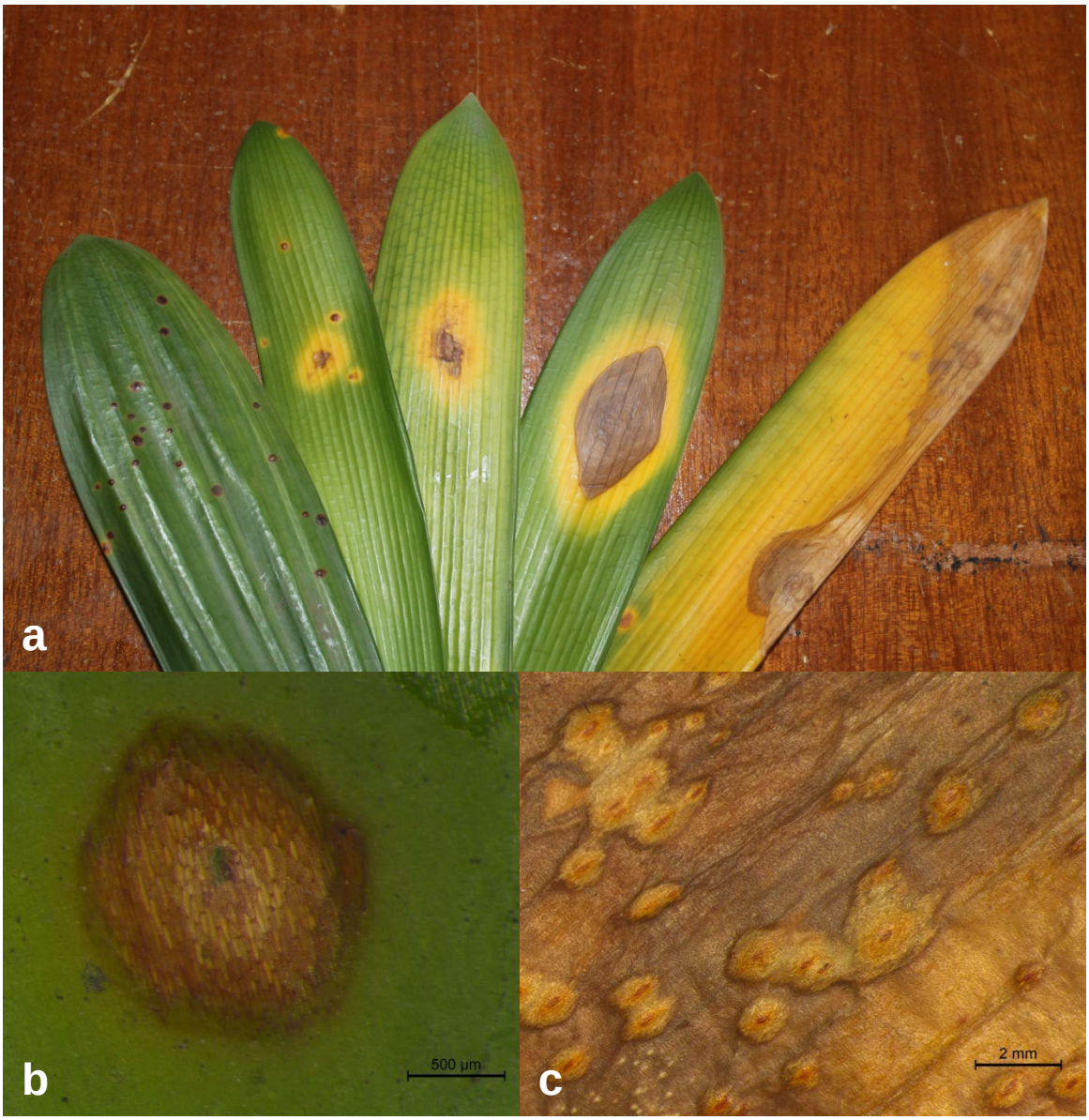


Figure 2.1 Symptoms associated with brown leaf spot of *Clivia miniata*: (a) leaves from infected plants showing symptoms of spots, lesions and necrosis; (b) a single spot on a green leaf; (c) a number of older lesions evident on dead leaf tissue.

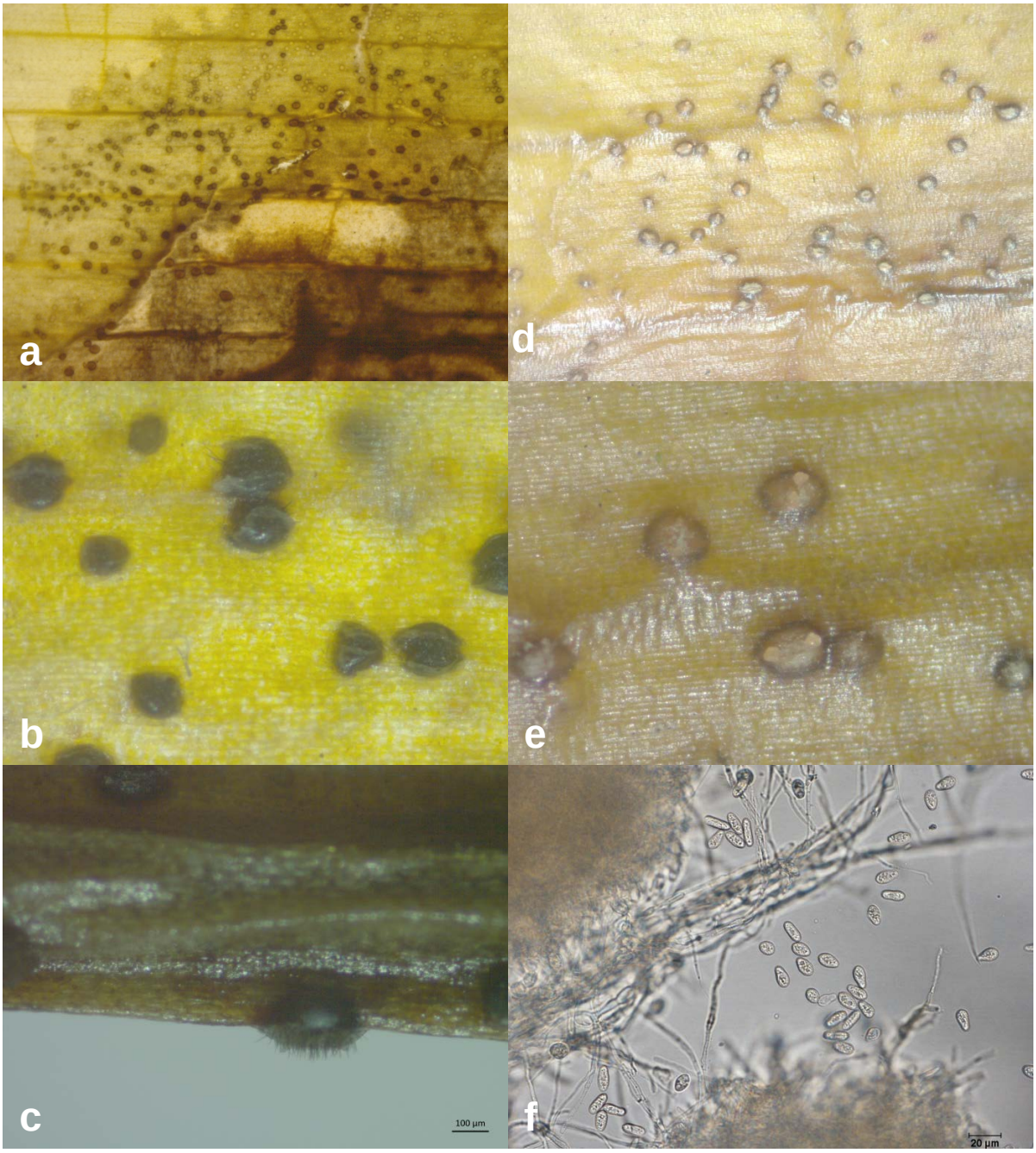


Figure 2.2 Development of fungal fruiting bodies (acervuli) and spores (conidia) from infected leaves: (a) acervuli appearing in a circular fashion towards the edge of the lesion; (b) some showed the presence of hair-like projections (setae); (c) an acervulus and setae seen from the side; (d) extrusion of conidial masses from acervuli; (e) close up view of acervuli and orange conidial masses; (f) wet mount of fungal mycelium and conidia.

The development of fungal fruiting bodies was evident on the leaves after 11-13 days incubation in the sporulation chamber (Figure 2.2a). These fruiting bodies appeared in a circular fashion towards the edge of the lesion and were smaller, but otherwise similar to, the raised lesions observed as initial symptoms of infection on *Clivia* leaves. Two pinkish spore

mass were observed to extrude from the opposite ends of each acervulus on days 14-15 (Figure 2.2d). Setae protruded from some, but not all, of the fruiting bodies (Figure 2.2b and c).

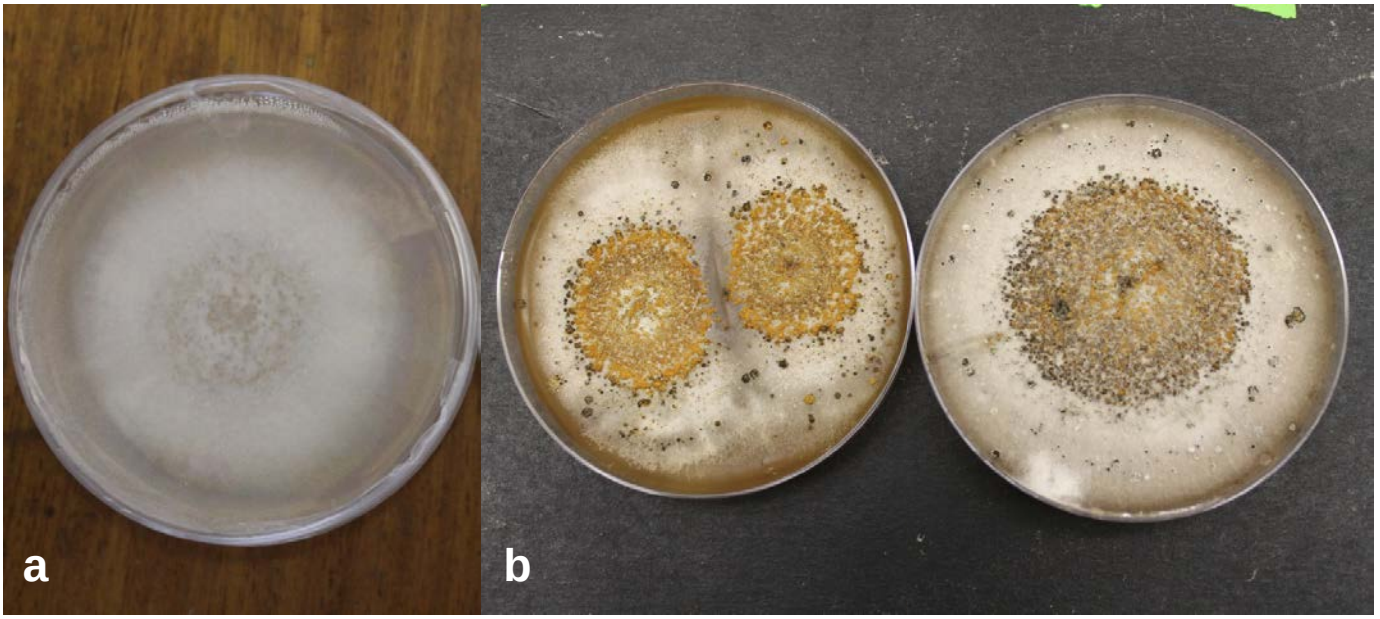


Figure 2.3 Cultural characteristics of the isolate: (a) a culture grown on PDA showing sparse conidial production in the culture centre; (b) cultures grown on MEA showing abundant orange conidial masses.

The mycelium produced by the isolate was white in colour while the conidial masses produced were orange in colour. Whilst there were no visible differences between the mycelial pigmentation of cultures grown on MEA or PDA, acervuli and conidia were produced much more abundantly when cultures were grown on MEA than when grown on PDA.

Figure 2.4 Symptoms that developed on *C. miniata* seedlings following inoculation: (a) raised areas on the adaxial leaf surface; (b) the reverse of these lesions on the abaxial leaf surface; (c) expansion of raised lesions into areas showing chlorosis and a reddish-brown tinge to infected tissues.

The inoculated plants all developed symptoms similar to those seen originally on the infected leaves 12-15 days after inoculation. All the fungal isolates obtained from the inoculated plants were morphologically identical to the original isolate obtained from the spore masses produced on a symptomatic leaf. The plants sprayed with water did not develop any symptoms during the observation period.

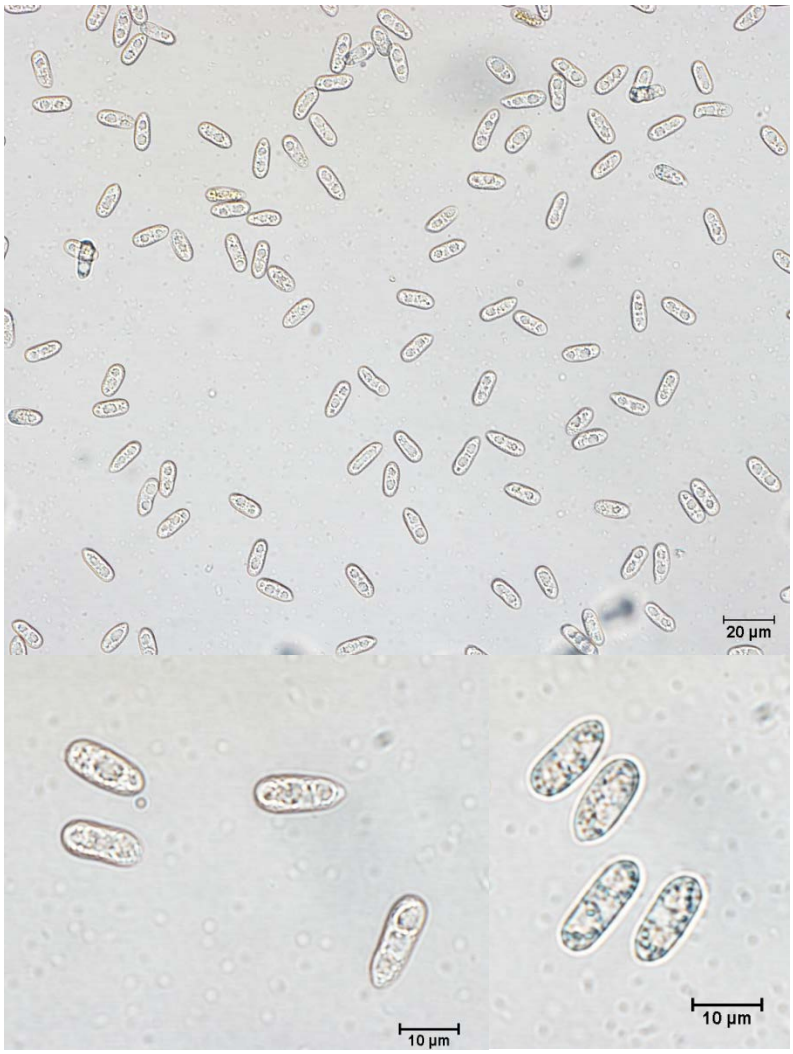


Figure 2.5 Bright field images of the conidia of the *Colletotrichum* species associated with brown leaf spot/anthracnose of *Clivia miniata*.

The conidia of this isolate are hyaline, aseptate and straight or cylindrical in profile. Their content appears granular. The conidial walls are smooth and the ends are rounded. Most, but not all, of the conidia have a hilum-like protuberance at one end.

Table 2.4 Summary of morphological data of *Colletotrichum* isolate.

Colony diameter (mm after 7 days)	Colony growth rate (mm day^{-1})	Conidial dimensions (µm)	Conidial shape	Conidial L/W	Conidial characterisitics
63 - 68 (66 ± 5.2 mm; n = 6)	8.8 - 9.7 (9.4 ± 0.4; n= 6)	Length: 14.9 - 20.2 (17.7 ± 1.2); n = 50 Width: 7.1 - 9.6 (8.2 ± 0.7); n = 50	Cylindrical	2.2	Hyaline, rounded ends, hilum (in most cases)

The morphological data of this isolate show similarities to *Colletotrichum* species in the *C. boninense* (Moriwaki, Sato & Tsukib.) species complex, as defined by Damm *et al.* (2012).

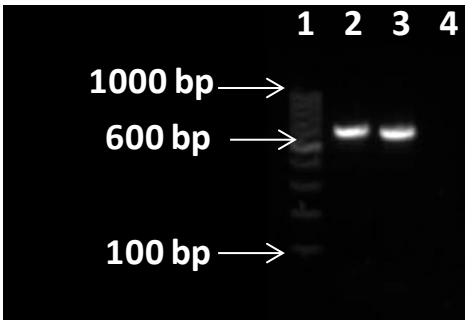


Figure 2.6 1.5% agarose gel showing PCR products amplified from total genomic DNA of the fungal isolate using ITS region specific primers: Lane 1 – 100 bp MWM, Lanes 2 and 3 - approximately 600 bp PCR products, Lane 4 – negative control.

As was expected, no products were present for the negative control and both reactions gave a single product of the correct predicted size, indicating a successful amplification of the ITS region of the fungal isolate.

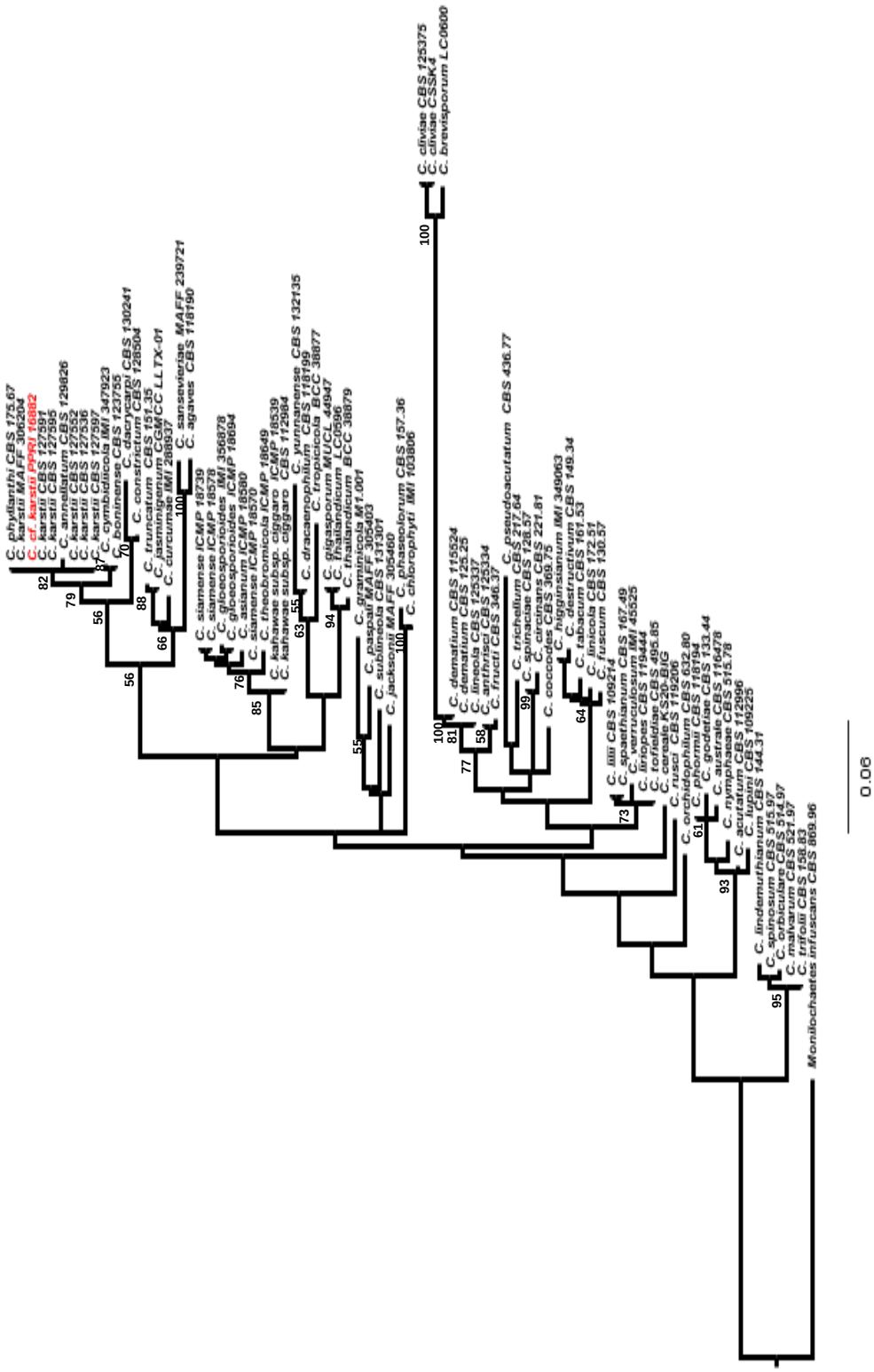


Figure 2.7 Maximum likelihood phylogeny of the isolate (highlighted in red) based on an alignment of the ITS region of various *Colletotrichum* species as listed in Table 2.3 (bootstrap branch support values < 50 not shown).

Phylogenetic analysis of the ITS region of the isolate indicates that it falls within a clade comprised various isolates of *C. karstii*. This clade is supported by a moderate bootstrap branch support value of 82.

2.5 DISCUSSION

The cultural, morphological and molecular characteristics of the fungal isolate obtained from the diseased *C. miniata* leaves shown in Figure 2.1 indicated that it is a *Colletotrichum* (Corda) species. The results of the pathogenicity test fulfilled Koch's postulates and confirmed that the isolate derived from the fruiting bodies shown in Figure 2.2 was capable of causing disease on healthy *Clivia* plants (Figure 2.4). A fungus with morphological characteristics consistent with the original isolate was re-isolated from the lesions on infected plants (Figure 2.3). It is thus evident that the brown leaf spot and anthracnose-like symptoms observed in this study were caused by this fungal isolate.

The preliminary identification of this fungus as a *Colletotrichum* species was based on a morphological approach. The morphology of the spores of the fungal isolates examined throughout the course of this study (Figure 2.5) matched those of the conidia of *Colletotrichum* species in the keys to fungal genera presented by Baxter *et al.* (1994) and Williams-Woodward (2001). The appearance of the fungal fruiting bodies that developed from the symptomatic *Clivia* leaves was characteristic of the acervuli of a *Colletotrichum* species (Agrios, 2005). A comparison of the symptoms observed in this study (on the initial infected leaves and on inoculated plants) (Figure 2.1 and Figure 2.4 respectively) with those symptoms described and/or depicted in the literature presented in Table 2.2 indicated that similar anthracnose and leaf spot disease have been observed on *C. miniata* in prior studies and were all caused by various *Colletotrichum* species.

The ITS region sequence data generated from the isolate supports the morphological identification of this as a *Colletotrichum* species, as indicated by the NCBI GenBank BLAST search results shown in Appendix 2.7.1. However, Cannon *et al.* (2012) have warned against using the data set available in GenBank for phylogenetic analysis, citing is as 'uncurated'. They stated that researchers should use the CBS-KNAW Fungal Biodiversity Centre Q-Bank *Colletotrichum* database as an alternative to Genbank for 'robust, phylogeny-based online identification'. The ITS sequence data gathered was thus shown via comparisons in the Q-Bank database to definitively belong to a species of *Colletotrichum*, showing a closest ITS nucleotide sequence similarity to isolates of *C. karstii* and some others in the *C. boninense* species complex, as shown in Appendix 2.7.1.

Cannon *et al.* (2012) have stated that sequence data of the ITS region alone cannot effectively differentiate between the species within each *Colletotrichum* species complex, but that the data is variable enough to allow successful identification of a *Colletotrichum* isolate to the species complex level. The phylogenetic tree shown in Figure 2.7 indicates an affinity of this isolate with other isolates of *C. karstii*, which form a relatively well supported clade in the dendrogram. Resolute identification of the isolate for species typification purposes would benefit from concatenated gene sequence data analysis (Cannon *et al.,* 2012). The best choice of genes for concatenated phylogenetic analysisis still under debate i.e. the studies of Damm *et al*. (2012) vs. Lima *et al.* (2013) vs. Sharma *et al.* (2013a).

The conidial dimensions of this isolate fall within the range reported for *C. karstii* isolates by Yang *et al.* (2011) and Damm *et al.* (2012). The L/W ratio of *C. karstii* has been reported as ranging from 2.2 to 2.6 depending on isolate and culture medium (Damm *et al.,* 2012). The L/W ratio of this isolate (2.2) thus falls in the reported range for *C. karstii*. In culture, the isolate grew as aerial, white mycelium when incubated in darkness and turned rosy pink when exposed to 24 h UV illumination. The conidial masses produced by this isolate are orange on PDA and the cultures depicted in Figure 2.3 show visual similarities to the *C. karstii* isolate cultures depicted by Damm *et al.* (2012) and Lima *et al.* (2013). Conidia were produced more abundantly when the isolate was grown on MEA than on PDA but the isolate produced orange conidial masses and occasional setae, regardless of the medium on which it was cultured.

Colletotrichum karstii was originally isolated and described from anthracnose lesions on various orchids in China (Yang *et al.,* 2011). Based on the polyphasic study conducted by Damm *et al.* (2012), many *Colletotrichum* isolates previously identified as *C. boninense,* or even *C. gloeosporioides,* have now been reclassified as *C. karstii*. *Colletotrichum karstii* has thus been found to be the 'most common and geographically diverse species in the *C. boninense* complex' (Damm *et al.,* 2012; Lima *et al.,* 2013).

Colletotrichum karstii has previously been recorded in South Africa, isolated from *Citrus* species (CBS 126532), *Eucalyptus grandis* Hill ex Maiden (CBS 110779, CBS 127535, CBS 129830 and CBS 127536) and wheat (*Triticum* sp.)(CBS 128540) (Damm *et al.,* 2012). Although this is not the first report of *C. karstii* from South Africa, it does constitute the first record of *C. karstii* on *Clivia* in South Africa. This report thus confirms the pathogenicity of *C. karstii* towards *C. miniata* and corroborates the report made in Japan by Moriwaki *et al.*

(2003) and amended by Damm *et al.* (2012) reporting anthracnose of *Clivia* to be caused by *C. karstii*.

2.6 REFERENCES

Aubrey, A. (2001) *Clivia miniata* [Lindl.] Regel. http://www.plantzafrica.com/plantcd/cliviaminiata.htm. Accessed: 07/06/2013.

Agrios, G.N. (2005) *Plant Pathology* 5th ed. Elsevier Academic Press, Burlington, USA. pp. 487-500.

Baxter, A.P., Rong, I.H., Roux, C., Schutte, L., van der Linde, E. & van Niekerk, E. (1994) *Elementary Keys to Common Fungi in South Africa*. Plant Protection Research Institute Handbook No. 2. Plant Protection Research Institute, Pretoria, South Africa. pp. 11, 18-19.

Braithwaite, M., Hill, C.F., Ganev, S., Pay, J.M., Pearson, H.G. & Alexander, B.J.R. (2006) A survey of subtropical nursery plants for fungal diseases in Northland. New Zealand Plant Protection 59: 132-136.

Cannon, P.F., Damm, U., Johnston, P.R. & Weir, B.S. (2012) *Colletorichum* –current status and future directions. Studies in Mycology 73: 181-213.

Cai, L., Hyde, K.D., Taylor, P.W.J., Weir, B.S., Waller, J., Abang, M.M., Zhang, J.Z., Yang, Y.L., Phoulivong, S., Liu, Z.Y., Prihastuti, H., Shivas, R.G., McKenzie, E.H.C. and Johnston, P.R. (2009) A polyphasic approach for studying *Colletotrichum*. Fungal Diversity 39: 183-204.

Choi, Y.W., Hyde, K.D. & Ho, W.H. (1999) Single spore isolation of fungi. Fungal Diversity 3: 29-38.

Crous, P.W. & Groenewald, J.Z. (2012) *Strelitziana cliviae* Crous, *sp. nov.* Fungal Planet description sheet 136. Persoonia 29: 164-165.

Crous, P.W., Phillips, A.J.L. & Baxter, A.P. (2000) Phytopathogenic Fungi from South Africa. University of Stellenbosch, Department of Plant Pathology Press, South Africa. pp. 7-50.

Crous, P.W., Shivas, R.G., Wingfield, M.J., Summerell, B.A., Rossman, A.Y., Alves, J. L., Adams, G.C., Barreto, R.W., Bell, A., Coutinho, M.L., Flory, S.L., Gates, G., Grice, K.R.,

Hardy, G.E.St.J., Kleczewski, N.M., Lombard, L., Longa, C.M.O., Louis-Seize, G., Macedo, F., Mahoney, D.P., Maresi, G., Martin-Sanchez, P.M., Marvanová, L., Minnis, A.M., Morgado, L.N. & Noordeloos, M. E. (2012) Fungal Planet description sheets: 128-153. Persoonia 29:146-201.

Damm, U. Cannon, P.F., Liu, F., Barreto, R.W., Guatimosim, E. & Crous, P.W. (2013) The *Colletotrichum orbiculare* species complex: important pathogens of field crops and weeds. Fungal Diversity 61: 29-59.

Damm, U., Cannon, P.F., Woudenberg, J.H.C., Johnston, P.R., Weir, B.S., Tan, Y.P., Shivas, R.G. & Crous, P.W. (2012) The *Colletotrichum boninense* species complex. Studies in Mycology 73: 1-36.

Damm, U., Woudenberg, J.H.C., Cannon, P.F. & Crous, P.W. (2009) *Colletotrichum* species with curved conidia from herbaceous hosts. Fungal Diversity 39: 45-87.

Darriba, D., Taboada, G.L., Doalla, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9: 772.

Dean, R., Van Kan, J.A.L., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J. & Foster, G.D. (2012) The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology 13: 414–430.

Duncan, G.D. (1999) Grow Clivias: A Guide to the Species, Cultivation and Propagation of the Genus Clivia. South African National Botanical Institute, Cape Town, South Africa. pp 4-8.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792-97.

Gardes, M. & Bruns, T.D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2:113-118.

Guidon, S. & Gascuel, O. (2003) A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Systematic Biology 52: 696-704.

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

Hill, C.F. (1982) New plant disease records in New Zealand, 1974-1982. New Zealand Journal of Botany 20: 355-359.

Hyde, K.D., Cai, L., Cannon, P.F., Crouch, J.A., Crous, P.W., Damm, U., Goodwin, P.H., Chen, H., Johnston, P.R., Jones, E.B.G.,, Liu, Z.Y., McKenzie, E.H.C., Moriwaki, J., Noireung, P., Pennycook, S.R., Pfenning, L.H., Prihastuti, H., Sato, T., Shivas, R.G., Tan, Y.P., Taylor, P.W.J., Weir, B.S., Yang, Y.L. and Zhang, J.Z. (2009) *Colletotrichum* – names in current use. Fungal Diversity 39: 147-182.

Kim, W.G., Hong, S.K. & Cho, W.D. (2001) Occurrence of anthracnose on English Ivy caused by *Colletotrichum trichellum* in Korea. Mycobiology 29: 107-109.

Lima, N.B., de A. Batista, M.V., De Morais Jr, M.A., Barbosa, M.A.G., Michereff, S.J., Hyde, K.D. & Câmara, M.P.S. (2013) Five *Colletotrichum* species are responsible for mango anthracnose in northeastern Brazil. Fungal Diversity 61: 75-88.

Mirabolfathy, M. (1989) The incidence of anthracnose of *Clivia miniata* in Tehran. Iranian Journal of Plant Pathology 25: 1-4.

Moriwaki, J., Sato, T. & Tsukiboshi, T. (2003) Morphological and molecular characterisation of *Colletotrichum boninense* sp. nov. from Japan. Mycoscience 44: 47-53.

Noireung, P., Phoulivong, S., Liu, F., Cai, L., McKenzie, E.H.C., Chukeatirote, E., Jones, E.B.G., Bakhali, A.H. & Hyde, K.D. (2012) Novel species of *Colletotrichum* revealed by morphology and molecular analysis. Cryptogamie Mycologie 33: 347-362.

Oprea, M. & Neamtu, M.S. (2001) Foliage diseases of ornamental plants induced by pathogenic fungi. Analele Institutului de Cercetări pentru Cereale Protecţia Plantelor 31: 25-28.

Pooley, E. (2005) A Field Guide to Wildflowers of KwaZulu-Natal and the Eastern Region. The Flora Publications Trust, South Africa. pp. 36-39.

Punithalingam, E. & Spooner, B.M. (2005) New taxa and new records of coelomycetes from *Agapanthus* in the U.K. Kew Bulletin 60: 149-158.

Reinten, E.Y., Coetzee, J.H. & Van Wyk, B.E. (2011) The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77: 934-946.

Sharma, G., Pinnaka, A.K. & Shenoy, B.D. (2013) ITS-based diversity of *Colletotrichum* from India. Current Research in Environmental and Applied Mycology 3: 194-220.

Silva-Rojas, H.V. & Avila-Quezada, G.D. (2011) Phylogenetic and morphological identification of *Colletotrichum boninense*: a novel causal agent of anthracnose in avocado. Plant Pathology 60: 899-908.

Stroh, H. (2002) Pests and disease affecting *Clivia* in South Africa. Clivia 4: 69-80.

Swart, W.J. (2004) Diagnosing plant disease: What the grower needs to know. Clivia Society Yearbook 6: 61-66.

Swart, W. (2005) Fungal pathogens associated with *Clivia*. Clivia Society Yearbook 7: 91-96.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Method. Molecular Biology and Evolution 28: 2731-2739.

Thompson, J,D., Higgens, D.G. & Gibson, T.J. (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673–4680.

Weir, B.S., Johnston, P.R. & Damm, U. (2012) The *Colletotrichum gloeosporioides* species complex. Studies in Mycology 73: 115-180.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*. (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White eds). Academic Press, New York: 315–322.

Whitlock, V.H. (1986) *Colletotrichum gloeosporioides* on *Clivia* in South Africa. Plant Disease 70: 800.

Williams-Woodward, J. (2001) Simplified Fungi Identification Key. The University of Georgia Special Bulletin 37: 1-12.

Yang, Y.L., Liu, Z.Y., Cai, L., Hyde, K.D., Yu, Z.N. & McKenzie, E.H.C. (2009) *Colletotrichum* anthracnose of *Amaryllidaceae.* Fungal Diversity 39: 123-146.

Zhang, N.T. (1990) Report of a national survey on diseases of urban ornamental plants. Forest Pest and Disease 1: 44-46.

Zwickl, D.J. (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. The University of Texas at Austin.

CHAPTER 3

FIRST REPORT OF THE OCCURRENCE OF LEAF BLIGHT ON *STRELITZIA REGINAE* (AITON) CAUSED BY A *PESTALOTIOPSIS* (STEYAERT) SPECIES IN SOUTH AFRICA

3.1 ABSTRACT

Strelitzia reginae is a monocotyledonous plant, native to the eastern region of South Africa, which has become a highly desirable flower and foliage plant throughout the world. A foliar disease (leaf spotting and necrosis) was observed on plants growing in Pietermaritzburg, KwaZulu-Natal, South Africa. Microscopic examination of leaf lesions indicated the presence of fungal acervuli and conidia characteristic of the fungal genus *Pestalotiopsis*. A *Pestalotiopsis* species allied to *P. clavispora* was isolated from diseased leaf tissues and verified to be pathogenic towards *S. reginae* through the implementation of Koch's postulates with *S. reginae* seedlings. The dimensions of the versicolorous conidia of the *Pestalotiopsis* isolate were estimated as 22 ± 1 µm long x 8 ± 0.5 µm wide. Three genes of this isolate (ITS, β-tubulin and *tef1*) were amplified and sequenced. A neighbour-joining phylogenetic analysis of the ITS sequence data for the isolate placed it within a well-supported clade comprised of *Pestalotiopsis* species with versicolorous conidia. The relationship between this isolate and the other *Pestalotiopsis* species within the versicolorous clade was, however, not well resolved by the ITS-based phylogenetic inference. The cultural characteristics of the isolate and its conidial dimensions shown similarities to those reported for the epitype culture of *P. clavispora*, with which it grouped in the ITS dendrogram presented in this study. However, concatenated phylogenetic analysis of the ITS, β-tubulin and *tef1* sequence data for this isolate will be required for accurate taxonomic placement of this isolate within the versicolorous *Pestalotiopsis* species group. This is the first report of a leaf blight of *S. reginae* caused by a *Pestalotiopsis* species in South Africa. A representative culture of the isolate implicated in this disease has been deposited at the fungal culture collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI), with the accession number *Pestalotiopsis* sp. PPRI 16883.

3.2 INTRODUCTION

South African flora is richly diverse and many South African plants have become established in the international horticultural trade, with many well-known cut flowers, indoor plants, flowering bulbs and foliage plants originating from within the borders of the country (Pooley, 2005; Van Jaarsveld, 2006; Moyo *et al.,* 2011; Reinten *et al.,* 2011). A comprehensive list of South African plant species of commercial interest was provided by Reinten *et al.* (2011),

with more than 150 taxa being listed as of ‘current or historical commercial interest’ in the international floricultural trade. Amongst these records, the bird-of-paradise or crane flower (*Strelitzia reginae* Aiton), is rated as a highly desirable flower and foliage plant.

Strelitzia reginae is a banana-like plant native to the eastern, temperate regions of South Africa, particularly in the Eastern Cape and KwaZulu-Natal provinces bordering the Indian Ocean (Pooley, 2005; Cron *et al.,* 2012). *Strelitzia reginae* is the best known member of its genus, and is grown as a cut flower and pot plant worldwide and also used extensively for outdoor landscaping purposes in temperate areas with climates similar to its native range (Hensley *et al.,* 2008; Hoffman *et al.,* 2011; Xaba, 2011). *Strelitzia reginae* is valued in the cut flower industry because its orange and blue flowers are brightly coloured, large, develop on a long, sturdy stem and exhibit good postharvest durability and longevity (Vieira *et al.*, 2012).

The horticultural industry is rigorous in its maintenance of quality standards and any irregularities in the quality of the product reduce its value significantly. Martinez (2007) estimated economic losses of approximately US$ 62 million (equating to a 6.8% reduction in crop value) for ornamental plant production in the US state of Georgia in 2006 due to phytopathogenic organisms. Williams-Woodward (2012) reported that losses to the same industry for 2010 due to pathogens approached 7.5%. If the losses recorded by Martinez (2007) and Williams-Woodward (2012) for one state, in one country and in one year were to be extrapolated to a global scale, then plant pathogens can be described as a significant constraint on the ornamental plant industry because of the damaging effects they can have on the health of the plants, and the subsequent losses of plant product quality from those diseased plants and costs required to control them.

Eighteen genera of phytopathogenic fungi have been reported in available literature to cause disease in the genus *Strelitzia*. These reports have been collated and compiled into Table 3.1, presented below. Information on this subject does not appear to have been documented.

Table 3.1 Known fungal pathogens of *Strelitzia*

Genus	Species	Common name of disease and/or host organs affected	*Strelitzia* species	Reference
Armillaria	*mellea*		*S. reginae*	Davino (1984); Coetzee *et al.* (2001)
Botrytis	*cinerea*	grey mould of flowers	*S. reginae*	Halevy *et al.* (1978); Criley & Paull (1993); McKenzie & Dingley (1996); Finger *et al*. (2003); Braithwaite *et al*. (2006)
Calonectria	*ilicicola*	brown leaf spot	*S. reginae*	Hirooka *et al.* (2008)
Colletotrichum	*gloeosporiodes*	anthracnose	*S. reginae*	Korade *et al.* (2001); Lin *et al.* (2006)
Cylindrocarpon	*destructans*	root rot and leaf wilt	*S. reginae*	Grasso & Cutuli (1972)
Cylindrocladium	*clavatum*		*S. reginae*	Barnard *et al.* (1989)
	pteridis		*S. reginae*	Crous & Wingfield (1994) ; Kubota (2001)
Fusarium	*culmorum*	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	moniliforme	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	oxysporum	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	roseum	flowers	*S. reginae*	Garibaldi (1964)
	solani	root necrosis and plant wilt	*S. reginae*	Orlikowski (1977)
	sporotrichiodes		*S. reginae*	Braithewaite *et al.* (2006)
Microsphaeropsis	sp.	leaf and peduncle spot	*S. reginae*	Agosteo & Pennisi (1996)
Penicillium	sp.		*S. reginae*	Finger *et al.* (2003)
Pestalotiopsis	*versicolor*	leaf spot and blight	*S. nicolai*	Braithewaite *et al.* (2006)
Phoma	*nigricans*		*Strelitzia* sp.	Braithewaite *et al.* (2006)
Phyllacora	*strelitziae*	tar spot	*S. alba*	Doidge (1942); Crous *et al.* (2000)
Phytophthora	*nicotianae*	leaf blight and root and foot rot	*S. reginae*	Luongo *et al.* (2010)
Sclerotium	*rolfsii*	southern blight	*S. reginae*	Polizzi *et al.* (2007)
Strelitziana	*africana*		*Strelitzia* sp.	Arzanlou & Crous (2006); Crous & Groenewald (2012)
Thanatephorus	*cucumeris*		*S. nicolai*	Braithewaite *et al.* (2006)
Toxicocladosporium	*strelitziae*		*S. reginae*	Crous *et al.* (2012b)
Ulocladium	*chartarum*	leaf spot	*S. reginae*	Babelegoto *et al*. (1987); Vannini & Vettraino (2000)

This study aimed to identify a pathogen infecting the leaves of plants of *S. reginae* growing both outdoors and in greenhouses at the University of KwaZulu-Natal's Pietermaritzburg Campus, Pietermaritzburg, South Africa.

3.3 MATERIALS AND METHODS

3.3.1 Microscopic examination of diseased plant tissues and fungal spores

Regions of discolouration, chlorosis and necrosis were observed on the leaf blades and petioles of *S. reginae*. Freshly harvested leaves from a symptomatic plant were examined for fungal structures using a Leica MZ 16 stereomicroscope. Images were taken using a Leica DFC 450 C camera and Leica Application Suite V. 4.0 imaging program.

Other leaves were harvested and left to dry out before being examined. Wet mounts were prepared by scraping a flame-sterilised scalpel over the diseased areas and immersing the blade in a drop of distilled water on a microscope slide. Slides were examined using an Olympus AX70 light microscope and images taken using a Nikon DS-Ri1 camera and NIS-elements BR 4.10.00 imaging system.

3.3.2 Initial isolation of fungus from fruiting structures

Initial isolation of the fungus was carried out by scraping the necrotic and blackened lesions on infected *S. reginae* leaf material with a sterile scalpel to pick up fungal spores. The blade

was then run over the surface of a malt extract agar (MEA) plate, amended with chloramphenicol at a concentration of 10 mg μl^{-1} to exclude bacterial contamination. The plate was incubated at 25 ± 2°C for four days and portions of the growing edges of any fungi present were subcultured onto fresh MEA plates to ensure that pure cultures were established. After seven days growth at 25 ± 2°C, representatives of each subculture were placed in a UV box with 24h illumination for five days to induce sporulation. The spores that were produced were examined microscopically, as described above, and the length and width of 30 spores were recorded to estimate their dimensions.

3.3.3 Isolation of fungi from symptomatic plant tissue

Fungal isolation from symptomatic leaf material was carried out by aseptically excising six 1 cm^2 leaf lesions and sterilising them in a 0.35% sodium hypochlorite solution for one minute. The plant material was then rinsed in sterile, distilled water, plated individually on MEA plates and incubated at 25 ± 2°C until fungal growth from the lesions was evident. Sporulation of the isolates was induced in the manner described above A single spore was obtained following the methods outlined by Choi *et al.* (1999) and was used to initiate a pure culture of the fungal isolate for further analysis.

3.3.4 Pathogenicity testing

Healthy *S. reginae* seedlings with 3-5 leaves were inoculated with a conidial suspension of the isolate as follows: an MEA plate showing spore production was irrigated with 10 ml distilled water. A flame-sterilised hockey stick was run over the surface of the plate to agitate the conidia into suspension. The conidial concentration of the suspension was determined with a haemocytometer and adjusted to $1x10^6$ conidia ml^{-1} with additional distilled water prior to inoculation.

Seedlings were inoculated using a 1.5 L Pressure Sprayer to apply the suspension to their leaves such that both the adaxial and abaxial surfaces were thoroughly covered. The plants were covered with polyethylene bags immediately after inoculation. The bags were removed after 24 h and plants observed daily for possible symptom development. Seedlings were misted with distilled water and bagged in the same manner to serve as negative controls. Fungi were re-isolated from symptomatic seedling tissues in the same manner as described for the initial isolations above.

3.3.5 DNA extraction

A representative culture of the fungus implicated in this disease has been deposited at the fungal culture collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI), with the accession number PPRI 16883.

Total genomic DNA was extracted from approximately 100 mg of fresh mycelium using a DNeasy Plant Mini Kit. Mycelial samples were prepared for DNA extraction by scraping seven day old mycelial tissue off a plate and aseptically transferring it to a sterile 1.5 ml Eppendorf tube containing 400 µl DNA extraction buffer provided with the kit, and five sterile plastic beads. The sample was homogenised with a Biospec Minibeadbeater for 30 s and the homogenate then applied to the column provided in the DNeasy Plant Mini Kit. The protocol followed thereafter was as per the manufacturer's instructions.

3.3.6 PCR amplification, cloning and sequencing of internal transcribed spacer (ITS), β-tubulin and *tef1* genes of the *Pestalotiopsis* isolate

A portion of the nuclear-encoded ribosomal RNA gene region of the isolate, spanning the 18S ribosomal RNA gene (partial sequence), the internal transcribed spacer 1, the 5.8S ribosomal RNA gene, the internal transcribed spacer 2 and the 28S ribosomal RNA gene (partial sequence), was amplified using the primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). Portions of the fungal genome spanning the partial β-tubulin and *tef1* gene regions of the isolate were successfully amplified using the forward/reverse primer pairs BT2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') / BT2B (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') and EF1-526F (5'-GTCGTYGTYATYGGHCAYGT-3') / EF1-1567R (5'-ACHGTRCCRATACCACCRATCTT-3'), respectively. These genes were chosen for phylogenetic analysis based on the advice put forward to *Pestalotiopsis* researchers by Maharachchikumbura *et al.* (2012) and Maharachchikumbura *et al.* (2013a). Thermal cycling protocols followed those of Maharachchikumbura *et al.* (2012).

PCR reactions were made up to a total volume of 20 µl as follows: 1 µl of extracted DNA, 10 µl 2X KAPA2G Fast HS ReadyMix, 1.5 µl each of the forward and reverse primers (10 µM) and 6 µl nuclease-free water. Negative controls (sterile nuclease-free water added instead of extracted DNA) were concurrently prepared and run with each PCR and electrophoretic process.

All PCR reactions were run using a G-Storm Goldblock GS1 thermocycler. PCR products were analysed on 1.5 % agarose gels, stained with SYBR® Safe DNA gel stain. The size of PCR products was assessed by comparison with concurrently run samples of the GeneRuler 1 kb DNA Ladder. The gels were run at 100 V for 50 min and the resulting PCR products were viewed using a Syngene G:Box and captured using GeneSnap image acquisition software.

Fresh PCR products of the expected size that were generated for each gene were manually excised from agarose gels and individually purified using a MinElute Gel Extraction Kit and a microcentrifuge. Once purified, the amplicons were individually cloned using a TA Cloning® Kit. A pCR™ 2.1 plasmid vector and One Shot® TOP10 chemically competent *E. coli* cells supplied with the kit were used for each cloning procedure. The protocol followed for ligation of the PCR products into the vector and transformation of the cells was as per the manufacturer's instructions. Five successfully transformed clones were selected from each transformation and bulked up overnight in LB broth containing 50 $\mu g.ml^{-1}$ kanamycin that was incubated at 37°C and 225 rpm. The plasmids from these bulked up clones were extracted using a QIAprep® Spin Miniprep Kit and analysed for the presence of an insert through reamplification with the initial sets of PCR primers and thermocycling conditions. The presence and size of any reamplified products was evaluated on 1.5% agarose gels as described above.

The presence of inserts in the extracted vectors was also tested for by a restriction digest using the Fast Digest EcoRI restriction enzyme. A 2 µl sample of the extracted plasmid solution, 1 µl EcoRI enzyme, 15 µl sterile nuclease-free water and 2 µl 10X digest buffer were prepared for each transformation and incubated in the thermocycler for 5 min at 37°C. The samples were then loaded onto a 1.5% agarose gel stained with SYBR® Safe DNA gel stain and run at 100 V for 50 min. The size of any restriction products was estimated through comparison with a concurrently run sample of the GeneRuler 100 bp DNA Ladder. The gels were viewed using the Syngene G:Box GeneSnap image acquisition software.

Sequencing of the vector insert amplicons from each successful PCR and cloning reaction (as determined by both tests performed to verify amplicon presence) was carried out at the Central Analytical Facility (CAF) at the University of Stellenbosch, South Africa (http://academic.sun.ac.za/saf/about.html) used a BigDye Terminator V3.1 sequencing kit (Applied Biosystems). and the M13 Foward (-20) and Reverse plasmid primers to guide the sequencing process.

3.3.7 Phylogenetic analysis

The DNA sequence files supplied by the CAF were edited with BioEdit Sequence Alignment Editor V7.0.9.0 (Hall, 1999). The forward and reverse sequence files were for each gene each manually trimmed to include the region bounded by the forward and reverse primers, aligned using the CLUSTALW (Thompson *et al.,* 1994) add-on in BioEdit. Congruency between the forward and reverse sequence data was verified by eye. A nucleotide BLAST search in the GenBank database of the National Centre for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/genbank) was initiated with the forward sequence file of each amplified gene from the isolate to assess their homology with other sequence data records in the database. This was done to verify that the correct genes of the isolate had been amplified and sequenced and could be used for further analyses.

Taxonomic placement of the isolate within the *Pestalotiopsis* genus was carried out based on an ITS-inferred phylogeny. Homologous *Pestalotiopsis* accessions indicated by the BLAST search of the ITS region of the database were not included in the analysis, due to the uncurated nature of the GenBank database. The taxa that were chosen instead from the study of Maharachchikumbara *et al.* (2012) and subsequent studies that have built on this. The taxa chosen for analysis are presented in Table 3.2 below.

The ITS dataset was assembled using BioEdit V7.0.9.0 and aligned using CLUSTALW. The alignment was manually optimised before the dataset was used for phylogenetic assessment. The final aligned dataset comprised 470 characters (of which 105 were variable and 66 parsimony informative) from 66 strains, representing 65 *Pestalotiopsis* taxa and *Seiridium* sp. SD096 as the outgroup.

An ITS-based phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) using MEGA 6.06 (Tamura *et al.,* 2013). Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.,* 2004). Gaps and missing data were treated as complete deletions and branching pattern confidence intervals were estimated based on 500 bootstrap resampling replicates. The resultant tree generated from this analysis, shown in Appendix 3.7.4 below, was annotated with FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and is presented in Figure 3.7 below.

Table 3.2 NCBI GenBank accession data of the ITS region of *Pestalotiopsis* taxa used for phylogenetic study

Species	Isolate	GenBank Accession Number	Reference
Pestalotiopsis sp.	PPRI 16883	n/a	This study
P. adusta	ICMP6088	JX399006	Maharachchikumbara *et al.* (2012)
P. adusta	MFLUCC 10-0146	JX399007	Maharachchikumbara *et al.* (2012)
P. anacardiacearum	IFRDCC 2397	KC247154	Maharachchikumbara et al. (2013a)
P. asiatica	MFLUCC 12-0286	JX398983	Maharachchikumbara *et al.* (2012)
P. camelliae	MFLUCC 12-0277	JX399010	Maharachchikumbara *et al.* (2012)
P. camelliae	MFLUCC 12-0278	JX399011	Maharachchikumbara *et al.* (2012)
P. cf. *algeriensis*	CGMCC 3.9164	JQ683718	Maharachchikumbara et al. (2013b)
P. cf. *disseminata*	CGMCC 3.9160	JQ683716	Maharachchikumbara et al. (2013b)
P. cf. *menezesiana*	CGMCC 3.9134	JQ683719	Maharachchikumbara et al. (2013b)
P. cf. *menezesiana*	CGMCC 3.9181	JQ683713	Maharachchikumbara et al. (2013b)
P. cf. *microspora*	CGMCC 3.9199	JQ683722	Maharachchikumbara et al. (2013b)
P. cf. *versicolor*	CGMCC 3.9107	JQ683712	Maharachchikumbara et al. (2013b)
P. cf. *versicolor*	CGMCC 3.9135	JQ683715	Maharachchikumbara et al. (2013b)
P. cf. *versicolor*	CGMCC 3.9159	JQ683714	Maharachchikumbara et al. (2013b)
P. cf. *virgatula*	CGMCC 3.9202	JQ683723	Maharachchikumbara et al. (2013b)
P. chinensis	MFLUCC 12-0273	JX398995	Maharachchikumbara *et al.* (2012)
P. chrysea	MFLUCC 12-0261	JX398985	Maharachchikumbara *et al.* (2012)
P. chrysea	MFLUCC 12-0262	JX398986	Maharachchikumbara *et al.* (2012)
P. clavata	MFLUCC 12-0268	JX398990	Maharachchikumbara *et al.* (2012)
P. clavata	MFLUCC 12-0269	JX398991	Maharachchikumbara *et al.* (2012)
P. clavispora	IFRDCC 2391	KC537808	Maharachchikumbara et al. (2013a)
P. clavispora	MFLUCC 12-0280	JX398978	Maharachchikumbara *et al.* (2012)
P. clavispora	MFLUCC 12-0281	JX398979	Maharachchikumbara *et al.* (2012)
P. coffeae-arabicae	HGUP4015	KF412647	Song *et al.* (2013)
P. diversiseta	MFLUCC 12-0287	JX399009	Maharachchikumbara *et al.* (2012)
P. ellipsospora	MFLUCC 12-0283	JX398980	Maharachchikumbara *et al.* (2012)
P. ellipsospora	MFLUCC 12-0284	JX398981	Maharachchikumbara *et al.* (2012)
P. ericacearum	IFRDCC 2439	KC537807	Maharachchikumbara et al. (2013a)
P. foedans	CGMCC 3.9123	JX398987	Maharachchikumbara *et al.* (2012)
P. foedans	CGMCC 3.9178	JX398989	Maharachchikumbara *et al.* (2012)
P. foedans	CGMCC 3.9202	JX398988	Maharachchikumbara *et al.* (2012)
P. furcata	MFLUCC 12-0054	JQ683724	Maharachchikumbara *et al.* (2012)
P. gaultheria	IFRD 411-014	KC537805	Maharachchikumbara et al. (2013a)
P. hainanensis		GQ869902	Maharachchikumbara *et al.* (2012)
P. inflexa	MFLUCC 12-0270	JX399008	Maharachchikumbara *et al.* (2012)
P. intermedia	MFLUCC 12-0259	JX398993	Maharachchikumbara *et al.* (2012)
P. intermedia	MFLUCC 12-0260	JX398997	Maharachchikumbara *et al.* (2012)
P. jesteri		AF377282	Maharachchikumbara *et al.* (2012)
P. jesteri	MFLUCC 12-0279	JX399012	Maharachchikumbara *et al.* (2012)
P. karstenii	IFRDCC OP13	KC537806	Maharachchikumbara et al. (2013a)
P. kunmingensis		AY373376	Maharachchikumbara *et al.* (2012)
P. licualacola	HGUP4057	KC492509	Geng et al. (2013)
P. linearis	MFLUCC 12-0271	JX398992	Maharachchikumbara *et al.* (2012)
P. linearis	MFLUCC 12-0272	JX398994	Maharachchikumbara *et al.* (2012)
P. magna	MFLUCC 12-652	KF582795	Maharachchikumbara et al. (2013a)
P. pallidotheae		AB482220	Maharachchikumbara *et al.* (2012)
P. rhododendri	IFRDCC 2399	KC537804	Maharachchikumbara et al. (2013a)
P. rhodomyrtus	HGUP 4230	KF412648	Song *et al*. (2013)
P. rosea	MFLUCC12-0258	JX399005	Maharachchikumbara *et al.* (2012)
P. samarangensis	MFLUCC 12-0233	JQ968609	Maharachchikumbara *et al.* (2012)
P. saprophyta	MFLUCC 12-0282	JX398982	Maharachchikumbara *et al.* (2012)
P. steyaertii	IMI 192475	KF582796	Maharachchikumbara et al. (2013a)
P. theae	MFLUCC 12-0055	JQ683727	Maharachchikumbara *et al.* (2012)
P. theae	SC011	JQ683726	Maharachchikumbara *et al.* (2012)
P. trachicarpicola	MFLUCC 12-0263	JX399000	Maharachchikumbara *et al.* (2012)
P. trachicarpicola	MFLUCC 12-0264	JX399004	Maharachchikumbara *et al.* (2012)
P. trachicarpicola	MFLUCC 12-0265	JX399003	Maharachchikumbara *et al.* (2012)
P. trachicarpicola	MFLUCC 12-0266	JX399002	Maharachchikumbara *et al.* (2012)
P. trachicarpicola	MFLUCC 12-0267	JX399001	Maharachchikumbara *et al.* (2012)
P. trachicarpicola	OP068	JQ845947	Maharachchikumbara *et al.* (2012)
P. umberspora	MFLUCC 12-0285	JX398984	Maharachchikumbara *et al.* (2012)
P. unicolor	MFLUCC 12-0275	JX398998	Maharachchikumbara *et al.* (2012)
P. unicolor	MFLUCC 12-0276	JX398999	Maharachchikumbara *et al.* (2012)
P. verruculosa	MFLUCC 12-0274	JX398996	Maharachchikumbara *et al.* (2012)
Seiridium sp.	SD096	JQ683725	Maharachchikumbara *et al.* (2012)

3.4 RESULTS

Symptoms of *Pestalotiopsis* infection observed on *Strelitzia reginae* are depicted below.

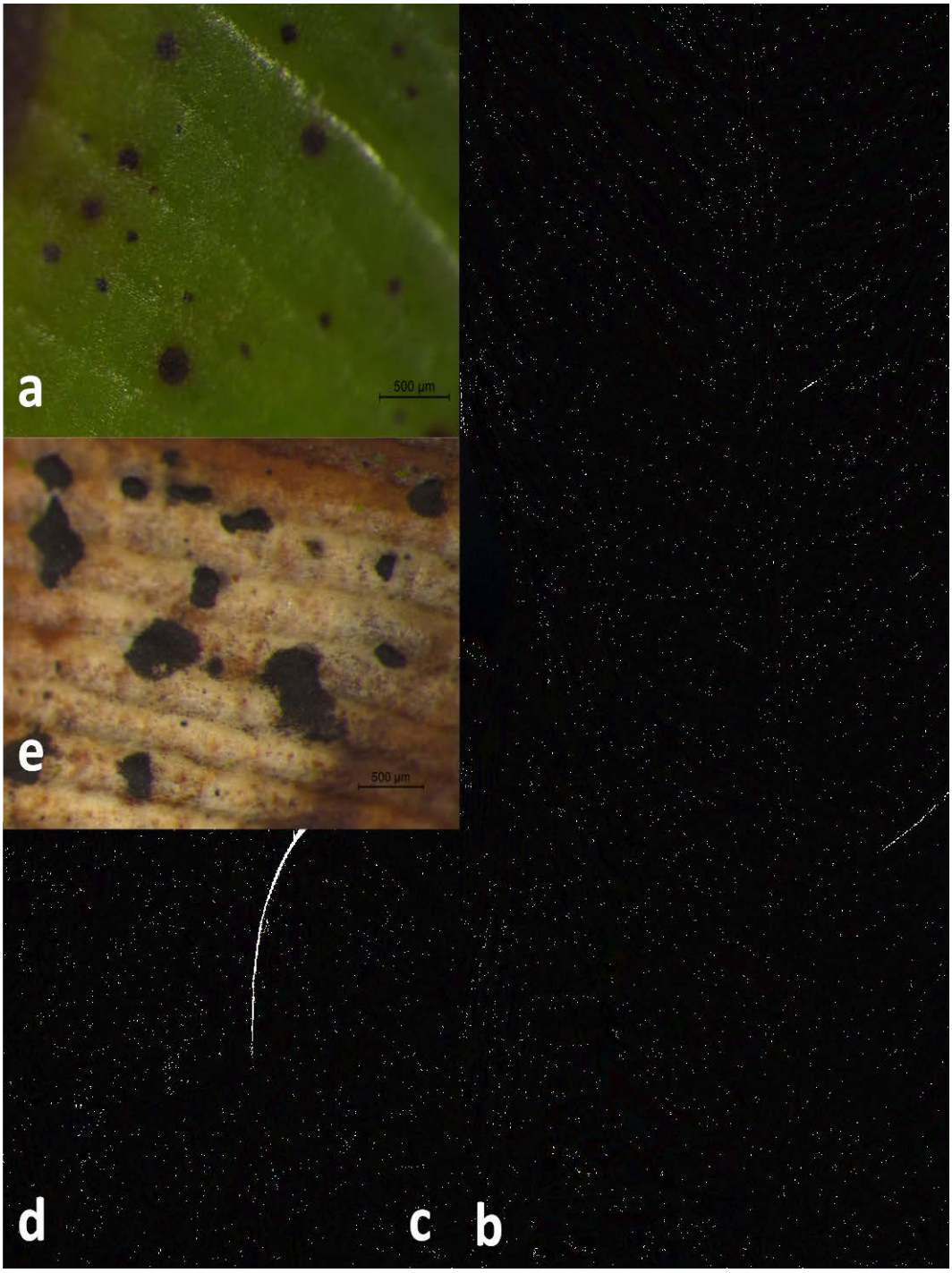


Figure 3.1 Initial symptoms on infected leaves are small, black circular lesions (a). Infected leaves subsequently show bleached tissue (b), which progresses to localised (c) or extensive leaf necrosis (d). Distinctive black spore-producing bodies develop on the newly necrotic leaf tissue (e).

The symptoms observed on *S. reginae* are prominent and can be very persistent, probably due to the slow-growing nature of the plants. Observation of the pieces of necrotic leaf lesions used in this study (Figure 3.1 d) indicated that any fungal conidia were readily picked up and transferred with a damp inoculating loop but not with dry equipment, suggesting that water-assisted dispersal of fungal propagules is important in the epidemiology of this disease.

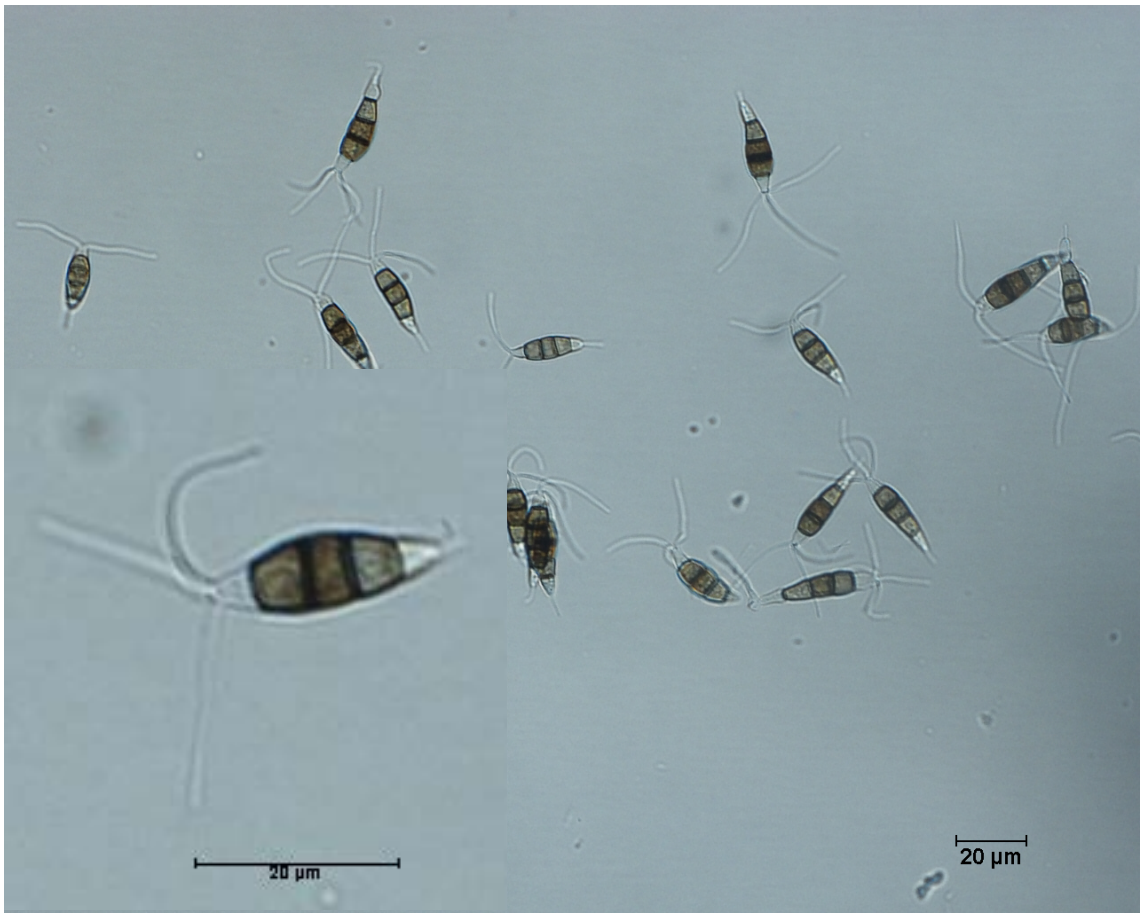


Figure 3.2 Bright-field images of the conidia of the fungal isolate implicated in the foliar disease observed on *S. reginae*

The spores observed in Figure 3.2 are morphologically consistent with the asexual conidial spores of the fungal genus *Pestalotiopsis* Steyaert when characterised using the dichotomous key to *Pestalotiopsis* and allied genera from Jeewon *et al.* (2002). The mean conidial dimensions of this isolate were measured as 22 ± 1 µm long x 8 ± 0.5 µm wide.

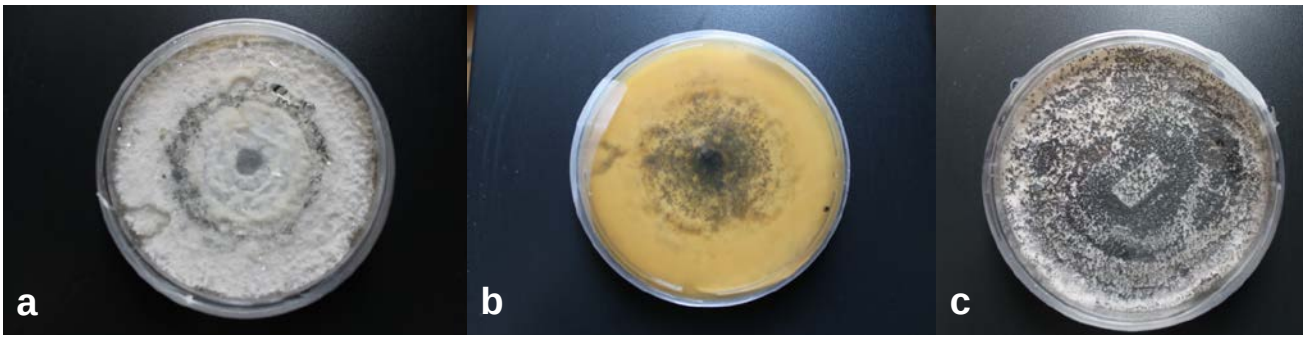


Figure 3.4 Colony morphology of the *Pestalotiopsis* isolate after eight days incubation on MEA at 25 ± 2°C: (a) from above; (b) from below. (c) Sporulation of the *Pestalotiopsis* isolate on MEA after five days exposure to 24 hour UV illumination.

The mycelium of the *Pestalotiopsis* isolate growing on MEA is initially white and cottony as illustrated in Figure 3.4(a) above. The development of conidia results in the mycelial surface becoming extensively covered in black spore masses as illustrated in Figure 3.4(c).

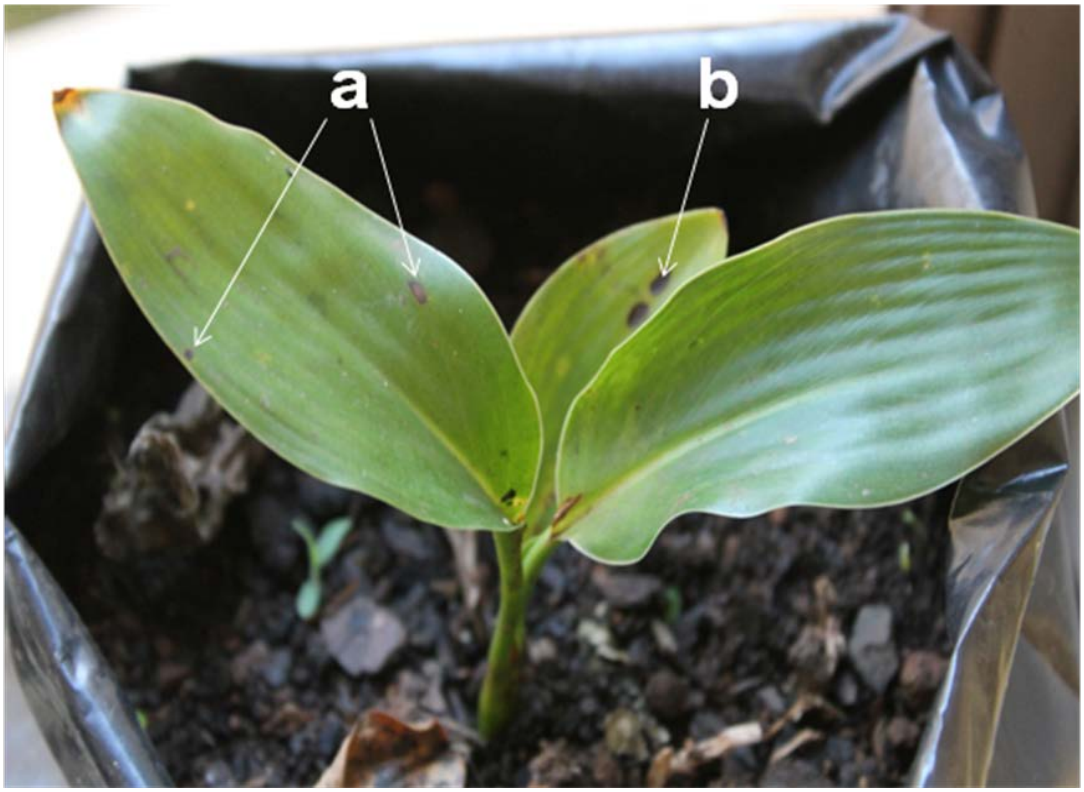


Figure 3.5 Lesions on leaves on infected *Strelitzia reginae* seedlings: (a) new lesions, (b) older lesions.

New lesions begin as small brown spots that enlarge and develop a black margin. As the lesions age, they become black and sunken, as illustrated in Figure 3.1(a).

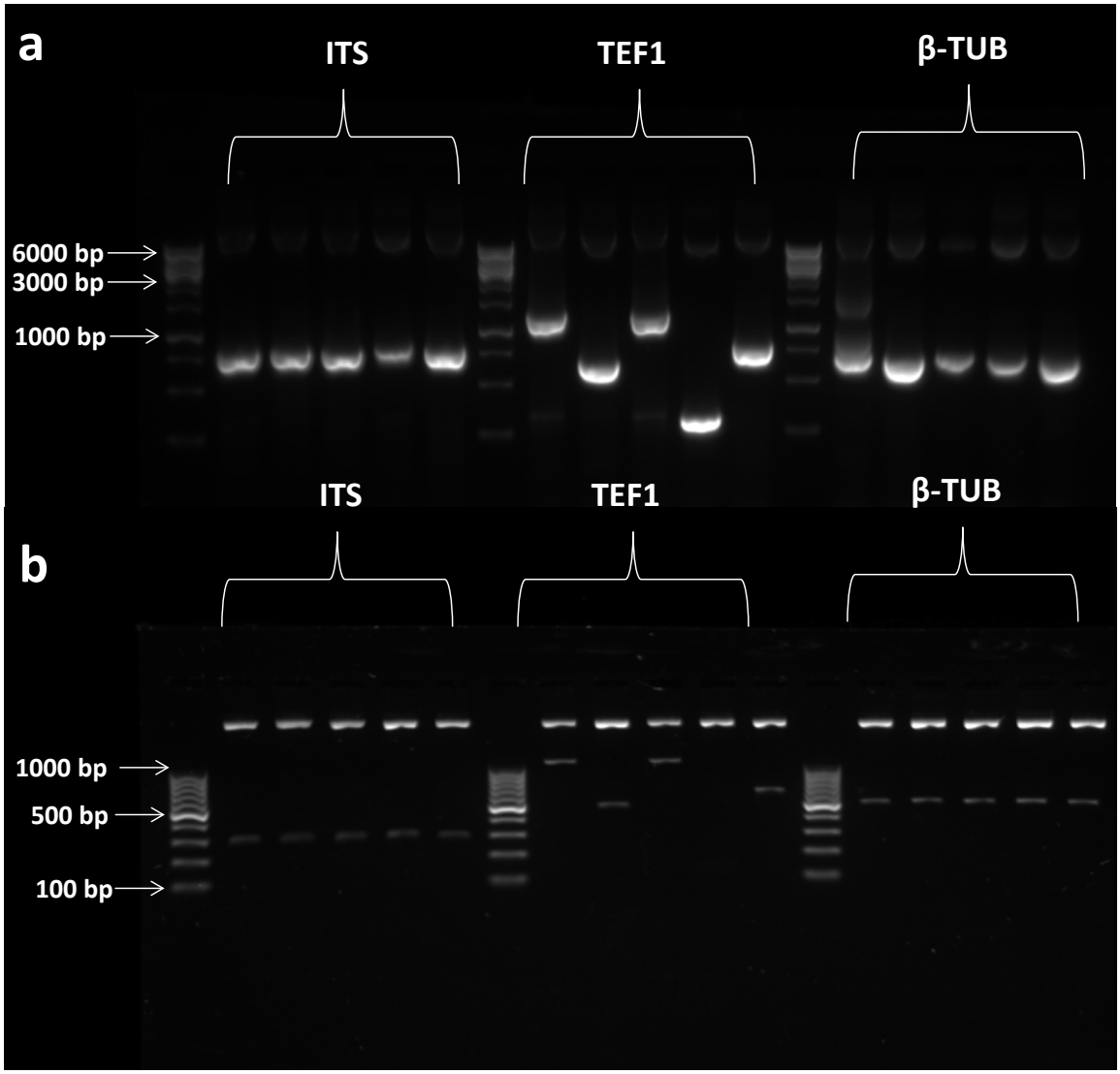


Figure 3.6 (a) PCR products re-amplified from plasmids transformed with three gene products of the isolate; (b) products obtained from a restriction digest of samples of the same plasmids analysed for the presence of an insert via PCR in (a).

At least one clone for each gene transformation showed the correct size PCR and restriction digest to be present in the extracted plasmids, indicating a successful amplification of the fungal gene sequences.

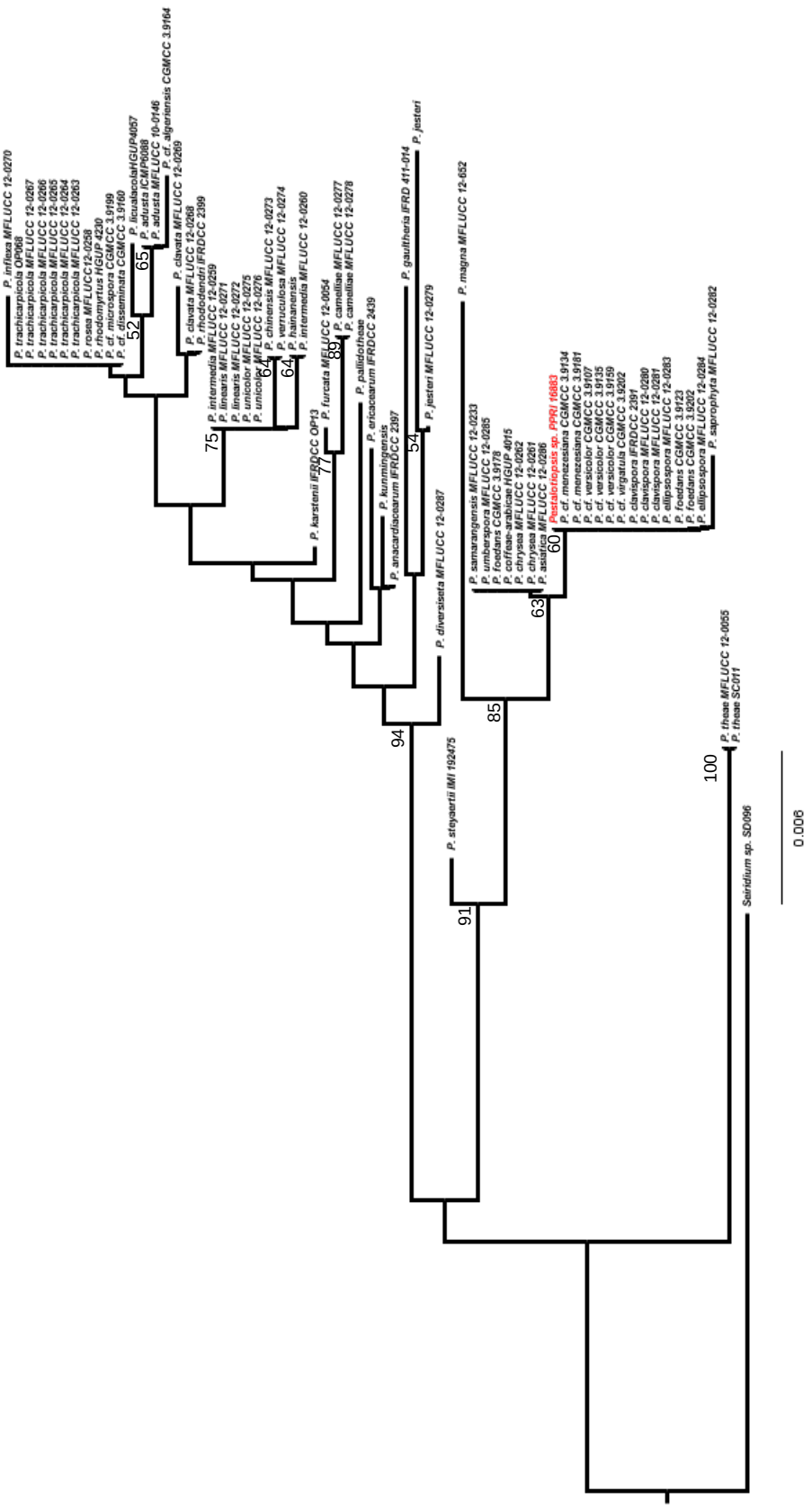


Figure 3.7 NJ phylogenetic tree generated based on the ITS sequence data for this isolate and the ITS region of other members of the *Pestalotiopsis* genus (bootstrap values < 50 not shown).

The topology of the dendrogram generated using the ITS sequence data of the isolate indicates that it falls within a clade comprised of *Pestalotiopsis* species that can be characterised by their versicolorous median conidial cells, defined as Clade B by Maharachchikumbura *et al.* (2012).

3.5 DISCUSSION

Eighteen genera of fungi have been found to cause disease in *Strelitzia* and have been reported in the available literature (Table 3.1). All organs of *Strelitzia* plants (roots, leaves and flowers) appear to be susceptible to fungal diseases. The disease identified in this study appears to be confined the leaves of infected plants, with both the leaf blades and petioles displaying symptomatic areas (Figure 3.1).

The conidia observed on wet mounts made from the blackened areas of the dried *S. reginae* leaves displayed a distinctive morphology, as illustrated in Figures 3.2 and 3.3. When compared with a dichotomous key to *Pestalotiopsis* and allied genera (Jeewon *et al.,* 2002) and a key to some commonly used conidial characteristics for *Pestalotiopsis* species identification (Maharachchikumbura *et al.,* 2011), the morphology of these spores is characteristic of a *Pestalotiopsis* species.

Jeewon *et al.* (2003) has described *Pestalotiopsis* species as ubiquitous saprobes, plant endophytes or phytopathogens. *Pestalotiopsis* sp. are anamorphic ascomycotes and produce asexual conidia in pycnidia (Maharachchikumbura *et al.,* 2011). It is apparent that that the blackened areas shown in Figure 3.1(e) are aggregations of pycnidia, containing many conidia, as illustrated in Figure 3.2.

The *Pestalotiopsis* sp. isolated from *S. reginae* produced conidia that were 4-septate and 22 ± 1 µm long x 8 ± 0.5 µm wide. The three euseptate median cells were versicolorous (Jeewon *et al.,* 2003), whilst the apical cells were hyaline. Two or three apical appendages (without knobbed ends) and a single, centric basal appendage are present on each conidium (Figures 3.2 and 3.3).

The isolate shows white, cottony mycelial growth on MEA (Figure 3.4a) and was found to produce conidia in black droplets on MEA, regardless of whether the culture was exposed to UV light or not during incubation. Cultures exposed to UV light did, however, sporulate quicker than those kept in a dark incubator. Sporulation was prolific and plates became covered in black spore-bearing masses soon after sporulation began (Figure 3.4c). All isolates induced to sporulate were confirmed to be the same *Pestalotiopsis* sp., based on their spore morphology.

Strelitzia reginae seedlings inoculated with a conidial suspension of the *Pestalotiopsis* isolate began to exhibit the development of small brown leaf lesions with a black border 8-10 days post inoculation (Figure 3.5a). The lesions became entirely black and sunken as they aged. These older lesions were similar to those observed in already infected plants and illustrated in Figure 3.1b. Plants inoculated with sterile distilled water remained healthy during and beyond the same time period.

A fungus producing a white cottony mycelium similar to that of the initial isolate was consistently re-isolated from the lesions of inoculated plants and produced conidia that were morphologically identical to those with which the plants were inoculated. This indicates that the *Pestalotiopsis* sp. originally isolated from a mature plant was capable of causing leaf blight on *S. reginae* seedlings and hence can be considered pathogenic towards *S. reginae.*

A number of *Pestalotiopsis* species have previously been recorded in South Africa (Jeewon *et al.,* 2002; 2003; 2004; Lee *et al.,* 2006), but none from *S. reginae*. Comparisons between the conidial and apical appendage morphologies of this isolate and those mentioned in the studies above exclude all the *Pestalotiopsis* species recorded in South Africa as the identity of this isolate, except possibly for *P. sydowiana* (Bres.) B. Sutton. Conidial dimensions of this isolate fall within the variable reported ranges for *P. sydowiana* in the studies conducted by Hopkins & McQuilken (2000), Remlein-Starosta (2004), Chen *et al.* (2012), Kamil *et al.* (2012) and Fang *et al.* (2013) but DNA sequence data from the isolates in these studies has not been made available for further comparison.

Leaf spotting of another *Strelitzia* species, *S. nicolai*, has been reported in New Zealand by Braithwaite *et al.* (2006). This leaf spotting was attributed to a *Pestalotiopsis* species, *P. versicolor* (Speg) Steyaert. *Pestalotiopsis versicolor* has not previously been reported from *S. reginae* or from South Africa but the conidial dimensions of this isolate fall within the range of those reported for *P. versicolor* by Wei *et al.* (2007), Feng *et al.* (2011), Kamil *et al.* (2012) and Maharachchikumbura *et al.* (2013b).

The fact that the conidial dimensions of this isolate overlap with those of a number of *Pestalotiopsis* species indicates that the accurate identification of *Pestalotiopsis* isolates based on solely on their conidial dimensions is unlikely. Recent research on the genus has indicated that it is taxonomically poorly understood at both an inter- and intraspecies level (Maharachchikumbura *et al.*, 2011). Researchers studying the genus have found that a combination of ITS, β-tubulin and *tef1* gene data gives the best phylogenetic species resolution when compared to single gene and/or morphology-only analyses. They have thus attempted to resolve this problem by generating a multi-locus backbone phylogenetic tree for

the genus (Maharachchikumbura *et al.,* 2012; 2013a) to which congruent gene data from new *Pestalotiopsis* isolates can be compared.

The ITS sequence data of this isolate indicates that it clusters in a clade with at least four *Pestalotiopsis* species that all possess versicolorous median conidial cells (Figure 3.7). Morphologically, the conidial dimensions of this isolate and its appearance when grown on an agar plate are most similar to those shown for the epitype of *P. clavispora* by Maharachchikumbura *et al.* (2012), with which it clusters in this versicolorous clade in Figure 3.7. Concatenated phylogenetic analysis of the ITS, β-tubulin and *tef1* gene sequence data from this isolate would provide the best indication of its taxonomic position within the genus and would allow for more accurate species identification.

To the best of our knowledge, this is the first report of a *Pestalotiopsis* sp. being a pathogen of *S. reginae* and is the first record of a *Pestalotiopsis*-disease on *Strelitzia* in South Africa. The economic impact of this disease is unknown at present but infected plants can exhibit large areas of discolouration and necrosis on leaf blades and petioles. As such, their appearance would likely discourage consumers from purchasing infected plants. Weakened, diseased plants may be more susceptible to other pathogens and possibly display reduced flower yield and quality compared to healthy plants. Studies on the epidemiology and control of this disease of *S. reginae* are warranted.

3.6 REFERENCES

Agosteo, G.E. & Pennisi, A.M. (1996) Infections of *Microsphaeropsis* sp. on *Strelitzia reginae*. Informatore Fitopatologico 46: 20-22.

Arzanlou, M. & Crous, P.W. (2006) *Strelitziana* M. Arzanlou & Crous, gen. nov. *Fungal Planet* 8: 1-2.

Babelegoto, N.M., Frisullo, S. & Varvaro, L. (1987) Alterazioni sulle parti epigee di *Strelitzia reginae* Banks causate da *Ulocladium chartarum* (Preuss) Simmons. La Difesa Delle Piante 10: 289-292.

Barnard, E.L., El-Gholl, N.E., Schubert, T.S., Leahy, R.M. & Schoulties, C.L. (1989) *Cylindrocladium clavatum* in Florida. Plant Disease 73: 273.

Braithwaite, M., Hill, C.F., Ganev, S., Pay, J.M., Pearson, H.G. & Alexander, B.J.R. (2006) A survey of subtropical nursery plants for fungal diseases in Northland. New Zealand Plant Protection 59: 132-136.

Chen, X.R., Xing, Y.P., Zhang, T.X., Zheng, J.T., Xu, J.Y., Wang, Z.R. & Hong, Y.H. (2012) First report of *Pestalotiopsis sydowiana* causing leaf spot necrosis of *Myrica rubra* in China. Plant Disease 96: 764.

Choi, Y.W., Hyde, K.D. & Ho, W.H. (1999) Single spore isolation of fungi. Fungal Diversity 3: 29-38.

Coetzee, M.P.A., Wingfield, B.D., Harrington, T.C., Stiemel, J., Coutinho, T.A. & Wingfield, M.J. (2001) The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. Molecular Ecology 10: 387-396.

Criley, R.A. & Paull, R.E. (1993) Review: Postharvest handling of bold tropical cut flowers – *Anthurium, Alpinia purpurata, Heliconia* and *Strelitzia.* Acta Horticulturae 337: 201-212.

Cron, G.V., Pirone, C., Bartlett, M., Kress, W.J. & Specht, C. (2012) Phylogenetic relationships and evolution in the Strelitziaceae (Zingiberales). Systematic Botany 37: 606-619.

Crous, P.W. & Groenewald, J.Z. (2012) *Strelitziana cliviae* Crous, *sp. nov.* Fungal Planet description sheet 136. Persoonia 29: 164-165.

Crous, P.W. & Wingfield, M.J. (1994) A monograph of *Cylindrocladium*, including anamorphs of *Calonectria*. Mycotaxon LI: 341-435.

Crous, P.W., Summerell, B.A., Shivas, R.G., Burgess, T.I., Decock, C.A., Dreyer, L.L., Granke, L.L., Guest, D.I., Hardy, G.E.S.J., Hausbeck, M.K., Hüberli, D., Jung, T., Koukol, O., Lennox, C.L., Liew, E.C.Y., Lombard, L., McTaggart, A.R., Pryke, J.S., Roets, F., Saude, C., Shuttleworth, L.A., Stukely, M.J.C., Vánky, K., Webster, B.J., Windstam, S.T. & Groenewald, J.Z. (2012b) Fungal Planet description sheets: 107-127. Persoonia 28: 138-182.

Crous, P.W., Phillips, A.J.L. & Baxter, A.P. (2000) Phytopathogenic Fungi from South Africa. University of Stellenbosch, Department of Plant Pathology Press, South Africa. pp. 7-50.

Davino, M. (1984) Infections of *Armillaria mellea* on *Strelitzia* plants. Colture Protette 13: 63-65.

Doidge, E.M. (1942) Revised descriptions of *Phyllachora* and related fungi. Bothalia 4: 421-463.

Fang, L., Wang, H.R. & Feng, J.J. (2013) Branch blight of Loquat caused by *Pestalotiopsis sydowiana* in China. Plant Disease 97: 990.

Feng, L., Yang, S.J., Zhan, R.L. & Zhao, Y.L. (2011) Identification and biological characteristics of macadamia leaf spot caused by *Pestalotiopsis versicolor*. Acta Phytophylacica Sinica 38: 437-442.

Finger, F.L., de Moraes, P.J., Barbosa, J.G. & Grossi, J.A.S. (2003) Vase life of bird-of-paradise flowers influenced by pulsing and term of cold storage. Acta Horticulturae 628: 863-867.

Garibaldi, A. (1964) *Fusarium roseum* infection on flowers of *Strelitzia reginae*. Rivista della Ortoflorofrutticoltura Italiana 48: 531-534.

Geng, K., Zhang, B., Song, Y., Hyde, K.D., Kang, J. & Wang, Y. (2013) A new species of *Pestalotiopsis* from leaf spots of *Licuala grandis* from Hainan, China. Phytotaxa 88: 49-54.

Grasso, S. & Cutuli, G. (1972) A decline of *Strelitzia reginae* caused by *Cylindrocarpon destructans*. Rivista di Patologia Vegetale IV 8: 207-214.

Halevy, A.H., Kofranek, A.M. & Besemer, S.T. (1978) Postharvest handling methods for bird-of-paradise flowers (*Strelitzia reginae* Ait.). Journal of the American Society for Horticultural Science 103: 165-169.

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

Hensley, D., Deputy, J., Yogi, J. & Leonhardt, K. (2008) Bird-of-Paradise. CTAHR-University of Hawaii Ornamentals and Flowers 27: 1-2.

Hirooka, Y., Takeuchi, J., Horie, H. & Natsuaki, K.T. (2008) Cylindrocladium brown leaf spot on *Howea belmoreana* caused by *Calonectria ilicicola* (anamorph: *Cylindrocladium parasiticum*) in Japan. Journal of General Plant Pathology 74: 66-70.

Hoffmann, F., Daniel, F., Fortier, A. & Hoffmann-Tsay, S.S. (2011) Efficient avian pollination of *Strelitzia reginae* outside of South Africa. South African Journal of Botany 77: 503-505.

Hopkins, K.E. & McQuilken, M.P. (2000) Characteristics of *Pestalotiopsis* associated with hardy ornamental plants in the UK. European Journal of Plant Pathology 106: 77-85.

Jeewon, R., Liew, E.C.Y. & Hyde, K.D. (2002) Phylogentic relationships of *Pestalotiopsis* and allied genera inferred from ribosomal DNA sequences and morphological characters. Molecular Phylogenetics and Evolution 25: 378-392.

Jeewon, R., Liew, E.C.Y. & Hyde, K.D. (2004) Phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association. Fungal Diversity 17: 39-55.

Jeewon, R., Liew, E.C.Y., Simpson, J.A., Hodgkiss, I.J. & Hyde, K.D. (2003) Phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species. Molecular Phylogenetics and Evolution 27: 372-383.

Kamil, D., Devi, T.P., Mathur, N., Singh, O.P., Pandey, P., Prabhakaran, N. & Patil, V. (2012) Addition of two new species of *Pestalotiopsis* to the fungal diversity in India. Journal of Mycopathological Research 50: 185-191.

Korade, B.B., Pawar, D.R. & Joshi, M.S. (2001) Anthracnose disease of spider lily. Journal of Maharashtra Agricultural Universities 26: 70-71.

Kubota, M. (2001) Occurrence of leaf spot on *Strelitzia reginae* caused by *Cylindrocladium pteridis*. Annual Report of the Kanto-Tosan Plant Protection Society 48: 89-91.

Lee, S., Crous, P.W. & WIngfield, M.J. (2006) Pestaloid fungi from *Restionaceae* in the Cape Floral Kingdom. Studies in Mycology 55: 175-187.

Lin, Q., Lin, G., Liu, F., Zhang, N. & Huang, W. (2006) Biological characteristics of anthracnose of *Strelizia reginae* and control efficiencies of fungicides against the pathogen. Fujian Journal of Agricultural Sciences 21: 14-19.

Liu, A., Chen, S., Wu, S., Xu, T., Guo, L., Jeewon, R. & Wei, J. (2010) Cultural studies coupled with DNA based sequence analyses and its implications on pigmentation as a phylogenetic marker in *Pestalotiopsis* taxonomy. Molecular Phylogenetics and Evolution 57: 528-535.

Luongo, L., Galli, M., Riccioni, L. & Belisario, A. (2010) First report of leaf blight and root and foot rot of a *Strelitzia* caused by *Phytophthora nicotianae* in Italy. Plant Disease 94: 134.

Maharachchikumbura, S.S.N., Guo, L.D., Cai, L., Chukeatirote, E., Wu, W.P., Sun, X., Crous, P.W., Bhat, D.J., McKenzie, E.H.C., Bakhali, A.H. & Hyde, K.D. (2012) A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterisation of 14 new species. Fungal Diversity 56: 95-129.

Maharachchikumbura, S.S.N., Guo, L.D., Chukeatirote, E., Bahkali, A.H. & Hyde, K.D. (2011) *Pestalotiopsis* – morphology, phylogeny, biochemistry and diversity. Fungal Diversity 50: 167-187.

Maharachchikumbura, S.S.N, Guo, L.D., Chukeatirote, E. & Hyde, K.D. (2013a) Improving the backbone tree for the genus *Pestalotiopsis*; addition of *P. steyaertii* and *P. magna* sp. nov. Mycological Progress 13: 617-624.

Maharachchikumbura, S.S.N, Guo, L.D., Chukeatirote, E., McKenzie, E.H.C. & Hyde, K.D. (2013b) A destructive new disease of *Syzigium samarangense* in Thailand caused by the new species *Pestalotiopsis samarangensis*. Tropical Plant Pathology 38: 227-235.

Martinez, A. (2007) Georgia plant disease loss estimates. University of Georgia Cooperative Extension Special Bulletin 41: 1-24.

McKenzie, E.H.C. & Dingley, J.M. (1996) New plant disease records in New Zealand: miscellaneous fungal pathogens III. New Zealand Journal of Botany 34: 263-272.

Moyo, M., Bairu, M.W., Amoo, S.O. & Van Staden, J. (2011) Plant biotechnology in South Africa: micropropagation research endeavours, prospects and challenges. South African Journal of Botany 77: 996-1011.

Orlikowski, L. (1977) Certain fungal diseases of *Strelitzia reginae* Banks. Prace Instytutu Sadownictwa w Skierniewicach, B 2: 163-167.

Polizzi, G., Aiello, D., Castello, I., Vitale, A. & Parlavecchio, G. (2007) First report of southern blight on firewheel tree, bay laurel, bird of paradise, Mediterranean fan palm and liverwort caused by *Sclerotium rolfsii* in Italy. Plant Disease 91: 1199.

Pooley, E. (2005) A Field Guide to Wildflowers of KwaZulu-Natal and the Eastern Region. The Flora Publications Trust, South Africa. pp. 12-16, 36-38, 46, 340.

Reinten, E.Y., Coetzee, J.H. & Van Wyk, B.E. (2011) The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77: 934-946.

Remlein-Starosta, D. (2004) *Pestalotiopsis* associated with *Erica* spp. ornamental plants in nurseries near Poznan – Increasing problem. Journal of Plant Protection Research 44: 307-312.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Song, Y., Geng, K., Zhang, B., Hyde, K.D., Zhao, W.S., Wei, J.G., Kang, J.C. & Wang, Y. (2013) Two new species of *Pestalotiopsis* from Southern China. Phytotaxa 126: 22-30.

Tamura, K., Nei, M. & Kumar, S. **(**2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences, USA 101:11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725-2729.

Thompson, J,D., Higgens, D.G. & Gibson, T.J. (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673–4680.

Van Jaarsveld, E. (2006) The southern African *Plectranthus* and the Art of Turning Shade to Glade. Fernwood Press, South Africa. pp. 9-10.

Vannini, A. & Verttraino, A.M. (2000) *Ulocladium chartarum* as the causal agent of a leaf necrosis on *Quercus pubescens*. Forest Pathology 30: 297-303.

Vieira, M.R., Lima, G.P., de Medeiros, D.C., de Souza, A.V. & de Oliveira, E.C. (2012) Genus: *Strelitzia*. Journal of Horticulture and Forestry 4: 178-180.

Wei, J.G., Pan, X.H., Li, Q.Q., Qin, W.M., Chen, J.N. & Xiong, Y. (2007) First report of *Pestalotiopsis versicolor* causing leaf-tip blight on acacia in China. Plant Pathology 56: 348.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*. (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White eds). Academic Press, New York: 315–322.

Williams-Woodward, J. (2012) 2010 Georgia plant disease loss estimates. University of Georgia Cooperative Extension Annual Publication 102: 1-20.

Xaba, P. (2011) *Strelitzia reginae* Aiton. http://www.plantzafrica.com/plantqrs/strelitziareginae.htm. Accessed: 07/06/2013.

CHAPTER 4

BACTERIAL FLOWER BLIGHT OF *STRELITZIA REGINAE* (AITON) IN KWAZULU-NATAL, SOUTH AFRICA

4.1 ABSTRACT

Strelitzia reginae is a monocotyledonous plant that originates in South Africa and has become a well-known floricultural crop. It is highly valued as a cut flower and garden plant in suitable climates. A damaging blight of the flowers of a number of *S. reginae* plants was observed in Pietermaritzburg, KwaZulu-Natal, South Africa. Isolation of potential phytopathogens from symptomatic flower petals consistently associated a Gram-negative, catalase-positive and oxidase-negative rod-shaped bacterium with symptomatic tissues. An assessment of Koch's postulates with a representative isolate of this bacterial strain confirmed its pathogenicity towards *S. reginae.* The morphological and cultural characteristics of the isolate indicated an affinity with the bacterial family the Enterobacteriacaeae. PCR amplification of the 16S rRNA region of the isolate generated a DNA fragment that exhibited BLAST similarities to the 16S rRNA region of various species of the enterobacterial genus *Pantoea*, chiefly *P. agglomerans.* Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) of the peptidic profile of isolate corroborated the 16S rRNA identification of the pathogen and indicated an affinity to *P. agglomerans.* This is the first report of a *Pantoea* species as a phytopathogen of *S. reginae* and also the first report of a floral disease of *S. reginae* in South Africa. A representative culture of the isolate has been deposited at the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) Culture Collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI), with the accession number *Pantoea* sp. BD 1293.

4.2 INTRODUCTION

Strelitzia reginae is an evergreen monocot native to South Africa and is the most widely known of the five members of its genus (Pooley, 2005; Cron *et al.,* 2012). Today, it is known and grown worldwide as a cut flower and somewhat as a foliage plant. (Reinten *et al*., 2011). It grows well outdoors in warm climates, similar to that of its native range in the Eastern Cape and KwaZulu-Natal provinces, and has even naturalized in southern California, USA (Hoffman *et al.,* 2011).

In common with all floricultural crops, damage to *S. reginae* products can drastically reduce the marketability of the crop. If this damage occurs on the flowers, the product is immediately compromised. If the damage is to the rest of the plant, its potential to produce top quality

flowers is compromised. Either way, a reduction in the potential profit to the *Strelitzia* grower occurs. A number of studies on the pathogens of *S. reginae* have thus been carried out wherever it is grown, of which prior reports of *Strelitzia* diseases induced by bacterial pathogens is summarized in Table 4.1 below.

Table 4.1 Bacterial diseases of *Strelitzia*

Genus	Species	Common name of disease and/or host organs affected	*Strelitzia* species	Reference
Acidovorax	*avenae subsp. avenae*	Bacterial leaf stripe	*S.nicolai*	Wehlburg (1971); Seijo & Peres (2011)
	avenae subsp. avenae	Bacterial leaf stripe	*S. reginae*	Seijo & Peres (2011)
Burkholderia	*gladioli*	Leaf spot and blight	*S. reginae*	Cirvilleri *et al.* (2006a)
Pectobacterium	*carotovorum* subsp. *caratovorum*	Brown rot	*S. reginae*	Ogorodnik *et al.* (1996); Abd-El-Khair & Nofal (2001)
Pseudomonas	*syringae pv. delphinii*	Necrosis and soft rot of flowers	*S. reginae*	Abd-El-Khair & Nofal (2001)
	syringae pv. syringae		*S. reginae*	Cirvilleri *et al.* (2005); Cirvilleri *et al.* (2006b).
	sp.	Bacterial leaf stripe	*S. reginae*	Wehlburg (1970); Miller (1976)
Ralstonia	*solanacearum*	Bacterial wilt	*S. reginae*	Liu *et al.* (2009); Rodrigues *et al.* (2011); Chandrashekara *et al.* (2012)
Xanthomonas	*campestris*		*S. nicolai*	Chase & Jones (1987)
	campestris		*S. reginae*	Chase & Jones (1987); Miller & Chase (1987); Graham *et al*. (1990)

Members of six bacterial genera have been reported to cause disease on *Strelitzia*. It seems that all organs of the plants are susceptible to bacterial infection, based on the location of symptoms mentioned by the authors of the studies cited in Table 4.1. The only prior report of a floral disease of *S. reginae* caused by bacterial infection is that made by Abd-El-Khair & Nofal (2001), where *Pseudomonas syringae* was implicated.

Symptoms of spotting and necrosis have been observed on the flowers of many *S. reginae* plants growing in Pietermaritzburg, KwaZulu-Natal, South Africa for a number of years. The aim of this study was to investigate the cause of the observed symptoms and identify the suspected phytopathogen responsible for the disease.

4.3 MATERIALS AND METHODS

4.3.1 Isolation of bacteria from infected flowers

Isolation of microbes from symptomatic *S. reginae* petals was carried out by aseptically excising four 1 cm^2 portions of symptomatic flower lesions from three separate plants to give 12 portions of petal tissue from symptomatic areas, sterilizing them in a 0.35% sodium hypochlorite solution for one minute and then triple-rinsing them in distilled water. Each piece was then individually placed onto 90 mm tryptone soy agar (TSA) plates and incubated at 25 ± 2°C until bacterial growth from the lesions was evident. The morphotype of the single bacterial strain that grew from all portions was identical. A single colony was thus obtained from a three-way streak (of the bacterial ooze observed around one of the petal portions onto a fresh TSA plate), suspended in a sterile 30% glycerol solution and stored at -80°C. A single

fresh loopful of this stored isolate was used as the progenitor of the stock used for all further analyses.

A representative culture of the isolate has been deposited at the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) Culture Collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) with the accession number BD 1293.

4.3.2 Koch's postulates and pathogenicity studies

Since the disease appeared to be widespread on the plants in the university grounds, six plants obtained from a locality with no disease history were housed in a polyethylene tunnel for one month before inoculation to confirm that they were not infected. All six plants failed to develop any symptoms of disease during this period and were thus used to apply Koch's postulates to test for any causative relationship between the bacterial isolate and the disease symptoms observed.

A 24 h old sample of the isolate grown on TSA plates was used to inoculate 100 ml of sterile tryptone soy broth (TSB). The broth was incubated overnight at 25 ± 2°C and then used to make up a 500 ml inoculation mix, containing approximately 1 x 10^6 cfu ml^{-1}, by mixing a sample of the broth with an appropriate amount of sterile, distilled water. The mix was then aseptically added to a sterile 1.5 L Pressure Sprayer and used to inoculate all flowers produced by five of the six plants in the tunnel. The inoculation mix containing the isolate was applied in the form of a fine mist evenly to all surfaces of the flowers until the point of run off. All flowers of the 6^{th} plant were inoculated with sterile distilled water, to serve as a negative control for the test. The plants were observed daily for symptom development. Microbes were re-isolated from symptomatic tissues in the same manner as described above for the initial isolation.

The inoculation trial was repeated with the same six plants to assess the pathogenic potential of the isolate under varying environmental conditions. Two of these plants served as controls for the duration of the trial. All the flowers of one were left untouched, whereas those of the 2^{nd} were misted with distilled water and covered with clear polyethylene bags (held in place with elastic bands). The remaining four plants were selected for inoculation and all of their flowers were surface sterilized with a thorough spray of 70% ethanol approximately ten minutes prior to inoculation. An inoculation mix was then prepared in the manner described above. Post-sterilization but pre-inoculation, the flowers of two sterilized plants were scratched with a sterile dissecting needle to induce extra damage to the tissues.

Thus, the flowers of two of these four sterilized plants were wounded and inoculated. One had its flowers bagged, the other not. The flowers of the two other sterilized plants were inoculated but not wounded (one was bagged, the other not). The plants were assessed daily for any symptom development over a period of 7 days, when day temperatures were in the region of 25 to 30°C.

4.3.3 Microbiological characterization of isolate

4.3.3.1 Morphological assessments

Single colonies of the isolate were selected after 24 hours of growth on TSA at 25 ± 2°C and aseptically transferred to fresh 90 mm TSA and Nutrient Agar (NA) plates to investigate any possible variation in the morphological characteristics of the isolate due to differences in the culture medium utilized. A wet mount of the isolate was prepared and viewed using a Zeiss Axio Scope.A1 light microscope.

4.3.3.2 Biochemical assessments

Single colonies of the isolate were selected after 24 hours of growth on TSA at 25 ± 2°C and aseptically smeared on clean microscope slides for assessment. A 3% KOH solution was used to assess the gram reaction of the isolate, while catalase and oxidase enzyme activities were tested according to standard methods – catalase using a pharmacy-bought 3% v/v H_2O_2 solution and oxidase using self-prepared Kovac's reagent (1% aqueous N,N,N',N'-tetramethyl-p-phenylenediamine). Strains of *Escherichia coli* Castellani and Chalmers and *Staphylococcus aureus* Rosenbach with known biochemical reactions were concurrently cultured and assessed alongside the isolate to serve as controls. These strains gave the expected results for all tests.

BioMérieux API 50 CH strips were used to develop a fermentative metabolic profile of the isolate. The biolog analysis was carried out and the results were interpreted as per the manufacturer's instructions, with API 50 CHB/E Medium used to rehydrate the carbohydrate substrates. The isolate was incubated at 30 ± 1°C for the duration of the assessment and carbohydrate substrate usage assessed and recorded at 24 and 48 hrs post inoculation.

4.3.4 DNA extraction, polymerase chain reaction (PCR) of the 16S rRNA gene region of the isolate

A method of crude lysis was used to obtain template DNA for PCR analysis from the isolate by suspending a single, 24 h old bacterial colony in 10 µl sterile 1 x TE buffer and then heating the sample to 95°C for 10 min using a G-Storm Goldblock GS1 thermocycler. The

sample was then spun down at 12 000 rpm for 2 min using a benchtop centrifuge. After centrifugation, the resulting supernatant was used as a template for PCR amplification.

PCR amplification of the conserved bacterial 16S rRNA gene region of the isolate was carried out using a KAPA 2G Fast HS ReadyMix PCR Kit. Two 20 µl reaction mix each consisting of 1 µl freshly prepared template DNA, 10 µl 2X KAPA2G Fast HS ReadyMix, 1.5 µl each of the forward (fD1) [5'- CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG - 3'] and reverse (rP2) primers [5'- CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT - 3'] (Weisburg *et al.,* 1991) and 6 µl sterile nuclease-free water were prepared for the isolate. A separate sample prepared with 1 µl of sterile nuclease-free water added instead of template DNA was prepared to serve as a negative control and concurrently subjected to the same PCR and electrophoretic processes as the samples from the isolate.

The 16S rRNA PCR was run with the following protocol: an initial denaturation step of 95°C for 2 min, followed by 35 cycles of 95°C for 20 s, 42°C for 35s and 72°C for 45s and a final elongation step of 72°C for 5 min. The PCR protocol used was based on that outlined by Weisburg *et al.* (1991) but optimized for the conditions of this study.

All PCR reactions were run using a G-Storm Goldblock GS1 thermocycler. Products from all PCR reactions were analysed on a 1.5 % agarose gel, with 1 µl SYBR® Safe DNA gel stain added per 10 ml of gel. GeneRuler 1kb DNA Ladder was used as a molecular weight marker to estimate the size of any PCR products obtained. The gel was run at 100 V for 50 min and PCR products were viewed using a Syngene G:Box and captured using GeneSnap image acquisition software.

4.3.5 DNA sequencing and phylogenetic analysis of the 16S rRNA region

A Zymo DNA Clean & Concentrator - 500 Kit was used to remove excess reagents from a successful PCR reaction showing a single 1500 bp band. The clean PCR product was sent to the Central Analytical Facility (CAF) of the University of Stellenbosch (http://www.sun.ac.za/saf) for DNA sequencing, using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems).

The DNA sequence file supplied by the CAF was opened and edited with BioEdit Sequence Alignment Editor V7.0.9.0 (Hall, 1999). Areas of the trace file with poor resolution outside of the region bound by the forward and reverse primers were removed before the sequence data was compared using a nucleotide BLAST search algorithm to that of other bacterial 16S rRNA homologues housed in a public-access DNA sequence database, the curated, type-

strain referenced Ribosomal Database Project (RDP) repository maintained by the Michigan State University (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

4.3.6 Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) of the isolate

Samples of a number of bacterial isolates were prepared and simultaneously analysed using a Bruker Microflex MALDI-TOF MS bench top Biotyper system. The isolate (ST1 in the MALDI-TOF MS output shown in Figure 4.6) was cultured for 24 hours on TSA at 25 ± 2°C. A single colony was picked off using a sterile toothpick and added to a 1.5ml Eppendorf tube containing 300 µl of sterile distilled water. An ethanol/formic acid extraction procedure was performed on the sample for profiling as per the methods laid out in the supplementary material provided by Bruker Daltonics. Of the resulting supernatant, 1 µl was pipetted onto a MSP 96 target spot polished steel plate and left to air dry, then 1 µl of $HCCA_{portioned}$ matrix solution was overlaid onto each sample and also left to air dry before analysis. Once dry, the steel target plates were loaded into a MALDI Biotyper and the analysis run.

4.4 RESULTS

Symptoms of this floral disease of *S. reginae* manifest as small, dark brown or black circular lesions on the petals of *S. reginae* cultivars.

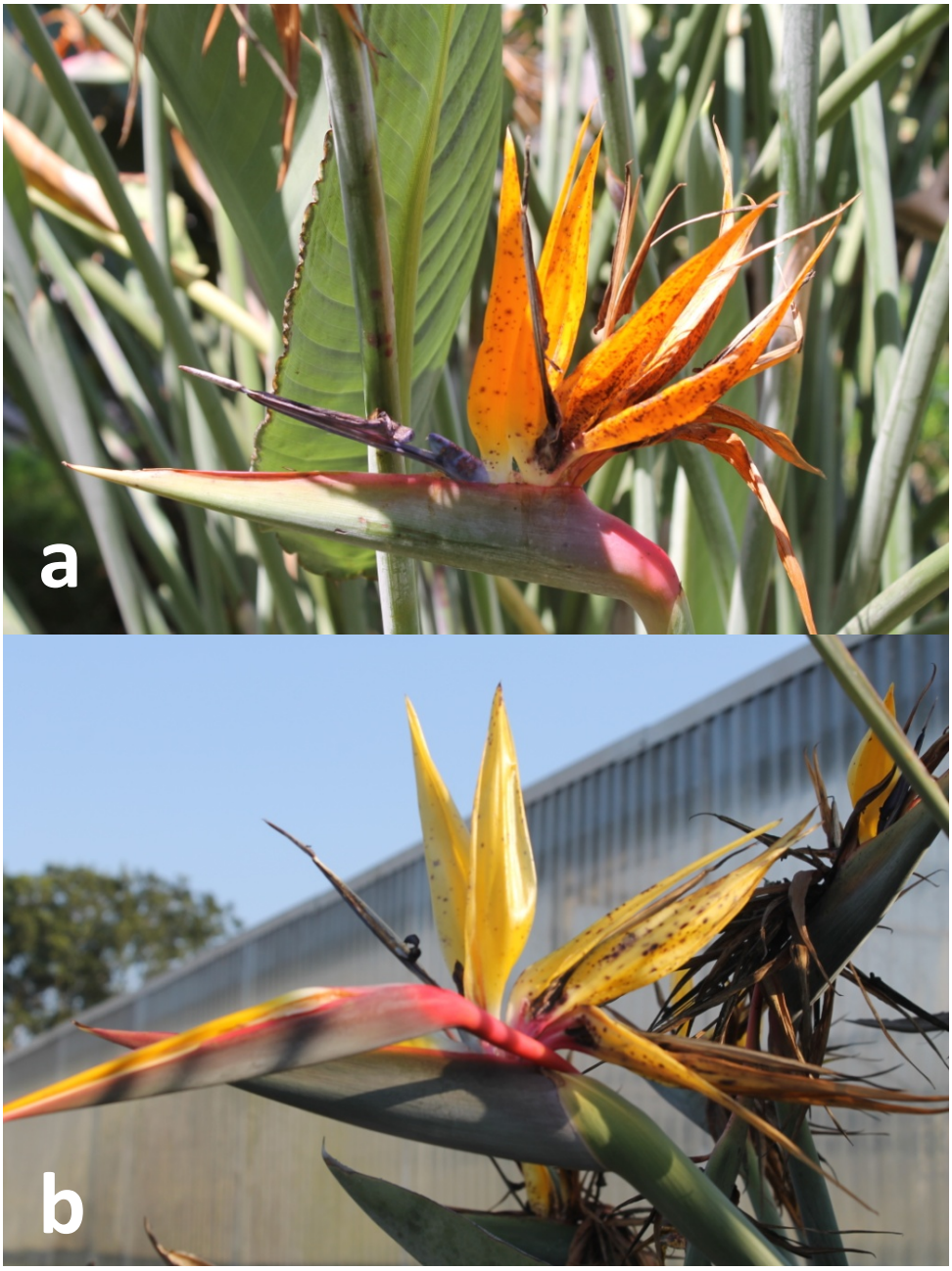


Figure 4.1 Disease symptoms on the petals of (a) *Strelitzia reginae* and (b) *S. reginae* 'Mandela's Gold'.

These symptoms first become apparent on the older flowers (as they senesce) and then progress to newer flowers. Disease outbreaks occur during periods of hot and humid weather (typically during spring and summer) in Pietermaritzburg, KwaZulu-Natal, South Africa. If these favourable environmental conditions are prolonged, the lesions coalesce to form irregular necrotic areas on the petals.

Figure 4.2 Tunnel-grown *S. reginae* flowers that developed disease symptoms following inoculation with the bacterial isolate

All flowers inoculated in the tunnel developed disease symptoms 6 days post-inoculation. The symptoms observed on these plants were very similar to those initially observed and shown in Figure 4.1.

Figure 4.3 Symptom severity increased: (a) under very humid conditions and (b) when flowers were wounded. Photographs taken 7 days post inoculation.

Flowers that were inoculated and bagged with a polyethylene bag showed much more extensive lesion coalescence when compared to flowers that were not bagged after inoculation. Flowers that were wounded with a sterile needle and inoculated (whether bagged or not) developed even greater levels of disease. It was thus evident that high humidity around the petals and any wounding of the petals can greatly increase disease severity.

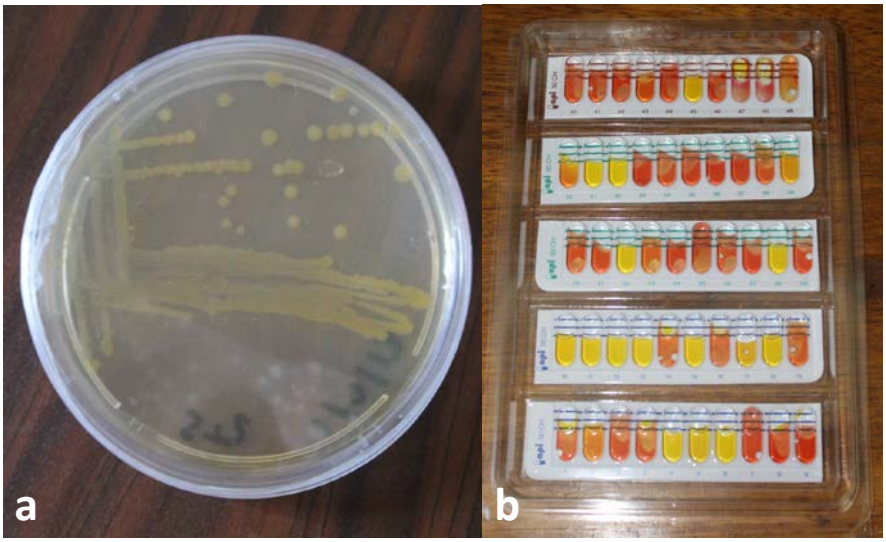


Figure 4.4 (a) Colonies of isolate on TSA after 48 h incubation at 25 ± 2°C; (b) API 50 CH metabolic profile of isolate after 48 h incubation at 30 ± 1°C.

The isolate grew rapidly and presented circular colonies that were convex in cross section with entire margins and had smooth, mucoid surfaces regardless of culture media. A yellow

appearance to the colonies was characteristic but the intensity of this yellow colouring varied with culture medium (lighter on NA, darker on TSA), age (became more intense as the culture aged) and/or cooler storage conditions. The isolate was able to grown aerobically on TSA and anaerobically under biolog test conditions, verifying its nature as a facultative anaerobe.

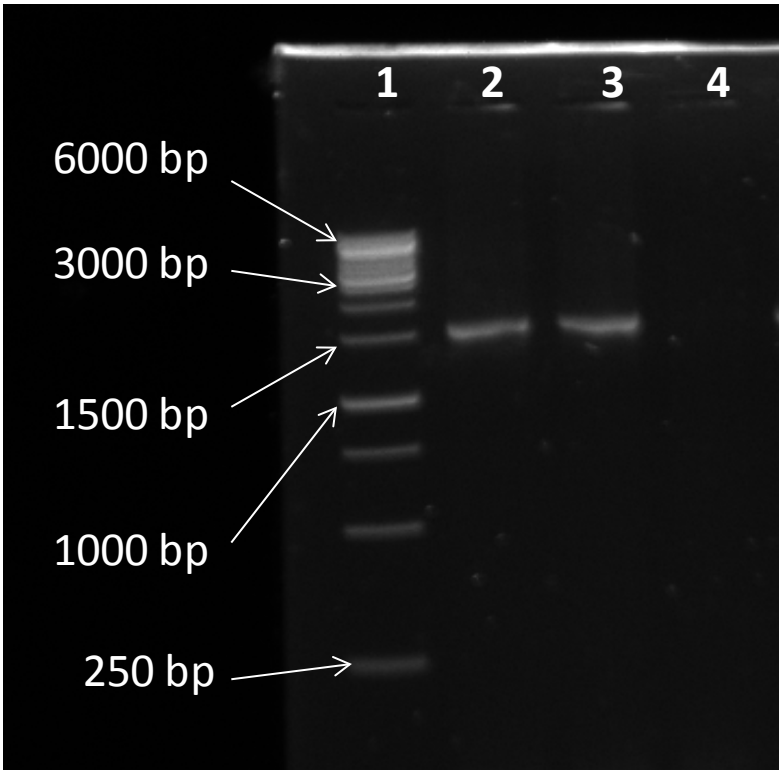


Figure 4.5 1.5% agarose gel showing PCR products amplified from total genomic DNA of the bacterial isolate using 16S rRNA region specific primers: Lane 1 – 1 kb MWM, Lanes 2 and 3 - approximately 1500 bp PCR products, Lane 4 – negative control.

A single band of the right size was evident for both PCR reactions containing total genomic DNA of the isolate. Amplification of the 16S rRNA region of this bacterial isolate was considered successful, since no PCR product was evident for the negative control.

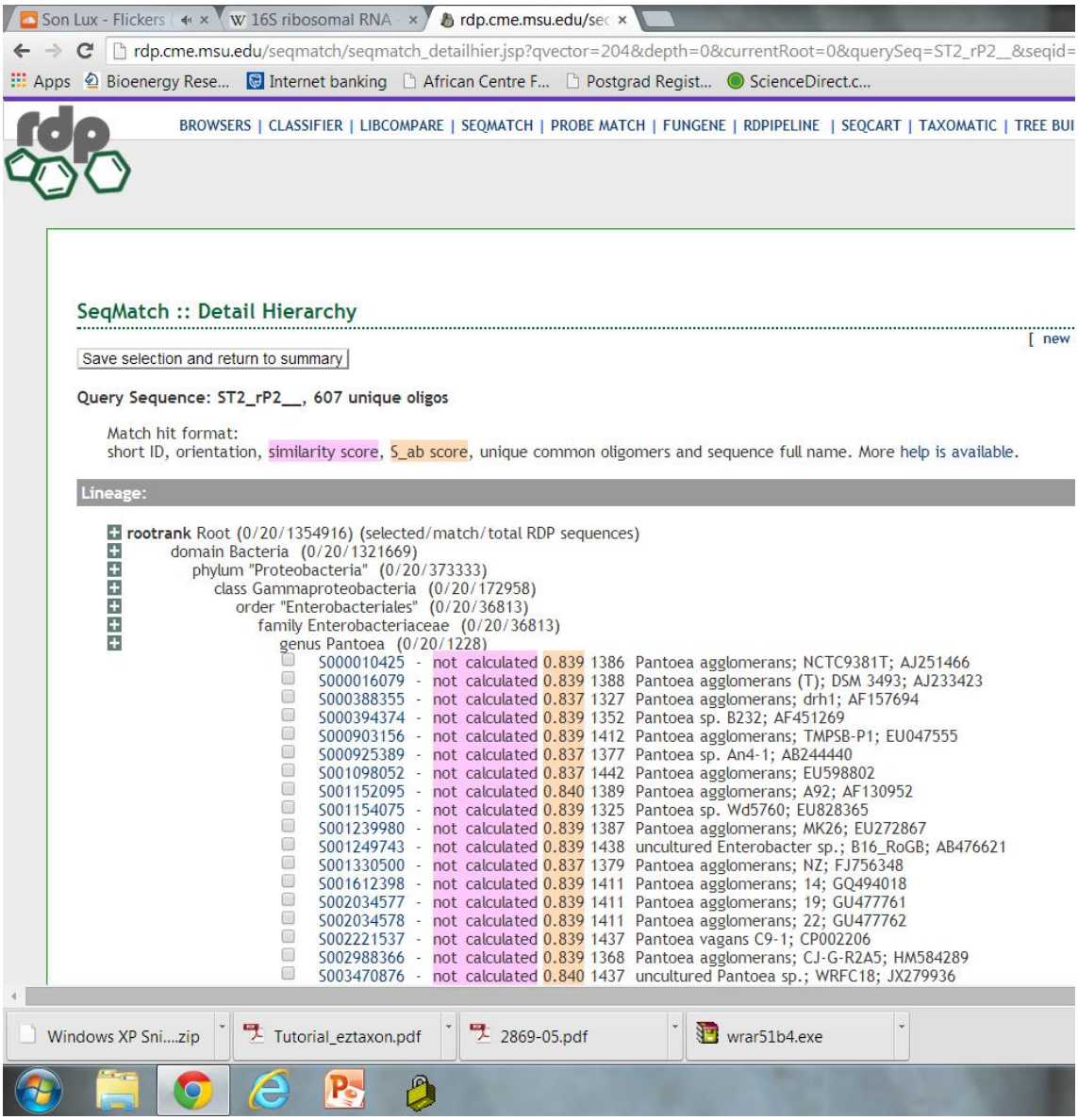
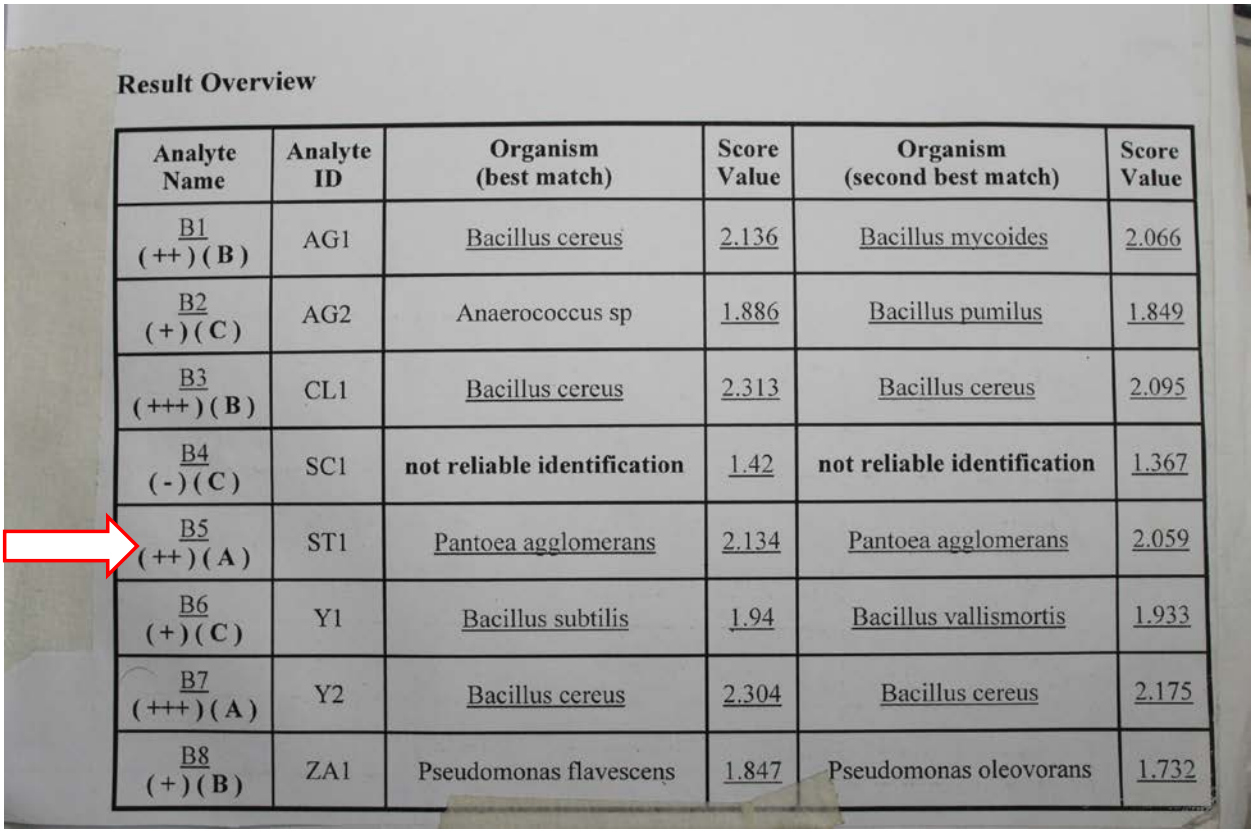


Figure 4.5 RDP database BLAST results for the16S rRNA region of the isolate

Sequence data obtained from the 16S rRNA region of the isolate indicated similarities to various species of *Pantoea* Gavini *et al.*., particularly *P. agglomerans* Gavini *et al.*



Result Overview

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
B1 (++) (B)	AG1	Bacillus cereus	2.136	Bacillus mycoides	2.066
B2 (+) (C)	AG2	Anaerococcus sp	1.886	Bacillus pumilus	1.849
B3 (+++) (B)	CL1	Bacillus cereus	2.313	Bacillus cereus	2.095
B4 (-) (C)	SC1	**not reliable identification**	1.42	**not reliable identification**	1.367
B5 (++) (A)	ST1	Pantoea agglomerans	2.134	Pantoea agglomerans	2.059
B6 (+) (C)	Y1	Bacillus subtilis	1.94	Bacillus vallismortis	1.933
B7 (+++) (A)	Y2	Bacillus cereus	2.304	Bacillus cereus	2.175
B8 (+) (B)	ZA1	Pseudomonas flavescens	1.847	Pseudomonas oleovorans	1.732

Figure 4.6 Bruker MALDI Biotyper output from the analysis of a number of bacterial strains. The isolate pertinent to this study (ST1) is indicated with the arrow.

According to the MALDI-TOF MS analysis of the isolate, both the first and second best matching organisms for the isolate were *P. agglomerans* (Ewing & Fife). The support score values (2.134 and 2.059 respectively) assigned to the isolate fell within the range of a 'secure genus identification, probable species identification', as outlined by the analysis report. Categorically, the values assigned to this isolate fall below the range MALDI-TOF MS analysis would assign to a 'highly probable species identification', namely 2.300-3.000.

4.5 DISCUSSION

Isolations from symptomatic *S. reginae* petals consistently yielded bacteria of a single morphotype, representatives of which are illustrated in Figures 4.1 and 4.4 respectively. The prevalence of the pathogen in association with diseased tissues and its survival of the surface-sterilization process in all cases pointed strongly towards the causal nature of this bacterium in the disease observed. Testing Koch's postulate tests confirmed this, as again only one type of bacteria was consistently re-isolated from symptomatic, inoculated plant petals. Flowers that were not inoculated remained healthy throughout the duration of the study. These re-isolated bacterial strains were morphologically identical to the original isolate

and displayed the same morphological and biochemical characteristics as the original isolate. Koch's postulates were thus fulfilled.

It was discerned in the brief pathogenicity trial that disease severity (and thus symptoms of the disease) can vary based on the environmental conditions prevalent at the time of infection. As illustrated in Figure 4.3, flowers that were kept under very humid conditions and/or wounded developed more severe symptoms faster than plants observed under natural infection or spray post inoculation in the greenhouse trials. In all cases, none of the flowers of plants kept in the greenhouse that were not inoculated developed any symptoms, whether they were bagged or not.

The isolate obtained in this study produces entire, yellow colonies when grown on TSA (Figure 4.4a) and is gram-negative, catalase positive and oxidase negative. It was found to be a rod-shaped, facultative anaerobe, revealed by its ease of culture on TSA and its ability to produce acid but not gas from the following carbohydrate substrates: D-arabitol, D-fructose, D-galactose, D-glucose, D-maltose, D-mannitol, D-mannose, D-ribose, D-saccharose (sucrose), D-trehalose, D-xylose, inositol, L-arabinose, L-rhamnose and N-acetylglucosamine (Figure 4.4b)

These morphological and biochemical results are consistent with those reported for bacterial strains belonging to the family Enterobacteriaceae and genus *Pantoea* Gavini *et al.* [Brady *et al.* (2010a); De Maayer *et al*. (2012) and Holt *et al.* (2000)]. The metabolic profile generated by using the API 50 CH kit (Figure 4.4b) was not used to assign a specific identity to the isolate, as Coutinho & Venter (2009), Rezzonico *et al.* (2010) and Rezzonico *et al.* (2012) have all highlighted the inaccuracy of using biologs to discriminate between *Pantoea* species and strains, but were rather used to generate a preliminary assessment of the biochemical profile of the isolate.

A single PCR product of approximately 1500 bp, corresponding to the 16S rRNA gene region of the isolate was generated, evident in Figure 4.3. A BLAST search for homologues indicated a close match with various 16S rRNA sequences assigned to various *Pantoea* species, largely *P. agglomerans*. This was apparent in the BLAST similarity results produced by both databases used. The 16S rRNA data of this isolate strongly supports its identification as a *Pantoea* species, but is less reliable for discerning what *Pantoea* species this isolate actually is. This finding echoes the research of Coutinho & Venter (2009), Rezzonico *et al.* (2010) and Brady *et al.* (2012) who observed that it is unlikely that reliable identification of the isolate to a species level can be made within the genus based solely on the 16S rRNA

gene sequence data of the isolate. Brady *et al.* (2012) recommended amplification and sequencing of the *atpD, gyrB*, *infB* and *rpoB* genes of *Pantoea* isolates in conjunction with the 16S rRNA gene, followed by concatenated phylogenetic analysis of all gene data, as the most reliable method to identify *Pantoea* isolates to a species and/or strain level.

Results from the MALDI-TOF MS analysis indicated that both the best and second best matching organism for the isolate was *P. agglomerans*. Rezzonico *et al*. (2010) studied the reliability of MALDI-TOF MS identification of *Pantoea* species and found that 'MALDI-TOF MS analysis was able to discriminate strains within *Pantoea* and to segregate related strains into separate species/clades with the same level of accuracy as *gyrB* sequencing and more sensitively than either biochemical or 16S rRNA gene sequencing approaches'. Rezzonico *et al*. (2010) also found that the use of different media for isolate growth prior to analysis had an effect on the composition of the mass spectra obtained for the isolate but not enough to blur the distinctions between species. Thus, although MALDI-TOF MS tentatively identifies this isolate as *P. agglomerans*, the supporting data is not strong enough to be conclusive. Additionally, the isolate did not exhibit the ability to hydrolyse esculin in its metabolic profiling, which Holt *et al.* (2000) cited as characteristic for *P. agglomerans*.

It is evident that a bacterial strain of the genus *Pantoea* is involved in the disease observed on *S. reginae and S. reginae* 'Mandela's Gold' plants growing in Pietermaritzburg. Personal observations of the progression of this disease on plants around the university campuses indicate that the petals are the only organ of infected plants to show symptoms but that a possible source of infection appears to come from the outside environment as the opening flowers leave the spathe. Spraying developing flower buds with 70% ethanol the day before the first petals break free from the sheaths making up the spathe led to disease-free flowers.

Wounded petals and/or very humid environmental conditions lead to the rapid expansion of the lesions into the wounded areas of the petals but not onto the spathe and scape. As they began to senesce, symptomless flowers that were harvested from outdoor plants and kept indoors in the laboratory, but not sterilized, consistently displayed symptoms identical to those observed in the field. Symptomless flowers harvested at the same time and from the same plants, but sprayed with a 70% ethanol solution, remained symptomless under the same conditions. This suggests that this bacterium is epiphytic on the spathe and moves onto the newly exposed petal tissues, which it colonises and subsequently invades, when environmental conditions are highly conducive to bacterial growth.

These symptoms always initially appear earliest on older flowers in spring and progress from older flowers to younger ones as the growing season progresses towards summer. Removal of symptomatic, senescing flowers appeared to reduce disease incidence on newer flowers. Symptoms appear much more heavily on plants growing as big clumps surrounded by many conspecifics, while plants that grow as smaller clumps and/or are more isolated in the landscape present much fewer symptoms. Effective control of this disease can probably be achieved by observing appropriate plant spacing and fertilizing schemes and taking steps to reduce excessive humidity in the growing environment, combined with frequent debris removal and periodic treatment of plant surfaces with appropriate disinfectants to reduce bacterial population levels.

4.6 REFERENCES

Abd-El-Khair, H. & Nofal, M.A. (2001) Flower bacterial soft rot of bird of paradise (*Strelitzia reginae*, Banks) in Egypt and its control. Arab Universities Journal of Agricultural Sciences 9: 397-410.

Brady, C.L., Cleenwerck, I., van der Westhuizen, L., Venter, S.N., Coutinho, T.A. & De Vos, P. (2012) *Pantoea rodasii* sp. nov., *Pantoea rwandensis* sp. nov. and *Pantoea wallisii* sp. nov., isolated from *Eucalyptus*. International Journal of Systematic and Evolutionary Microbiology 62: 1457–1464.

Brady, C.L., Cleenwerck, I., Venter, S.N., Engelbeen, K., De Vos, P. & Coutinho, T.A. (2010) Emended description of the genus *Pantoea*, description of four species from human clinical samples, *Pantoea septica* sp. nov., *Pantoea eucrina* sp. nov., *Pantoea brenneri* sp. nov. and *Pantoea conspicua* sp. nov., and transfer of *Pectobacterium cypripedii* (Hori 1911) Brenner *et al*. 1973 emend. Hauben *et al*. 1998 to the genus as *Pantoea cypripedii* comb. nov. International Journal of Systematic and Evolutionary Microbiology 60: 2430–2440.

Brady, C.L., Venter, S.N., Cleenwerck, I., Engelbeen, K., Vancanneyt, M., Swings, J. & Coutinho, T.A. (2009) *Pantoea vagans* sp. nov., *Pantoea eucalypti* sp. nov., *Pantoea deleyi* sp. nov. and *Pantoea anthophila* sp. nov. International Journal of Systematic and Evolutionary Microbiology 59: 2339–2345.

Chandrashekara, K.N., Prasannakumar, M.K., Deepa, M., Vani, A. & Khan, A.N. (2012) Prevalence of races and biotypes of *Ralstonia solanacearum* in India. Journal of Plant Protection Research 52: 53-58.

Chase, A.R. & Jones, J.B. (1987) Leaf spot and blight of *Strelitzia reginae* (bird-of-paradise) caused by *Xanthomonas campestris*. Plant Disease 71: 845-847.

Cirvilleri, G., Bonaccorsi, A., Scuderi, G. & Scortichini, M. (2005) Potential biological control activity and genetic diversity of *Pseudomonas syringae* pv. *syringae* strains. Journal of Phytopathology 153: 654-666.

Cirvilleri, G., Bonaccorsi, A., Vitale, A., Castello, I. & Polizzi, G. (2006a) First report of leaf spot and blight of *Strelitzia reginae* caused by *Burkholderia gladioli* in Italy. Plant Disease 90: 1553.

Cirvilleri, G., Scuderi, G., Bonaccorsi, A. & Scortichini, M. (2006b) Molecular characterisation of *Pseudomonas syringae* pv. *syringae* strains from different host plants using fluorescent amplified fragment length polymorphism. Journal of Plant Pathology 88: 329-333.

Coutinho, T.A. & Venter, S.N. (2009) *Pantoea ananatis*: an unconventional plant pathogen. Molecular Plant Pathology 10: 325-335.

Cron, G.V., Pirone, C., Bartlett, M., Kress, W.J. & Specht, C. (2012) Phylogenetic relationships and evolution in the Strelitziaceae (Zingiberales). Systematic Botany 37: 606-619.

De Maayer, P., Chan, W.Y., Blom, J., Venter, S.N., Duffy, B., Smits, T.H.M. & Coutinho, T.A. (2012) The large universal *Pantoea* plasmid LPP-1 plays a major role in biological and ecological diversification. BMC Genomics 13: 625.

Graham, J.H., Hartung, J.S., Stall, R.E. & Chase, A.R. (1990) Pathological, restriction-fragment length polymorphism and fatty acid profile relationships between *Xanthomonas campestris* from citrus and noncitrus hosts. Phytopathology 80: 829-836.

Hoffman, F., Daniel, F., Fortier, A. & Hoffman-Tesfay, S.S. (2011) Efficient avian pollination of *Strelitzia reginae* outside of South Africa. South African Journal of Botany 77: 503-505.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (2000) Bergey's Manual of Determinative Bacteriology 9th Edition. Lippincott, Williams & Wilkins, Philadelphia, USA. pp. 184, 202-213.

Liu, Y., Kanda, A., Yano, K., Kiba, A., Hikichi, Y., Aino, M., Kawaguchi, A., Mizoguchi, S., Nakaho, K., Shiomi, H., Takikawa, Y. & Ohnishi, K. (2009) Molecular typing of Japanese strains of *Ralstonia solanacearum* in relation to the ability to induce a hypersensitive reaction in tobacco. Journal of General Plant Pathology 75: 369-380.

Miller, J.W. (1976) Bacterial leaf stripe of Bird-of-Paradise. Plant Pathology Circular 169: 1-2.

Miller, J.W. & Chase, A.R. (1987) Leaf spot and blight of bird-of-paradise caused by *Xanthomonas campestris*. Plant Pathology Circular 300: 1-2.

Ogorodnik, L.E., Gvozdyak, R.I., Kabashnaya, L.V. & Yakovleva, L.M. (1996) *Erwinia carotovora* subsp. *carotovora* – a causal agent of brown rot of *Strelitzia*. Mikrobiologichnii Zhurnal 58: 7-10.

Pooley, E. (2005) A Field Guide to Wildflowers of KwaZulu-Natal and the Eastern Region. The Flora Publications Trust, South Africa. pp. 46-47.

Reinten, E.Y., Coetzee, J.H. & Van Wyk, B.E. (2011) The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77: 934-946.

Rezzonico, F., Vogel, G., Duffy, B. & Tonolla, M. (2010) Application of whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification and clustering analysis of *Pantoea* species. Applied and Environmental Microbiology 76: 4497-4509.

Rezzonico, F., Stockwell, V.O., Tonolla, M., Duffy, B. & Smits, T.H.M. (2012) *Pantoea* clinical isolates cannot be accurately assigned to species based on metabolic profiling. Transplant Infectious Disease 14: 220-221.

Rodrigues, L.M., Destefano, S.A., Diniz, M.C., Comparoni, R. & Neto, J. (2011) Pathogenicity of Brazilian strains of *Ralstonia solanacearum* in *Strelitzia reginae* seedlings. Tropical Plant Pathology 36: 409-413.

Seijo, T.E. & Peres, N.A. (2011) First report of *Acidovorax avenae* subsp. *avenae* causing bacterial leaf stripe of *Strelitzia nicolai*. Plant Disease 95: 1474.

Wehlburg, C. (1970) Bacterial leaf stripe of *Strelitzia reginae* Banks. Phytopathology 60: 589.

Wehlburg, C. (1971) Bacterial leaf stripe of *Strelitzia reginae* caused by *Pseudomonas* sp. Plant Disease Reporter 55: 447-448.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173: 697-703.

CHAPTER 5

ETIOLOGY OF A BACTERIAL SOFT ROT OF *CLIVIA MINIATA* (REGEL).IN SOUTH AFRICA

5.1 ABSTRACT

Clivia miniata is an evergreen monocotyledonous plant native to the eastern regions of South Africa. It has become a popular pot plant and garden plant in suitable climates. Two outbreaks of a soft rot disease were observed in commercial nurseries where *C. miniata* was being grown, one in Howick, KwaZulu-Natal and the other in White River, Mpumalanga, South Africa. Available literature reports have indicated similar disease outbreaks to be caused by soft rot bacteria. Two bacterial isolates that were able to cause soft rots of potato slices were obtained from diseased *C. miniata* plants, one from each locality. Koch's postulates were evaluated with each isolate and indicated that each caused soft rot symptoms like those observed in the initial outbreaks. Both bacterial isolates were Gram-negative, catalase- and oxidase-positive straight rods and exhibited cultural and morphological similarities to members of the bacterial genus *Pseudomonas*. PCR amplification of the 16S rRNA region of the White River isolate indicated similarities to a number of *Pseudomonas* species when a BLAST search of the Ribosomal Database Project database was performed, corroborating the morphological and cultural evaluations of these soft rot bacteria. A representative culture of each soft rot isolate has been deposited at the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) Culture Collection of the South African Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI) with the accession number *Pseudomonas* sp. BD 1294 for the Howick isolate and *Pseudomonas* sp. BD 1295 for the White River isolate, respectively. This is the first report of soft rot of *C. miniata* caused by a *Pseudomonas* species in South Africa.

5.2 INTRODUCTION

Although not the first species of its genus to be discovered, *Clivia miniata* Reg. is probably the best known of the six *Clivia* species (Aubrey, 2001; Dixon, 2011). It occurs naturally in the subtropical eastern regions of South Africa and Swaziland and grows in partial shade in forests and bush clumps, generally in substrates high in organic matter (Pooley, 2005). As a floricultural crop, it is grown principally as a flowering pot plant that is often discarded after the flowers have faded (Duncan, 1999; Reinten *et al.,* 2011).

Bacterial diseases of *C. miniata* have been reported four times in the available literature (Table 5.1 below). The principle symptom mentioned by the authors of these studies is a soft rot of infected tissues and sometimes the death of affected plants. Laing (2009) has

previously reported a soft rot disease experienced by growers of *C. miniata* in South Africa to be caused by *Pectobacterium carotovorum* subsp. *carotovorum* Waldee.

Table 5.1 Bacterial species reported to cause disease on *Clivia miniata*

Genus	Species	Common name of disease	*Clivia* species	Reference
Erwinia	*cypripedii*	brown rot	Clivia spp.	Han & Choi (1994)
Pectobacterium	*carotovorum* subsp. *carotovorum*	soft rot	*Clivia miniata*	Choi & Lee (2000); Laing (2009)
Pseudomonas	*syringae* pv. *syringae*		*Clivia miniata*	Khan & Rudolph (1997)

Bacterial soft rots are caused by a number of bacterial genera, including *Pectobacterium, Dickeya* Samson *et al., Pseudomonas* Migula*, Bacillus* Cohn and *Clostridium* Prazmowski (Lunt, 2013). Soft rot diseases affect many crops worldwide, both in the field and during storage, and can cause significant crop losses (Charkowski, 2009; Lunt, 2013). Symptoms consistent with bacterial soft rot (oozing lesions and rapid rot of infected tissues) that were observed on several *C. miniata* plants during a hot and humid growing season prompted the investigations detailed in this study.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial isolation, purification and storage

Clivia miniata seedlings displaying symptoms of a foliar soft rot were observed in Howick, KwaZulu-Natal, South Africa in early spring (September 2012). A soft rot of the leaves (from the edges inwards and downwards towards the base of the stem) was also observed on mature, cultivated plants of *C. miniata* near White River, Mpumalanga, South Africa in late summer (February 2013). Environmental conditions at the time of both outbreaks were hot (day temperatures exceeding 30°C) and very humid (rainfall occurring daily).

Four decaying leaf samples of approximately 1 cm^2 were aseptically excised from one representative symptomatic plant in each outbreak and surface sterilized in a 0.35% sodium hypochlorite for one minute. These segments were then each rinsed three times with sterile distilled water and individually aseptically macerated in a drop of sterile distilled water using a sterile scalpel on sterile glass microscope slides. The macerated segments were left for five minutes before a single loopful of inoculum from each segment was aseptically streaked onto fresh 90 mm tryptone soy agar (TSA) plates and incubated at 25 ± 2°C for 48 hours.

Representative single colonies of each bacterial morphotype that were observed on each plate were streaked onto fresh TSA plates and also incubated at 25 ± 2°C for 48 hours to check for strain purity. A single colony of each pure isolate was individually suspended in a

sterile 30% glycerol solution and stored at -80°C. A loopful of these stored isolate stocks was used to grow up the cultures [plated on TSA incubated at 25 ± 2°C for 24 hours] used for all subsequent analyses. No fungal growth was observed from the diseased plant material that was used for microbe isolation.

5.3.2 Evaluation of the soft rot ability of bacterial isolates

A total of 11 bacterial isolates were obtained from the diseased plants collected in both outbreaks and purified by subculturing separate, single colonies onto fresh TSA plates. The bacterial isolates obtained from the infected plants were evaluated for their ability to rot potato tuber slices, using the method outlined by Perombelon & Van der Wolf (2002). The potato slices were incubated at 20 ± 2°C for 48 hours.

5.3.3 Inoculation of healthy plants and determination of Koch's postulates

Two of the eleven bacterial isolates displayed the ability to rot inoculated potato slices; one from each disease outbreak. Koch's postulates were thus evaluated individually for each of these isolates as follows: three healthy *C. miniata* seedlings were surface sterilized by misting them thoroughly with a 70% ethanol solution before they were lightly wounded on their stem bases with a sterile scalpel (two parallel vertical incisions approximately 10 mm long and 2 mm deep were made on each plant). An inoculation solution containing each isolate was made just prior to inoculation by aspetically suspending several 24 h old colonies in sterile distilled water and adjusting the concentration of each inoculation broth to approximately 1 x 10^5 cells ml^{-1}. A volume of 100 µl of pre-prepared bacterial suspension was pipetted into the wounds and the plants covered with polyethylene bags for 24 hours following inoculation.

Three additional healthy plants that were sterilized and wounded in the same manner but infiltrated with 100 µl sterile distilled water served as negative controls for the duration of the experiment. The plants were housed in a polyethylene tunnel with an automated misting system that created a warm and highly humid environment during the inoculation trails, reminiscent of the environmental conditions at the time the diseased plants were first observed. Bacteria were re-isolated from diseased plant tissues in the manner described above.

A representative culture of each soft rot isolate from Howick and White River has been deposited at the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) Culture Collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) with the accession number BD 1294 for the Howick isolate and BD 1295 for the White River isolate respectively.

5.3.4 Microbiological characterization of isolates

5.3.4.1 Morphological assessments

The isolates were streaked onto fresh TSA plates and grown for 48 hr at 25 ± 2°C. A wet mount of each isolate was prepared and viewed using a Zeiss Axio Scope.A1 light microscope.

5.3.4.2 Biochemical assessments

Single colonies of the isolates were selected after 24 hours of growth on TSA at 25 ± 2°C and aseptically smeared on clean microscope slides for assessment. A 3% KOH solution was used to assess the Gram Stain reaction of each isolate, while catalase and oxidase enzyme activities were tested according to standard methods – catalase using a pharmacy-bought 3% v/v H_2O_2 solution and oxidase using self-prepared Kovac's reagent (1% aqueous N,N,N',N'-tetramethyl-p-phenylenediamine). Strains of *Escherichia coli* Castellani and Chalmers and *Staphylococcus aureus* Rosenbach with known biochemical reactions were concurrently cultured and assessed alongside the isolates to serve as controls. These strains gave the expected results for all tests.

BioMérieux API 50 CH strips were used to develop a fermentative metabolic profile of the isolate. The Biolog® analysis was carried out and the results were interpreted as per the manufacturer's instructions, with API 50 CHB/E Medium used to rehydrate the carbohydrate substrates. Each isolate was incubated at 30 ± 1°C for the duration of the assessment and carbohydrate substrate usage assessed and recorded at 24 and 48 hrs post-inoculation.

The isolates were also assessed for their ability to degrade pectin as follows: loopfuls of inoculum were aseptically streaked onto two step crystal violet pectate (CVP-S2) medium [composition and formulation as per Perombelon & Van der Wolf (2002)] and incubated at 25 ± 2°C for 48 hours. This test is carried out to distinguish soft rot *Erwinia* and *Pectobacterium* species from other soft rot bacterial genera, based on the formation and appearance of any pits that develop on the medium (Perombelon & Van der Wolf, 2002; Ma *et al.,* 2007).

5.3.5 DNA extraction and polymerase chain reaction of the 16S rRNA region of the soft rot isolates

A method of crude lysis was used to obtain template DNA for PCR analysis from each isolate by suspending a single, 24 h old bacterial colony in 10 µl sterile 1 x TE buffer and then heating the sample to 95°C for 10 min using a G-Storm Goldblock GS1 thermocycler. The sample was then spun down at 12 000 rpm for 2 min using a benchtop centrifuge. After centrifugation, the resulting supernatant was used as a template for PCR amplification.

PCR amplification of the conserved bacterial 16S rRNA gene region of the isolates was carried out using a KAPA 2G Fast HS ReadyMix PCR Kit. Two 20 µl reaction mixes, each consisting of 1 µl freshly prepared template DNA, 10 µl 2X KAPA2G Fast HS ReadyMix, 1.5 µl each of the forward (fD1) and reverse (rP2) primers (Weisburg *et al.,* 1991) and 6 µl sterile nuclease-free water, were prepared for each isolate. A separate sample prepared with 1 µl of sterile nuclease-free water added, replacing template DNA, was prepared to serve as a negative control and was concurrently subjected to the same PCR and electrophoretic processes as the samples from the isolates.

The 16S rRNA PCR was run with the following protocol: an initial denaturation step of 95°C for 2 min, followed by 35 cycles of 95°C for 20 s, 42°C for 35s and 72°C for 45s and a final elongation step of 72°C for 5 min. The PCR protocol used was based on that outlined by Weisburg *et al.* (1991) but optimized for the conditions of this study.

All PCR reactions were run using a G-Storm Goldblock GS1 thermocycler. Products from all PCR reactions were analysed on a 1.5 % agarose gel, with 1 µl SYBR® Safe DNA gel stain added per 10 ml of gel. GeneRuler 1kb DNA Ladder was used as a molecular weight marker to estimate the size of any PCR products obtained. The gel was run at 100 V for 50 min and PCR products were viewed using a Syngene G:Box and captured using GeneSnap image acquisition software.

5.3.6 Sequencing of PCR products and phylogenetic analysis

A Zymo DNA Clean & Concentrator - 500 Kit was used to remove excess reagents from a successful PCR reaction showing a single 1500 bp band for each isolate. The clean PCR products were sent to the Central Analytical Facility (CAF) of the University of Stellenbosch (http://www.sun.ac.za/saf) for DNA sequencing, using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems).

The DNA sequence files supplied by the CAF were opened and edited with BioEdit Sequence Alignment Editor V7.0.9.0 (Hall, 1999). The chromatogram generated for the Howick isolate was noisy and had low quality scores and was thus not suitable for further phylogenetic analysis. However, it was evident from the chromatograms provided for the White River isolate that good quality 16S rRNA sequence data was available and could be used for further analyses.

Areas of the White River isolate trace file with poor resolution outside of the region bound by the forward and reverse primers were removed before the sequence data was compared to that of other bacterial 16S rRNA homologues housed in the curated, type-strain referenced

bacterial Ribosomal Database Project (RDP) repository maintained by the Michigan State University (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp), using a nucleotide BLAST search algorithm.

5.4 RESULTS

Infection of *C. miniata* seemed to begin in sunken, circular foci on the leaves of susceptible plants and infected plants showed rapid development of disease symptoms

Figure 5.1 Soft rot symptoms on *Clivia miniata*: (a) an infected seedling from Howick, KwaZulu-Natal; (b) an infected older plant from White River, Mpumulanga.

The rotting observed spread from the leaves into the stems and rhizomes of many plants. A strong, putrid smell emanated from diseased plant tissues.

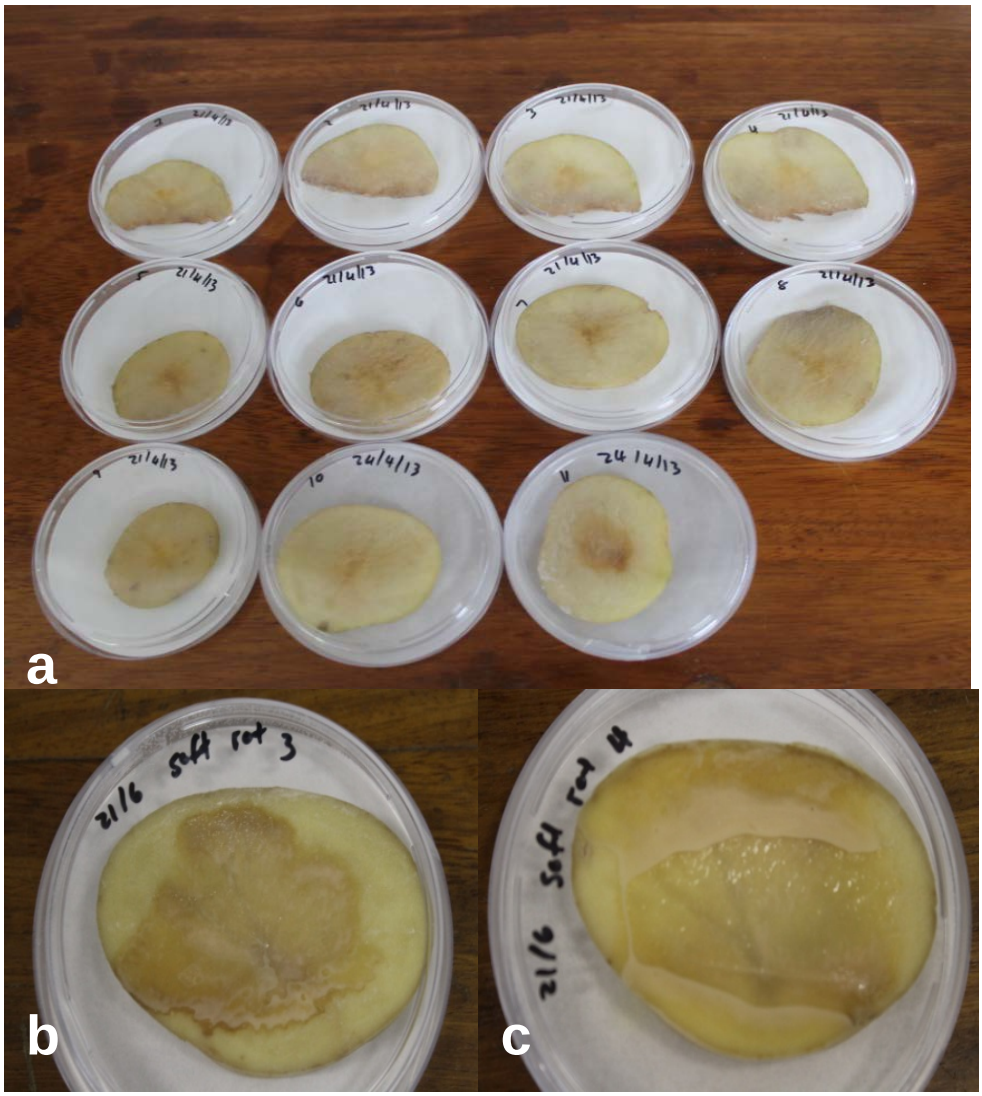


Figure 5.2 (a) Evaluation of all eleven bacterial isolates for their soft rot ability; (b) soft rot induced by the isolate obtained from Howick and (c) by the isolate obtained from White River.

Two bacterial isolates were found to exhibit the ability to rot potato tuber slices - one isolate from each locality. Differences in the physical appearance of the rots observed were evident, namely that that induced by the Howick isolate (b) was drier than that caused by the White River isolate (c).

Figure 5.3 Development of soft rot on *Clivia miniata* seedlings: (a) seedling inoculated with the isolate from Howick; (b) seedling inoculated with the isolate from White River.

The tissues surrounding the points of inoculation on all seedlings inoculated with each isolate showed collapse and liquefaction, typical symptoms of bacterial soft rot. Plants inoculated with distilled water instead of a bacterial isolate remained healthy. Apparent differences in the physical appearance of each rot (i.e. wetter for the White River isolate) mirror those observed on the inoculated potato slices shown in Figure 5.2.

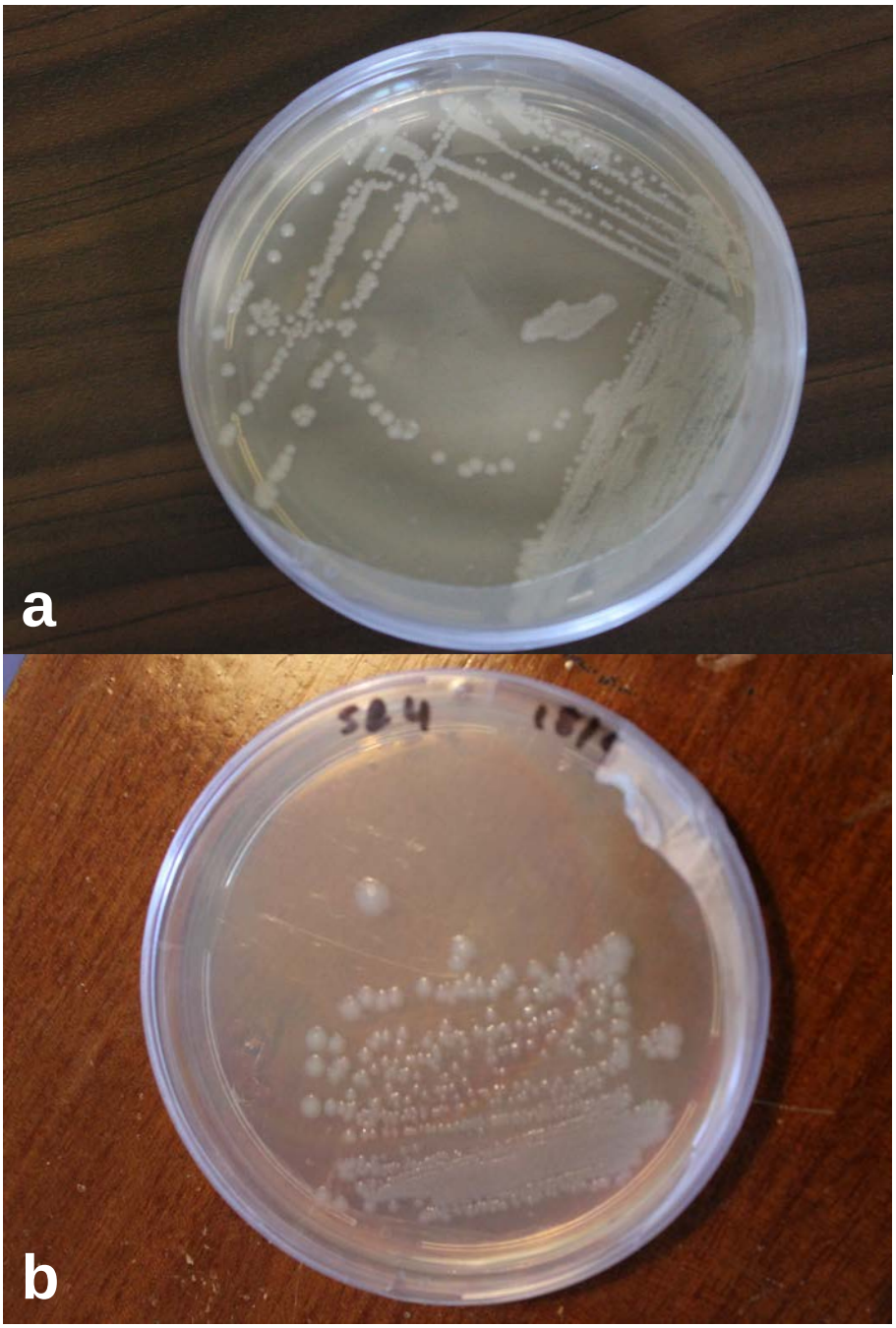


Figure 5.4 72 h old colonies of (a) Howick isolate on TSA; (b) White River isolate on TSA.

Colonies of both isolates were creamy white, circular, raised and mucoid, with lobed margins when cultured on TSA. Those of the White River isolate grew more rapidly than those of the Howick isolate when incubated concurrently, evident by the larger individual colony size in 5.4 (b) than 5.4 (a). The colonies of the White River isolate also appeared slightly more translucent than those of the Howick isolate when backlit. Visible inclusion bodies were observed to develop in colonies of both isolates as the cultures aged, giving some older colonies a resemblance to fried eggs.

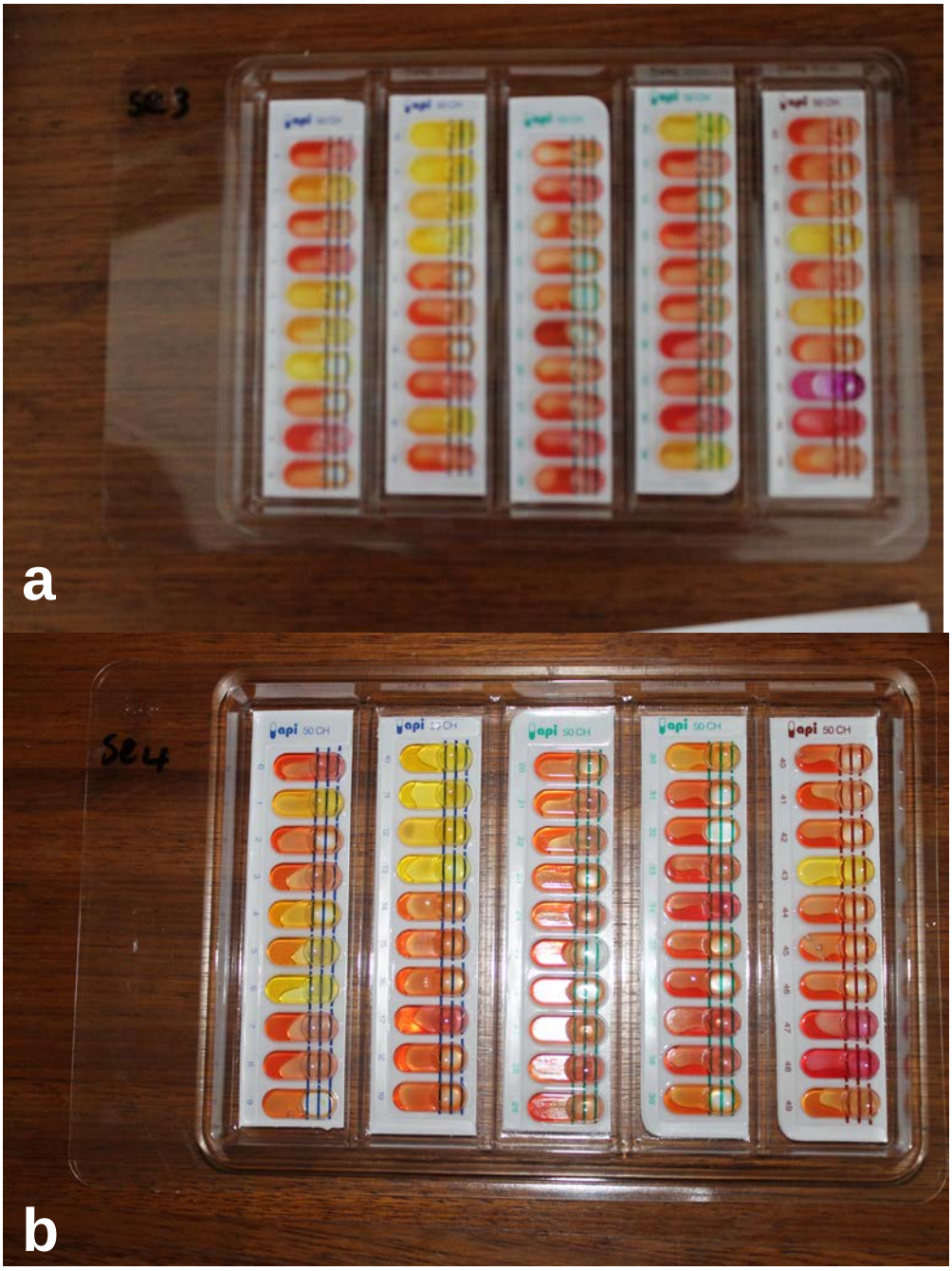


Figure 5.5 API 50 CH fermentative metabolic profile of isolate from Howick (a) and White River (b) after 48 h incubation at 30 ± 1°C.

The Biolog® profiles generated for each isolate were very similar: both isolates were able to produce acid but not gas from glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose D-melibiose, gentiobiose and D-fucose. They differed in that the Howick isolate additionally was able to utilize D-mannitol and D-arabitol; the White River isolate was not able to do so.

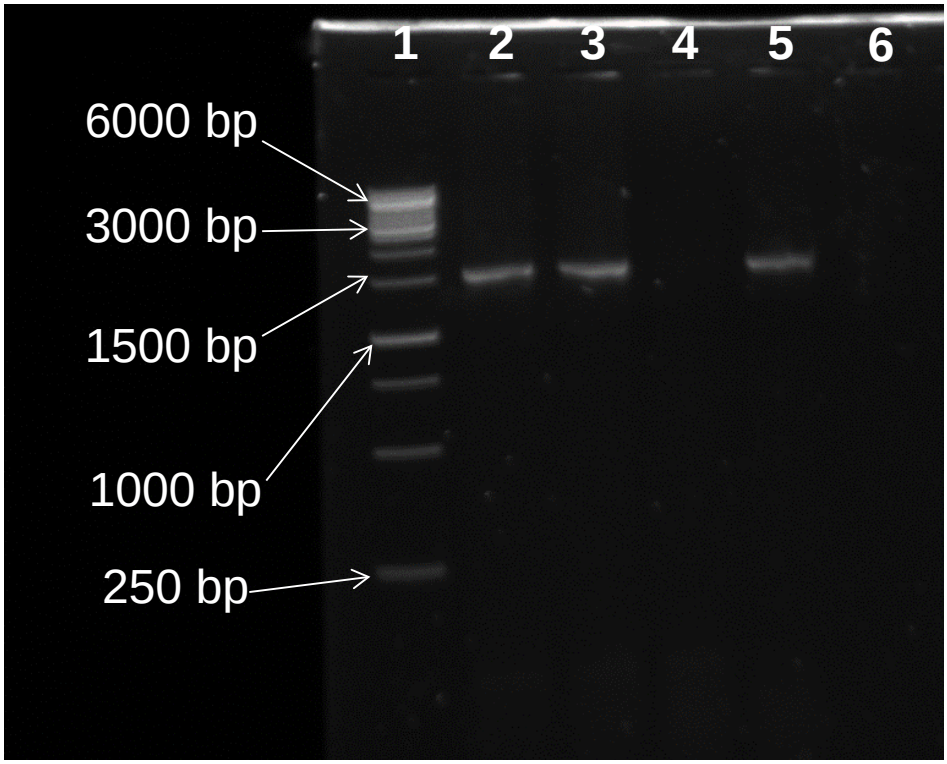
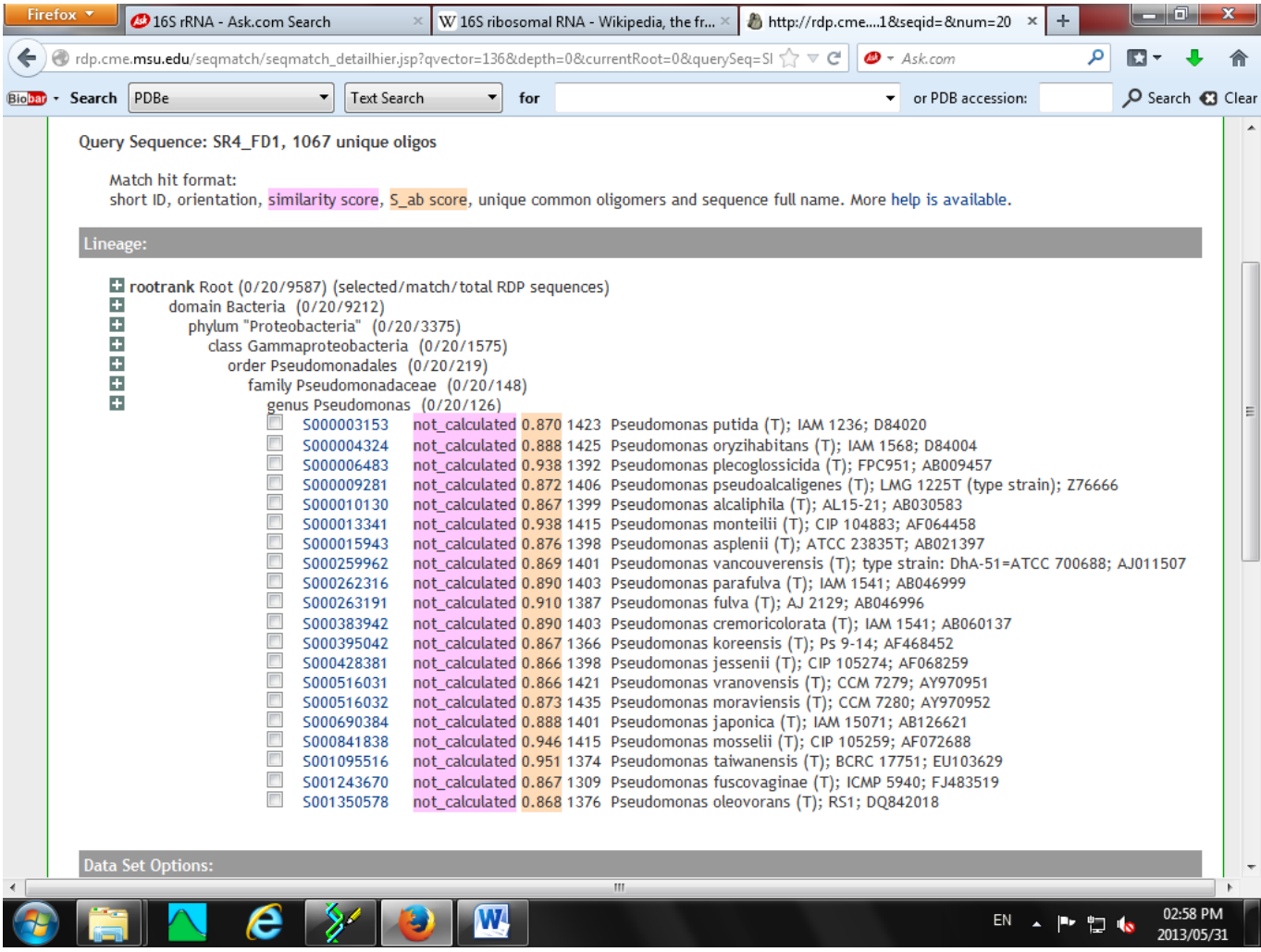


Figure 5.6 1.5% agarose gel of PCR products obtained from soft rot isolates: Lane 1: molecular weight marker; Lanes 2 and 3: samples containing DNA of the White River isolate; Lanes 4 and 5: samples containing DNA of the Howick isolate; Lane 6: negative control.

A single band of the expected size (approximately 1500 bp) was evident for the lanes containing bacterial DNA. Lane 6 (the negative control) did not show any products, as expected. Amplification of the 16S rRNA region of the White River isolate was thus considered successful and PCR product from the reaction in Lane 2 was used for DNA sequencing purposes. Only one of the samples from the Howick isolate indicated a successful amplification (Lane 5). Sequencing results from this PCR product indicated a mixed template trace, likely due to contamination with extraneous bacterial DNA during the preparation process. This sequencing result could thus not be used for further analysis.

Table 5.2 Top 20 type strain bacterial 16S rRNA database BLAST matches of White River isolate



The top 20 RDP BLAST search results for the White River isolate all belong to one genus, *Pseudomonas* Migula. All entries listed in the results above are from type-strain isolates of authentic *Pseudomonas* species.

5.5 DISCUSSION

Bacteria were readily isolated from decaying tissues of *C. miniata* plants. Eleven morphologically distinct isolates were obtained from the Howick seedlings and the White River plant. Two of the eleven bacterial isolates (one from each locality) were found to possess soft rot abilities (Figure 5.2) when evaluated using the method described by Perombelon & Van der Wolf (2002) and also to be pathogenic towards inoculated *C. miniata* plants.

Although Perombelon & Van der Wolf (2002) stated that the ability of a bacterial isolate to induce rot of potato slices is not necessarily indicative of pathogenicity, the implementation of Koch's postulates with the isolates showed that both were pathogens of *C. miniata*. The soft rot observed to develop on the seedlings after inoculation (Figure 5.3) eventually spread into the leaf bases and the seedlings withered and collapsed. Bacteria re-isolated from these plants were consistently identical to the original isolates, indicating that these isolates were responsible for causing the soft rot symptoms observed on the plants in both Howick and White River.

Soft rot *Erwinia* and *Pectobacterium* spp have the characteristic ability to produce cavities on CVP. Since neither isolate in this study was able to form cavities when grown on CVP, they were excluded as belonging to either the *Erwinia* or *Pectobacterium* genera. According to Perombelon & Van der Wolf (2002), the trisodium citrate added to CVP media inhibits the pit forming growth of *Pseudomonas* species on this medium. This is consistent with the lack of pit formation and poor growth of the isolates observed on CVP.

The morphological, biochemical and cultural characteristics observed for both these isolates are consistent with bacteria of the genus *Pseudomonas*, as defined by Holt *et al.* (2000). Both isolates were observed to be gram-negative, catalase- and oxidase-positive, straight rods. They also showed similar morphological characters (Figure 5.4) and almost identical biolog profiles (Figure 5.5). The congruency between both isolates in morphological, biochemical, cultural and epidemiological factors possibly indicates that they belong to the same bacterial genus. Adequate 16S rRNA DNA sequence data from the Howick isolate would allow for comparison between the DNA of both isolates to confirm this hypothesis.

The 16S rRNA sequence data of the White River isolate indicates an affinity with a number of bacterial species, all of the genus *Pseudomonas* (Table 5.2). It is, however, also evident that the 16S rRNA of the isolate does not indicate any further definitive relationships between this taxon and other species within the genus.

As indicated in Table 5.1, three species of bacteria (belonging to three genera) have previously been reported to cause soft rot of *C. miniata.* One of these reports [Laing (2009] was made in South Africa, but the causal organism implicated in the disease observed in that study was not the same as those observed in this study. This is the first report of soft rot of *C. miniata* in South Africa to be caused by a *Pseudomonas* species.

5.6 REFERENCES

Aubrey, A. (2001) *Clivia miniata* [Lindl.] Regel. http://www.plantzafrica.com/plantcd/cliviaminiata.htm. Accessed: 07/06/2013.

Charkowski, A.O. (2009) Decaying signals: will understanding plant-bacterial communications lead to control of soft rot? Current Opinion in Biotechnology 20: 178-184.

Choi, J.E. & Lee, E.J. (2000) New bacterial soft rot of ornamental foliage plants by *Erwinia carotovora* subsp. *carotovora* in Korea. Plant Disease Research 6: 19-22.

Dixon, R. (2011) Relationships in *Clivia*. Clivia 13: 63-81.

Duncan, G.D. (1999) Grow Clivias: A Guide to the Species, Cultivation and Propagation of the Genus Clivia. National Botanical Institute, Kirstenbosch Cape Town, South Africa. pp 4-8.

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

Han, K.S. & Choi, J.E. (1994) Bacterial brown rot of Scarlet Kafir Lily (*Clivia* spp.) caused by *Erwinia cypripedii*. The Plant Pathology Journal 4: 333-335.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (2000) Bergey's Manual of Determinative Bacteriology 9th Edition. Lippincott, Williams & Wilkins, Philadelphia, USA. pp. 93-94.

Khan, I.D. & Rudolph, K. (1997) Pathochemical investigations on a phytotoxin secreted by *Pseudomonas syringae* pv. *syringae* in bush bean leaves (*Phaseolus vulgaris* L.). Developments in Plant Pathology 9: 261-264.

Laing, M. (2009) Soft rot of agapanthus, bulbine, clivia and wild ginger. In: Bacterial Diseases of Plants in South Africa. T.A. Coutinho, T. Goszczynska, C. Lennox and S.N. Venter (eds.). Briza Publications, South Africa. pp. 163-164.

Lunt, T. (2013) Bacterial soft rot. University of Wisconsin-Extension Garden Facts XHT1224.

Ma, B., Hibbing, M.E., Kim, H.-S., Reedy, R.M., Yedidia, I., Breuer, J., Breuer, J., Glasner, J.D., Perna, N.T., Kelman, A. & Charkowski, A.O. (2007) Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. Phytopathology 97: 1150-1163.

Perombelon, M.C.M. & Van Der Wolf, J.M. (2002) Methods for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium carotovorum* subsp. *atrosepticum*)
on potatoes: a laboratory manual. Scottish Crop Research Institute Occasional Publication 10: 22-23, 66-68.

Pooley, E. (2005) A Field Guide to Wildflowers of KwaZulu-Natal and the Eastern Region. The Flora Publications Trust, South Africa. pp. 36-38.

Reinten, E.Y., Coetzee, J.H. & Van Wyk, B.E. (2011) The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77: 934-946.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173: 697-703.

CHAPTER 6

ETIOLOGY OF A LEAF SPOT DISEASE OF *ZANTEDESCHIA AETHIOPICA* (SPRENG.) OBSERVED IN KWAZULU-NATAL, SOUTH AFRICA

6.1 ABSTRACT

Zantedeschia aethiopica is a South African monocotyledonous plant that has been grown in Europe as a pot plant and cut flower since the late 1600s. Phytopathogenic organisms have been widely reported to infect *Z. aethiopica* wherever it is grown. A foliar blight has been observed on *Z. aethiopica* growing in Pietermaritzburg, KwaZulu-Natal, South Africa for a number of years. The etiology and symptoms of the foliar disease observed in Pietermaritzburg are similar to those previously reported to be caused by the bacterium *Xanthomonas campestris* pv. *zantedeschiae* in South Africa and Taiwan. A bacterial strain was consistently isolated from diseased plant material collected in Pietermaritzburg and implementation of Koch's postulates indicated that it was responsible for causing the foliar blight observed. PCR amplification of the 16S rRNA region of this Gram-negative, catalase-positive and oxidase-negative rod-shaped bacterial isolate indicated similarities to various species of *Pseudomonas,* chiefly *P. putida*, but not to any *X. campestris* isolates. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) of the peptidic profile of the isolate corroborated the 16S rRNA based assessment of its identity as a *Pseudomonas* sp but did not provide a strong indication of species identity. This is the first report of a foliar blight of *Z. aethiopica* caused by a *Pseudomonas* sp. in South Africa. A representative culture of the isolate has been deposited at the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) Culture Collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) with the accession number *Pseudomonas* sp. BD 1296.

6.2 INTRODUCTION

Zantedeschia aethiopica (Spreng.) is a rhizomatous monocotyledonous plant, native to South Africa, and is the type species of its genus (Pooley, 2005). It has been known in European horticultural commerce since the late 1600s and today is popular worldwide for its conspicuous white 'flowers', which are either displayed as cut flowers or admired on potted plants (Aubrey, 2001; Pooley, 2005; Reinten *et al.,* 2011). Reinten *et al.* (2011) also noted that, as a genus, *Zantedeschia* is 'currently the subject of international interest among breeders' and that 'there is considerable research interest in selecting new species and breeding new cultivars for the cut flower industry'.

Although commonly grown for its flowers, the quality of the foliage produced by *Z. aethiopica* plants is of importance in determining their overall aesthetic appeal to consumers, diseased foliage making plants undesirable and unmarketable. Diseased foliage also weakens plants and sick plants are likely to produce fewer flowers than healthy plants. The identification of foliar pathogens of *Z. aethiopica* is thus of importance to the industry that has developed around this floricultural crop.

Bacterial, fungal and viral diseases of *Z. aethiopica* have been widely reported in the available literature. Bacteria are the most commonly reported cause of *Zantedeschia* diseases and a unique pathovar of the phytopathogenic bacterium *Xanthomonas campestris* Dowson has been reported from diseased *Z. aethiopica* in South Africa and Taiwan. Reports of bacterial diseases of the genus *Zantedeschia* are summarised in Table 6.1 below.

Table 6.1 Bacterial species reported to cause disease on *Zantedeschia* species and hybrids

Genus	Species	Common name of disease	*Zantedeschia* species	Reference
Chryseobacterium	*indologenes*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Chryseobacterium	sp.		*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Erwinia	*chrysanthemi*	soft rot of tubers	*Zantedeschia* hybrids	Lee & Chen (2002); Mikicinski *et al*. (2010a)
Paenibacillus	*polymyxa*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al.* (2010a)
Pectobacterium	*carotovorum* subsp. *atrosepticum*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Pectobacterium	*carotovorum* subsp. *carotovorum*	soft rot of tubers	*Zantedeschia* hybrids	Wright (1998); Lee & Chen (2002); Snijder & van Tuyl (2002); Snijder *et al*. (2004); Cho *et al*. (2005); Janse (2006); Mikicinski *et al*. (2010a)
Pseudomonas	*marginalis*	soft rot of tubers	*Zantedeschia* hybrids	Krejzar *et al*. (2008); Mikicinski *et al*. (2010a); Mikicinski *et al*. (2010b)
Pseudomonas	*putida*	soft rot of tubers	*Zantedeschia* hybrids	Krejzar *et al*. (2008); Mikicinski *et al*. (2010a)
Pseudomonas	*veronii*	soft rot of tubers	*Zantedeschia* hybrids	Mikicinski *et al*. (2010b)
Xanthomonas	*campestris* pv. *zantedeschiae*	bacterial leaf spot and blight	*Z. aethiopica*	Joubert & Truter (1972); Lee *et al.* (2005); Coutinho & Goszczynska (2009)

The symptoms observed on plants during this study are similar to those reported by Joubert & Truter (1972), Lee *et al.* (2005) and Coutinho & Goszczynska (2009), which were caused by *X. campestris* pv. *zantedeschiae* Dye, namely leaf spotting and blight. Infected plants did not die from infection but were less floriferous than healthy plants and were aesthetically unappealing. The disease symptoms observed in this study were widespread in the Pietermaritzburg, KwaZulu-Natal during periods conducive to disease development.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial isolation, strain purification and storage

Symptomatic leaves of *Z. aethiopica* were collected from a home garden in Pietermaritzburg during a hot (day temperatures > 30°C) and humid period (daily rainfall) of the late summer season. The symptomatic leaves are shown in Figure 6.1 below. The disease symptoms appeared to mostly be confined to the older leaves of infected plants, worsening as the leaves approached senescence.

Half of a symptomatic leaf (Figure 6.1d) was used to isolate suspected pathogens. The leaf sample was aseptically immersed in a 0.35% sodium hypochlorite solution for one minute and then rinsed three times with sterile, distilled water. Ten leaf segments, each approximately 0.5 cm^2, were aseptically excised from the chlorotic regions surrounding the leaf lesion and individually placed on a 90 mm tryptone soy agar (TSA) plate. The plates were incubated at 25 ± 2°C until microbial growth from the segments was evident. The only microbes to grow from the leaf segments were bacteria, all of a single morphotype as shown in Figure 6.2. A loopful of inoculum was obtained from a representative area of bacterial ooze and streaked onto a fresh 90 mm TSA plate and incubated at 25 ± 2°C. A single colony from this plate was then further streaked onto a new plate and incubated at 25 ± 2°C to ensure strain purity. A single colony from this streak was suspended in a sterile 30% glycerol solution and stored at -80°C. A loopful of this stored isolate stock was used to grow up the cultures used for all subsequent analyses (plated on TSA and incubated at 25 ± 2°C for 24 hours). A representative culture of the isolate has been deposited at the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) Culture Collection of the South African Agricultural Research Council Plant Protection Research Institute (ARC-PPRI) with the accession number BD 1296.

6.3.2 Assessment of Koch's postulates and pathogenicity studies

Koch's postulates were assessed by inoculating ten healthy *Z. aethiopica* seedlings with the isolate. These seedlings were obtained from a locality with no disease history and transferred to a polyethylene tunnel two weeks prior to inoculation to ensure that they remained healthy. The tunnel was fitted with an automated misting system that created a warm and highly humid environment during the inoculation trial.

Potential modes of infection were investigated alongside Koch's postulates. Half the seedlings were inoculated by misting with a suspension of the isolate. The suspension was prepared by aseptically suspending five 24 h old colonies in sterile distilled water. The concentration of the suspension was adjusted to approximately 1 x 10^6 cells ml^{-1} and aseptically transferred to a sterile 1.5 L Pressure Sprayer before being applied thoroughly to both surfaces of all their leaves. The seedlings were covered with a polyethylene bag for 24 hours following inoculation.

The other five seedlings were inoculated through the creation of wounds and the introduction of bacterial suspension into these wounds. Small incisions were made with a sterile scalpel on the upper surface of a leaf of each seedling. A volume of 100 µl of pre-prepared

suspension (preparation details as above) was pipetted into the wounds after they were made. These seedlings were not bagged.

Six further seedlings served as the negative controls for each experiment; three were misted with distilled water and bagged, the other three were wounded and inoculated with 100 µl sterile distilled water. These were housed next to the inoculated plants in the tunnel and did not develop symptoms for the duration of the experiment.

6.3.3 Microbiological characterization of the isolate

6.3.3.1 Morphological assessment

The isolate was streaked onto a fresh TSA plate and grown for 48 hr at 25 ± 2°C. A wet mount was prepared and viewed using a Zeiss Axio Scope.A1 light microscope .

6.3.3.2 Biochemical assessments

Single colonies of the isolate were selected after 24 hours of growth on TSA at 25 ± 2°C and aseptically smeared on clean microscope slides for assessment. A 3% KOH solution was used to assess the Gram Stain reaction of the isolate, while catalase and oxidase enzyme activities were tested according to standard methods: catalase by using a pharmacy-bought 3% v/v H_2O_2 solution; oxidase by using self-prepared Kovac's reagent (1% aqueous N,N,N',N'-tetramethyl-p-phenylenediamine). Strains of *Escherichia coli* Castellani and Chalmers and *Staphylococcus aureus* Rosenbach with known biochemical reactions were concurrently cultured and assessed alongside the isolates to serve as controls. These strains gave the expected results for all tests.

BioMérieux API 50 CH strips were used to develop a fermentative metabolic profile of the isolate. The Biolog® analysis was carried out and the results were interpreted as per the manufacturer's instructions, with API 50 CHB/E Medium used to rehydrate the carbohydrate substrates. The isolate was incubated at 30 ± 1°C for the duration of the assessment and carbohydrate substrate usage assessed and recorded at 24 and 48 hrs post inoculation.

6.3.4 DNA extraction, polymerase chain reaction (PCR) of the 16S rRNA gene region and DNA sequencing of the isolate

A method of crude lysis was used to obtain template DNA for PCR analysis from the isolate by suspending a single, 24 h old bacterial colony in 10 µl sterile 1 x TE buffer and then heating the sample to 95°C for 10 min using a G-Storm Goldblock GS1 thermocycler. The sample was then spun down at 12 000 rpm for 2 min using a benchtop centrifuge. After centrifugation, the resulting supernatant was used as a template for PCR amplification.

PCR amplification of the conserved bacterial 16S rRNA gene region of the isolate was carried out using a KAPA 2G Fast HS ReadyMix PCR Kit. Four 20 µl reaction mixes, each consisting of 1 µl freshly prepared template DNA, 10 µl 2X KAPA2G Fast HS ReadyMix, 1.5 µl each of the forward (fD1) and reverse (rP2) primers (Weisburg *et al.,* 1991) and 6 µl sterile nuclease-free water, were prepared for the isolate. A separate sample prepared with 1 µl of sterile nuclease-free water added instead of template DNA was prepared to serve as a negative control and concurrently subjected to the same PCR and electrophoretic processes as the samples from the isolate.

The 16S rRNA PCR was run with the following protocol: an initial denaturation step of 95°C for 2 min, followed by 35 cycles of 95°C for 20 s, 42°C for 35s and 72°C for 45s and a final elongation step of 72°C for 5 min. The PCR protocol used was based on that outlined by Weisburg *et al.* (1991) but optimized for the conditions of this study.

All PCR reactions were run using a G-Storm Goldblock GS1 thermocycler. Products from all PCR reactions were analysed on a 1.5 % agarose gel, with 1 µl SYBR® Safe DNA gel stain added per 10 ml of gel. GeneRuler 1kb DNA Ladder was used as a molecular weight marker to estimate the size of any PCR products obtained. The gel was run at 100 V for 50 min and PCR products were viewed using a Syngene G:Box and images captured using GeneSnap image acquisition software.

A Zymo DNA Clean & Concentrator - 500 Kit was used to remove excess reagents from a successful PCR reaction showing a single 1500 bp band. The clean PCR product was sent to the Central Analytical Facility (CAF) of the University of Stellenbosch (http://www.sun.ac.za/saf) for DNA sequencing, using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems).

The DNA sequence files supplied by the CAF were opened and edited with BioEdit Sequence Alignment Editor V7.0.9.0 (Hall, 1999). Areas of the trace file with poor resolution outside of the region bound by the forward and reverse primers were removed before the sequence data was compared (using a nucleotide BLAST search algorithm) to that of other bacterial 16S rRNA homologues housed in the public-access DNA sequence database of Ribosomal Database Project (RDP), maintained by the Michigan State University (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). Only the 16s rRNA data from bacterial type strains was selected for comparison to that of this isolate.

6.3.5 Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) of the isolate

Samples of a number of bacterial isolates were prepared and simultaneously analysed using a Bruker Microflex MALDI-TOF MS bench top Biotyper system. The isolate (ZA1 in the MALDI-TOF MS output shown in Figure 6.7) was cultured for 24 hours on TSA at 25 ± 2°C. A single colony was picked off using a sterile toothpick and added to a 1.5ml Eppendorf tube containing 300 µl of sterile distilled water. An ethanol/formic acid extraction procedure was performed on the sample for profiling, as per the methods laid out in the supplementary material provided by Bruker Daltonics. Of the resulting supernatant, 1 µl was pipetted onto a MSP 96 target spot polished steel plate and left to air dry, then 1 µl of $HCCA_{portioned}$ matrix solution was overlaid onto each sample and also left to air dry before analysis. Once dry, the steel target plates were loaded into a MALDI Biotyper and the analysis run.

6.4 RESULTS

The foliar symptoms observed on infected *Z. aethiopica* plants began as small, chlorotic circles of diseased tissue.

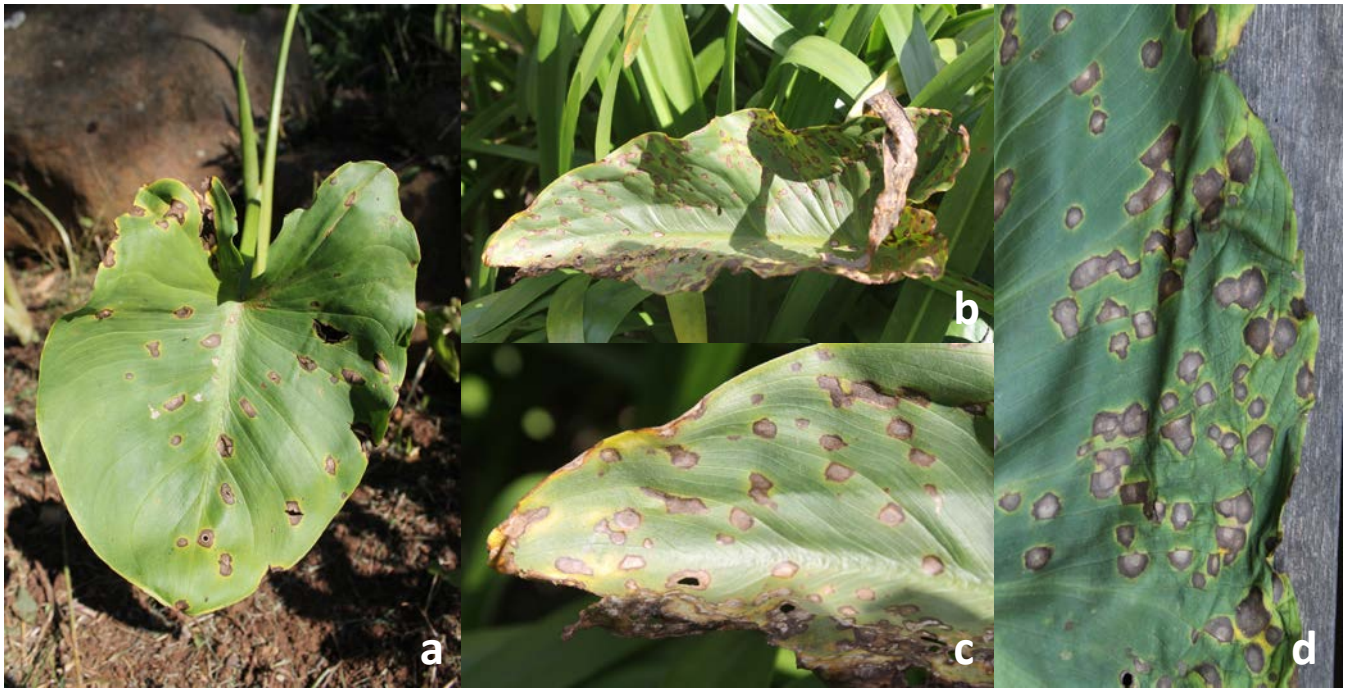


Figure 6.1 Foliar disease symptoms observed on *Zantedeschia aethiopica:* (a) a moderately symptomatic leaf soon after symptom appearance; (b) a severely symptomatic leaf showing widespread spotting and necrosis; (c) a close-up of the infected leaf tip ; (d) leaf lesions from which the suspected pathogen was isolated.

If the hot and humid conditions that trigger symptom appearance persist, the chlorotic lesions rapidly enlarge and become necrotic. When the environmental conditions become less humid, the necrotic areas dry out and often out of the centre of the lesions. This causes the leaves to develop a shot-hole appearance and drastically reduces their aesthetic appeal.

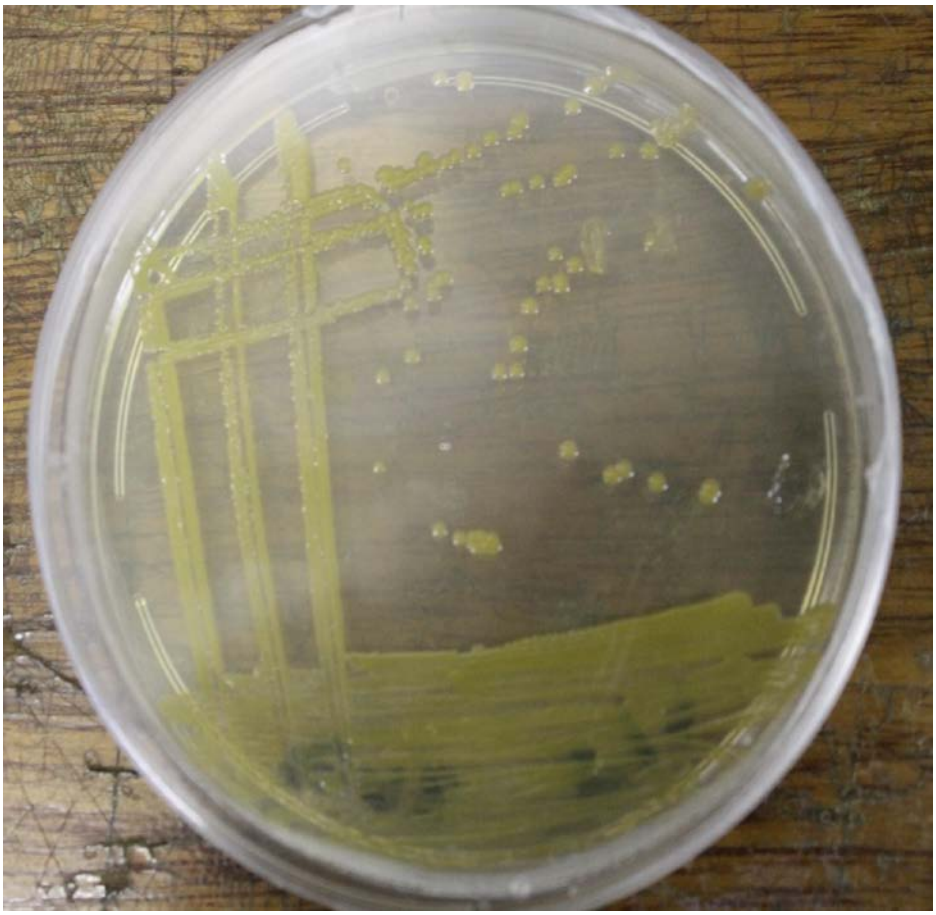


Figure 6.2 A 48 h old colony of the bacterial isolate obtained from the diseased leaf tissue examined in this study, incubated on TSA at 25 ± 2°C.

Colonies of this isolate were a bright, clear yellow. They were circular, raised and convex in cross section, with entire margins. No inclusion bodies were observed.

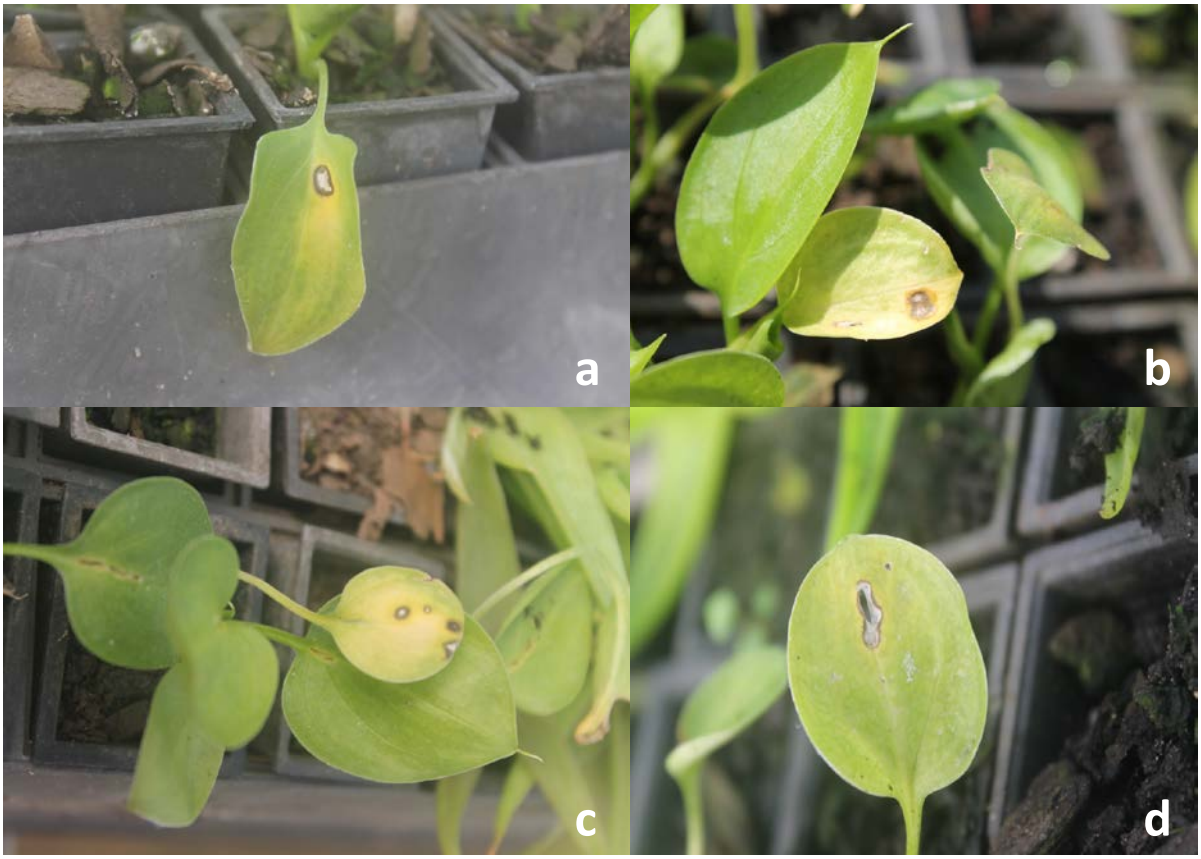


Figure 6.3 (a) and (b) Disease symptoms observed to develop on *Z. aethiopica* seedlings after misting with the bacterial isolate; (c) and (d) symptoms that developed on seedlings after the introduction of a suspension of the isolate into wounds on their leaves.

The leaf lesions observed to develop on all inoculated seedlings were very similar to those initially observed in this study, albeit smaller on the seedlings than on the adult plants. It is apparent that the bacterial isolate was able to induce disease development via at least two entry mechanisms (natural openings and wounds). These are consistent with documented methods by which phytopathogenic bacteria invade plant tissues.

Figure 6.4 API 50 CH fermentative metabolic profile of the isolate after 48 h incubation at 30 ± 1°C.

The Biolog® profile generated for the isolate indicated that it was able to produce acid, but not gas, from glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-sorbitol, D-melibiose, gentiobiose, D-fucose and potassium 5-ketogluconate.

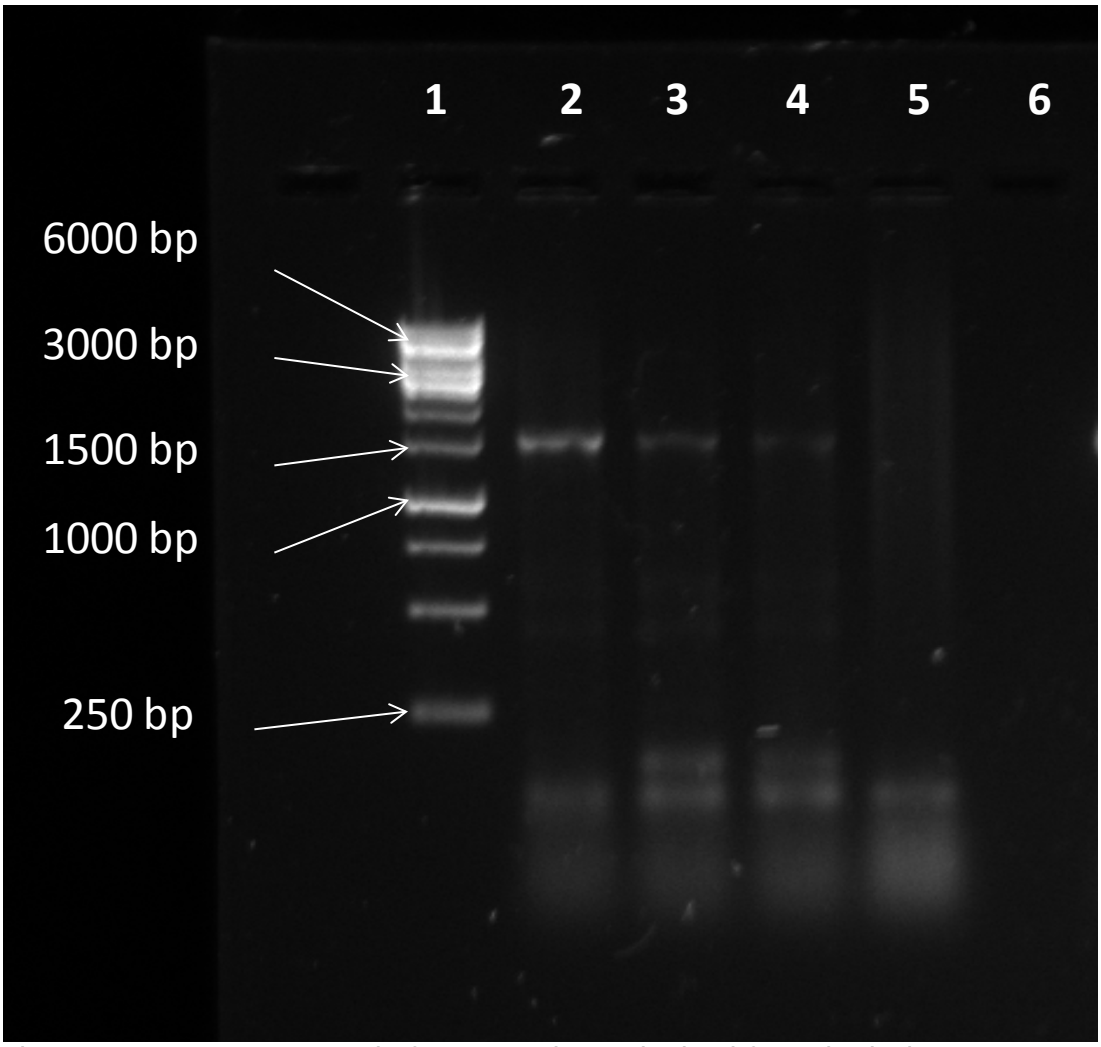


Figure 6.5 1.5% agarose gel of PCR products obtained from the isolate: Lane 1: molecular weight marker, Lanes 2-5: samples containing DNA of the isolate, Lane 6: negative control.

A single band of the expected size (1500 bp) was evident for three of the reactions containing the isolate DNA. No product was evident for the negative control and amplification of the 16S rRNA region of the isolate was considered a success. PCR product from Lane 2 was used for DNA sequencing purposes.

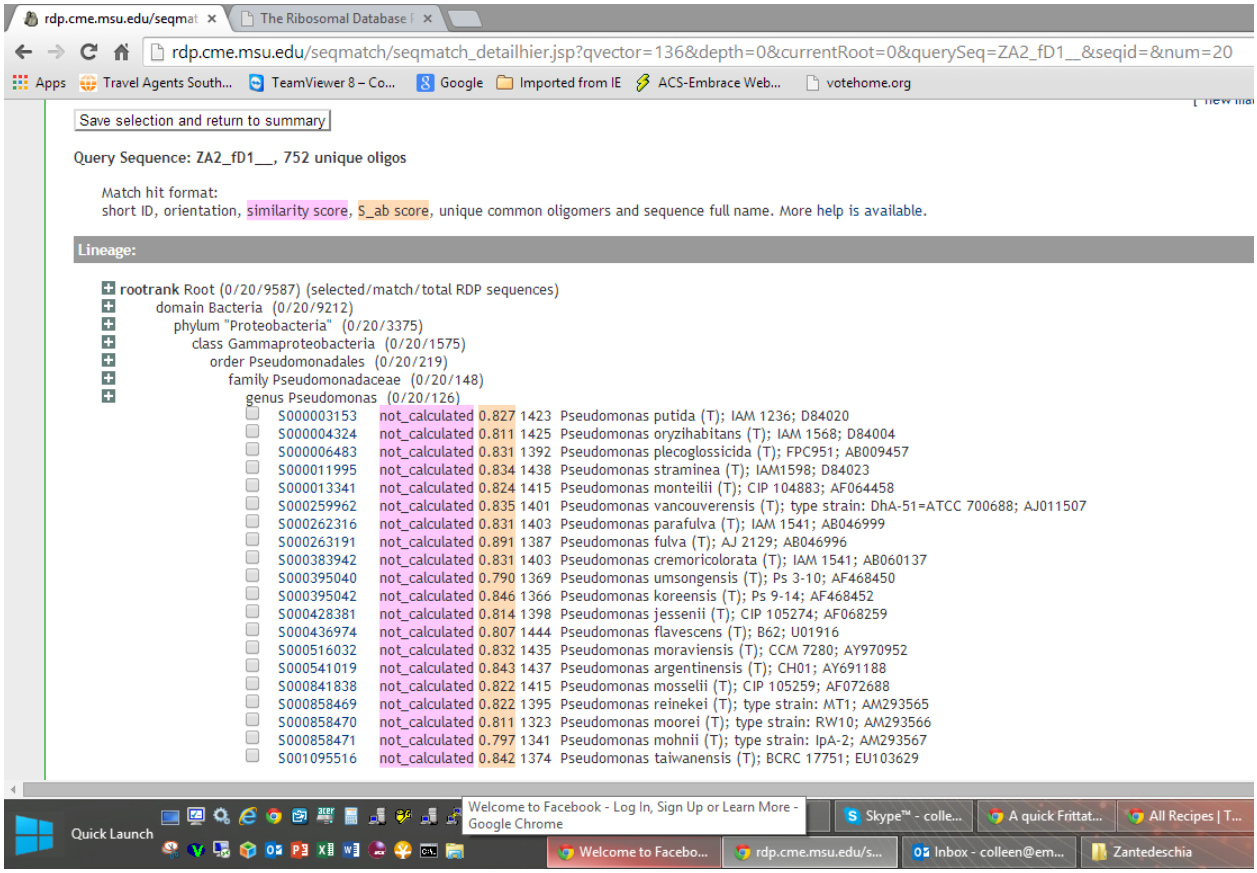
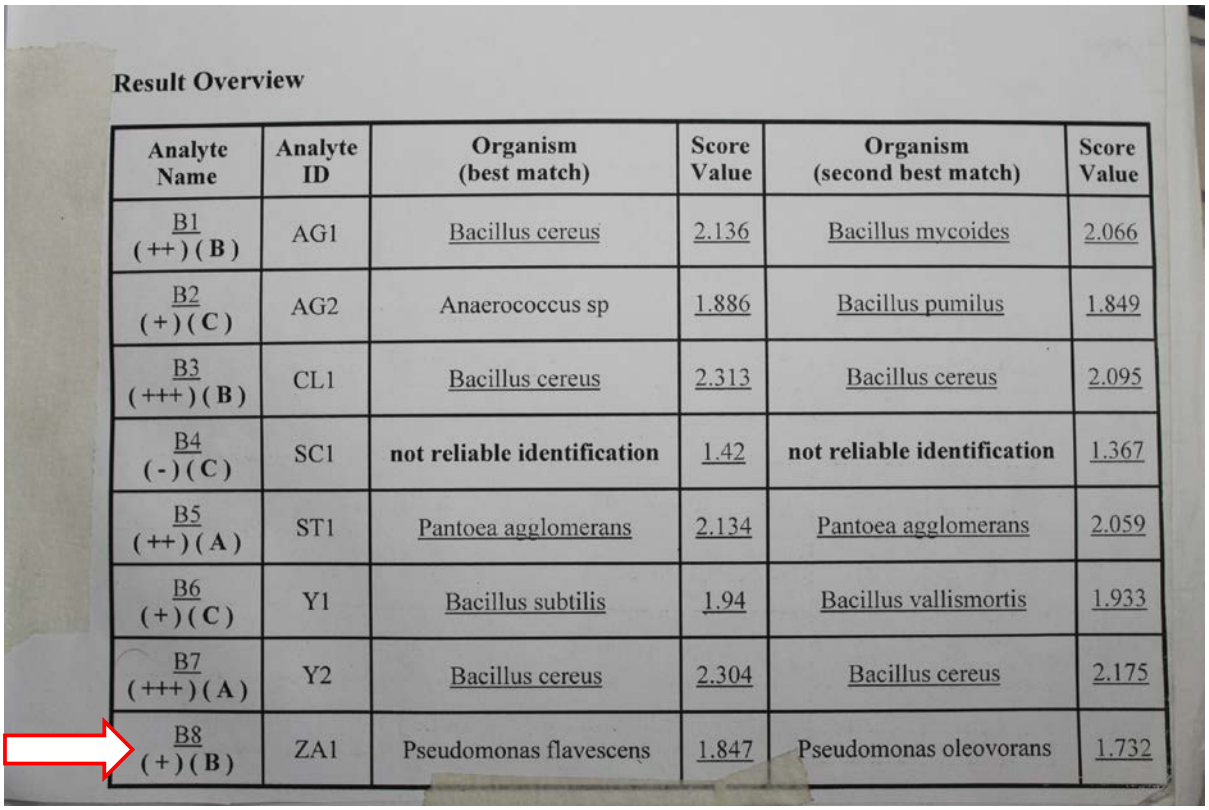


Figure 6.6 The top 20 16S rRNA bacterial matches generated for the isolate obtained in this study.

All the bacterial species identified by the BLAST search as being similar in their 16S rRNA regions to this isolate belonged to the genus *Pseudomonas* Migula. It was apparent that the 16S rRNA gene sequence data is not sufficient to resolve a clear species identity when compared to the *Pseudomonas* isolates.



Result Overview

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
B1 (++) (B)	AG1	Bacillus cereus	2.136	Bacillus mycoides	2.066
B2 (+) (C)	AG2	Anaerococcus sp	1.886	Bacillus pumilus	1.849
B3 (+++) (B)	CL1	Bacillus cereus	2.313	Bacillus cereus	2.095
B4 (-) (C)	SC1	**not reliable identification**	1.42	**not reliable identification**	1.367
B5 (++) (A)	ST1	Pantoea agglomerans	2.134	Pantoea agglomerans	2.059
B6 (+) (C)	Y1	Bacillus subtilis	1.94	Bacillus vallismortis	1.933
B7 (+++) (A)	Y2	Bacillus cereus	2.304	Bacillus cereus	2.175
B8 (+) (B)	ZA1	Pseudomonas flavescens	1.847	Pseudomonas oleovorans	1.732

Figure 6.7 Bruker MALDI Biotyper output from the analysis of a number of bacterial isolates. The isolate pertinent to this study (ZA1) is indicated with the arrow.

The MALDI-TOF MS analysis of this isolate indicated that both the first and second best matching organisms for this isolate are *Pseudomonas* species (*P. flavescens* and *P. oleovorans* respectively). The support score values (1.847 and 1.732 respectively) assigned to the isolate fell within the range of 'probable genus identification', as outlined by the analysis report but, like the 16S rRNA data of the isolate, this analysis did not provide a clear indication of the *Pseudomonas* species this isolate was.

6.5 DISCUSSION

The reports cited in Table 6.1 described similar symptoms to those observed in this study on *Z. aethiopica* and indicated that the bacterial pathogen responsible for these symptoms can be *X. campestris* pv. *zantedeschiae*. Koch's postulates were successfully demonstrated with the yellow-pigmented bacterial isolate obtained from the diseased *Z. aethiopica* leaf tissues observed in this study. Its ability to induce disease on inoculated plants demonstrated that its role as the pathogen was confirmed. The morphological and biochemical characteristics were investigated for this isolate. The bacterial cells were observed to be straight rods that were Gram- and oxidase-negative and catalase positive. These match those reported for the genus *Xanthomonas* by Holt *et al.* (2000) and for the above *X. campestris* pathovar by Coutinho & Goszczynska (2009).

It is likely that the strain isolated in this study would be classified as another isolate of *X. campestris* pv. *zantedeschiae* if the two more modern bacterial techniques (16S rRNA and MALDITOF-MS) used in this study were not also implemented. The results obtained from both these studies are concordant in that they disagree with its placement within *X. campestris* pv. *zantedeschiae* and rather assign this isolate to the genus *Pseudomonas.* Since all the morphological and biochemical characters investigated and reported above have also been reported for the genus *Pseudomonas* by Holt *et al.* (2000), their use in discriminating between the two bacterial genera is limited. No DNA sequence data has been made available for the *X. campestris* pv. *zantedeschiae* strains reported by Lee *et al.* (2005) and Coutinho & Goszczynska (2009) and it would be pertinent to investigate whether DNA sequence data from these isolates would also assign them to the genus *Pseudomonas* and also how closely related all these isolates are.

Multigene phylogenetic analyses have been recommended for accurate *Pseudomonas* species identification, using the sequences of core 'housekeeping' genes, such as the 16S rRNA, *gyrB, rpoB and rpoD* genes, for isolate identification (Yamamoto *et al.,* 2000; Mulet *et al.,* 2010; Ghyselinck *at al.,* 2013). Amplification, sequencing and concatenated phylogenetic analysis of the sequence data of the above genes for the isolate obtained in this study will allow better taxonomic placement of the isolate and indicate whether it should be recognised as a new *Pseudomonas* species or pathovar.

Pseudomonas species have been reported to infect *Zantedeschia* before, but only to cause soft rots of the tubers of hybrid cultivars (Krezjar *et al.,* 2008; Mikicinski *et al.,* 2010a; Mikicinski *et al.,* 2010b). This is thus the first report of a *Pseudomonas* species causing a leaf spot and blight of *Z. aethiopica*, pending further investigation of the bacterial isolates

assigned to the species epithet *X. campestris* pv. *zantedeschiae*. Coutinho & Goszczynska (2009) stated that the epidemiology of this bacterial leaf spot is not well known and that the bacterium 'can probably survive in infected plant parts and spread in propagation material'.

The development of a robust isolation and identification protocol for this pathogen would allow growers whose plants are affected by the disease to pre-emptively screen for the pathogen and also to screen uninfected plants for use as propagative material. It is likely that good cultural management practices such as roguing of infected plants, removal of infected debris and maintenance of good plant health would also help reduce the severity of this disease. Plants fertilised with calcium and silicon-containing fertilisers or those treated with resistance inducers, such as Bion® may offer further methods that *Zantedeschia* growers can utilise to control the disease.

6.6 REFERENCES

Aubrey, A. (2001) *Zantedeschia aethiopica* (L.) Spreng. http://www.plantzafrica.com/plantwxyz/zantedeschaeth.htm. Accessed: 10/08/2014.

Cho, H.R., Lim, J.H., Yun, K.J., Snijder, R.C., Goo, D.H., Rhee, H.K. & Kim, K.S. (2005) Virulence variation in 20 isolates of *Erwinia carotovora* subsp. *carotovora* on *Zantedeschia* cultivars in Korea. Acta Horticulturae 673: 653-659.

Coutinho, T.A. & Goszczynska, T. (2009) Leaf spot of arum lilies. In: Bacterial Diseases of Plants in South Africa. T.A. Coutinho, T. Goszczynska, C. Lennox & S.N. Venter (eds.). Briza Publications, South Africa. pp. 85-86.

Ghyselinck, J., Coorevits, A., Van Landschoot, A., Samyn, E., Heylen, K. & De Vos, P. (2013) An *rpoD* gene sequence based evaluation of cultured *Pseudomonas* diversity on different growth media. Microbiology 159: 2097-2108.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (2000) Bergey's Manual of Determinative Bacteriology 9th Edition. Lippincott, Williams & Wilkins, Philadelphia, USA. pp. 100.

Janse, J.D. (2006) Phytobacteriology: Principles and Practice. CAB International, UK. pp. 105-107.

Joubert, J.J. & Truter, S.J. (1972) A variety of *Xanthomonas campestris* pathogenic to *Zantedeschia aethiopica*. New Zealand Journal of Crop and Horticultural Science 78: 212-217.

Krezjar, V., Mertelik, J., Pankova, I., Kloudova, K. & Kudela, V. (2008) *Pseudomonas marginalis* associated with soft rot of *Zantedeschia* spp. Plant Protection Science 44: 85-90.

Lee Y.A. & Chen K.P. (2002) First report of bacterial soft rot of white flowered calla lily caused by *Erwinia chrysanthemi* in Taiwan. Plant Disease 86: 1273.

Lee, Y.A., Chen, K.P & Chang, Y.C. (2005) First report of bacterial leaf blight of white-flowered calla lily caused by *Xanthomonas campestris* pv. *zantedeschiae* in Taiwan. Plant Pathology 54: 239.

Mikicinski, A., Sobiczewski, P., Pulawska, J. & Treder, J. (2010a) Involvement of *Paenibacillus polymyxa* in the etiology of bacterial soft rot of calla lily. Journal of Plant Pathology 92: 375-380.

Mikicinski, A., Sobiczewski, P., Sulikowska, M., Pulawska, J. & Treder, J. (2010b) Pectolytic bacteria associated with soft rot of calla lily (*Zantedeschia* spp.) tubers. Journal of Phytopathology 158: 201-209.

Mulet, M., Lalucat, J. & Garcia-Valdes, E. (2010) DNA sequence based analysis of the *Pseudomonas* species. Environmental Microbiology 12: 1513–1530.

Pooley, E. (2005) A Field Guide to Wildflowers of KwaZulu-Natal and the Eastern Region. The Flora Publications Trust, South Africa. pp. 84-86.

Reinten, E.Y., Coetzee, J.H. & Van Wyk, B.E. (2011) The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77: 934-946.

Snijder, R.C., Cho, H., Hendriks, M.M., Lindhout, P. & Van Tuyl, J.M. (2004) Genetic variation in *Zantedeschia* spp. (Araceae) for resistance to soft rot caused by *Erwinia carotovora* subsp. *carotovora*. Euphytica 135: 119-128.

Snijder, R.C. & Van Tuyl, J.M. (2002) Evaluation of tests to determine resistance of *Zantedeschia* spp. (Araceae) to soft rot caused by *Erwinia carotovora* subsp. *carotovora.* European Journal of Plant Pathology 108: 565-571.

Wright, P.J. (1998) A soft rot of calla (*Zantedeschia* spp.) caused by *Erwinia carotovora* subspecies *carotovora*. New Zealand Journal of Crop and Horticultural Science 24: 331-334.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A. & Harayama, S. (2000) Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of gyrB and rpoD genes. Microbiology 146: 2385–2394.

CHAPTER 7

INVESTIGATIONS INTO VIRUS-LIKE DISEASES OF THE GENUS *CLIVIA* LINDL.

7.1 ABSTRACT

The South African plant genus *Clivia* is a widely cultivated floricultural crop. *Clivia* plants in nurseries and private collections often exhibit foliar symptoms of streaking and mottling that have been speculated to be caused by viral infection. Transmission electron microscopy (TEM) of sap samples obtained from symptomatic *C. caulescens, C. miniata* and numerous interspecific hybrid plants showed the presence of filamentous viral particles in the leaf sap preparations. A successful purification protocol was devised to isolate viral particles from *Clivia* leaf material. Attempts were made to amplify viral nucleic acids from infected leaves and purified virus samples using reverse transcription polymerase chain reaction (RT-PCR). PCR primers reported to amplify nucleic acids from closteroviruses, potexviruses and potyviruses were tested against viral samples but did not result in any successful amplifications of viral nucleic acids. Viral transmissibility was assessed through mechanical inoculation of *Nicotiana tabacum* cv. Xanthi with leaf material of a symptomatic interspecific *Clivia* hybrid and also through mechanical inoculation of healthy plants of *C. caulescens* with sap from symptomatic plants of *C. caulescens*. The results of these tests were inconclusive as neither transmission method induced symptoms in the inoculated plants. The investigations outlined in this study highlight the presence of virus-like particles in association with virus-like disease symptoms on *Clivia* plants but were not able to provide an elucidation of the identity of the viruses observed. This raises the possibility that these viruses are novel entities and highlights the lack of detailed study on the subject of viral disease of *Clivia*. This study is the first to report viral infection of *Clivia* in South Africa.

7.2 INTRODUCTION

Clivia is a genus of monocotyledonous plants that belong to the family Amaryllidaceae J.St.-Hil. (Aubrey, 2001). The six species, numerous cultivars thereof and many interspecific hybrids are widely cultivated (Smith, 2011). Common concerns expressed by those with an interest in growing the plants, commercially or in private collections, is whether the plants can become infected with viruses, what effects the presence of viral infection can have on plants and how any viral pathogens may be spread. Research in these areas is scant and reports of viral infection of *Clivia* available in the literature are outlined below.

Table 7.1 Reports of viral infection of *Clivia*

Genus	Species	Symptoms of infected plants	*Clivia* species	Country reported	Reference
Potexvirus	Undetermined		*Clivia* hybrids	New Zealand	Hammet (2004)
Potyvirus	Narcissus late season yellows virus (NLSYV)	Streaking and mottling of leaves	*C. gardenii, C. miniata* 'Belgium hybrid', *C. miniata* var. *citrina*	Australia	Adcock (2007)
Tospovirus	Iris yellow spot virus (IYSV)		*Clivia* spp.	Japan	Jones (2005); Gent *et al.* (2006)
	Tomato spotted wilt virus (TSWV)		*Clivia* spp.	Holland, USA	McDonough *et al.* (1999)

Clivia plants assessed in this study for viral infection displayed foliar symptoms of streaking and mottling that raised suspicions about their health. The symptomatic leaves of each plant studied are shown in Figures 7.1 and 7.2 below. The plants were obtained from various locations, including gardens, private collections or in habitat. The details of the plants examined are summarised in the table below.

Table 7.2 Details of the Clivia plants that were examined in this study

Species/Cultivar	Identifier	Locality	Habitat
C. miniata	CMB	Benoni, Gauteng	Home garden
C. 'Karkloof Yellow'	CIH	Howick, KwaZulu-Natal	Clivia nursery
C. caulescens	CCM	Mariepskop, Mpumalanga	Montane forest
C. miniata 'Ella van Zijl'	CEVZ	Nelspruit, Mpumalanga	Private collection
C. 'USA Yellow'	CUY	Nelspruit, Mpumalanga	Private collection
Interspecific hybrid	CIN	Nelspruit, Mpumalanga	Private collection

Many Clivia cultivars and interspecific hybrids do not produce uniform progeny when grown from seed; division of clumps is thus the most common method employed by Clivia growers to propagate selected cultivars and clones (Duncan, 2008; Wang *et al.,* 2012). This method is advantageous in that it allows for true-to-type propagation of desired cultivars but disadvantageous in that it allows for the transmission of diseases to the propagules if the mother plant is not healthy. This study was carried out to address the concerns cited above and aimed to provide evidence of viral infection in Clivia plants and then to attempt to identify any viruses observed in association with symptomatic plants.

7.3 MATERIALS AND METHODS

7.3.1 Use of transmission electron microscopy (TEM) to visualise viral particles

Transmission electron microscopy was used to look for the presence of viral particles in preparations of leaf sap obtained from plants showing virus-like symptoms. A formvar-coated copper grid was inverted onto a droplet of leaf sap (obtained from a freshly cut leaf) for one

minute to allow for adhesion of viral particles. The grid was then placed on a droplet of 2% uranyl acetate for 30 s to allow for negative staining of any adherent particles. The grid was then left to dry fully before being loaded onto the stage of a JEOL 1400 transmission electron microscope for analysis.

7.3.2 Virus purification from symptomatic plant material

A search of the available literature did not indicate any protocols used by prior researchers of viral Clivia diseases for purification of viral particles from leaf material. Viral particles were thus purified from Clivia leaf samples using a protocol based on that utilised by Crescenzi *et al.* (1997), but amended to suit the laboratory equipment and conditions that were available for use in this study. 100 g of symptomatic leaf tissue was ground into a fine powder using liquid nitrogen and a sterile mortar and pestle. Care had to be taken to thoroughly grind the leaf material as finely as possible because it had a tendency to shatter into small blocks instead of pulverising easily into a fine powder.

The ground leaf material was homogenised in 0.1M sodium citrate extraction buffer pH 8.3, that also contained 0.02 sodium sulphite and 0.002M EDTA. The homogenate was filtered through cheesecloth and then centrifuged in an Avanti® J-26 XPI centrifuge at 10 000 rpm for 10 minutes. The resulting supernatant was then mixed on ice with 3% Triton X-100 for one hour. Thereafter, the solution was centrifuged at 75 600 x *g* for 120 min. The resulting pellet was resuspended overnight in 0.01M sodium citrate buffer pH 8.3 containing 0.5M urea and 0.1% 2-mercaptoethanol.

The suspension was then layered onto a 30% sucrose cushion in 0.01M sodium citrate buffer pH 8.3 and centrifuged at 50 000 x *g* for 120 min. The resulting pellet was resuspended in 0.01M sodium citrate buffer pH 8.3 overnight and then spun a final time at 14 800 rpm for five minutes to precipitate any remaining debris. The supernatant suspected to contain purified viral particles was transferred to a clean Eppendorf and stored at 4°C. The presence of viral particles in the purified samples was evaluated using the same TEM protocol as described above.

7.3.3 Inoculation of tobacco (*Nicotiana tabacum* L.) and *Clivia caulescens* Dyer with leaf material from symptomatic *Clivia* plants

Three seed grown *N. tabacum* cv. Xanthi plants were inoculated with a suspension of powdered leaf material obtained from a symptomatic interspecific Clivia hybrid (CIH). The leaf material was ground up with liquid nitrogen in a sterile mortar and pestle and suspended in sterile distilled water before being rubbed into silicon carbide induced abrasions on the

leaves of the tobacco plants. A fourth tobacco plant was similarly abraded and inoculated with distilled water instead of leaf suspension and a fifth tobacco plant was not inoculated at all. These plants were kept in a glasshouse and monitored for four weeks post inoculation. TEM examination of leaf sap from these plants was carried out according to the protocol described above.

Five healthy, non-symptomatic *C. caulescens* seedlings were inoculated with sap obtained from the leaf of a symptomatic adult plant of *C. caulescens* (CCM). A sterile scalpel was used to make three parallel incisions on a young leaf of each plant. These incisions were each approximately 10 mm long and did not extend all the way through the leaf blade. Sap was obtained from the symptomatic plant by cutting off a leaf tip and allowing the sap to bead on the cut surface. The sap was then transferred into the wounds on each seedling with a sterile scalpel. Three other seedlings were wounded in the same manner but inoculated with sterile distilled water instead of leaf sap. All plants were kept in a greenhouse and monitored for four weeks post inoculation.

7.3.4 Attempts to use reverse transcription polymerase chain reaction (RT-PCR) to amplify viral nucleic acids

Attempts were made to amplify viral nucleic acids from symptomatic leaf samples and purified virus samples. The morphology of the viral particles observed in this study indicated that the viral nucleic acid type was most likely RNA and would thus require reverse transcription prior to PCR amplification. An SV Total RNA Isolation Kit was used to isolate RNA from symptomatic leaf samples, as per the manufacturer's instructions. RNA was released from virions present in the purified virus samples by heating them to 65°C for 5 min prior to RT. A RevertAid First Strand cDNA Synthesis Kit was used to transcribe any RNA templates present in the samples into their cDNA analogues before PCR amplifications were attempted. This procedure was carried out as per the manufacturer's instructions.

Three viral genera were chosen for investigation with RT-PCR, based on the morphology of the virions observed using TEM, the reports of viral infection of the genus *Clivia* summarised in Table 7.3 and the availability of PCR primers in the research environment in which the study was conducted. These genera were *Closterovirus* (family Closteroviridae), *Potexvirus* (family Alphaflexiviridae) and *Potyvirus* (family Potyviridae). The specific viral gene targeted for amplification, the forward/reverse primer pair used for amplification and reference for the PCR protocols are as follows:

Table 7.3 Viral genera selected for RT-PCR testing

Viral genus	Gene target	Primer pair	Reference
Closterovirus	Heat shock protein (Hsp70)	CL43U-F	
		CL43L-R	Winter *et al.* (1997)
Potexvirus	Viral replicase	Potex 4	
		Potex 5	Miglino *et al.* (2004)
Potyvirus	Nuclear inclusion protein b (Nlb)	Nlb2F	
		Nlb3R	Zheng *et al.* (2010)

PCR amplifications were carried out using a KAPA 2G Fast HS ReadyMix PCR Kit and on a G-Storm Goldblock GS1 thermocycler. Each reaction mix consisted of 10 µl 2X KAPA2G Fast HS ReadyMix, 1.5 µl of each forward and reverse primer, 3 µl cDNA template and 4 µl sterile nuclease-free water. A negative control was prepared for each PCR run, with sterile nuclease-free water instead of cDNA, and concurrently run with each PCR reaction.

Products from all PCR reactions were analysed on 1.5 % agarose gels, with 1 µl SYBR® Safe DNA gel stain added per 10 ml of gel. The gels were run at 100 V for 50 min and PCR products were viewed using a Syngene G:Box and GeneSnap image acquisition software.

7.4 RESULTS

It was evident that virus-like particles were present in association with the symptomatic Clivia plants shown below. These particles were all similar in morphology (flexuous rods) but varied widely in length.

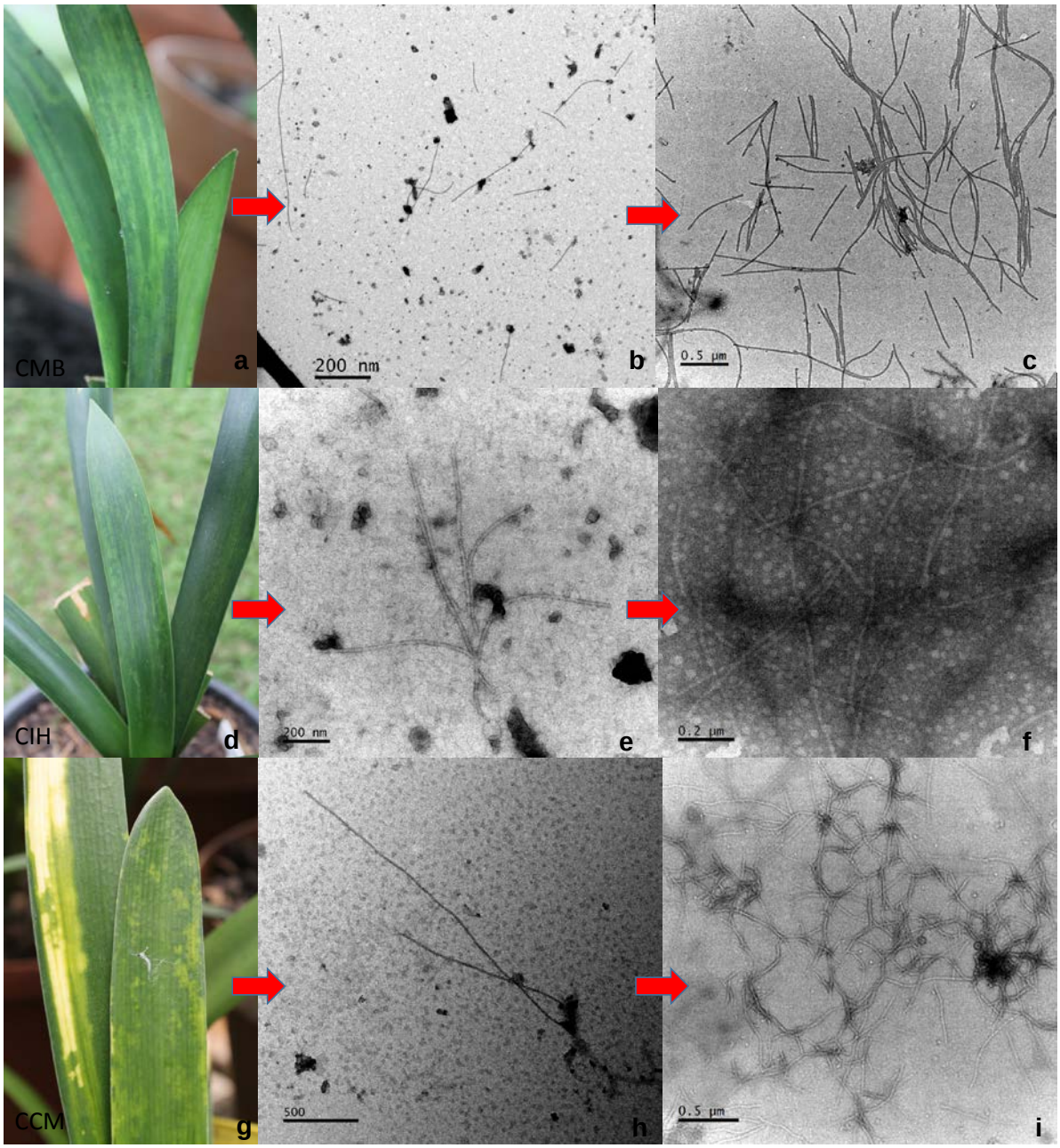


Figure 7.1 (a), (d) and (g): Virus-like symptoms observed on Clivia plants; (b), (e) and (h): viral particles observed in TEM preparations of leaf sap from symptomatic leaves; (c), (f) and (i): concentrated viral particles observed in TEM preparations of purified virus samples obtained from symptomatic plants.

The highly concentrated aggregations of viral particles observed in the purified samples indicated that the purification protocol implemented in this study was successful at isolating viral particles from Clivia leaf material.

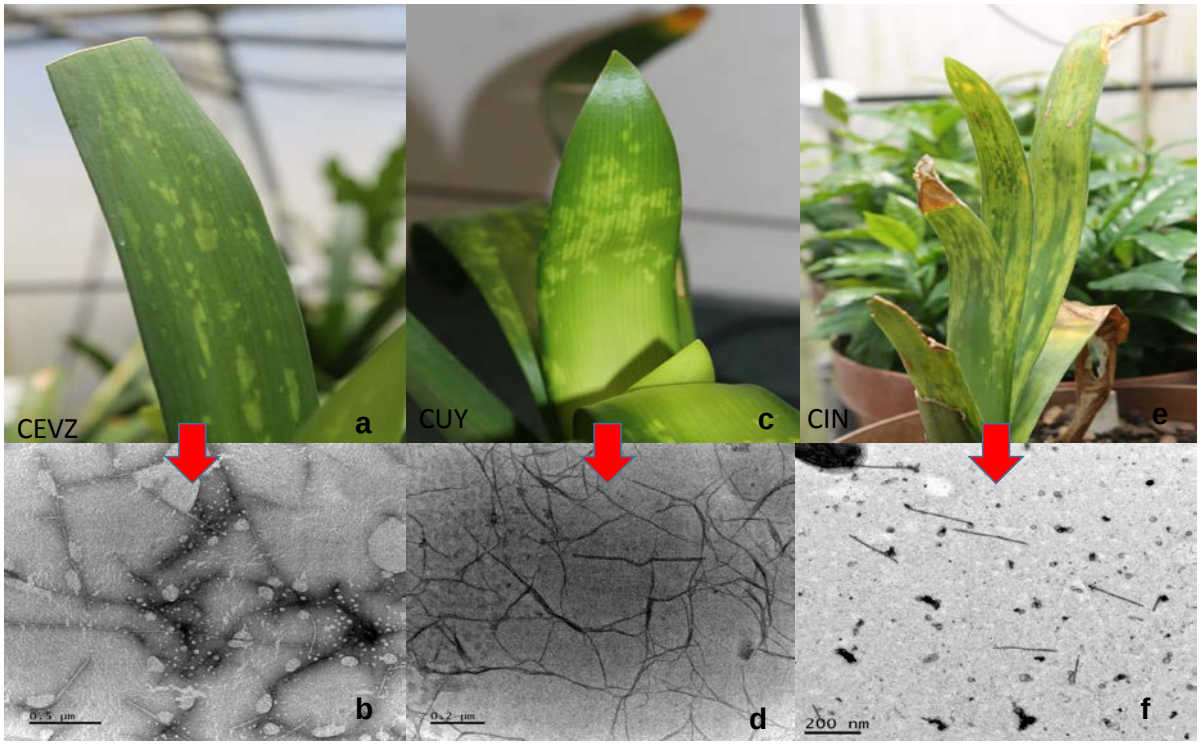


Figure 7.2 Further examples of symptomatic Clivia plants and virus-like particles observed in preparations of their leaf sap.

Virus-like particles displaying a filamentous morphology were evident in the crude leaf sap preparations of the plants shown above. Attempts to purify viral particles from these plants were not made due to the lack of sufficient leaf material during the study period.

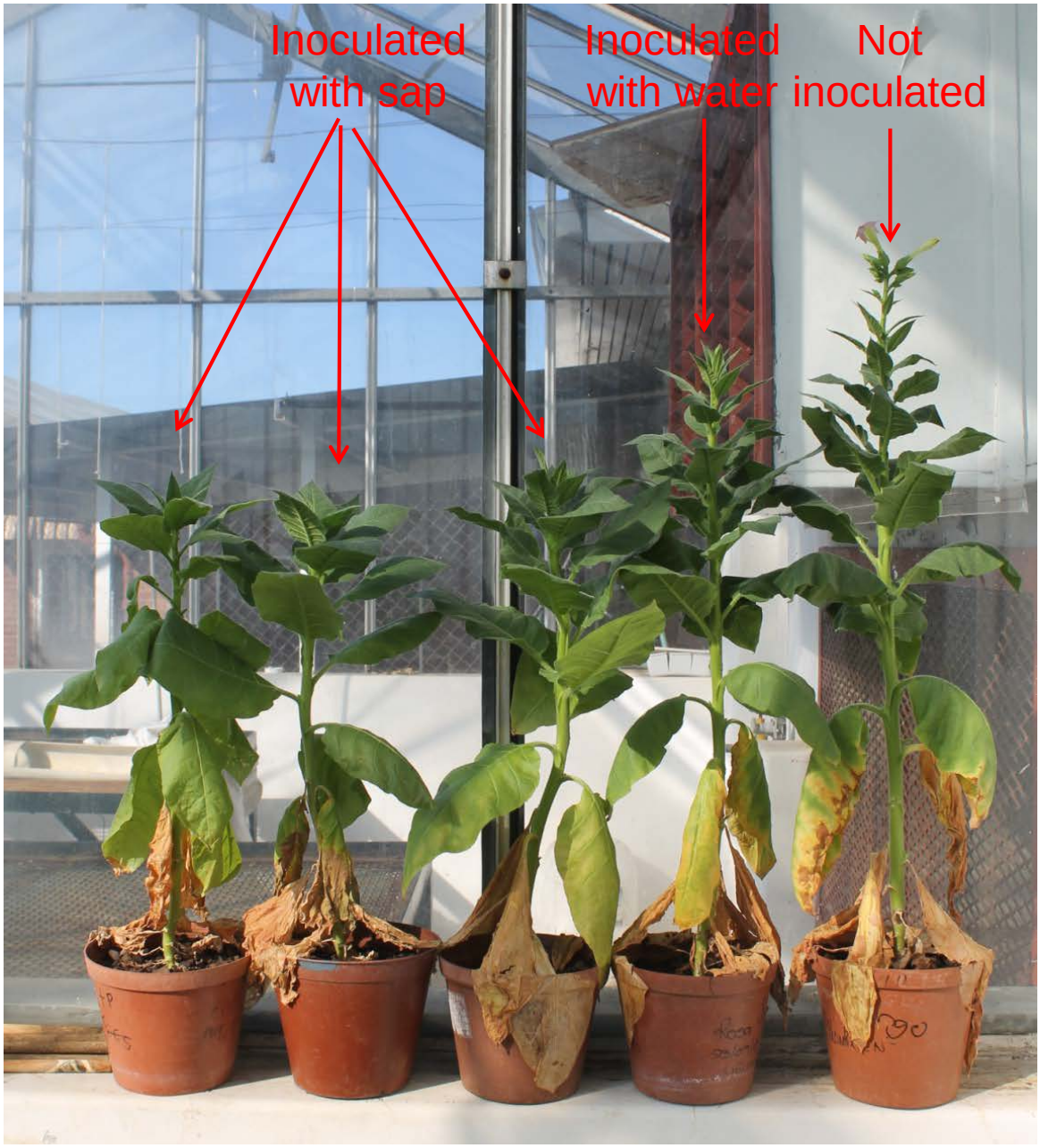


Figure 7.3 *Nicotiana tabacum* cv. Xanthi mechanically inoculated with symptomatic leaf material of interspecific Clivia CIH.

The tobacco plants inoculated with CIH leaf material appeared stunted relative to the plant inoculated with water or to the plant not inoculated at all. The inoculated plants did not show any other symptoms that would normally be associated with viral infection, such as leaf mottling or distortion, aside from the apparent stunting.

Figure 7.4 Inconclusive attempt at mechanically inoculating *C. caulescens* seedlings: (a) symptomatic adult *C. caulescens* plant used to provide inoculum, (b) leaf sap that collected on the cut surfaces of a symptomatic leaf, (c) healthy *C. caulescens* seedlings that were inoculated with the sap seen in (b) and (d) the wounds created on the seedling plants into which the sap evident in (b) was transferred.

At four weeks post inoculation, none of the seedlings inoculated with sap from the plant shown above in Figure 7.4 (a) displayed any abnormal symptoms. The seedlings wounded and inoculated with water also remained healthy during the observation period.

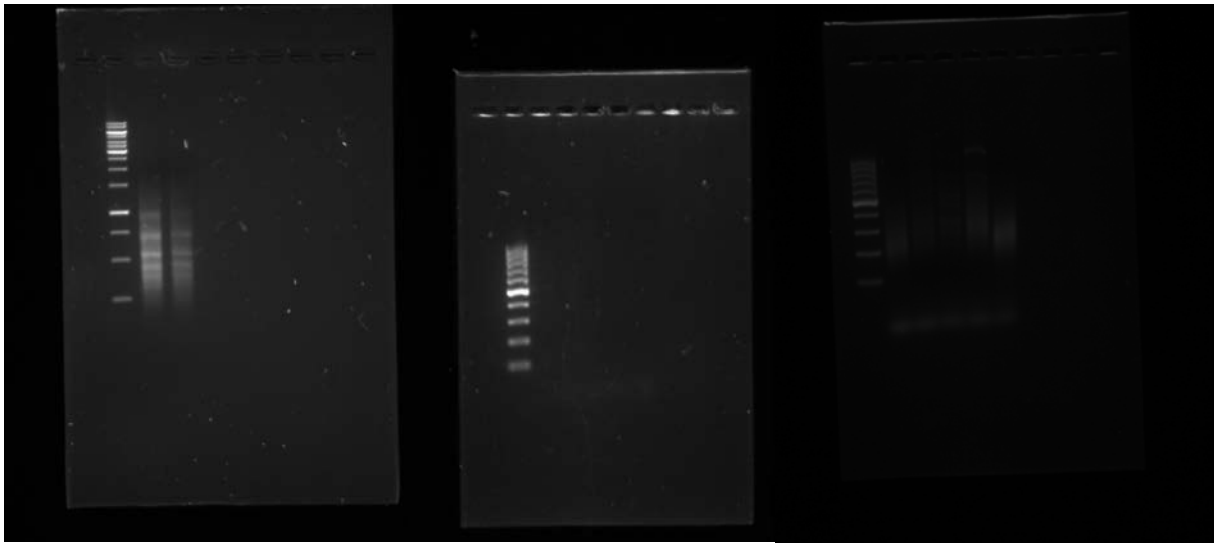


Figure 7.5 Examples of unsuccessful PCR attempts to amplify viral nucleic acids

Analysis of the potential PCR products generated from samples of symptomatic Clivia leaves and purified virus samples on agarose gels indicated that none of the tested combinations of host Clivia plant/purified virus sample and viral primer set successfully produced clear products of the expected sizes. Attempts at molecular identification of the viruses observed with the use of PCR were thus unsuccessful.

7.5 DISCUSSION

Clivia plants from a wide range of locations showed symptoms of leaf mottling and streaking (Figures 7.1 and 7.2) that contrast clearly with the evenly pigmented leaves of healthy plants. These symptoms are similar to those described by Adcock (2007) in Clivia plants infected with potyviruses. The presence of virus-like particles in association with these foliar confirms the presence of viral pathogens in the symptomatic plants.

The titre of viral particles observed in the TEM micrographs obtained from the purified samples was much higher than that observed in the TEM micrographs of leaf sap preparations (Figure 7.1). There was also far less cellular debris in the purified samples, making it easier to visualise the viral particles present. The implications of these observations are that the purification protocol used in this study was successful at concentrating viral particles out of leaf material and is thus a good starting point for future researchers of Clivia viruses to utilise in their research.

The remaining studies were inconclusive in their attempts to provide evidence for the identity of the viruses observed. Not all the possible combinations of the Clivia accessions listed in Table 7.2 and PCR primers and protocols listed in Table 7.3 were tested and it is possible

that an untested combination of the two variables could have led to successful amplification of viral nucleic acids. Future studies on this subject would benefit from a repetition of the experiments to cover all possible combinations. Another possible explanation for the lack of PCR products for molecular analysis is that the viruses observed do not belong to any of the viral families tested for and may represent novel entities.

DePaulo and Powell (1995) noted that the presence of double-stranded RNA (dsRNA) in plant tissues may be used to diagnose viral infections and furthermore, to characterise the viruses that produce the dsRNA encountered. Numerous extraction protocols have been developed to isolate dsRNA from plant tissue, including those outlined by Balijja *et al.* (2008) and Tzanetakis & Martin (2008). The application of this technique to diseased Clivia samples may allow for the isolation of viral nucleic acids from symptomatic tissues and for the elucidation of clearer results based on the results of downstream enzymatic reactions of any dsRNA encountered.

The aim of this study was partially fulfilled in that evidence was provided that Clivia plants showing symptoms thought to indicate viral infection do have virus-like particles associated with these symptoms. However, progress in determining their identity and potential modes of their transmissibility were not definitive and should remain the focus of future studies.

7.6 REFERENCES

Adcock, K. (2007) Potyvirus infection in Clivias. Clivia News 16: 11-12.

Aubrey, A. (2001) *Clivia miniata* [Lindl.] Regel. http://www.plantzafrica.com/plantcd/cliviaminiata.htm. Accessed: 09/10/2014.

Balijja, A., Kvarnheden, A. & Turchetti, T. (2008) A non-phenol-chloroform extraction of double-stranded RNA from plant and fungal tissues. Journal of Virological Methods 152: 32-37.

Crescenzi, A., d'Aquino, L., Nuzzaci, M., Ostuni, A., Bavoso, A., Comes, S., De Stradis, A. & Piazzolla, P. (1997) Production of strain specific antibodies against a synthetic polypeptide corresponding to the N-terminal region of the plum pox potyvirus coat protein. Journal of Virological Methods 69: 181-189.

DePaulo, J.J. & Powell, C.A. (1995) Extraction of double-stranded RNA from plant tissues without the use of organic solvents. Plant Disease 79: 246-248.

Duncan, G. (2008) Grow Clivias. South African National Botanical Institute, South Africa. pp. 11-12.

Gent, D.H., du Toit, L.J., Fichtner, S.F., Mohan, S.K., Pappu, H.R. & Schwartz, H.F. (2006) Iris yellow spot virus: an emerging threat to onion bulb and seed production. Plant Disease 90: 1468-1480.

Hammet, K. (2004) Virus symptoms and particles – correspondence from the Clivia enthusiasts email group. Clivia News 13: 19-22.

Jones, D.R. (2005) Plant viruses transmitted by thrips. European Journal of Plant Pathology 113: 119-157.

McDonough, M.J., Gerace, D. & Ascerno, M.E. (1999) Western flower thrips feeding scars and tospovirus lesions on petunia indicator plants. http://purl.umn.edu/51809. Accessed 05/07/2013.

Miglino, R., Jodlowska, A. & van Schadewijk, A.R. (2004) Detection and identification of a novel potexvirus infecting *Allium* by paramagnetic beads ssRNA isolation and one tube RT-PCR assay with a new potexvirus genus primer set. Acta Horticulturae 722: 285-292.

Smith, K.R. (2011) The Clivia Society Clivia Register. http://www.cliviaregister.com/. Accessed: 09/10/2014.

Tzanetakis, I.E. & Martin, R.R. (2008) A new method for extraction of double-stranded RNA from plants. Journal of Virological Methods 149: 167-170.

Wang, Q.-M., Gao, F.-Z., Gao, X., Zou, F.-Y.,Sui, X., Wang, M., Hui, Y.-J. & Wang, L. (2012) Regeneration of *Clivia miniata* and assessment of clonal fidelity of plantlets. Plant Cell, Tissue and Organ Culture 109: 191-200.

Winter, S., Korbler, M., Kollner, U., (1997) Detection and differentiation of sweet potato closteroviruses by RT-PCR and single-stranded conformational polymorphisms. In: Diagnosis and Identification of Plant Pathogens, Proceedings of the 4th International Symposium of the European Foundation for Plant Pathology. Kluwer Academic Publishers, Dordrecht, pp. 473-475.

Zhang, L., Rodoni, B.C., Gibbs, M.J. & Gibbs, A.J. (2010) A novel pair of universal primers for the detection of potyviruses. Plant Pathology 59: 211-220.

DISSERTATION OVERVIEW

The goal of the research presented in this dissertation was to identify the causes of several diseases of important indigenous ornamental crops grown in South Africa; namely a putative fungal and viral disease of *Clivia*; a putative bacterial and fungal disease of *Strelitzia* and a putative bacterial disease of *Zantedeschia*. The research presented in the study aimed at addressing three components: firstly, to review and summarise the available literature pertaining to phytopathological studies on the plant species studied because this has not previously been carried out; secondly, to investigate whether phytopathogenic microbes were responsible for causing the selected diseases; and, thirdly, to clearly identify the phytopathogens that were implicated in the diseases of *Clivia, Strelitzia* and *Zantedeschia* that were studied.

The goals outlined above were met by the studies presented in this dissertation. The tabular summaries of fungal, bacterial and viral diseases of these crops presented together in Chapter 1, and individually in Chapters 2-7, provide an easily accessible reference for future researchers into the diseases of these crops, with which their research can be compared. It is hoped that this will facilitate the initiation of further studies relating to the epidemiology and control of these diseases.

The fulfilment of Koch's postulates in five of the six plant disease studies presented above verified the suspected phytopathogenic nature of these diseases and satisfied the second component of the research goal. A uniting factor in the isolation of disease-causing organisms in these studies was the apparent novelty of each of these plant-pathogen relationships. Some chapters represent the first report of these plant diseases in South Africa and others represent the first report of the studied diseases globally. This highlights the need for more research on the pathogens of indigenous plants and assessments of the presence of phytopathogens in the local floricultural industry.

The apparent lack of transmissibility of the virus-like diseases in *Clivia* plants detailed in Chapter 7 did not conclusively point to the cause of the symptoms of the plants as being due to the observed viruses. Future studies will be necessary to provide conclusions about the topic of viral infection in *Clivia*. Potential future research can focus on modes of viral transmission, more extensive attempts at the identification of viruses associated with *Clivia* plants and assessments of the distribution and impacts of viral diseases in the *Clivia*-growing industry.

The final component of the research goal for this dissertation was to characterise any phytopathogens isolated in the studies. Where Koch's postulates were fulfilled,

morphological, cultural and molecular characterisation were used to provide a secure genus-level identification of the phytopathogenic isolates of *Colletotrichum, Pestalotiopsis, Pantoea* and *Pseudomonas* that were implicated in the observed disease complexes. Accurate identification of these fungal and bacterial isolates at a species-level would be necessary in the further studies into the epidemiology and control of the diseases that they cause. Future research focussing on concatenated phylogenetic analysis of the sequence data from multiple genes of each isolate would allow for accurate assignment of these pathogens within their respective genera.

South African flora has made significant contributions to the global floriculture industry, and the continued interest in the development of new floricultural crops from South Africa's exceptionally diverse plant community highlights the importance of our plants to the industry. A conspicuous trend illustrated by the research presented in this dissertation is the general lack of modern studies pertaining to the phytopathogens of South African indigenous plants. There is a clear need in the future for comprehensive, ongoing studies surveying the diseases that affect floricultural crops in South Africa.