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Identification of alternative hosts to citrus of "*Candidatus* Liberibacter africanus" amongst indigenous Rutaceae of South Africa

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Submitted in partial fulfilment of the requirements for the degree
Magister Scientiae
in the Faculty of Natural and Agricultural Science
University of Pretoria
Pretoria
South Africa

Supervisor: Professor G. Pietersen

February 2010

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Declaration

I declare that the thesis/dissertation, which I hereby submit for the degree Masters in Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Acknowledgements

I acknowledge with greatest appreciation the following people and organisations:

Professor Gerhard Pietersen my supervisor for your utmost patience and guidance through the years.

Professor L Korsten for all her inputs towards this study.

Dr SP van Vuuren for the wealth of knowledge transferred to me and assistance with collection and provision of samples.

Citrus Research International and Agricultural Research Council in partnership with Department of Science and Technology (mentorship programme) for funding and supporting my Magister Scientiae study.

Dr JL Staphorst and Dr I Rong for their mentorship through the years.

Lowveld and Kirstenbosch National Botanical Gardens and private property owners for permission to collect samples on their premises.

My colleagues at ARC-PPRI and fellow students at UP for their assistance at all times.

My family; mama, papa and sister – a pillar of strength in every sense of the words, without you none of this would be possible. My loving parents, without your continuous motivation and enormous respect for education I would not be where I am today. My most heartening brother, this is in your memory. *O ke mošomo wa lena Barolong ba bina tšhipi*.

Abstract

Title: Identification of alternative hosts to citrus of "*Candidatus* Liberibacter africanus" amongst indigenous Rutaceae of South Africa

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Supervisor: Professor Gerhard Pietersen

Department: Department of Microbiology and Plant Pathology; University of Pretoria

Degree: Master of Science

Citrus greening or Huanglongbing is a severe disease affecting all citrus cultivars and rootstocks and is responsible for causing severe economic losses in many citrus producing countries. It is caused by various fastidious phloem-restricted, Gram negative bacteria belonging to the "*Candidatus* Liberibacter" genus. Currently only three species that affects citrus have been identified, "*Candidatus* Liberibacter africanus" (Laf) found in Africa, Mascarene Islands and the Middle East, "*Candidatus* Liberibacter asiaticus" (Las) present in Asia, and only recently emerged in Florida USA, São Paulo Brazil and Cuba and a new species "*Candidatus* Liberibacter americanus" (Lam) found only in São Paulo Brazil. Due to the continuing spread of this serious and destructive disease into areas of South Africa previously regarded as disease-free and disease-managed, the role of alternative hosts in the transmission of the disease is questioned. The aim of this study was to identify possible alternate hosts of Laf amongst plants of Rutaceae indigenous to South Africa. Identification of these alternate hosts forms a critical part of the integrated management and control strategies of the disease.

Leaf material from symptomatic and asymptomatic indigenous rutaceous and non-rutaceous plants were collected from various sites in South Africa, from different environments which included vegetation adjacent to citrus orchards, in areas with high incidence of the citrus greening disease, from natural habitats, botanical gardens and private properties. A multiplex polymerase chain reaction (PCR) was developed for the simultaneous detection of Laf DNA and ubiquitous ribulose bisphosphate carboxylase oxygenase (Rubisco) gene which served as an internal control for the presence and quality of extracted DNA and potential amplification inhibition by compounds of the DNA extract. The multiplex PCR oligonucleotide primers were used to amplify fragments of 669bp from the β ribosomal operon of Laf and 179bp of Rubisco gene. Petioles and/or leaf midrib tissues were tested for the presence of Laf using the newly developed multiplex PCR and β operon specific primers of a published conventional PCR method.

In this study seventeen *Calodendrum capense* Thunb. (Cape chestnut) plants from various geographic areas of South Africa were identified to be naturally infected with Liberibacter DNA. Sequence analysis revealed the DNA to be that of "*Candidatus* Liberibacter africanus subspecies capensis" (LafC). Although this subspecies of Liberibacter has previously been detected on *C. capense* in the Stellenbosch area of the Western Cape Province, this is the first report indicating the natural occurrence of LafC in *C. capense* in Gauteng, Limpopo and Mpumalanga Provinces of South Africa. The geographic distribution of the LafC bacterium associated with Cape chestnut appears to be more widespread than initially expected. No Laf DNA was detected from *C. capense* or any other indigenous plants surveyed.

Graft-inoculation procedure was further used to assess the host range of Laf using a selected number of healthy Rutaceae seedlings. These were inoculated with bark patches using a known infected inoculum source for the transmission of the bacterium. The inoculated seedlings were monitored for the presence of Laf using conventional, multiplex and TaqMan real-time PCR. Conventional and multiplex PCR failed to amplify Laf DNA from the inoculated samples 1 year post inoculation (PI). Real-time PCR yielded low cycle threshold (Ct) values for *C. capense* and high Ct values for *Clausena anisata* (Wild) Hook. f. ex Benth. and *Zanthoxylum capense* (Thunb.) Harv. inoculated samples 2 years PI. This may be indicative of high concentration of Laf within *C. capense* and low concentrations of the bacterium within *C. anisata* and *Z. capense* plants. This is the first report on the ability, under experimental conditions, of *C. capense and Z. capense* to host Laf. Detection of the pathogen from these inoculated rutaceous plants might indicate that these plants could have a natural ability to host and play a role in the transmission of the disease to citrus species.

The findings of this study suggest that the alternate hosts of Laf identified under experimental conditions must be considered within the management and control strategies for citrus greening control. The role of these hosts in natural epidemic of the disease must still be confirmed and the epidemiology of LafC be studied to assess whether it includes *Citrus* species.

List of abbreviations

ARC	Agricultural Research Council
Amp	Ampicillin
bp	Base pairs
CG	Citrus greening
CRI	Citrus Research International
cm	Centimetre
^{o}C	Degrees Celsius
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid disodium salt
FAO	Food and Agriculture Organization of the United Nations
HCl	Hydrochloric acid
HLB	Huanglongbing
ITSC	Institute for Tropical and Subtropical Crops
KNBG	Kirstenbosch National Botanical Gardens
Laf	"*Candidatus* Liberibacter africanus"
LafC	"*Candidatus* Liberibacter africanus subspecies capensis"
Lam	"*Candidatus* Liberibacter americanus"
Las	"*Candidatus* Liberibacter asiaticus"
LNBG	Lowveld National Botanic Gardens
mAbs	Monoclonal antibodies
ml	Millilitre
mM	Millimolar
M	Molar
MLO	Mycoplasma-like organism
n	Nanometer
NaCl	Sodium Chloride
NaOAC	Sodium acetate

NaOH	Sodium hydroxide
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
pmol	Picamole
PPRI	Plant Protection Research Institute
rpm	Resolutions per minute
RSA	Republic of South Africa
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Rubisco	Ribulose bisphosphate carboxylase oxygenase
RubiscoL	Ribulose bisphosphate carboxylase oxygenase large subunit
SB	Sodium boric acid
subsp.	Subspecies
UK	United Kingdom
USA	United States of America
UP	University of Pretoria
UV	Ultra-violet
V	Volts

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Chapter 1

Introduction and objectives of the study

1.1 Introduction

Citrus greening disease is considered the most serious vector-transmitted bacterial disease of *Citrus* species and some of its related genera within Rutaceae family of plants. The disease is also known as Huanglongbing (HLB) (da Graça & Korsten, 2004) but in this thesis it will be referred to as citrus greening in order to differentiate it from HLB caused by a different "*Candidatus* Liberibacter" species.

Citrus greening is caused by a fastidious phloem-limited Gram-negative bacterium. The bacterium is taxonomically classified under the alpha subdivision of the *Proteobacteria*. There are three known species of the "*Candidatus* Liberibacter" genus that infect *Citrus* species – "*Candidatus* Liberibacter africanus" (Laf), "*Candidatus* Liberibacter asiaticus" (Las) and "*Candidatus* Liberibacter americanus" (Lam). The most recent addition to the genus is a "*Candidatus* Liberibacter" species which infects Solanaceae family plants and so far has not been shown to infect Rutaceae plants (Li *et al*., 2008; 2009). A subspecies of Laf was identified from an indigenous Rutaceae plant of South Africa – *Calodendrum capense* Thunb. The name "*Candidatus* Liberibacter africanus subspecies capensis" (LafC) was proposed for this subspecies. In South Africa and other African countries, Laf is vectored by the citrus psylla *Trioza erytreae* (Del Guercio).

The origin of the disease is not well understood and is a topic that still requires further investigation. It has been hypothesized that the disease may have originated from Africa, possibly in an asymptomatic host such as *Vepris lanceolata* (Lam) G. Don. and then later spread by an insect vector to citrus in the east coast of the continent and then taken to the Indian subcontinent through infected budwood or plants where it later

spread to China (Gottwald *et al*., 2007). This however remains a hypothesis until proven.

It is possible for plants of the same family to host the same pathogen regardless of whether the pathogen can cause disease in the host or not. The ability of the citrus psylla, *T. erytreae*, to feed and breed on both the indigenous Rutaceae plants and citrus (van den Berg *et al*., 1992) supports the possibility that Rutaceae plants could serve as alternate inoculum sources to citrus.

The management of the disease requires an integrated approach that includes removal of infected plant material that serve as inoculum, the effective usage of insecticides for psylla vector control, quarantine measures to prevent movement of citrus and citrus relatives material, and the propagation and planting of disease-free material.

Identification of potential alternative host plants to citrus of Liberibacter species, that might influence the epidemiology of the disease in citrus should form an integral part of the control and management strategies of the disease worldwide. The occurrence of possible alternate hosts would negatively affect psylla control measures on citrus as these hosts would continue to support psylla growth thus re-infestation onto citrus. The identification of possible alternate host plants of the disease will improve the critical integrated approach of the disease's management.

Control and management of the disease is reliant on accurate detection of the bacterial pathogen from citrus and alternate host plants. Diseased citrus plants often harbour low titres of the bacterium and distribution of it is uneven throughout the plants. Proliferation of the bacterium is experienced during the cooler seasons hence blotchy mottle symptoms are observed during the cooler months of the year. This greatly

influences the detection ability of various detection techniques (da Graça, 1990). Another factor that influences detection techniques is the presence, in extracts, of phenolic compounds and polysaccharides associated with different plant species, which may act as inhibitors to the techniques.

1.2 Summary of the objectives of the study

The main aim of this study is to identify alternative hosts to citrus of the citrus greening bacterium, Laf, amongst indigenous Rutaceae plants occurring in South Africa.

The study was conducted by dividing the project into different sections as described below:

1. Development of a multiplex PCR standard protocol for the simultaneous specific and sensitive detection of the Laf and an internal control plant specific DNA. In this case a ubiquitous ribulose bisphosphate carboxylase oxygenase housekeeping gene was used as an internal control in the PCR. DNA extracted from citrus plants known to be infected with the greening pathogen was used for the optimization of the PCR. Various aspects that should be determined for this protocol include:

   - Selection of an internal control DNA that can be amplified from DNA extracts of various plants.

   - Optimization of the PCR to co-amplify Laf and internal control DNA simultaneously in one reaction.

- Determination of whether the multiplex PCR could be used as an indicator for successful extraction of DNA from various hosts other than citrus.

- PCR could be used to indicate the non-inhibition of the PCR by inhibitors.

2. Determine whether alternate host plants to commercial citrus plants for Laf occur by:

    - Identifying and selecting a site with high citrus greening infections in commercial citrus plants.

    - Collect mostly symptomatic but also non-symptomatic samples from areas surrounding citrus orchard blocks and from their other natural habitats. The alternate samples would compromise mainly of *Rutaceae* plants, especially those that have been shown to host the citrus greening vector *T. erytreae*, and other indigenous plants of South Africa.

    - Extract the DNA of the possible alternate host samples and testing them using either multiplex PCR with the internal control developed in this study or the conventional published PCR.

    - Determine the nucleotide sequence of the Liberibacter DNA detected from the alternate host plants and compare their variability to known Liberibacter DNA sequences.

3. To determine the capability of selected rutaceous plants to host the Laf bacterium by:

    - Graft-inoculating bark patches and/or buds cut from a citrus greening infected citrus plant onto selected rutaceous plant seedlings and maintaining the seedlings in a vector-free, temperature controlled glasshouse.

    - DNA extraction and PCR testing, using the newly developed multiplex PCR and published PCR protocols, of samples from the inoculated seedling to determine the presence of Liberibacter DNA.

    - Regular monitoring of the seedlings for typical citrus greening symptoms or other symptoms expression.

Chapter 2

Literature review

2.1 Introduction

Citrus greening or Huanglongbing (HLB) is a devastating disease of citrus world wide. To date the disease is found in the African, Asian, South and North American continents that host the biggest citrus production. It has been recently detected in countries where it has previously not been reported e.g Brazil, Florida United States of America, Timor-Leste and Papua New Guinea (Weinert *et al*., 2004; Teixeira *et al*., 2005). The spread of this disease to new countries threatens the production of citrus world-wide. Major economic losses are reported in countries affected by the disease due to reduced yields of quality fruit and a decrease in the lifespan of infected trees.

This literature review offers an insight into the current situation of the citrus greening disease in South Africa, with the causal agent being "*Candidatus* Liberibacter africanus" (Laf). It focuses on the description and historical background of the disease, economic effects experienced in South Africa, disease and vector spread in Africa, advancement in the detection techniques used, plant host range of both the vector and pathogen, and lastly, the current control and management strategies of the disease.

The pathogen and insect vector relationship is important in the transmission of the disease concerned. Citrus greening disease is transmitted primarily by infected psyllid *T. erytreae* in the fields (McClean & Oberholzer, 1965 b) and by grafting infected material onto non-infected material as previously shown by McClean and Oberholzer (1965 a). The disease can also be transmitted transovarially by the citrus psylla (van den Berg *et al*., 1991-1992).

2.2 Name variations and their meaning

The disease has a number of name variations depending on the country where it occurs, the symptoms observed on the diseased citrus plants and the causal agent responsible for the disease. The name Huanglongbing was first used by farmers in China to describe yellow shoots or yellow dragon as portrayed by the symptoms on an infected tree and used in first report on the graft-transmission of the disease by Lin Kung Hsiang in 1956 (Bové, 2006). HLB might have originated from China in the 1890's (da Graça, 1991). In South Africa the disease was described in the 1920's and has subsequently been known as citrus greening due to the immature discoloration of the fruits (da Graça, 1991). It was also called "yellow branch" in the western Transvaal which described the yellow colour appearing on the infected shoots while "greening" was used in the eastern Transvaal (van der Merwe & Andersen, 1937).

In the Philippines, yellow-green patterns on old leaves, similar to that of yellow shoots described in South Africa were observed, and the disease was then called mottle leaf (Altamirano *et al*., 1976). Die-back in India was noticed when die-back of twigs, slow death and wilting of citrus occurred in some plants. In Indonesia the phloem necrosis and vein phloem degeneration names were brought about by the observation of localized pockets of necrotic phloem which led to the necrotic leaves. Likubin, referring to decline, was adopted in Taiwan (Raychaudhuri *et al*., 1969; da Graça, 1991; Bové, 2006).

2.3 Citrus production and economic impact of the disease in South Africa

Citrus production and export in South Africa have always competed with some of the world's major citrus industries. According to the Food and Agriculture Organization of the United Nations (FAO) in 2005/2006 South Africa's fresh citrus production reached 1,743 thousand tons of citrus of which 1,213 thousand tons were exported to other countries. South Africa is ranked as the twelfth major citrus producing and the second largest citrus exporting country in the world (FAO, 2006).

Preliminary estimates in 2005/2006 by the South African Citrus Growers Association (CGA) of the overall production area for citrus in South Africa was 56, 623 hectares, with the Limpopo (17,452), Western Cape (11,852), Mpumalanga (10,762) and Eastern Cape (10,054) Provinces providing the largest areas for production. The main varieties that are planted include Valencia, navel, grapefruit, soft citrus, lemon and lime, mid-season oranges and pummelos (CGA, 2007). These varieties are susceptible to the citrus greening disease which could consequently lead to massive losses in production with the further spread of the disease.

Economical losses experienced in the citrus industry of South Africa due to citrus greening are not thoroughly recorded on an ongoing basis but some incidences of estimated crop losses have been previously made. Estimated crop losses of 30-100% were recorded between 1932 and 1960 (Pretorius & van Vuuren, 2006) and 100 000 sweet oranges trees were considered to be commercially unprofitable in the early 1960's (Oberholzer *et al*., 1965). In the mid 1970's an estimated 4 million citrus plants were infected with the disease (Buitendag & Von Broembsen, 1993; le Roux *et al*., 2006) while in the 1980's an annual estimated loss of R35 million in production was due to the disease (van den Berg *et al*.,

1991-1992). On a global scale, it is estimated that by the early 1990's more than 60 million trees had been destroyed by the disease (da Graça & Korsten, 2004).

2.4 Morphology of the bacterium

The aetiology of the prokaryote associated with citrus greening organism was first described by Laflèche & Bové in 1970 (Lafléche & Bové, 1970 b). At that time these organisms were described as mycoplasma-like organisms (MLO) and were seen residing within the phloem tissue of sweet orange seedlings obtained from South Africa (Laflèche & Bové, 1970 b) and later from citrus with an Indian origin (Garnier *et al*., 1976; da Graça, 1991). The haemolymph and salivary glands of the citrus greening transmitting psylla vector, *Trioza erytreae*, were also observed to harbour these MLO (Moll & Martin, 1973).

Mycoplasma-like structures were also associated with citrus stubborn disease in California and citrus decline in India. Interest developed around the issue of confirming whether the organism associated with greening and stubborn diseases were of a mycoplasma-like nature. In 1970 Lafléche and Bové (Lafléche & Bové, 1970 b) showed, by electron microscopy, that the two organisms were different. The greening structures appeared to be more rigid, longer and filamentous whilst the structures associated with stubborn disease were bell-shaped, rounder, pleomorphic and sinusoidal (Laflèche & Bové, 1970 b; Garnier *et al*., 1976). In 1971 the agent of the stubborn disease was obtained in culture and but only characterized as *Spiroplasma citri* in 1973 (Saglio *et al*., 1973).

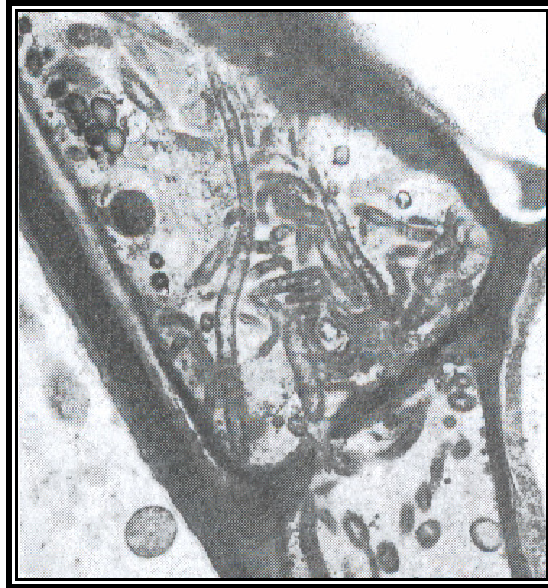
According to the Subcommittee on the Taxonomy of Mycoplasmatales, recognized by the International Committee on Nomenclature of Bacteria, which prevailed in 1976, a mycoplasma organism had to have certain

properties relative to its envelope structure. These are: (1) The organism had to be surrounded by a triple-layered unit membrane (2) Could sometimes be covered by a thin layer of electron-dense material; (3) Be devoid of a cell wall and have no cell wall precursors. The stubborn organism complied with the minimum standards in contrast to that of the greening organism (Garnier *et al*., 1984).

The general thickness of the unit membrane envelope of MLO's is 7-10 nm. The envelope of greening was found to be more complicated as seen by electron microscopy. It consisted of a triple-layer of approximately 25nm thickness with an electron dense outer layer, an electron clear middle layer and an electron dense inner layer (Garnier *et al*., 1976; Bové & Garnier, 1998). The inner membrane appeared to be the cytoplasmic membrane and the outer membrane a cell wall. The middle layer provoked curiosity as this would later determine how the greening organism was to be classified.

Cytochemical tests were used to visualize the peptidoglycan layer (PG) of citrus greening as previously done for the Gram negative *Escherichia coli*. Papain digestion of the cells revealed three membranous layers assumed to be that of the outer, inner membrane and the PG located between the two layers. Papain enzyme was used to visually separate the R-layer of the PG from the outer membrane of the cell. Lyzozyme treatment of the papain digested leaf midribs resulted in the disappearance of the middle layer. Similar results were obtained when *E. coli* cells were treated with the papain and lyzozyme enzymes. The same treatment was applied to a PG layer deficient Gram positive bacterium *Staphylococcus aureus* with the expected opposite result (Garnier *et al*., 1984).

Su and Huang (1990) describe the organisms as flexible elongated rods of growing form (100-250 X 500-2500 nm), rigid rods of mature form (350-550 600-1500 nm) and spherical bodies of old form with a thin cytoplasm (diameter 700-800 nm).



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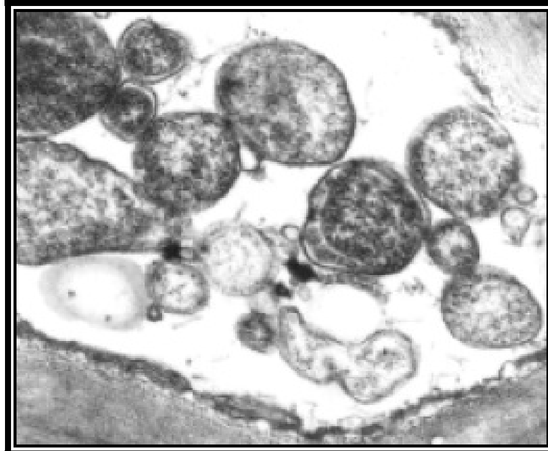

b

Figure 2.1 Pleomorphic morphology of the Liberibacter bacterium within the phloem tissue of (a) periwinkle (Garnier *et al*., 1984) and (b) *Citrus* species (Tanaka *et al*., 2006).

2.5 Liberibacter species infecting *Citrus*: Africanus, Asiaticus and Americanus

The disease is wide-spread throughout the world in Asia, the Indian Subcontinent, Oceania, South- and East- African, North- and South- American countries and the Arabian Peninsula (Bové & Garnier, 1984; Bové, 2006; Li *et al*., 2006).

Laf is the citrus greening pathogen that is found in most African countries and is vectored by a psylla *Trioza erytreae*. Laf occurs and tolerates cool moderate temperatures (20 – 23°C) and high altitudes. A subspecies of the Liberibacter genus was recently detected, characterized and named "*Candidatus* Liberibacter africanus subspecies capensis" (LafC) (Garnier *et al*., 2000 b). The name was proposed after phylogenetic analyses of the β operon genes subspecies revealed an 82.1% sequence homology to Laf and a homology of 79.9% to the "*Candidatus* Liberibacter asiaticus" (Las), and after the Cape chestnut bacterium reacted to monoclonal antibodies specifically to the serotype of the Laf species (Garnier *et al*., 2000 b).

Las is the causal agent of HLB prevalent in Asian, North and South American countries. *Diaphorina citri* is the psylla vector of Las and both the bacterium and vector are more tolerant to hot conditions (30 – 35°C) and can occur in low altitude areas. *T. erytreae* has been shown, under experimental conditions to transmit the heat tolerant and destructive Las (Massonie *et al*., 1976) and the possible introduction of this Liberibacter species into areas where *T. erytreae* is prevalent could result in detrimental consequences for the citrus industries concerned.

The initial names proposed for the two species were "*Candidatus* Liberobacter africanum" and "*Candidatus* Liberobacter asiaticum". However the names were revised to Liberibacter africanus and Liberibacter asiaticus in order to comply with the rules of the International Code of Nomenclature of Bacteria (Garnier *et al*., 2000 b; da Graça & Korsten, 2004).

"*Candidatus* Liberibacter americanus" (Lam) has recently been characterized in Brazil after it was detected in citrus plants in the São Paulo State (Coletta-Filho *et al*., 2004; Teixeira *et al*., 2005). This species is also transmitted by the psylla vector *D. citri* and by budding with infected plant materials (Li *et al*., 2006). This species was shown to be heat sensitive, tolerating lower temperatures (22 - 24 ^{o}C) but not high temperatures (27 – 32°C) on naturally infected and graft-inoculated sweet orange seedlings (Lopes & Frare, 2009). Not only is Lam a threat, but the presence of Las, the most severe form of the Liberibacters, could be detrimental to the citrus industry of Brazil (Teixeira *et al*., 2005; Bové, 2006).

2.6 Symptomology

The disease is mainly characterized by the mottling or blotchy mottle symptom which entails leaves developing a pattern of blotchy or patchy light green to yellow colour. The primary and secondary veins of the leaves become yellow and enlarged. Small, upright and leathery leaves are associated with young infected leaves. Chlorotic patterns are noted and the asymmetrical mottle distinguishes the disease from zinc, manganese, iron and other mineral deficiencies which were previously associated with the disease (Bové, 2006; McClean & Oberholzer, 1965 b). The non-specific nature of the foliage symptoms makes the disease difficult to distinguish from nutrient deficiencies or other plant diseases

that exhibit similar symptoms (Li *et al*., 2006). Foliage symptoms are better expressed in the winter than in the summer in South Africa (Garnier & Bové, 1993).

Symptoms associated with the fruits from known infected citrus plants vary. The fruit do not grow to normal size, exhibit a lopsided form showing an uneven axis of symmetry and the juice has an undesirable bitter acid taste due to lower levels of sugar (da Graça, 1991). Ripening fruit remain green on one side. The colouration of infected fruit starts at the peduncular end which results in colour inversion (Bové, 2006). The seeds are usually aborted. Fruit quality is further reduced by the disproportional colouring. A silver deposit appears when pressure is applied on the green area. The infected fruits fall from the branches earlier than the non-infected fruit (le Roux *et al*., 2006; Gottwald *et al*., 2007). A continuous fruit drop occurs from normal November fruit drop until harvest time. Fruit are not used as primary indicators for the infection of a citrus plant by citrus greening as the same symptoms can be associated with stubborn disease (Bové, 2006). Citrus greening infected fruit are not marketable in the citrus industry.

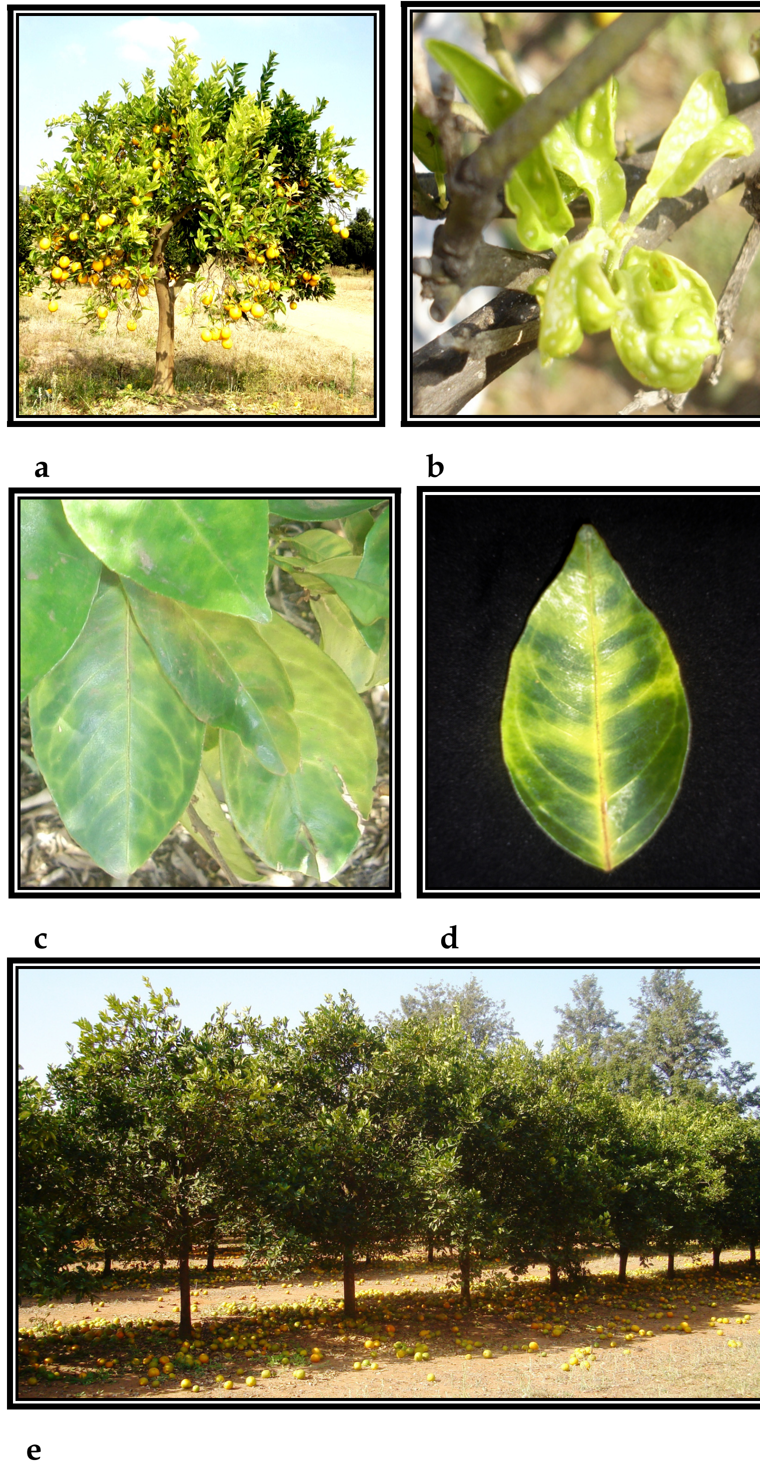

Figure 2.2 Various symptoms associated with greening disease in citrus plants: (a) typical infected shoots exhibiting yellow shoots; (b) psylla-like breeding marks on foliage (c and d); blotchy mottling on leaves; (e) fruit loss from greening infected trees.

2.7 Survey of the disease, vector and vector-parasitism in Africa

South Africa

Del Guercio described the *Trioza erytreae* psylla in 1918 in Eritrea, formerly a region that was part of Ethiopia. The vector was discovered invading citrus plants for the first time in the Eastern Cape and Stellenbosch areas of South Africa in 1897. It was suspected to have occurred in Ethiopia and moved down the east coast of Africa towards the Cape.

The disease was first described in South Africa in the 1920's (le Roux *et al*., 2006) and has since spread across the country, limiting areas for the production of citrus. The disease is present in citrus production areas in the North West, Limpopo, Mpumalanga, KwaZulu-Natal and most recently in the Western Cape Provinces (Garnier *et al*., 2000 a; Schwerdtfeger & Pietersen, 2007).

The first report of the psylla's ability to transmit the greening disease, then believed to be a viral disease, was in 1965 after experiments were done to transmit the disease to sweet orange seedlings (McClean & Oberholzer, 1965 a; 1965 b; Schwarz, 1965).

Areas where the disease occurs and incidences, based on symptoms severity were reported by Pretorius and van Vuuren (2006). The highest incidence includes the Karino-White River, Hazyview, Brondal, Nelspruit, Tzaneen, Rustenburg, Mokopane, Zebediela and certain magisterial districts of the Western Cape Province. Moderate incidences occur in Letsitele, Letaba Valley, Lydenburg-Ohrigstad, Muden-

Pietermarizburg-Richmond areas. Low incidences are found in Kaapmuiden-Malelane-Hectorspruit, Groblersdal-Marble Hall and Northern KwaZulu Natal including the Nkwaleni Valley.

The disease was reported to be absent in Citrusdal, the Eastern Cape and Northern Cape citrus growing areas (Pretorius & van Vuuren, 2006).

High temperature and low humidity were reported to be extremely detrimental to the population of the *T. erytreae* within the Letaba district (Catling & Annecke, 1968). Larger populations of the psylla insects were observed in the cooler, shaded and moist upland groves and this was supported by the experiment where a survival rate of 61-100% of the egg to first instar of the *T. erytreae* was noted when the mean daily maximum temperatures and relative humidity were 23.7°C and 54.5% respectively. Combinations of the two extreme conditions resulted in the subsequent desiccation of the eggs and first instar nymphs (Catling & Annecke, 1968).

Population fluctuations corresponded with the flush rhythms of the citrus trees, with no breeding activities occurring during the winter, while feeding occurred on young succulent flushes that appear during the cooler temperatures between July and the months of spring in the Letswalo experimental site situated in the area of the Letaba Valley, South Africa (Catling & Annecke, 1968).

Parasitism of the psylla was reported in South Africa by Annecke and Cilliers (1963) and the parasite was identified as *Tetrastichus dryi* which occurred as a common citrus psylla parasite in the former Transvaal. van den Berg *et al* (1987) gave a comprehensive report on the predators of *T. erytreae* in the lowveld and Rustenburg areas. The predators are recorded according to the life cycle stage of the psylla when predated i.e.

egg, nymph or adult and the predator is identified from the class to species level.

Other African countries

The Indian Ocean islands of Reunion Island and Mauritius harbour both *T. erytreae* and *D. citri* as well as the two forms of "*Candidatus* Liberibacter" species i.e Laf and Las. Each psylla can vector both Liberibacter species (Garnier *et al*., 1996).

In Cameroon large numbers of the *T. erytreae* occurred in the volcanic highlands region of Nkoundja (1,220 m) and the humid and high altitude (700 m) rainforest area of Sangmelima where climatic conditions favoured the occurrence of psylla outbreaks (Aubert *et al*., 1988).

The high altitudes areas found in Malawi (above 1,000 m) support the proliferation of the disease which is widespread. Parasitism of the *T. erytreae* by the endo-parasite *Tetrastichus* species and the ecto-parasite *Psyllaephagus* species were observed in some cases even though the natural control they exert on the vector was not showing satisfactory results (Aubert *et al*., 1988).

Ethiopia hosts both the psylla species and the Laf greening organism. Orchards located in Addis Ababa (2,400 m) attested the presence of the greening symptoms and *T. erytreae* whereas positive results of the greening bacterium were obtained from samples that were collected from the Awasa/Koka (1,600 m) and the Debre Zeit (1,850 m) vicinities. *Psyllaephagus* species and *Cheiloneurus cyanonotus* were the natural parasites for the psylla (Aubert *et al*., 1988).

Both *T. erytreae* and the Laf symptoms were observed in Rwanda and a high rate of endo-parasitism (90-92%) induced by *Tetrastichus dryi* was noted on collected psylla (Aubert *et al*., 1988).

Burundi was similar to Rwanda but an ecto-parasite belonging to the Psyllaesophagus group along with other unknown secondary parasites were found (Aubert *et al*., 1988).

Saint Helena (1967), Madeira (1996) (Jagoueix *et al*., 1996) and Tenerife (Canary Islands) (2002) (Gonzalez Hernandez, 2003) harbour both *T. erytreae* and *D. citri* but not the greening disease. Citrus orchards in Gabon contain typical leaf galls of *T. erytreae* (Aubert *et al*., 1988).

No greening symptoms or transmitting vector *T. erytreae* were found in Namibia (Swakopmund and Grootfontein) and Zambia (Lusaka) (Aubert *et al*., 1988).

Citrus in Comores Islands did not show any greening symptoms but an interesting finding of species of *Diaphorina* was made. The psyllid is not known to transmit Liberibacter species (Aubert *et al*., 1988).

Other countries in Africa that are reported to have greening disease include Central African Republic (Lafléche & Bové, 1970 a), Kenya (Bové & Garnier, 1984), Madagascar (Lafléche & Bové, 1970 a), Somalia (da Graça, 1991), Swaziland (Catling & Atkinson, 1974), Tanzania (Swai, 1988) and Zimbabwe (Lafléche & Bové, 1970 a).

2.8 Techniques utilised for detection of "*Candidatus Liberibacter*"

Over the years different molecular detection techniques have been used to detect the "*Candidatus* Liberibacter" species. Various DNA-based and other methods have been utilised to identify and characterize the then unknown agent of the citrus disease. Other technical techniques were used in to study biological characteristics portrayed by Laf pathogen in infected citrus. The techniques were successful in determining the graft-transmissibility of Laf, morphology and molecular characteristics of the bacterium. The improvement of such techniques has simplified detecting the pathogen, even though each technique has its own limitations.

Biological indexing

Biological indexing using seedlings as indicators has been used extensively to study the symptomology of the disease and the effect on different cultivars. Valencia sweet orange and Orlando tangelo seedlings are preferentially used in South Africa whereas Ponkan mandarin and Mosambi sweet orange or Darjeeling are used in Taiwan and India respectively (da Graça, 1991). Despite being a time consuming and labour intense method, it is still one of the most reliable methods for detection of graft transmissible pathogens (Singh & Ready, 2003).

Gentisoyl-β-D-glucose and chromatography

The detection of a fluorescent phenolic compound from the albido of greening infected sweet orange trees was used as a detection method for greening. Extract from infected tissue was spotted onto a

chromatographic paper and allowed to develop for eight hours with a n-butanol-acetic acid-water solvent (5:1:1) (Schwarz, 1965). A violet-blue fluorescent spot could be observed from greening-infected and not in healthy tissue under UV light at 365 nm (Schwarz, 1965). The fluorescent compound was later identified as gentisoyl glucose (Feldman & Hanks, 1968). Gentisoyl glucose could also be detected in stubborn infected material from California (Schwarz, 1968) but the technique was continued to be used as the concerned disease does not occur in South Africa.

Thin layer chromatography was used to index greening infected plants by reading plate profiles using either greening marker spots or gentisic glucoside as a standard marker (van Vuuren & da Graça, 1977; van den Berg *et al*., 1991-1992). Immunoaffinity chromatography has been used in conjunction with monoclonal antibodies to detect and purify greening organisms associated with the serotype specific monoclonal antibodies (Villenchanoux *et al*., 1992).

Electron microscopy

Electron microscopy has been used to confirm the presence of the bacteria and remains the only diagnostic tool that observes the non-culturable bacterium directly. Electron microscopic identification is based on two properties unique to the greening bacterium as compared to other bacteria in citrus – the restricted location within the sieve tube cells and the presence of a cell wall on the organism (Bové, 2006).

The first purified strain observed under the electron microscope was the Poona strain from an infected periwinkle plant, which was purified using immunoaffinity coupled with monoclonal antibodies (Villechanoux *et al*., 1990). This long standing technique is still being

used to confirm detection of Liberibacters in citrus plants in countries were the disease is newly discovered as in the case of the Lam species found in Brazil (Tanaka *et al*., 2006).

Monoclonal antibodies and ELISA

Monoclonal antibodies (MAbs) against specific greening isolates, developed by Garnier *et al* (1987), cannot be used as a universal detection tool since the monoclonals only react with the specific Liberibacter species used during the antiserum production. The MAbs allows for differentiation of Liberibacter serotypes obtained from different geographic regions (Villenchnoux *et al*., 1992; Ahlawat, 1997).

An attempt to produce MAbs that would recognize most or all strains of citrus greening was made by Gao and colleagues by *in vitro* immunizing spleen cells of mice with immunoaffinity chromatography purified antigenic protein of the Indian Poona strain (Gao *et al*., 1993). One of the three MAbs obtained reacted against all Las sources but not those obtained from China while the remaining two MAbs reacted with most Las sources but also not with the source from China. All three MAs failed to detect the South African Nelspruit Laf strain as expected (Gao *et al*., 1993) however Korsten *et al* (1993) reported that the Indian monoclonal 2D12 Poona source cross-reacted with greening infected material obtained from hotter citrus growing areas of Mooinooi and Pretoria in South Africa.

The inability to culture the bacterium has resulted in the production of only thirteen monoclonal antibodies against Liberibacter organisms. These two were against the Poona source, five were against the Chinese source and the other five were for the South African Nelspruit source (Bové, 2006).

DNA hybridization

DNA hybridization probes were designed and used in a DNA dot-blot hybridization system for the detection of both Laf and Las. This technique was used mostly for the detection of the disease in areas where it was not previously known to occur and when surveys are conducted. It proved to be successful in the discovery of the disease in countries such as Vietnam (Bové *et al*., 1996). The sensitivity of the technique is reported to be the same as that of the electron microscopy, with both techniques requiring high titers of the pathogen for detection (Villechanoux *et al*., 1992; Li *et al*., 2006). Southern blot hybridization using *rpl*KAJL-*rpo*BC probes for both Laf and Las have also been developed as detection tools (Planet *et al*., 1995). The need to utilise different probes for the detection of the Laf and Las and DNA extraction required for dot hybridization is the most limiting factor of this method (da Graça & Korsten, 2004).

Polymerase chain reaction

Different variations of the polymerase chain reaction (PCR) have been developed over the years in order to improve the sensitivity, specificity and reliability of detecting plant pathogens including the various "*Candidatus* Liberibacter" species.

PCR methods have been developed specific for all three "*Candidatus* Liberibacters". In 1996 primers targeting the 16S rDNA sequences were designed to detect the Laf and Las species but the method required digestion of the PCR product with Xba1 in order to differentiate the two species (Jagoueix *et al*., 1996; Li *et al*., 2006) therefore an alternate PCR method that would differentiate the two species was needed. This was

achieved when primers were designed to detect the ribosomal protein genes of the β-operon and to differentiate the Laf and Laf by their amplicon sizes (Hocquellet *et al*., 1999).

Hung *et al* developed primers that target the β-operon sequence of Las species only (Hung *et al*., 2000; Li *et al*., 2006) while nested PCR primers have also been developed to improve the sensitivity in the detection of the Liberibacters (Harakawa *et al*., 2000). Primers that detect the 16S rDNA of the Lam species have been recently developed after the emergence of the new species in Brazil (Colleta-Filho *et al*., 2005; Teixeira *et al*., 2005).

The Random Amplified Polymorphic DNA (RAPD) technique using single random 10 base primers has been used to reveal polymorphisms in genomic DNA and to isolate unknown genes of the uncultured bacterium. It has been able to distinguish organisms, species of the same organism without prior genome sequence knowledge or radio-labeling (Henson & French, 1993). Hocquellet and colleagues (2000) used 102 random primers in a PCR and consistently amplified eight DNA bands from plants infected with Las. After cloning and analyses, six PCR products were found to be of Liberibacter DNA and were identified as the *nus*G, *pgm*, *omp* and a hypothetical protein gene (Hocquellet *et al*., 1999; Hocquellet *et al*., 2000). The technique is limited in that purification of the target pathogen must first be achieved to avoid the amplification of the DNA of plants and other endogenous organisms, as random amplification of these is often encountered (Henson and French, 1993).

The conserved 16S rDNA of the Liberibacter bacteria contains sequence variations that are able to differentiate the three species. Quantitative real-time PCR primer-probe sets that are specific to all three Liberibacter

species were designed based on these conserved regions of the 16S rDNA (Li *et al*., 2006). A single probe, which can be used with species specific primers, was designed and used to detect all three Liberibacter species but only Las and Lam were amplified. A cytochrome oxidase primer probe was included to act as an internal control to assess the quality of DNA extracts and reaction reagents. This rapid, sensitive, reproducible and specific TaqMan quantitative PCR amplification assay has not been reported to be tested on Laf infected citrus samples to validate the ability of the probe to amplify all three species (Li *et al*., 2006).

Competitive PCR for the quantification of the Huanglongbing pathogen from various diseased citrus cultivars was developed in 2006 by Kawabe *et al* (2006). The quantification of the DNA was achieved by using image-analyses software, therefore the amplified DNA is only measured after the competitive PCR is completed (Kawabe *et al*., 2006).

Loop–mediated isothermal amplification (LAMP) of the *nus*G-*rpl*KAJL-*rpo*B gene cluster of citrus greening using primers that were previously designed to amplify this gene cluster was achieved (Okuda *et al*., 2005). Even though the loop-mediated PCR is sensitive, the concentration of the Liberibacter within infected plants and vectors determines the successful and consistent detection of the pathogen from infected plant material (Li *et al*., 2006). Another isothermal amplification method, cycleave isothermal and chimeric primer-initiated amplification, (cycleave ICAN) was developed for detection of 16S rDNA of Las (Urasaki *et al*., 2008).

The effective generation and characterization of unknown sequence data of the non-culturable bacterium can assist in the development of improved detection techniques and analyses of the genetic variability of

the bacterium (Bastianel *et al*., 2005; Lin *et al*., 2007) as was demonstrated with the variable 16S/23S ribosomal intergenic regions Las and Laf (Jagoueix *et al*., 1997) and through the use of PCR-based genomic walking approach which identified 8564bp unknown flanking genomic sequences of the *tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*BC gene cluster, *omp* gene and the 16/23S rRNA gene of the Las (Lin *et al*., 2007) .

2.9 Inhibition of Liberibacter PCR by plant extracts

PCR is the most used, reliable and stringent molecular method for detection of the greening pathogen but various factors undermine the functional capabilities thereof.

Wilson (1997) records three mechanisms in which inhibition of amplification may occur. The failure of lysis of the cell wall during RNA or DNA extraction procedure can result in no nucleic acids being released to serve as target for amplification. Nucleic acid degradation also results in the unavailability of the target DNA. Finally, polymerase inhibition by inactivation of the enzyme and interference in binding of the polymerase to the target molecule can cause amplification failure.

The capture of cultivable pathogens by antibodies has been used to avoid the inhibition of PCR by DNA extract contaminants which negatively affect most conventional PCR tests. However bacteria that cannot be cultured, require monoclonal antibodies to be produced and used against the specific serotype for immunisation. The detection of other serotypes can only be done if antibodies reacting with those specific serotypes are produced (Jagoueix *et al*., 1996).

A protocol involving the extraction of total DNA from a plant and the trapping thereof onto a column was developed (Jagoueix *et al*., 1996). The method however does not evaluate the presence of any extracted DNA and inhibition of the polymerase activity and false negatives are possible. A method that detects extracted DNA and potential inhibitory effects, will overcome the problem of false negatives and should be used. Multiplex (RT)-PCR assays have been developed to overcome the above-mentioned problems encountered during PCR amplifications of plant pathogens (Nassuth *et al*., 2000; Menzel *et al*., 2002). The use of internal control gene with the co-amplification of one or more plant viruses of staple commercial crops has been exploited and serves as a reliable detection method for the relevant plant viruses. This method of detection also serves as a cost, time and effort saving strategy when compared to individual PCR reactions (Nie *et al*., 2000; Sharman *et al*., 2000; Markoulatos *et al*., 2002; Menzel *et al*., 2002).

The real-time PCR developed by Li *et al* (2006) for the detection of the three known Liberibacters using a single probe and three primer sets together with an internal control, is the most technologically advanced method used for detection and diagnosis of citrus greening disease thus far. The endogenous internal control DNA targeted is the cytochrome oxidase genes of plants. This assay is able to assess the quality of the DNA extracts and monitors the presence of inhibitors that are found in various plant extracts by amplifying the internal control DNA. Even though this quantitative real-time PCR offers a robust and reliable detection method for citrus greening with lower cross contamination, the costs involved preclude its use in laboratories that can afford only basic molecular detection equipment (Li *et al*., 2006; Schaad & Frederick, 2002).

The use of PCR with internal controls for the detection of viral pathogens have been utilised previously. Reverse transcriptase PCR (RT-PCR) with an internal control was developed for the testing of plant and viral mRNA from grapevine (grapevine virus A and B, grapevine associated virus 3, arabis mosaic virus and rupestris stem pitting associated virus), apple (apple stem pitting virus), raspberry (tobacco ringspot virus), strawberry (strawberry mild yellow edge virus), peach, apricot, plum and wheat (wheat streak mosaic virus). The internal control used was designed from the Ribulose bisphophate carboxylase oxygenase genes (Rubisco L) (Nassath *et al*., 2000).

Mitochondrial DNA, *nad*-5 gene (NADH dehydrogenase subunit 5) from apple plants was used as an internal control within the PCR system for the amplification of apple chlorotic leaf spot virus, apple stem pitting virus, apple mosaic virus and apple stem grooving virus (Menzel *et al*., 2002).

For effective use, an internal control should have several properties: be a ubiquitous plant metabolic product; internal control primers should amplify non-degraded DNA; indicate any inhibition of polymerase activity and successful amplification thereof should eliminate false-negative results (Nassuth *et al*., 2000).

2.10 Rutaceae host range for *Trioza erytreae*

The development from egg to adult of *T. erytreae* has been recorded on a number of rutaceous plants indigenous to Southern Africa. These include *Clausena anisata* (Wild) Hook. f. ex Benth., *Vepris lanceolata* (Lam) G. Don., *Zanthoxylum capense* (Thumb) Harv., *Oricia* and *Fagara* species (van den Berg, 1990). Moran (1968 a, b) reported that *V. lanceolata* was the preferred host plant and also supports nymphal development of the

citrus psylla when compared to *C. anisata*, *Z. capense* and *Calodendrum capense*. The *C. capense* leaves attract the adult citrus psyllid for feeding but are not suitable for nymphal development. In another study, Moran and Buchan (1975) concluded that leaf hardness characteristics could not be a factor in host plant discrimination by *T. erytreae*, *between Citrus limon* and indigenous hosts, but that the larger soft flush leaves of lemon which flush regularly thus provided copious, soft flush for oviposition and nymphal development.

Van den Berg *et al* (1987) monitored indigenous plants near a citrus orchard and found citrus psylla *T. erytreae* on fifty *C. anisata*, twenty *Z. capense* and ten *V. lanceolata* plants. Adult citrus psylla were also found, in a feeding position, on *Casimiroa edulis* however it was uncertain as to whether the psylla could feed on the plant (van den Berg & Deacon, 1989). It is not mentioned whether these plants were monitored for the presence of the greening disease or the symptoms thereof. *T. erytreae* was observed to be feeding on the prevalent *C. anisata* trees in the highlands of Cameroon and Ethiopia (Aubert *et al*., 1988).

The continuous movement of citrus psylla between unsprayed citrus orchards and indigenous host plants surrounding the orchards has been observed (van den Berg *et al*., 1991; van den Berg *et al*., 1991-1992). Possible vector transmission of the greening pathogen from infected citrus to other rutaceous plants could lead to a sustained cycle of transmission of the pathogen. This possibility still needs to be determined experimentally to identify new hosts of the fastidious bacterium.

2.11 Host range of "*Candidatus* Liberibacter" species

Host range for Liberibacter species amongst *Citrus* species

All citrus species are considered susceptible to greening regardless of rootstock used (Bové, 2006). The cultivars can be divided into symptom groups. Severely affected ones include sweet orange, tangelo and mandarin; moderately affected ones include grapefruit, lemon and sour orange and the tolerant ones are lime, pummelo and trifoliate orange (le Roux *et al*., 2006). Rootstock cultivars are also susceptible to infection by greening with citrus cultivars planted on *Poncirus trifoliata* and its hybrid rootstocks producing more greening infected fruit than other rootstock cultivars (Pretorius & van Vuuren, 2006).

McClean and Schwarz (1970) produced a thorough description of citrus greening symptoms exhibited on various citrus cultivars in the field and seedlings exposed for natural infection by *T. erytreae*. Sweet oranges are amongst the most susceptible citrus varieties to greening infection in South Africa (McClean & Oberholzer, 1965 a). Mandarin (*C. reticulata* Blanco) show severe stunting, yellowing and mottling of leaves, but are less susceptible to infection compared to sweet oranges. All tangerines showed symptoms after the transmission of the pathogen by the vector (McClean & Schwarz, 1970; Miyakawa & Yuan, 1990).

Grapefruit (*C. paradisi* Macfadyen) are less affected by greening, probably due to low transmission rates. Sour orange (*C. aurantium*) seedlings that were graft-transmitted with greening reacted by producing blotchy-mottle symptoms on older leaves. Sub-inoculations to sweet orange also produced symptoms. Psylla could infect 5 out of 6 of the sour oranges with the positive results being confirmed by graft-

inoculations to other citrus seedlings. Eureka lemon (*C. limon* (Linn) Burman) showed typical uneven blotchy-mottling while rough lemon (*C. jambhiri*) produces blotchy-mottle or faint symptoms and is more tolerant to the disease than other vulnerable citrus cultivars (McClean & Schwarz, 1970).

Lime (*C. aurantifolia* (Christm.) Swing) is easily infected by greening when exposed to the transmitting psylla or when the pathogen is graft-transmitted from infected sources. Trifoliate orange (*Poncirus trifoliata*) does not demonstrate well defined symptoms but is able to host the bacterium. This was proved by McClean and Schwarz when they exposed trifoliate orange seedlings for three months in an orchard with greening infected citrus (McClean & Schwarz, 1970). Troyer citrange, a hybrid of *P. trifoliata* X *C. sinensis*, can also host greening naturally and this was confirmed by graft-transmission of the pathogen to seedlings of Troyer (McClean & Bové, 1970).

Host range of Liberibacter species amongst other Rutaceae

Efforts to detect the citrus greening pathogen from endemic plants in the different areas of South Africa have resulted in the discovery of various rutaceous plants that can host the pathogen. Alternate hosts are important in the introduction and distribution of plant pathogens in areas where the disease has not been reported.

Side-grafting of an healthy citrus indicator plants to *C. anisata*, located in a field close to infected citrus orchard, resulted in the development of symptoms on the indicator plant and positive testing of one of the indicator plants as analyzed by thin layer chromatography indicating that *C. anisata* hosts Laf (van den Berg *et al*., 1991-1992).

Korsten *et al* (1996) successfully used dot hybridization probes as well as PCR to detect the greening pathogen from a *V. lanceolata* cited by its previous name *Toddalia lanceolata* Lam in the article. Garnier *et al* (2000 a and 2000 b) detected Liberibacter DNA from a *C. capense* tree indigenous to the Western Cape Province. They further identified the bacterium as a subspecies of Laf designated "*Candidatus* Liberibacter africanus subspecies capensis". PCR amplification of the ribosomal 16s rDNA and ribosomal protein gene, MAbs serological test and restriction enzymes were used to detect the bacterium.

Detection of the Las and Lam from other rutaceous plants in other countries has also been recorded. Multiplication of the greening organism, after graft-inoculation, in plants that are suitable hosts for the Asian citrus psyllid was monitored by DNA hybridization and PCR. Liberibacter DNA was successfully detected in Chinese box orange *Severinia buxifolia* (Poiret) Ten and wood apple, *Limonia acidissima* L grafted with infected scions of a Valencia sweet orange plant (Hung *et al*., 2000). A southern Taiwan field sample of *S. buxifolia* also tested positive for greening after a dot hybridization test (Koizumi *et al*., 1996; Hung *et al*., 2000; Hung *et al*., 2001). The ornamental *S. buxifolia* is also known as *Atalantia buxifolia* (Poir.) Oliv. Graft transmission and PCR detection of Las was reported by Deng *et al* (2008) after inoculating two buds of a symptomatic *A. buxifolia* onto mandarin trees which later produced the mottling symptoms previously seen on the ornamental plant.

In Brazil, adult *Murraya paniculata* with yellow shoots, shoot die-back and an adult *D. citri* psylla that was feeding on the plant, tested PCR positive for the presence of Lam. As a result, a survey was conducted over 5 municipalities, and 2 of the 360 *Murraya* samples collected, tested positive for Las while 37 were infected with the Lam. The wide-spread

occurrence of *M. paniculata* represents a threat to the citrus production in Brazil due to the usage of the plant as an exotic tree mainly found along the municipality routes, in the parks and cemeteries (Lopes *et al*., 2006).

There has been reports on the transmission of Las from naturally infected *M. paniculata* to citrus via dodder (*Cuscuta pentagona*) (Zhou *et al*., 2007) and the detection of the pathogen from *M. paniculata* symptomatic leaves using nested-PCR in China (Deng *et al*., 2007).

Vector transmission of the bacterium was achieved by allowing healthy *D. citri* to feed and acquire Las from infected sweet orange plants and then transmitting it to *M. paniculata* seedlings. Reciprocal assays of transmitting the pathogen from infected *M. paniculata* to sweet orange seedlings via the psyllid vector were successful. Real time PCR was used to detect the pathogen during both assays (Damsteegt *et al*., 2007).

These reports clear the uncertainty with regards to the ability of *M. paniculata* to host the pathogen and the vector transmissibility thereof by *D. citri*. The pathogen could previously not be detected by microscopy and dot hybridization even though internal and external symptoms were observed from grafted citrus seedlings and after vector transmission of the pathogen to the plant was considered to be successful (Miyakawa & Yuan, 1990; Koizumi *et al*., 1996; Hung *et al*., 2000; Halbert & Manjunath, 2004).

The recent detection of the Huanglongbing pathogen in infected *Clausena lansium* (Lour.) Skeels (known as wampee) plants in China, threatens the production of one of the most popular species within the *Rutaceae* family grown in the tropical and subtropical parts, which is having an expanding cultivation due to their commercial value in China.

Las was amplified using single-step and nested PCR from *C. lansium* (Ding *et al*., 2005).

Clausena indica and *C. lansium* were previously regarded as host plants of the disease based on symptoms expressed, whereas *Clausena anisum*, *Clausena excavate*, *C. indica* and *C. lansium* are hosts to the Las vector *D. citri* but not the bacterium itself (Halbert & Manjunath, 2004).

The difficulty in detection of the pathogen due to low concentrations has created uncertainty as to whether a citrus relative plant is a host or non-host for the Liberibacter. Classical symptoms associated with the pathogen have been used as criteria to assign a citrus relative plant as a host as indicated in Table 2.1 (Halbert & Manjunath, 2004).

Host range of Liberibacter species amongst non-rutaceous plants

Cuscuta (dodder) is considered a host plant for greening since the pathogen can multiply within the dodder and supports higher titres of the bacterium than observed in citrus (da Graça., 1991). Dodder has been used experimentally to transmit the Las and Laf successfully to *Catharanthus roseus* (L.) G. Don (periwinkle) (Garnier & Bové, 1983).

Dodder has also been used to transmit Las, Laf and recently Lam species to *Nicotiana tabacum* L. cv. Xanthi (tobacco) seedlings (Olfato *et al*., 1991; Garnier *et al*., 1993 and Franscischini *et al*., 2007). The morphology of the organism does not appear different in citrus to that found in *C. roseus* and *N. tabacum* after transmission via a *Cuscuta campestris* bridge plant (Garnier & Bové, 1983; Chung *et al*., 1991; Franscischini *et al*., 2007).

Dodder transmission of Las to tomato *Lycopersicon esculentum* has been reported indicating further successful transmission of the Liberibacter bacterium to plants outside the Rutaceae family and its possible non-specific and known broad host range (Duan *et al*., 2008). The Manapal and FL47 tomato cultivars were draped with *Cuscata pentagona* dodder shoots that were already established on infected sweet orange plants. The tomato plants showed vein clearing and typical symptoms of blotchy mottle, thick and leathery leaves and lopsided fruits post transmission of the bacterium. PCR with primers OI1/OI2c and A2/J5 confirmed presence of Las DNA from the tomatoes (Duan *et al*., 2008).

The ability of adult citrus psylla to feed on non-rutaceous plants further suggests that these plants could possible be involved in the maintenance of the disease (van den Berg *et al*., 1991-1992) but can only be confirmed if the pathogen can be detected in the psylla and in the plant itself. The difficulty in detecting the pathogen from non-rutaceous plants maybe due to inhibitory compounds within different plant species. These plants, especially those growing near citrus orchards, could be a major factor, as an inoculum source, in the transmission or reintroduction of the disease within orchards or those that have been eradicated of the disease.

Table 2.1 Host range of Laf, Las and Lam as identified by molecular techniques or symptom observation (Halbert and Manjunath, 2004) with modifications.

Plant species	Comments
Aeglopsis chevalieri Swingle	Questionable symptoms
Atalantia missionis Oliver	Symptoms only, vector transmission
Balsamocitrus dawei Stapf.	Symptoms only, vector transmission
Calodendrum capense Thunb.	Molecular characterization
Catharanthus roseus (L.) G. Don	Symptoms, electron microscopy; (dodder transmission only)
X *Citroncirus webberi* J. Ingram & H.E. Moore	Symptoms (few) stunting, seed abortion, symptoms fairly intense
Citrus amblycarpa Ochse	
Citrus aurantifolia (Christm.) Swingle	Mild symptoms
Citrus aurantium L.	Symptoms
Citrus depressa Hayata	Symptoms
Citrus grandis (L.) Osbeck	Symptoms, pomelo-infecting strain prevalent since 1970's. *C. grandis* is considered a junior synonym of *C. maxima*
Citrus hassaku Hort. ex Tanaka	Symptoms
Citrus hystrix DC.	Symptoms
Citrus ichangensis Swingle	Symptoms
Citrus jambhiri Lushington	Symptoms (fair) (McClean & Schwarz, 1970)
Citrus junos Sieb. ex Tanaka	Symptoms
Citrus kabuchi Hort. ex Tanaka	Symptoms
Citrus limon (L.) Burm. f.	Symptoms, presence of putative pathogen in tissue; plant reported tolerant to disease, but source of vectors
Citrus x limonia Osbeck	Symptoms

Table 2.1 Continued

Plant species	Comments
Citrus x nobilis Lour. 'Ortanique'	Symptoms
Citrus x nobilis Lour.	Symptoms
Citrus x paradisi Macfad.	Symptoms
Citrus reticulata Blanco	Symptoms
Citrus sinensis (L.) Osbeck	Symptoms, presence of putative pathogen in tissue
Citrus sunki Hort. ex Tanaka	Symptoms
Citrus unshiu (Mack.) Marc	Symptoms
Citrus sp. (mandarins)	Symptoms
Citrus sp. (pomelo/shaddock)	Symptoms
Clausena anisata (Willd.) Hook. F. ex Benth,	Symptoms, graft transmission (van der Berg *et al*., 1991 -1992)
Clausena indica Oliver	Symptoms (stunting)
Clausena lansium (Lour.) Skeels	Symptoms only, vector transmission
Cuscuta australis R. Br.	Observed to multiply in stems, haustoria and flower stalks
Fortunella spp.	Symptoms
Limonia acidissima L.	Symptoms only; vector transmission; DNA hybridization; infection apparently temporary
Microcitrus australasica (F.J. Muell.) Swingle	Stunting
Murraya koenigii (L.) Sprengel	No detection by dot hybridization after attempted graft transmission; no symptoms
Murraya paniculata (L.) Jack	Mixed results: Detection by PCR (Lopes *et al*., 2006) , symptoms only (external and internal), vector transmission, can harbour greening organism, EM negative, no detection by dot hybridization after attempted graft transmission, no symptoms, not a host

Table 2.1 Continued

Plant species	Comments
Nicotiana tabacum L. 'Xanthii'	Symptoms, dodder transmission only
Poncirus trifoliata (L.) Raf.	Back inoculations
Severinia buxifolia (Pioret) Ten.	DNA hybridization with specific probe; symptoms
Swinglea glutinosa (Blanco) Merr.	Symptoms only, vector transmission
Triphasia trifolia (Burm. f.) P. Wilson	Severe stunting, vector transmission
Vepris lanceolata (Don) G. Don = *Toddalia lanceolata* Lam	DNA/DNA hybridization, PCR
Possible non-hosts:	
Citrus indica Tanaka	No symptoms in the endemic field
Citrus limetta Risso	No symptoms, non-specified laboratory inoculation
Citrus macroptera Montrons	No symptoms in the endemic field

2.12 Control and management of the disease

Biological control by cross-protection

In South Africa it was reported that a population of strains of *Citrus tristeza virus* (CTV)were able to cross-protect Valencia sweet orange trees from Laf (van Vuuren *et al*., 2000). Glasshouse work was done to investigate the causative agent(s), present in the GXI isolate [greening cross-protecting isolate, formerly known as citrus dwarfing isolate (CD 4)]. Potential agents within GXI included citrus viroids, citrus psorosis, citrus impietratura and citrus tatter leaf. CTV was the only agent found in the isolate and therefore this agent was thought to be responsible for the cross protection against citrus greening (van Vuuren *et al*., 2000).

The reason for this cross protection is not known but is thought to be a result of chemical changes in the plant cells, the plant producing protective substances onto the surfaces or the production of substances in the plant tissue which prohibits entrance or multiplication of the pathogen (van Vuuren *et al*., 2000; Halbert & Manjunath, 2004). This finding contradicts a report in which severe symptoms were noted on the mandarin (*Citrus reticulate* BL.) after simultaneous infection of CTV and Las in Thailand (Prommintara, 1990). This might have been due to the use of two different CTV sources, two Liberibacters Las and Laf and even two different scion cultivars that were used in the experiments

Control of *Trioza erytreae* by other insects

Tetrastichus species are parasites known to control psylla populations on citrus trees. *Tetrastichus* species are ectoparasites as they kill the *T. erytreae* from the outside. In Reunion Island *Tetrastichus dryi* alone or

together with *Psyllaephagus pulvinatus* wasps were reported to be successful in controlling *T. erytreae* and *D. citri*, mainly because of the absence of hyperparasitoids. These species were encountered in a survey for citrus greening diseases in African countries (Aubert & Quilici, 1984). The control of the psylla by the parasitoids was not sufficient to allow the conclusion that the psylla was being biologically controlled by these insects (Aubert & Quilici, 1984; Aubert *et al*., 1988; Etienne & Aubert, 1980; Pretorius & van Vuuren, 2006). The best predatory control on citrus psylla found on rutaceous plants in South Africa was achieved by ants rather than coccinellids, Hemiptera and hemerobiids insect species (van den Berg *et al*., 1987).

Predators of the psylla do reduce the population, but they are incapable of reducing the population to levels required to ease the losses caused by the citrus greening disease due to the presence of hyperparasitoids (van den Berg *et al*., 1987).

Cultural practices for citrus plants

No resistance mechanism is known against this disease and therefore management is largely based on: (1) propagation and planting of disease-free nursery material; (2) implementation of quarantine barriers to prevent the spread of the disease to disease-free areas; (3) pruning of the infected branches and treatment of the re-growth with insecticide to avoid psylla feeding sites on the plant; (4) the removal of neglected infected orchards reducing the greening inoculum (Pretorius & van Vuuren, 2006; le Roux *et al*., 2006).

In order to further augment the management and control strategies of the disease in South Africa, it is recommended that infected trees at the following stages be removed: trees up to 5 years that are showing

symptoms; trees that are 6 to 10 years old which are 50% or more infected; those that are 10 years and older or that have branches with 40% infection should be pruned (Buitendag & von Broembsen, 1993; le Roux *et al*., 2006).

Psylla control by means of trunk application of insecticides applied as or soil drenches is the most important facet of controlling greening disease. With efficient psylla control, other control measures become redundant (Pretorius & van Vuuren, 2006).

Soil applications of insecticides include Aldicarb (Temik®) (G) 12.5g/m^2 and imidacloprid (Confidor®) which offer 25- and 52-day protection respectively. For stem applications acetamiprid (Mospilan®) and methamidophos (Citrimet®) are used and offer 42- and 18-day protection respectively while various registered insecticides are used for foliar applications (Pretorius & van Vuuren, 2006).

Thermotherapy on infected budwood has been used to eliminate the pathogen in the past. Budwood was placed on a grid suspended over the water surface of a hot water bath at different temperature for a specified time but loss in viability of tissue was observed at higher temperatures (Schwarz & Green, 1972). In another experiment, exposure of symptomatic plants to 30°C temperature resulted in the disappearance of the symptoms and greening free plants were confirmed by thin layer chromatography of gentisic acid (Labuschagne & Kotze, 1988). Thermotherapy of orchard trees is regarded as impractical for large scale use (le Roux *et al*., 2006).

Chemotherapeutic treatment of infected plants by means of trunk application of tetracycline hydrochloride injections at various dosages

were utilised in the 1970s. Temporary reduction of the pathogen was achieved but with toxic consequences. Toxicity effects on injected citrus trees included narrow leaves, photosensitivity, occasional die-back of twigs, stained heartwood, small fruits and brown staining of heartwood (Moll *et al*., 1980). N-pyrolidinomethyl tetracycline was used as an alternative in order to avoid the toxic effects of the tetracycline hydrochloride injections but this antibiotic also only provided temporary relief from the disease (Buitendag & van Broembsen, 1993). Treatment of infected trees with penicillium was not successful in South Africa (Schwarz *et al*., 1974). Antibiotic treatment in South Africa is no longer applied (le Roux *et al*., 2006).

Management of indigenous hosts

van den Berg (1991-1992) recommended that all *C. anisata* growing in the proximity of citrus orchards ought to be eliminated as it had been shown to be a host plant of the bacterium and the *T. erytreae* psyllid vector. Korsten (1996) only found *V. lanceolata* as a natural reservoir of the greening organism but made no recommendations for the management of the plant.

2.13 Discussion

The impact of alternate host plants for pathogens of economically important crops can be significant. The ability of the host plant to serve as a reservoir of the pathogen can result in the transmission thereof to citrus thus maintaining and even increasing the cycle of infection between the citrus and indigenous plants. Effective monitoring of the possible alternate hosts by the use of the existing molecular techniques would aid in the identification of unknown hosts. The role and

identification of psyllid vectors in the possible transmission of the pathogen between citrus and alternate indigenous hosts also needs to be determined. Alternate host plants should be considered in the control and management of the disease.

Chapter 3

Development of a multiplex PCR protocol for the detection of Laf and plant internal control DNA

3.1 Introduction

Citrus greening disease is widely distributed across most citrus producing areas of South Africa. The eradication of the disease in countries with recent outbreaks have occurred and those countries that have had the disease for many years is critical. This bacterial citrus disease has been present in South Africa since the early 1920's and is regarded as a threat to the citrus production industry (Oberholzer *et al*., 1965). The detection of the pathogen in citrus plays a key role in the management and control of the disease, given that infected citrus plants need to be removed to reduce inoculum sources for the psylla and the planting of disease-free material.

The detection of the pathogen is done using molecular detection techniques such as DNA-DNA hybridization (Bové *et al*., 1993), ELISA, PCR, monoclonal antibodies, combination of PCR and restriction enzyme digestion using HindIII and XbaI (Villenchanoux *et al*., 1992; Jagoueix *et al*., 1996). The latter techniques are considered to be the most sensitive detection methods as they are based on the detection of DNA. They remain influenced, however, by the DNA concentration of the pathogen and other factors. The greening organism is normally present in low concentration and is erratically distributed within the infected citrus plants therefore correct sampling of tissue is critical in the detection of the pathogen.

False-negative results can be obtained when using conventional PCR possible due to the inhibition of the PCR by plant compounds that were extracted together with the DNA during the DNA extraction procedure. A more sensitive PCR technique and one that would reveal inhibition and plant extract quality is required for testing citrus samples for presence of Liberibacter species. A multiplex PCR with an internal

control to plant DNA can be used to indicate the successful extraction of quality DNA and inhibition of the amplification reaction.

By altering a conventional PCR method to detect two or more targeted sequence loci, by the addition of two or more primers to the reaction, a multiplex reaction is achieved. Multiplex PCR has been used as a valuable technique for identification of infectious viruses, bacteria, fungi and/or parasites (Elnifro *et al*., 2000). The two targeted sequence loci are represented, in this study, by an ubiquitous plant house-keeping gene, or internal control DNA, and the citrus greening bacterium ribosomal DNA. The presence of the internal control DNA product after PCR indicates that no significant inhibition to the PCR occurred while its absence indicates inhibition to the PCR or a failed DNA extraction process.

Even though a real-time qualitative PCR with a cytochrome oxidase internal control has been developed for the detection and identification of Liberibacter species (Li *et al*., 2006), its major disadvantage is the cost involved in acquiring the equipment and routine large scale usage for detection of the pathogen.

3.2 Detection of ribulose bisphosphate carboxylase oxygenase genes using PCR

Internal control, plant-specific primers have been used in the detection of plant viruses in various commercial plant crops in multiplex PCR systems. These primers are designed to amplify known metabolic RNA or DNA genes of plants as control templates. When using plant metabolic product genes as controls, it is important they are constantly produced by the plants and is accessible for amplification. The amplification of these ubiquitous genes is influenced by factors such as

extraction and/or PCR inhibitors, quality and quantity of the extracted RNA or DNA.

Ribulose bisphosphate carboxylase oxygenase (Rubisco) is an enzymatic protein that is involved in the photosynthesis process in plants and is one of the largest enzymes in nature with a molecular mass of 560kDa. Large amounts of the enzyme are found in the chloroplast stroma. It accounts for 50% of proteins within the cell and is responsible for catalyzing carbon dioxide and ribulose bisphosphate to phosphoglyceric acid which is further catalyzed to glucose (Spreitzer & Salvucci, 2002; Madigan *et al*., 2003; Parry *et al*., 2003).

Nassuth *et al* (2000) utilized the inclusion of an internal gene control in a RT-PCR for the detection of several viral RNA's. The Ribulose bisphosphate carboxylase oxygenase large subunit (RubiscoL) genes were used as internal controls which would show consistency in the yield of RNA extracted from the plant and in this way indicate the presence of both the RubiscoL and viral RNA and inhibitory effects on the activity of the reverse transcriptase or of the polymerase (Nassuth *et al*., 2000; Menzel *et al*., 2002). Two primer sets were designed, from several known plant Rubisco sequences, to universally amplify the targeted conserved regions of genes, also from plants whose Rubisco sequences are unknown (Nassuth *et al*., 2000).

In this study primers targeting the RubiscoL genes and the β operon ribosomal proteins of Las, Laf or LafC were used in a multiplex PCR to simultaneously amplify the Liberibacter and internal control DNA within known infected citrus plants used as models for the development of the multiplex PCR.

Table 3.1 Examples of commercially important plant pathogens that have been detected using multiplex PCR with internal control.

Plant	Pathogen	Source
Grapevine	*Grapevine virus A, Grapevine virus B, Grapevine leafroll associated virus 3, Arabis mosaic virus, Rupestris stem pitting associated virus*	Nassuth *et al.*, 2000
	Arabis mosaic virus, Grapevine fanleaf virus, Grapevine virus A, Grapevine virus B, Rupestris stem pitting-associated virus, Grapevine fleck virus, Grapevine leafroll-associated virus-1, -2, and -3	Gambino & Gribaudo, 2006
Strawberry	*Strawberry crinkle virus, Strawberry mild yellow edge virus, Strawberry mottle virus, Strawberry vein banding virus*	Thompson *et al.*, 2003
Peach	*Prune dwarf virus*	Sánchez-Navarro *et al.*, 2005
Apricot	*Apple mosaic virus*	Sánchez-Navarro *et al.*, 2005
Plum	*Apple chlorotic leaf spot virus, American plum line pattern virus, Plum bark necrosis stem pitting associated virus, Prunus necrotic ringspot virus* and *Plum pox virus*	Sánchez-Navarro *et al.*, 2005
Cherry	*Prunus necrotic ringspot virus*	Sánchez-Navarro *et al.*, 2005
Citrus	*Citrus exocortis viroid, Citrus bent leaf viroid, Hop stunt viroid, Citrus viroid-III and Citrus viroid-IV*	Wang *et al.*, 2009
GF305 (stone fruit)	*Apricot latent virus* and *Plum pox virus*	Sánchez-Navarro *et al.*, 2005
Potatoes	*Clavibacter michiganensis* subsp. *Sepedonicus*	Pastrik *et al.*, 2002
	Ralstonia solanacearum	Pastrik, 2000
	Potato virus Y	Nie and Singh, 2000

3.3 Materials and Methods

Total DNA extraction

Total DNA extraction from plants were performed using the CTAB extraction method by Doyle and Doyle (1990) with modifications according to Brown (University of Arizona) or Fundecitrus as described in appendix A1.1 and A1.2.

PCR amplification of internal control (RubiscoL) DNA

PCR amplification conditions similar to those used for the amplification of Laf and Las DNA as described by Hocquellet *et al* (1999) were used for the detection of RubiscoL DNA. RBCL-H535 forward and RBCL-C705 reverse primers were used for amplifying the Ribulose bisphosphate carboxylase oxygenase gene (Nassuth *et al.*, 2000).

The primer concentrations were optimized for amplification in the multiplex PCR. Primer concentrations tested were 0.5uM, 1.0uM, 2.0uM, 3.0uM, 5.0uM and 10.0uM. Aliquots of working solutions were prepared from a primer stock solution of 100uM and stored at -20°C. A no-DNA template reaction, used as negative control, and a healthy control were included in the PCR.

The reaction mixture was added to the final volume of 50.5µl containing 2.5µl of 2% Triton X-100, 5µl of 10x NH_4 reaction buffer (Bioline, Boston, USA), 5µl of 3.5mM deoxyribonucleotide triphosphate mix (dNTPs), 2µM RBCL-H535 primer, 2µM RBCL-C705 primer, 10µM A2 primer, 10µM J5 primer, 2µl of 0.05M $MgCl_2$, 5µl of 10mM 2-mercaptoethanol, 5µl of 2000ug/ml BSA, 0.5µl of 2.5 units Biotaq™ DNA polymerase

(Bioline, Boston, USA) and 20µl molecular grade water (SIGMA, Missouri, USA). Only 50µl of reaction mixture was used for PCR.

PCR thermal cycling programme was conducted using a GenAmp® PCR System 2700 (Applied Biosystems, California, USA) thermo-cycler. PCR amplification cycles programme consisted of 35 cycles each of denaturing step (92°C for 20s), annealing (62°C for 20s) and extension (72°C for 45s). Five microliters of each amplified product was mixed with 1µl of loading dye and loaded on a 1% (w/v) agarose gel of 1X SB or TAE buffer (appendix A1). The agarose gel was stained with 5µl ethidium bromide (5ug/ml) (appendix A1) and electrophoresed at 100V for 30 minutes and viewed under an Ultraviolet transilluminator.

Simultaneous amplification of Laf and internal control DNA

DNA primers for the detection of β ribosomal protein operon of Laf and the RubiscoL DNA were used in one reaction under PCR conditions adapted to amplify the two targeted fragments. A_2 forward and J_5 reverse primers (Hocquellet *et al*., 1999) were used together with the RBCL-H535 forward and RBCL-C705 reverse primer pair (Nassuth *et al*., 2000) at the pre-selected concentrations. Nucleic acid extracts that previously tested positive and negative for Laf were included as positive and healthy controls respectively in multiplex PCR tests. The PCR reaction mix and thermocycler conditions were done as in Annexure A2.2. Resulting PCR products were mixed with loading dye (appendix A1) and loaded onto a 1% agarose gel stained with ethidium bromide and viewed under a UV transilluminator.

Sensitivity test

To determine the sensitivity level of the multiplex PCR, 10-fold serial dilutions (10^{-1} – 10^{-10}) of total DNA extract from a Laf infected plant which was originally suspended in 100µl TE buffer, in total DNA extract of a healthy sample were done. From each dilution 1µl was used as template in the multiplex PCR. Figure 3.3 shows sensitivity of the multiplex PCR as observed on a 1% agarose gel.

Table 3.2 Primers for amplification of RubiscoL gene of plants and β ribosomal protein operon of Liberibacter DNA.

Primer	Primer sequence 5′ to 3′	Product size
RBCL-535	CTTTCCAAGGCCCGCCTCA	171 bp
RBCL-C705 Nassuth (2000)	CATCATCTTTGGTAAAATCAAGTCCA	
A_2	TATAAAGGTTGACCTTTCGAGTTT	669 bp
J_5 Hocquellet (1999)	ACAAAAGCAGAAATAGCACGAACAA	
CAL1 Garnier (2000)	GATTCGTAGAGGTGTTTTTTGAGG	588 bp

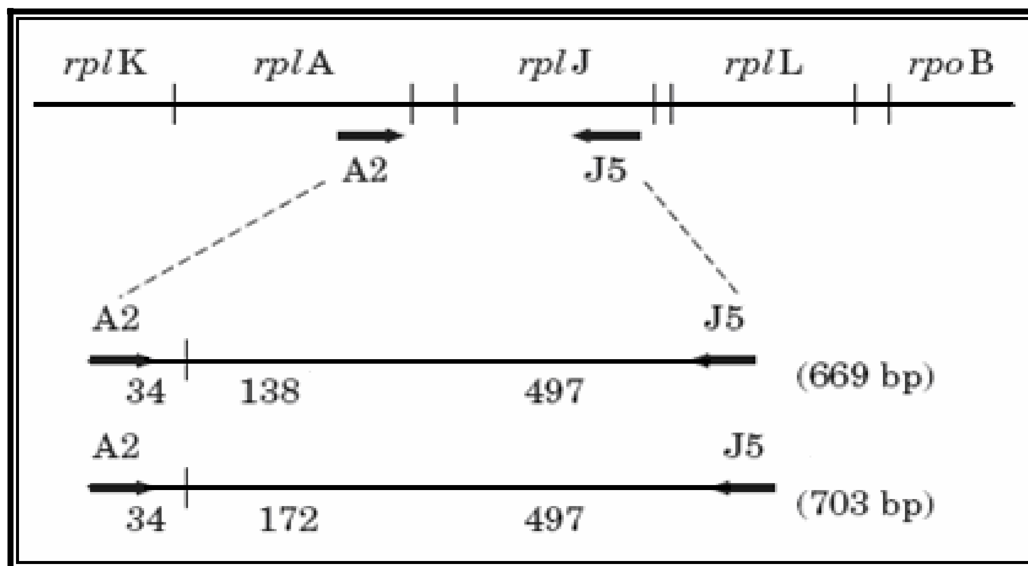

Figure 3.1 Ribosomal proteins of the β operon of Laf and Las and binding sites of the A2/J5 primers as adopted from Hocquellet *et al* (1999).

3.4 Results

PCR amplification of the RubiscoL internal control DNA

Nassuth *et al* (2000) obtained expected size fragments for RubiscoL from tissues of grapevine, apple, raspberry, strawberry, wheat, peach, apricot and plum. PCR was done to test whether these primers would amplify expected fragment from citrus extracts. PCR fragments of the correct size, compared to a 100bp DNA size ladder marker (Promega, Madison, USA), could be observed under ultraviolet transilluminator on a 1% (w/v) SB or TAE agarose gel in the presence of a positive control template after electrophoresis. The expected fragment of 171bp was amplified steadily from the DNA extract. Amplification of the internal control was obtained with all the various primer concentrations (0.5-10.0uM) tested but different amplification band intensities were observed. PCR products with low intensity were obtained with lower primer concentrations (0.5 - 2 uM) while the intensity of the products increased with the higher primer concentrations (Figure 3.2).

Simultaneous amplification of Laf and internal control DNA

RubiscoL and Liberibacter (Laf, Las and LafC) specific primers were added to a 25µl or 50µl PCR reaction mixture for the development of the multiplex PCR. Combining the two primer pairs and the use of the same cycling conditions resulted in co-amplification of 669bp and 171bp amplicons from a known citrus greening infected plant nucleic acid extract. The 669bp and 171bp amplicons represents the Laf and RubiscoL DNA respectively.

The concentration of the RubiscoL primers had to be lowered to 2µM to prevent high intensity products and competition for dNTP's by the two primer pairs A2/J5 and RBCL 535/RBCL C705. Concentration of the Laf primers were kept at 10µM. The two primer concentrations did not result in any non-specific hybridization, as seen by the lack of additional bands (Figure 3.4). Combining the greening and RubiscoL primers was successful in co-amplification of Laf and RubiscoL control DNA from a known infected source with varying band intensity levels.

Sensitivity test

The specificity of the two primer pairs were determined using extracted DNA with mixed bacterial infections and theoretically using primer design programs (Hocquellet *et al*., 1999; Nassuth *et al*., 2000). The detection limit of the primer pairs in this newly developed multiplex PCR had to be tested to avoid failure of the technique and false results. Total nucleic acid extracts from a plant that previously tested PCR positive for Laf was diluted in total DNA of a healthy extract. The highest dilution at which Laf could still be positively amplified was 10^{-6} while the amplicons for the internal control could be detected in all dilutions on the 1% (w/v) agarose gel. Figure 3.3 illustrates the sensitivity of the multiplex PCR as observed on a 1% agarose gel.

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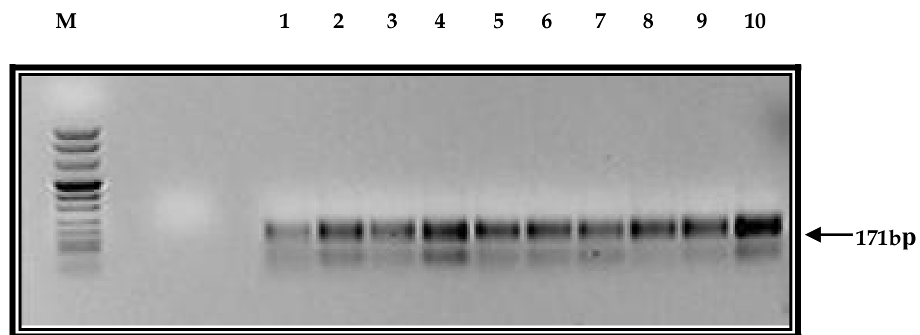


Figure 3.2 Agarose gel analyzing the PCR amplification of RubiscoL DNA from a citrus extract using various concentrations for RubiscoL DNA primers. DNA marker (lane M), 1µM (lane 1), 2µM (lane 2), 3µM (lane 3), 4µM (lane 4), 5µM (lane 5), 6µM (lane 6), 7µM (lane 7), 8µM (lane 8), 9µM (lane 9) and 10µM (lane 10).

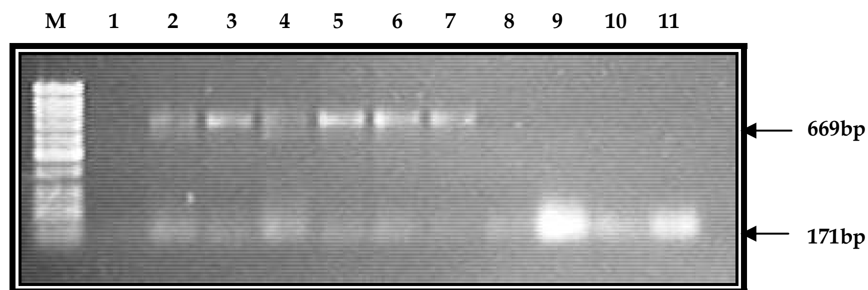


Figure 3.3 Sensitivity of multiplex PCR for Laf. Total DNA extract of Laf infected sample was serially diluted 10-fold in a healthy DNA extract. DNA Marker (lane M), no template (lane 1) and 10-fold dilution of Laf ranging from 10^{-1} to 10^{-10} (lanes 2 – 11).

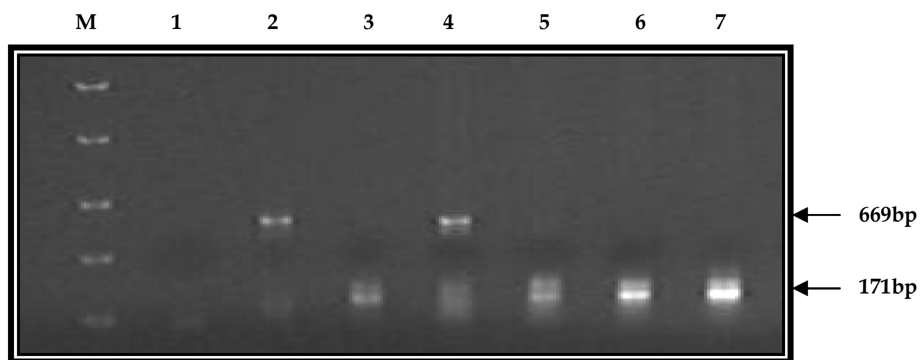


Figure 3.4 Agarose gel illustrating the simultaneous amplification of Laf and internal control DNA. DNA marker (lane M), no template (lane 1), positive control (lane 2), healthy control (lane 3), UPCRI 06-0100 (lane 4).

3.5 Discussion

Plant extracts often contain substances such as phenolic compounds and polysaccharides that can inhibit *Taq* polymerase activity in PCR reaction. An important characteristic of many phenolic compounds is to form complexes with nucleic acids (Koonjul *et al*., 1999). PCR inhibitors can be removed from samples using DNA purification procedures and DNA polymerase of commercially available kits (Trivedi *et al*., 2009) but is not cost effective for large-scale extraction of nucleic acid. Methods that can indicate inhibitory effects when detecting pathogens from various hosts are required for accurate diagnosis and management of diseases caused by the pathogens.

An effective method for verifying inhibition of PCR is the use of Multiplex PCR with internal control. Multiplex PCR with internal plant control target sequences have been developed for various disease causing agents of plants (Nassuth *et al*., 2000; Pastrik *et al*., 2001; Menzel *et al*., 2002; Thompson *et al*., 2003). It is desirable to use an internal positive control that would produce a fragment, indicative of successful PCR reaction, irrespective of the presence of a targeted pathogen or targeted pathogens (Bariana *et al*., 1994). The internal control primers to be used should target mRNA of a constitutively expressed gene that is conserved in plants (Bariana *et al*., 1994).

The number of sequences that can be amplified simultaneously in a multiplex PCR and the sizes of their amplicons are critical for achieving specific and high yielding products. The two amplicons produced should easily be distinguished from each other based on size upon gel electrophoresis. For the multiplex PCR developed in this study, mRNA encoding the large subunit of Rubisco (RubiscoL) was used as an internal control. Amplicon sizes could affect competition for the use of

dNTP's by the two primer sets used therefore the use of an internal control which produces a smaller amplicon is desirable. The internal control primers amplified an expected band of 171bp as designed by Nassuth *et al* (2000) while Laf primers amplified a 669bp band. The observation of the two expected amplicons on the gel (Figure 3.4) confirmed that the two primer pairs did not cross-react with each other during the reactions.

To achieve a robust multiplex PCR, optimization of reaction parameters and cycling conditions are required (Henegariu *et al*., 1997; Pastrik, 2000). Primer concentrations for pathogen and internal control were optimized to determine the primer combination that resulted in preferred amplification of expected fragments. Annealing temperature for primer pairs used in a multiplex PCR is essential to avoid suboptimal annealing rates of the primers to their target sequences. Annealing temperature can influence amplification of the desired target sequence, in this case Laf sequence, which can be outcompeted by amplification of internal control or other non-specific products (Elnifro *et al*., 2000) or it can influence the negative amplification of the less efficient target sequence (Markoulatos *et al*., 2002). Annealing temperature of 62°C was chosen for this multiplex PCR to avoid the mispriming of Laf primers A2/J5. Nassuth *et al* (2000) used 54°C as annealing temperature for RBCL 535/RBCL C705 primers but in this experiment the primers could still amplify the expected fragments of 171bp at the annealing temperature of 62°C.

Apart from annealing temperature and extension time, primer ratios can also affect outcomes of the reaction (Kapley *et al*., 2000). Different concentrations were selected for Laf primers A2/J5 and the internal control primers RBCL 535/RBCL C705. A high concentration was preferred for Laf primers while a lower concentration was used for

RubiscoL primers. This was done to achieve preferential amplification of Laf rather than the internal control DNA, by eliminating competition for the limited amount of dNTP's and *Taq* polymerase available in reaction mixture between the primer pairs used.

Combining two primer pairs in a reaction can result in additional spurious bands in addition to the expected internal control band. This can be due to pathogen primers amplifying segments of the plant internal gene or the formation of primer dimers (Bariana *et al*., 1994; Elnifro *et al*., 2000). Optimization of the multiplex to reduce non-specific interactions eliminates consumption of reaction components, destruction of rates of annealing and extension and non-efficient amplification of desired targeted genes (Elnifro *et al*., 2000).

Hocquellet *et al* (1999) reported the A2/J5 primers to detect 0.01ng of purified Laf DNA when added to the reaction mixture. Presence of Laf in alternate hosts could be difficult to detect due to the presence of inhibitors therefore the sensitivity of the detection method should be determined using DNA extracts, still containing plant material, as dilution medium for Laf template DNA. Detection limit of the multiplex PCR was determined by serial dilution of Laf infected total DNA extract in a healthy total DNA extract. Laf was detected reliably at 10^{-6} dilution from the original 100ul of the original total DNA extract. Internal control DNA was amplified in the highest dilution reactions.

The multiplex PCR developed in this study could amplify the targeted amplicons of Laf and RubiscoL internal DNA control of expected size. This PCR combines two tests in one reaction, the first is for specific and sensitive detection of a pathogen and second, an indicator test for successful amplification as indicated by internal PCR control (Pastrik, 2000). Reaction parameters were optimized to avoid the amplification of

spurious amplification products, cross reactivity of primers and formation of primer-dimers. In addition, the A2/J5 primers used in this multiplex PCR can also detect Las and LafC (Hocquellet *et al*., 1999; Garnier *et al*., 2000) from infected plants or even Las and Laf from plants simultaneously infected with both species as previously shown in Mauritius (Garnier *et al*., 1996). Laf and Las specific PCR products will be differentiated by size comparisons on an agarose gel. Inclusion of internal control validates presence of extracted DNA and absence of inhibitors which makes the method reliable, less laborious and cost effective. A newly developed multiplex PCR should be compared to other corresponding uniplex PCR protocols as was done for detection of clinical, food and plant pathogens, including that of citrus plants (Bubert *et al*., 1999; Elnifro *et al*., 2000; Markoulatos *et al*., 2002; Gorsane *et al*., 2005; Roy *et al*., 2005; Wang *et al*; 2009). The multiplex PCR developed in this study should be compared to uniplex PCR, especially those targeting Liberibacter β operon, to compare the sensitivity and specificity of the methods for the detection of Laf, LafC or even Las from alternate hosts. This multiplex PCR is able to detect Laf with the coamplification of host DNA and can be used for reliable screening of citrus and other plants for infection of the Laf/LafC bacteria.

Chapter 4

Molecular identification of alternate hosts to *Citrus* of Laf amongst indigenous plants of South Africa

4.1 Introduction

Minimal information on the alternative hosts to citrus of citrus infecting Liberibacter species is available (Hung *et al*., 2000) and as with other bacteria it is not easy to identify alternate hosts and potential inoculum sources (Costa *et al*., 2004). Nonetheless, efforts have to be made to identify possible alternatives. Unique genes that confer "basic compatibility" of a microorganism with its plant partner and thus enabling the pathogen to infect and replicate within the plant are required to determine host range of a pathogen (Keen & Staskawicz, 1988). A few genera of the Rutaceae and non-rutaceous plants have been identified as natural or experimental hosts of Liberibacter species with the use of molecular techniques such as PCR and DNA hybridization, inoculation tests and symptom recognition.

The existence of possible alternate hosts and the distribution of these might have a role in the epidemiology of the disease, and consequently may impact current implemented integrated control strategies for greening disease which includes; quarantine barriers, propagation and planting of disease-free material, pruning or removal of inoculum sources and psylla control. The occurrence of alternative hosts may make these cultural control practices futile if these hosts are not considered and included within the control strategies.

The host range of Liberibacter species, especially that of Laf outside the Rutaceae family, is not well known. Indigenous vegetation surrounding citrus orchards able to support the citrus psylla, *T. erytreae*, have previously been suspected to be possible sources of re-infestation despite insecticide treatment of the orchards (van den Berg, 1990) and thus the natural vegetation might be probable reservoirs of the citrus greening bacterium. The recent association of the new "*Candidatus*

Liberibacter" species with the diseases of non-rutaceous Solanaceae family plants, tomato (*Solanum lycopersicon* L.), capsicum, Cape gooseberry (*Physalis peruviana*) and potato (*Solanum tuberosum*), and transmission of Las to *Lycopersicon esculentum* tomato (Munyaneza *et al*., 2007; Duan *et al*., 2008; Hansen 2008 a; 2008 b; Liefting *et al*., 2008 a; 2008 b) further indicates the importance of investigating different plant families as possible additional host plants for any of the known and possibly unknown Liberibacter species. This Liberibacter species causes streaking and dark striping in the medullary ray tissue of affected potato tubers and fried potato chips hence its common name zebra chip (Li *et al*., 2009) and it can be transmitted by the vector *Bactericera cockerelli* (Hansen *et al*., 2008 b). Although this Liberibacter is closely related to the citrus infecting Liberibacters, it is not associated with citrus disease or the citrus psylla *D*. *citri* (Li *et al*., 2008; 2009).

In South Africa only three rutaceous plants were previously identified as alternate hosts to citrus of Liberibacter. Laf was transmitted from *C. anisata* to sweet orange indicator plant by side-grafting approach (van den Berg *et al.,* 1991-1992). Infection was confirmed by symptom development on indicator plant and thin layer chromatography. Laf was detected from a *V. lanceolata* plant using DNA hybridization probes (Korsten *et al*., 1996). In 2000 the first report on the detection of a Liberibacter, using PCR, from a *C. capense* (Cape chestnut) plant found in Stellenbosch area in the Western Cape Province of South Africa was conveyed (Garnier *et al*., 2000 a). The role of this Liberibacter in the outbreak of disease in the Western Cape was determined by characterizing the targeted 16S rDNA and ribosomal proteins and comparing them to that of Laf and Las (Garnier *et al.,* 2000 (b)). Comparison to the β operons of Laf and Las revealed that the newly found bacterium had high sequence homology with Laf. MAbs specific to Laf also reacted with the Liberibacter detected from the Cape chestnut

suggesting it has epitopes in common with Laf thus belonging to the same serogroup as Laf. However, differences in sequence were sufficient to led to the proposal that subspecies status be awarded to the bacterium, namely "*Candidatus* Liberibacter africanus subspecies capensis" (Garnier *et al*., 2000 (b)) (LafC).

The detection of the LafC and Laf from the three rutaceous plant prompted this part of the study. The aim being to survey and investigate whether these and other rutaceous plants, naturally occurring in different areas of South Africa, are infected with Laf or possibly LafC and Las. The potential of rutaceous to naturally host Laf/LafC/Las species was investigated by using PCR specific primers A2/J5 that target their β operon ribosomal protein genes.

In this chapter the various indigenous species, rutaceous and non-rutaceous, which were collected and tested for the presence of Laf are discussed. Plants that showed citrus greening-like symptoms, for instance mottling, yellowing of veins and any psylla-like breeding marks on the leaves, were recorded and leaf samples collected. Since it is not certain whether the disease displays similar symptoms in indigenous plants than in citrus, or that infection with the pathogen could be asymptomatic (Costa *et al*., 2004), any material indicative of greening-like symptoms or psylla-like breeding marks were collected. Plant material was collected from various geographical regions distributed over five provinces of South Africa where rutaceous plants are known to occur, irrespective of citrus greening disease occurrence.

Preservation and storage condition of the samples may affect the results of the PCR amplification of the Liberibacter DNA. Jagoueix and colleagues (1996) experimented with the collection and preservation of samples and how the different conditions would affect the PCR

outcomes thereof. Freezing the midribs for up to 3 weeks reduced the sensitivity of the PCR and no amplification of the Liberibacter specific 16S rDNA could be amplified from decaying or fermented leaves.

Possible indigenous host plants for the Liberibacter presented a challenge as the length of time that they could sustain the Liberibacter's presence is unknown. Time and temperature and other environmental or chemical factors could influence the outcome of DNA extraction from plant samples and ultimately the PCR results obtained. These factors were considered during sample collection, DNA extraction and storage of the plant samples.

4.2 Natural hosts of Laf and LafC in South Africa

Calodendrum capense

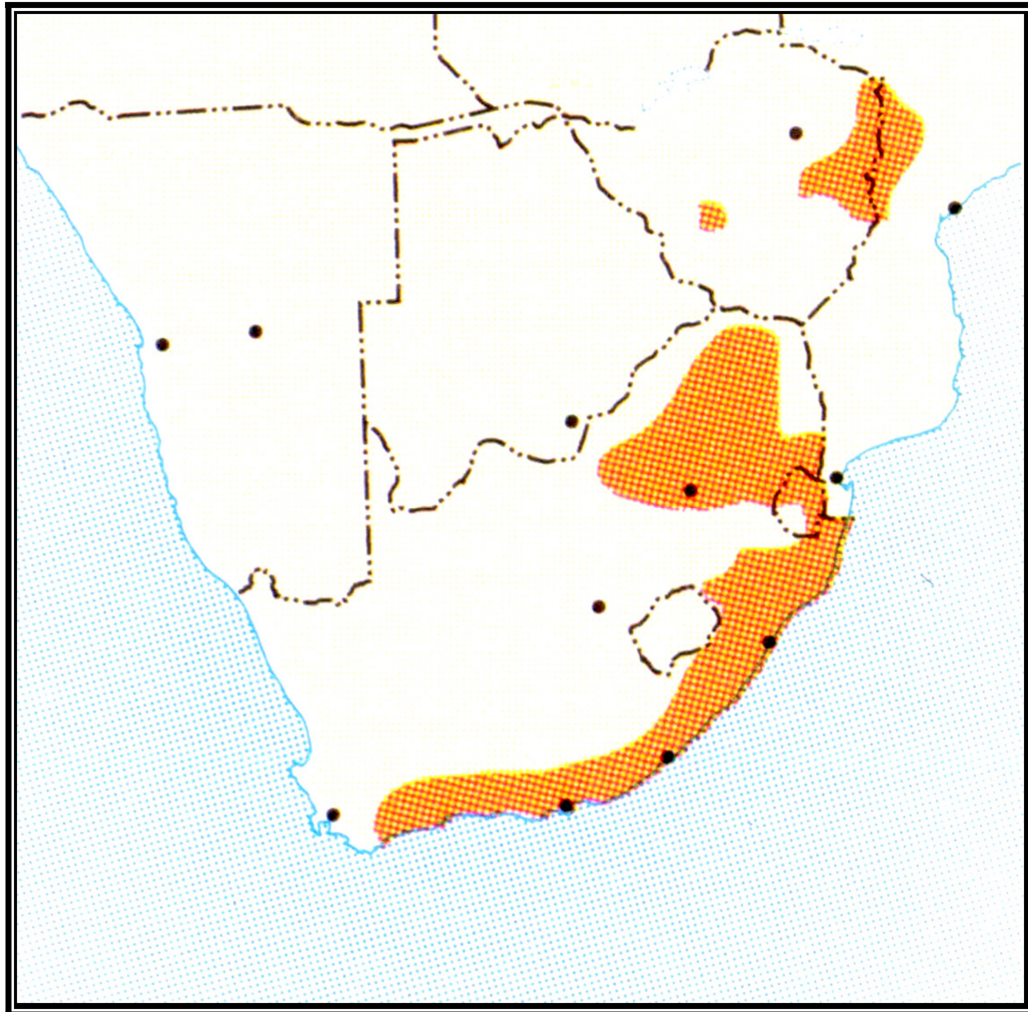
C. capense is a botanical name meaning "beautiful tree of the Cape" or simply "cape chestnut", is an indigenous ornamental rutaceous plant of the Cape region flora of South Africa. The plant is distributed in other parts of Southern Africa but it is believed to be native to the Cape area hence the species name. It is an evergreen deciduous to semi-deciduous plant that can reach 25 meters in height (Palmer & Pitman, 1972; Venter & Venter, 1996; van Wyk & van Wyk, 1997).

The bark is grey and relatively smooth. It is the only rutaceous plant in South Africa whose leaves are simple, elliptic and with an opposite leaf arrangement. The leaves are large, about 5-22cm long and 3-10cm wide. Their lateral veins are positioned parallel to each other and are at a right angle to the midrib. The lamina of the leaves have prominent secretory glands and if crushed have a typical, pungent, citrus-like odour. The flowers are large and light to dark pink in colour with 5 long curved

petals with dark maroon glands and flower mainly during the summer season. The fruits have 5 lobes with warty and splitting capsules that contain shiny black seeds (Palmer & Pitman, 1976; Venter & Venter, 1996; van Wyk & van Wyk, 1997).

Figure 4.1 Photo of a flowering *Calodendrum capense* "Cape chestnut" found in Pretoria South Africa. (Photo courtesy of Pietersen, G.)

Figure 4.2 Foliage and flowers (a) and warty 5-lobed fruit (b) of *Calodendrum capense*. (Photos courtesy of Pietersen, G.)



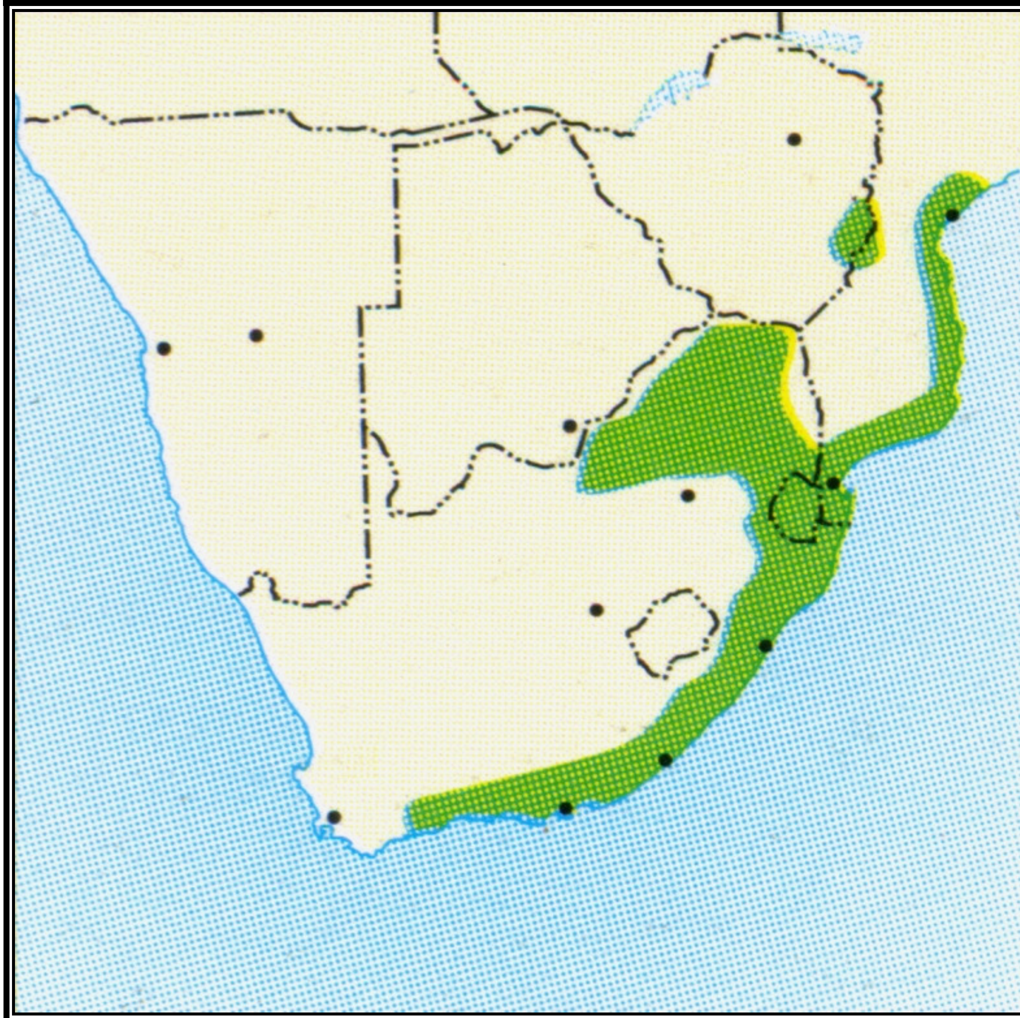
Distribution of *Calodendrum capense.*

Figure 4.3 A map indicating the natural distribution of *Calodendrum capense* in Southern Africa (van Wyk & van Wyk, 1997).

Vepris lanceolata

Commonly known as white ironwood, *V. lanceolata* is an evergreen plant with a smooth grey, slender and erect trunk which can reach 24 meters in height. It has a wide and rounded crown. The leaflets are lance-shaped but sometimes broader, leathery, stalkless with markedly wavy margins, gland dotted and aromatic when crushed. The unisex flowers are small and greenish yellow in colour on branched terminal heads. It bears black fruits that are about 5mm in diameter, round, gland-dotted and have 4 lobes (Palmer & Pitman, 1976; van Wyk & van Wyk, 1997).

V. lanceolata is found in most coastal and inland forests of South Africa, as far north as tropical Africa and also in Mauritius and Reunion (Palmer & Pitman, 1976), both of which harbour Las and Laf and their respective psyllid vectors *D. citri* and *T. erytreae* (Garnier *et al*., 1996).



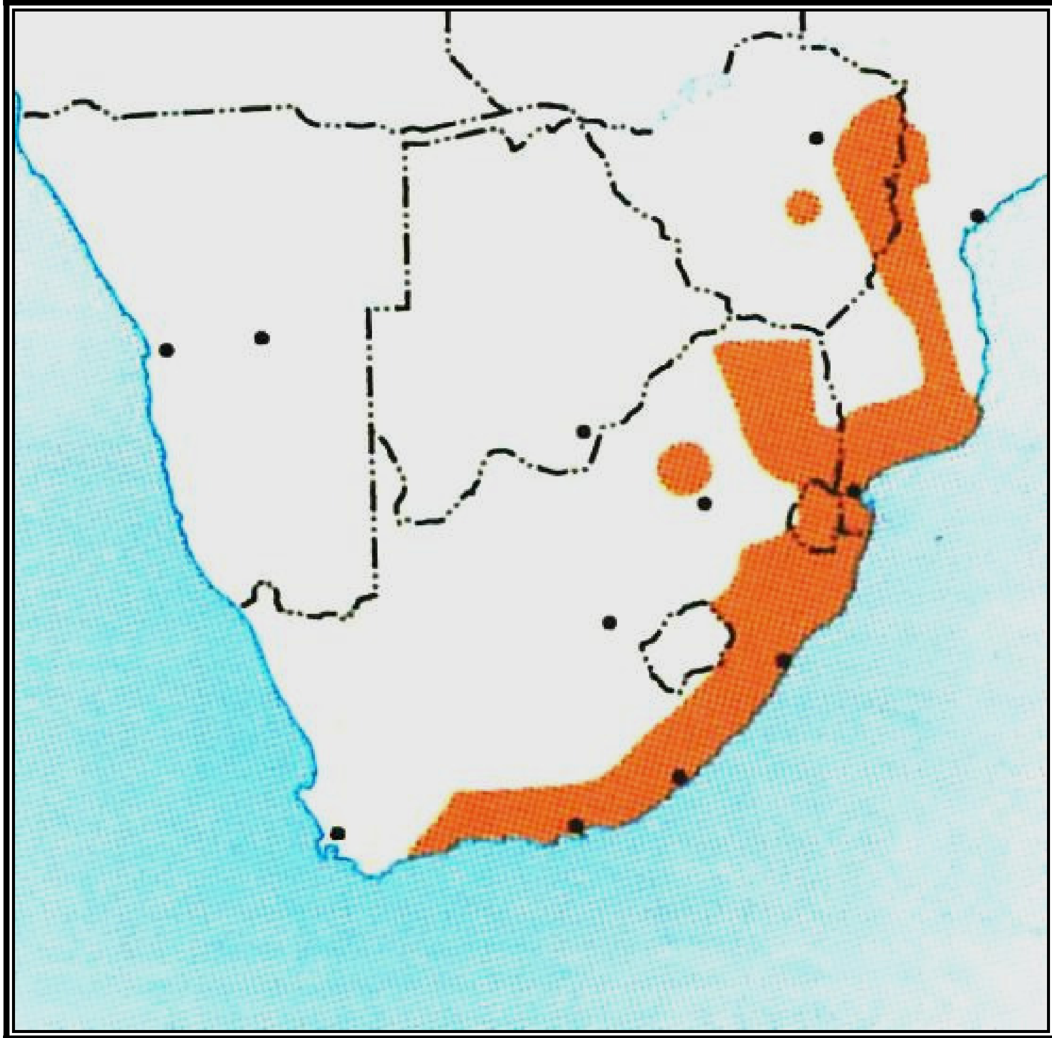
Distribution of *Vepris lanceolata.*

Figure 4.4 Distribution map of *Vepris lanceolata* through Southern Africa (van Wyk & van Wyk, 1997).

Clausena anisata

C. anisata or "horsewood" is a shrubby, slender and unarmed tree. It has dark green leaflets with an asymmetrical arrangement. The leaves smell like horse urine when touched or crushed hence the common name "horsewood". It has white or yellowish bisexual flowers borne in axillary sprays. The ripening fruits are red while the ripened fruits are black (Palmer & Pitman, 1976; van Wyk & van Wyk, 1997).

C. anisata is found along the coastal and inland forests and near streams in South Africa. It is also found in Swaziland, Mozambique, Zimbabwe and Malawi as indicated in Figure 4.5. (Palmer & Pitman, 1976; van Wyk & van Wyk, 1997). Greening has been reported to occur in citrus plants in Swaziland (Catling & Atkinson, 1974) and Zimbabwe (Lafléche & Bové, 1970 a).



Distribution of *Clausena anisata.*

Figure 4.5 Distribution map of *Clausena anisata* through Southern Africa (van Wyk & van Wyk, 1997).

4.3 Materials and method

Plant collection and total DNA extraction

Leaf samples of mainly indigenous rutaceous and non-rutaceous plants were collected from different geographical localities in South Africa. The collection sites included Nelspruit, Pretoria, Vredefort, Rhenosterpoort, and Cape Town areas. Samples were collected from naturally occurring vegetation situated near or adjacent to commercial citrus orchards, in their natural habitats and from two national botanical gardens during 2006, 2007 and 2008 seasons. The preferred time for collection of plants samples was during the cooler months of the year when Laf is known to proliferate within infected citrus. Most plant samples were collected during the months of March, May, June and July. The majority of the samples were rutaceous species, while a few non-rutaceous plants were also collected. The majority of the rutaceous species were collected from the Kirstenbosch National Botanical Gardens in the Western Cape as a number of rutaceous species are endemic to the Western Cape and are maintained at Kirstenbosch. A map indicating sample collection sites is shown in Figure 4.15.

About 5g of leaf material with greening-like symptoms was collected per plant, and placed into a plastic bag and immediately stored in a cooler-box. Leaf samples were also randomly collected from asymptomatic plants. The plant samples were marked appropriately and allocated an accession number. The plant materials were stored at 4°C until they were processed for DNA extraction. Total DNA from symptomatic or asymptomatic leaf petioles and/or midrib tissue was extracted using CTAB buffer (Doyle & Doyle, 1990) protocol as described in Appendix A1.2, and stored at -20°C until further use. The total DNA extracts

served as templates for PCR amplification using Liberibacter specific primers.

Identification of plant materials collected

Plant identities at least to the genus level, were confirmed using books that provide descriptive guidelines or keys to identification of plants that are found in the Southern African region (Palmer & Pitman, 1972; Venter & Venter, 1996; van Wyk & van Wyk, 1997; Germishuizen & Meyer, 2003). The names of the Rutaceae and non-Rutaceae plants sampled and the location from where they were sampled are given in Table 4.1 and 4.2 respectively.

PCR amplification and gel electrophoresis

The multiplex PCR with internal control, developed during this study, and conventional A2/J5 PCR (Hocquellet *et al*., 1999) were used to test for the presence of Liberibacter africanus DNA from extracts of the collected samples according to protocols in Appendix A2.1, A.2.2 and A2.3. In addition, a PCR using LafC specific primer CAL1, which binds to an insertion of 25bp in the LafC ribosomal gene that is not found in Laf, and reverse primer J5 as described by Garnier *et al* (2000) was done according to the protocol in Appendix A.2.3. The use of00 multiplex PCR on the nucleic acid extracts of all plant samples indicates at a minimum the presence of added DNA extract template and a successful amplification reaction that was uninhibited by contaminants and/or indigenous plant compounds. To view the PCR products obtained, the products were mixed with loading dye and electrophoresed at 100V for 30 minutes on 1% (w/v) agarose gel stained with ethidium bromide (5mg/ml) for visualising amplicons under ultraviolet transilluminator

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(Appendix A1). The size of the amplicons was determined by comparing their bands to a molecular DNA marker (Promega, Madison, USA) of which 5µl was loaded onto the agarose gel. Real-time PCR (Li *et al.*, 2006) was used on three *C. capense* plants according to protocol in Appendix A2.4.

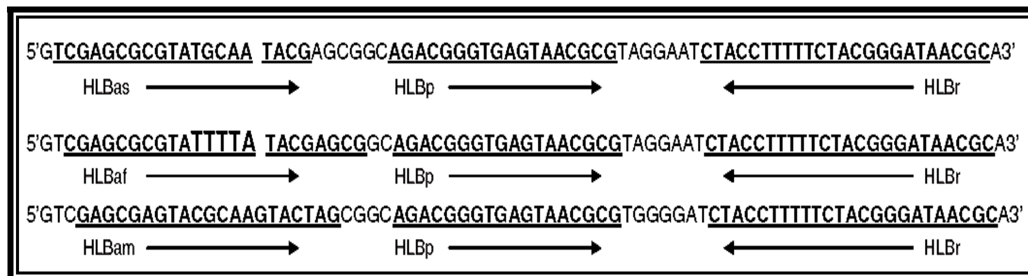


Figure 4.6 Sequences of the TaqMan primers and probe targeting the 16S rDNA of Laf as adopted from Li *et al* (2006).

DNA sequencing and analysis

PCR products of the expected size were cut from the gel and purified using the Wizard® SV gel and PCR clean-up system (Promega, Madison, USA) according to the manufacturer's instructions. Automated DNA sequencing of the amplified products was performed according to the protocol in Appendix A2.5 using the same primers as used in the amplification reaction. DNA from the sequencing reaction was precipitated using the EDTA/NaOAc/EtOH (BigDye Terminator V3.1 kit cycle sequence protocol) procedure in Appendix A2.5. DNA sequencing reactions were done at Inqaba biotech laboratories (Pretoria, RSA) or at the University of Pretoria DNA sequencing facility using the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, California, USA) or the 3130 DNA Analyzer (Applied

Biosystems, California, USA). Sequences were compared to those on GenBank database using BLAST analysis tool and similarities obtained (National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov). Sequences were edited and compiled using BioEdit sequence alignment editor version 7.0 (Ibis Biosciences, California, USA). The β operon sequence of LafC was obtained from GenBank (accession no. AF248498) and aligned with edited sequences in the multiple alignment. Multiple alignments were constructed using BioEdit sequence alignment editor version 7.0 (Ibis Biosciences, California, USA).

Table 4.1 Rutaceous plant species sampled from various geographic locations in South Africa.

Plant species	Total number of plants	Observations	Location
Acmadenia heterophylla Glover	3	-	KNBG
Acmadenia macropetala Glover	1	-	KNBG
Acmadenia mundiana Eckl. & Zeyh.	5	-	KNBG
Acmadenia obtusata Thunb.	2	-	KNBG
Adenandra fragrans (Sims) Roem. & Schult.	1	-	KNBG
Adenandra obtusata Sond.	2	-	KNBG
Adenandra uniflora Willd.	1	-	KNBG
Agathosma apiculata G. Mey	1	-	KNBG
Agathosma capensis (L.) Dummer	1	-	KNBG
Agathosma cerefolium (Vent.) Bartl. & H.L. Wendl.	1	-	KNBG
Agathosma ciliaris (L.) Druce	3	-	KNBG
Agathosma collina Eckl. & Zeyh.	1	-	KNBG
Agothosma crenulata (L.) Pillans	2	-	KNBG
Agathosma dielsiana Schltr. Ex Dummer	1	-	KNBG
Agathosma glabrata Bartl. & H.L. Wendl.	2	-	KNBG

Table 4.1 Continued

Plant species	Total number of plants	Observations	Location
Agathosma gonaquensis Eckl. & Zeyh.	5	-	KNBG
Agathosma imbricata (L.) Willd.	1	-	KNBG
Agathosma lanceolata (L.) Engl.	2	-	KNBG
Agathosma mucrunulata Sond.	3	-	KNBG
Agathosma ovata (Thunb.) Pillans	10	-	KNBG
Agathosma serpyllacea Licht. Ex Roem. & Schult.	3	-	KNBG
Calodendrum capense Thunb.	47	-	UP Campus
		-	ARC-PPRI
		Blotchy mottling	LNBG
		Yellowing of leaves	KNBG
		Blotchy mottling	Pretoria
		-	Rhenoster-poort
Citrus species	7	Blotchy mottling	ARC-ITSC
		Psylla-like breeding marks	
Clausena anisata (Willd.) Hook. F. ex Benth,	11	Psylla-like breeding marks	ARC-ITSC
			LNBG
		Mottling-like	KNBG
Coleonema album (Thunb.) Bartl. & H.L. Wendl	4	-	KNBG
Coleonema pulchellum I. Williams	1	-	KNBG

Table 4.1 Continued

Plant species	Total number of plants	Observations	Location
Diosma hirsuta L.	1	-	KNBG
Diosma prama I. Williams	2	-	KNBG
Euchaetis meridionales I. Williams	2	-	KNBG
Murraya paniculata (L.) Jack	6	Yellowing of leaves	Nelspruit
		Yellowing of leaves	UP Campus
Teclea natalensis (Sond.) Engl.	1	Psylla-like breeding marks	LNBG
Toddalia asiatica (L.) Lam.	1	Small leaves	LNBG
Toddaliopsis bremekampii I. Verd.		Large populations of adults & nymphs	LNBG
Vepris lanceolata (Lam) G.Don	10	Psylla-like breeding marks	LNBG
Vepris reflexa I. Verd.	1	Small leaves & psylla-like breeding marks	LNBG
Zanthoxylum capense (Thunb.) Harv.	20	Adult psylla population	LNBG
		Psylla-like breeding marks & blotchy mottling	UP Campus
		Psylla-like breeding marks	ARC-ITSC
		Blotchy mottling	KNBG
Zanthoxylum chalybeum Eng.	1	-	LNBG (seeds imported from Zimbabwe)

Table 4.2 Non-rutaceous plant species samples collected from various geographic locations in South Africa.

Plant species	Number of plants	Observations	Location
Catharanthus roseus (L.) G. Don	2	Yellowing of leaves	Croc Valley (PTY)
Myosotis arvensis (L.) Hill	1	Yellowing of leaves	ARC-ITSC
Ochna serrulata (Hochst.) Walp.	1	Yellowing of leaves	Fredenheim
Sterculia murex Hemsl.	2	Psylla-like breeding marks	Fredenheim
Strychnos madagascariensis Poir.	11	Mottling-like symptoms, kink growth, psylla-like breeding marks & lopsided fruits	Croc Valley (PTY) Fredeheim
Strychnos spinosa Lam.	2	Mottling-like leaves, kink growth, psylla-like breeding marks	Croc Valley (Pty) Fredenheim
Trichilia emetica Vahl	2	Yellowing of leaves	Fredenheim
Other	12	-	ARC-ITSC Croc Valley (PTY)

ARC-ITSC = Agricultural Research Council-Institute for Tropical and Sub-Tropical Crops

ARC-PPRI = Agricultural Research Council-Plant Protection Research Institute

KNBG = Kirstenbosch National Botanical Gardens, Cape Town.

LNBG = Lowveld National Botanic Gardens, Nelspruit.

UP campus = University of Pretoria, Pretoria.

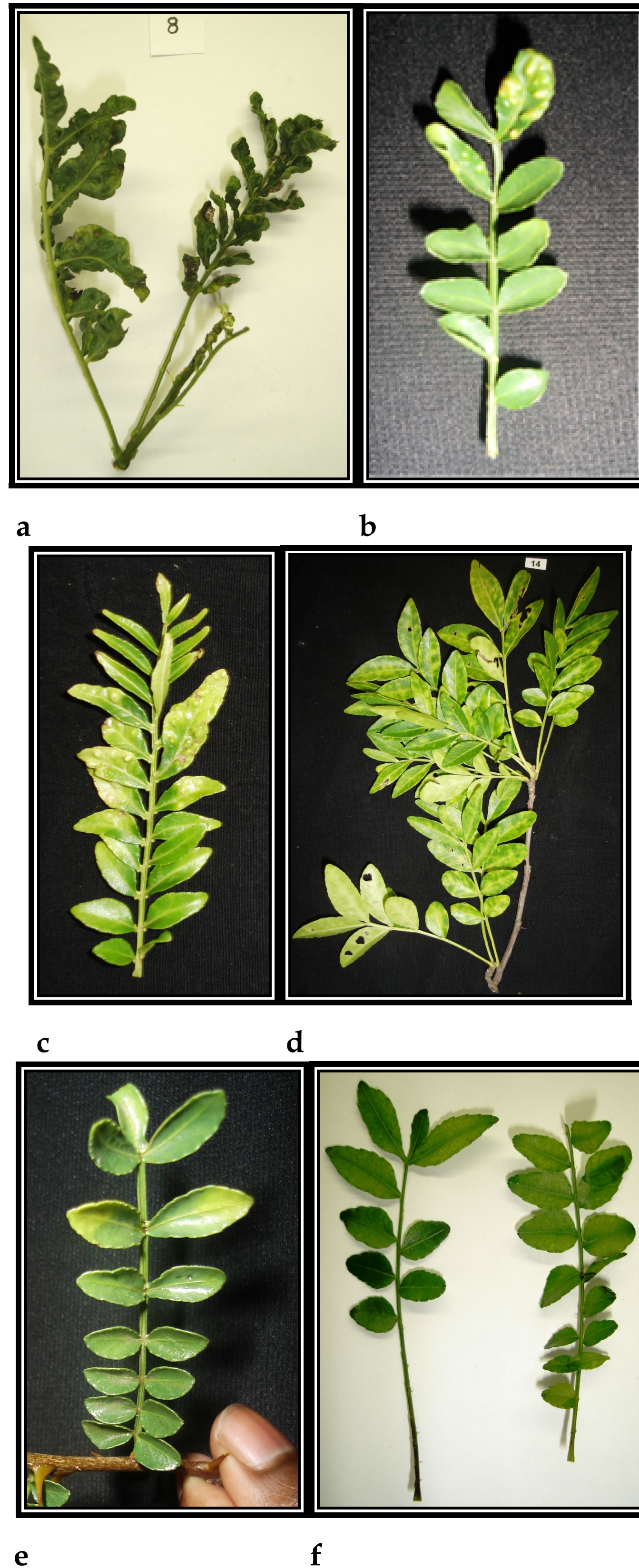
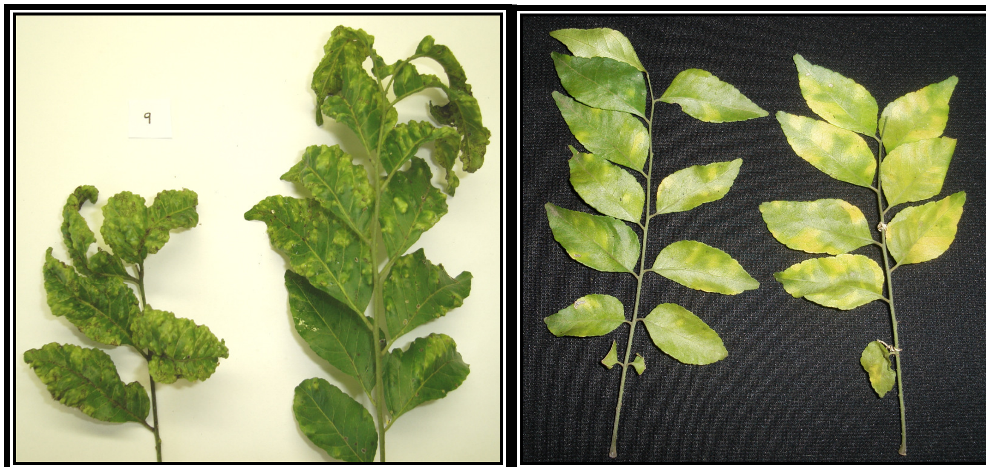


Figure 4.7 Greening-like symptoms exhibited on *Zanthoxylum capense*; *Trioza erytreae* feeding on leaf (a) psylla-like breeding marks (b and c) and mottling (d, e and f).

a **b**



c **d**

Figure 4.8 *Clausena anisata* leaves with psylla-like breeding marks (a and d) and mottling-like symptoms (b, c and d).

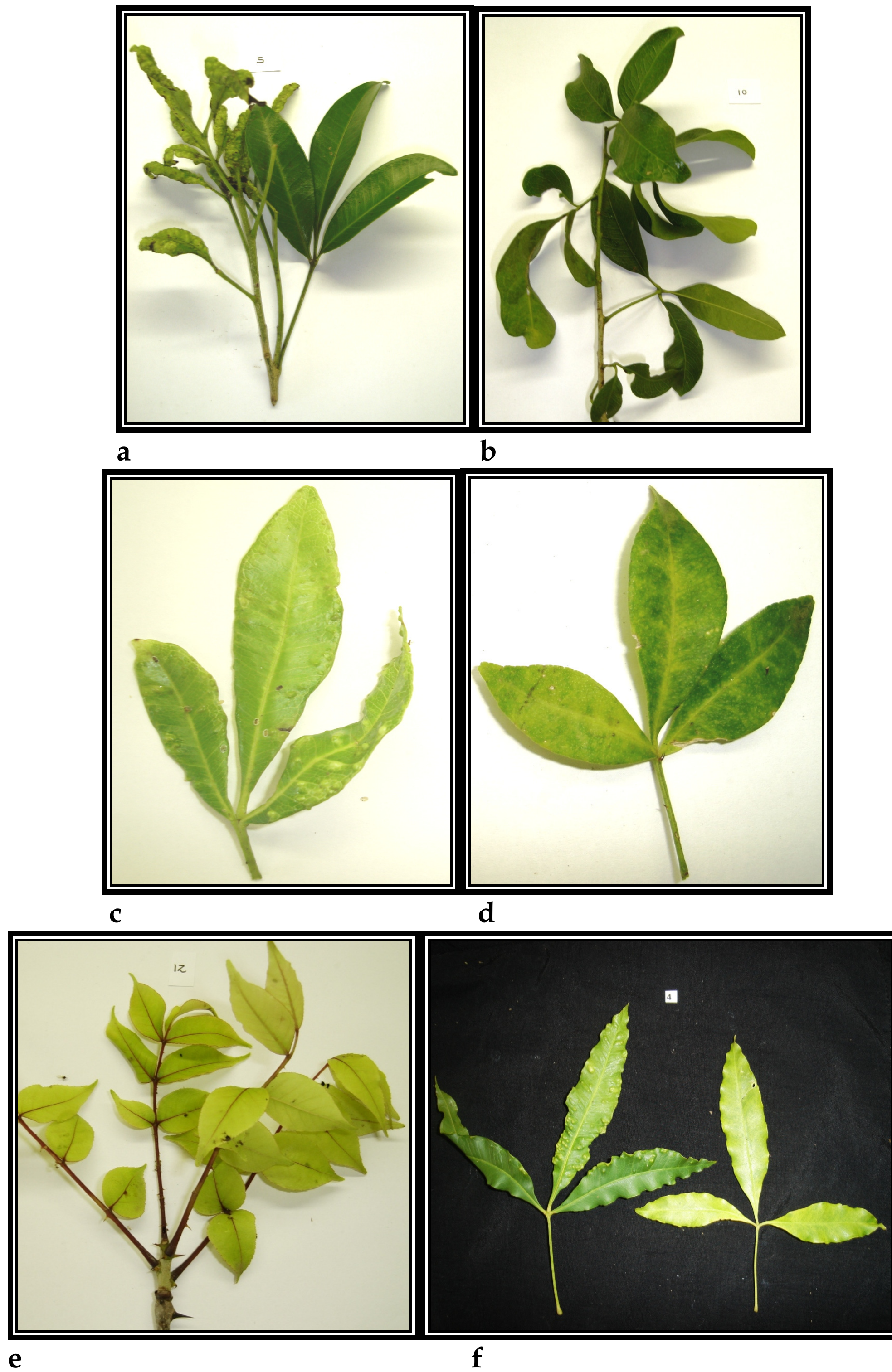


Figure 4.9 Common rutaceous species assessed for Laf: (a) *Toddaliopsis bremekampii;* (b) *Vepris reflexa;* (c) *Teclea natelensis;* (d) *Toddalia asiatica;* (e) *Zanthoxylum chalybeum;* (f) *Vepris lanceolata*.

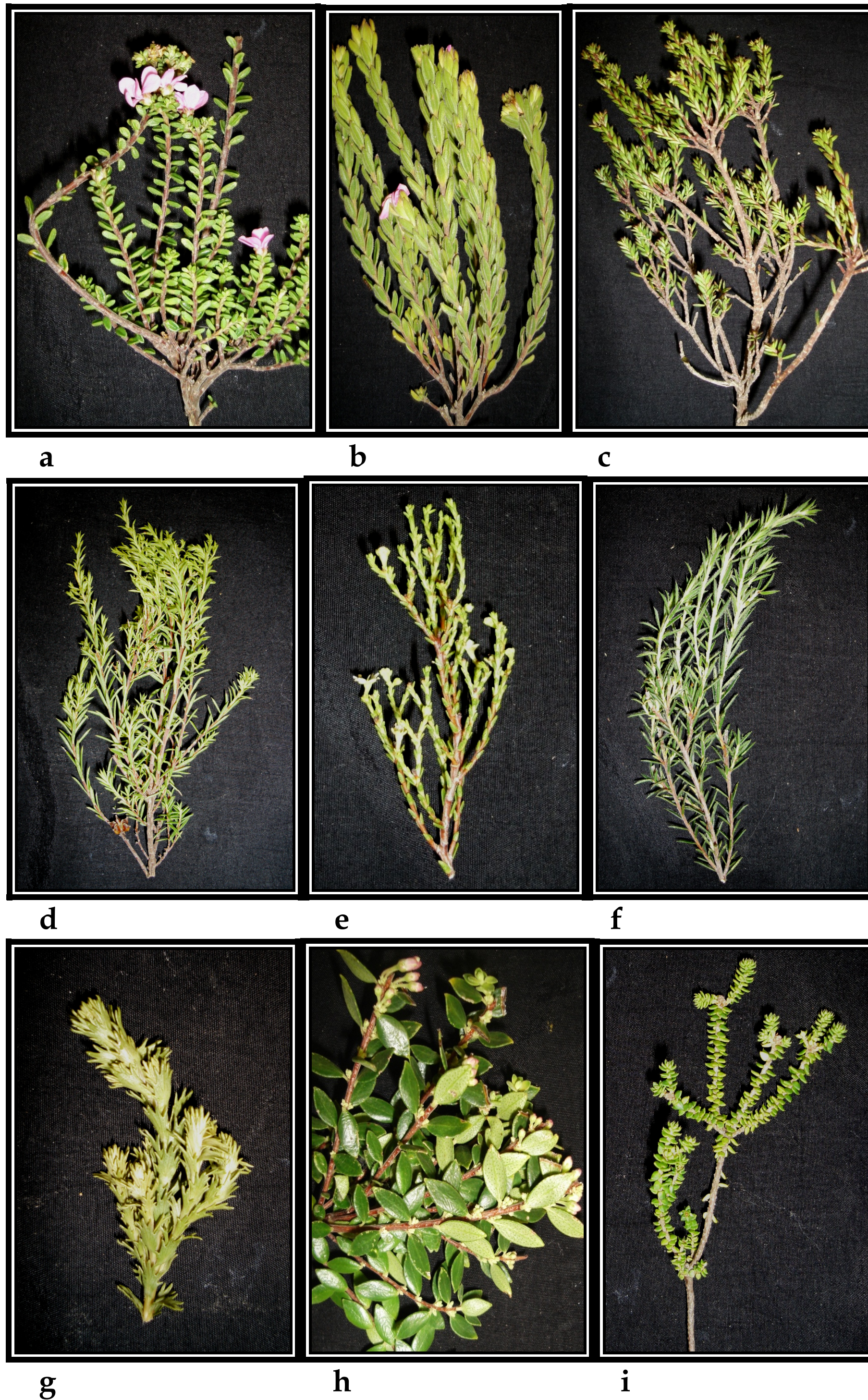


Figure 4.10 Native "buchu" species surveyed for Laf: (a) *Acmadenia heterophylla;* (b) *Acmadenia mundiana;* (c) *Acmadenia obtusata;* (d) *Coleonema album;* (e) *Diosma prama;* (f) *Diosma hirsute;* (h) *Agathosma lanceolata;* (h) *Agathosma ovata;* (i) *Agathosma gonaquensis*.

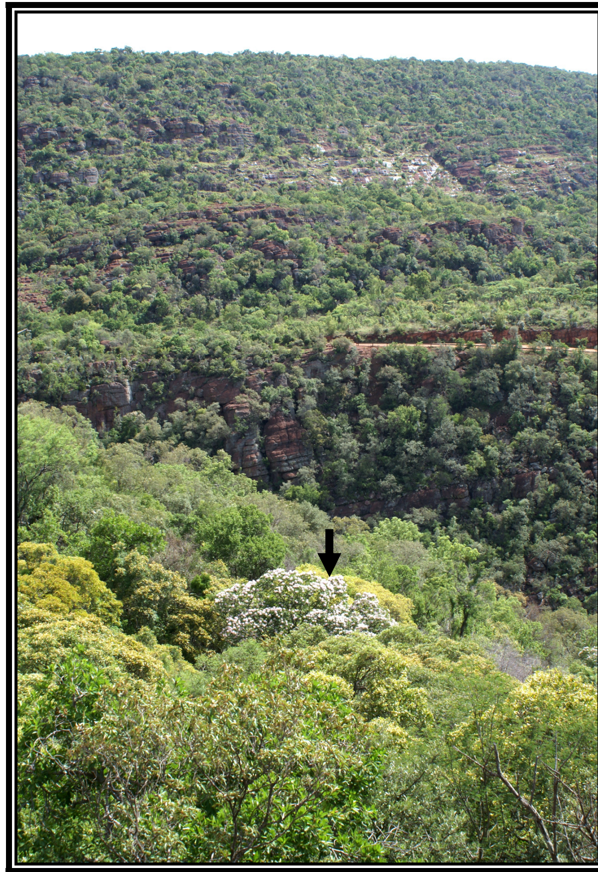


Figure 4.11 *Calodendrum capense*, as indicated by arrows, in their natural habitat at Rhenosterpoort, Limpopo province. (Photos courtesy of Pietersen, G.)

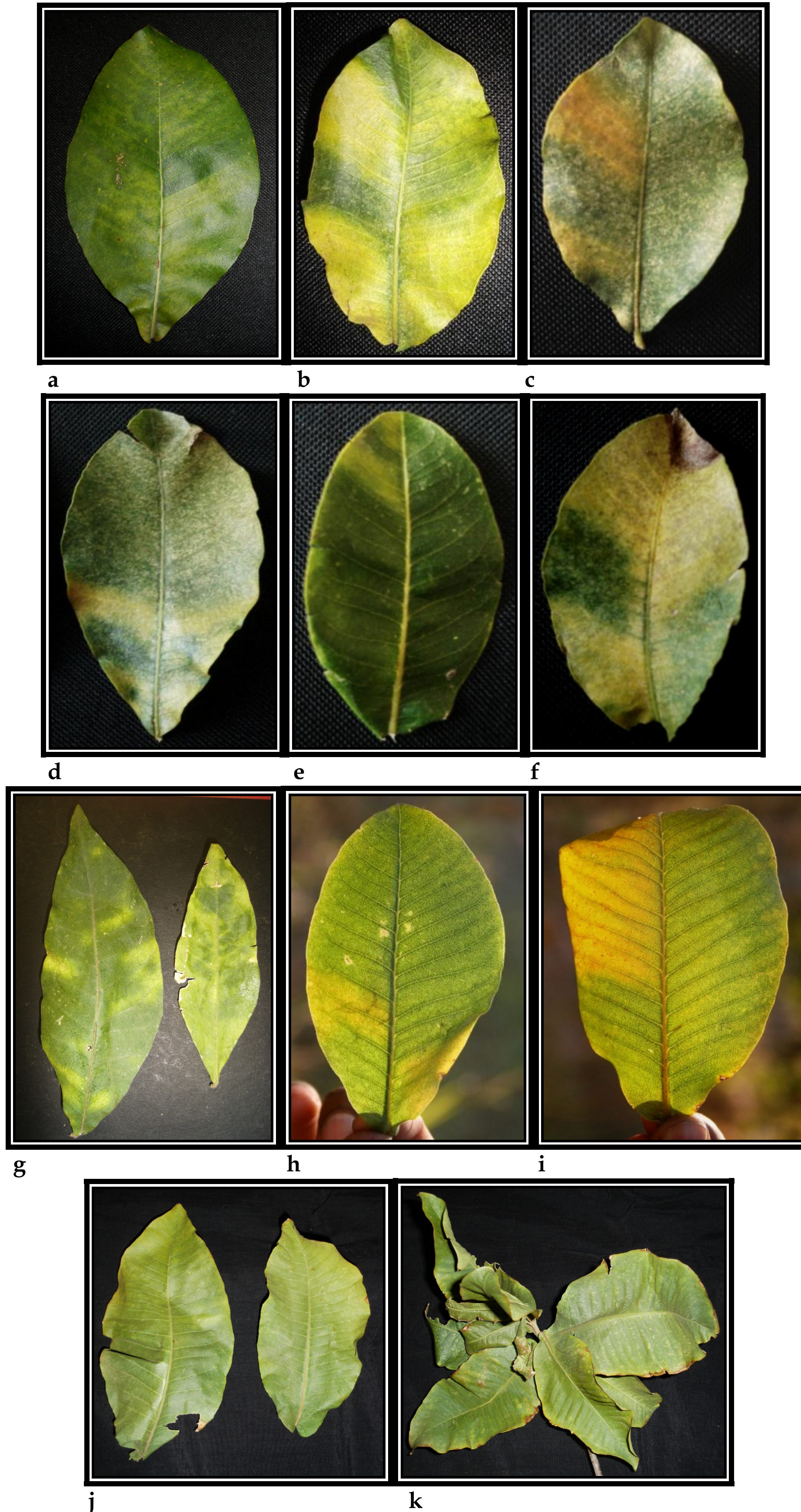


Figure 4.12 *Calodendrum capense* leaves with blotchy mottling symptoms (a – i) and with no symptoms (j - k).

4.4 Results

PCR amplification analysis

A total of 193 plants were collected from various geographic regions and processed for total DNA. These were subjected to both conventional and multiplex PCR to test for the presence of the greening bacterium. The plant samples consisted primarily of Rutaceae plants of the 14 genera and 35 species tested. Included within these rutaceous plants were a number of native dense shrubs commonly known as "buchu" of the genera *Acmadenia*, *Adenandra*, *Agathosma*, *Coleonema*, *Diosma* and *Euchaetis* which have not previously been surveyed as potential inoculum sources of any Liberibacter species. Other commonly known rutaceous genera tested included *Calodendrum*, *Clausena*, *Murraya*, *Teclea*, *Toddalia*, *Toddaliopsis*, *Vepris* and *Zanthoxylum*. Information regarding location of original plant specimen from which the samples were sourced is presented in Table 4.1 and 4.2. Total DNA was extracted from all 193 samples within one week of collection to obtain good quality total DNA as leaf samples had the propensity of wilting within days of storage.

Primers A2/J5, CAL1/J5 and A2/J5 together with RBCL 535/RBCL C705 (Hocquellet *et al.*, 1999; Nassuth *et al.*, 2000) were used in a conventional uniplex or multiplex PCR that detects Laf or Las and internal plant control DNA from the plant samples. In the multiplex PCR, healthy control and negative plant samples produced the internal control band of 179bp while no band was produced for the no template control due to the absence of template. Rubisco DNA could be consistently detected in most samples but intensity of the bands varied between the total DNA extracts of the different plant species. In all PCR tests performed the following were included: 1) a healthy plant extract;

2) negative control containing no DNA template; 3) Laf infected plant extract as positive control. In both the conventional and multiplex PCR neither the healthy nor the negative control yielded 669bp or other sized products. Conversely Liberibacter DNA of expected size was amplified in all positive control reactions. Multiplex PCR yielded expected internal control amplicon of 179bp in the healthy control and DNA extracts reactions therefore eliminating the possibility of false negative results.

PCR amplification with β operon specific primers pairs A2/J5 yielded DNA fragments of 669bp, while a combination of CAL1/J5 yielded DNA fragments of 588bp, only from the LafC infected sources. Amplicons of the expected size were yielded from 17 of a total of 47 *C. capense* DNA extracts when using conventional PCR amplification with A2/J5 or CAL1/J5 primer pairs. Of these 17 positive samples, characteristic mottling symptoms were observed while others were asymptomatic as shown in Figure 4.12. Only a few of these samples were collected during the summer, while the majority were collected during the cooler autumn and winter seasons. Amplicons were compared to a molecular marker on a 1% (w/v) agarose gel after electrophoresis in 1% SB or TAE buffer. Table 4.3 lists all positive samples, their allocated accession numbers and global positioning coordinates. The remaining 146 plants from the 13 rutaceous and 10 non-rutaceous genera did not yield amplicons of the expected size for Laf or LafC band signifying the absence of these bacteria from these samples.

To detect Liberibacter DNA from three *C. capense* extracts, 07-0308, 07-0309 and 07-0310 from the Limpopo Province, real-time PCR using primer-probe set HLBafpr specific for the African Liberibacter strain as designed by Li *et al* (2006) was used. Cycle threshold values of 47, 41

and 33 respectively where obtained for the respective samples. Electrophoresis of the products on a 1% (w/v) agarose gel revealed a band of about 70bp (Li *et al*., 2006). In this case HLBafpr was used without the inclusion of the internal control cytochrome oxidase gene primer-probe COXfpr. The inclusion of COXfpr will improve the assay as a detection tool for Laf and LafC.

The *C. capense* specimens testing positive for Laf were collected from various provinces of South Africa. Previously used names are presented in parenthesis e.g. Limpopo (Northern Province), Gauteng and Mpumalanga (Eastern Province).

The correlation between the presence of symptoms and infection of a plant by LafC was not consistent as a number of positive samples were asymptomatic. Of the plants that tested positive 80% were symptomatic with typical blotchy mottling while the remaining 20% were asymptomatic (Figure 4.12).

Occasionally non-rutaceous plants (Table 4.2) had typical psylla-like breeding marks but no adult psylla, nymphs nor eggs could be observed and therefore it cannot be assumed that the leaf distortions were caused by *T. erytreae*. Blotchy mottle is regarded as the classic symptom associated with greening infected citrus plants. Blotchy mottle symptoms were observed on a specific *Z. capense* (UPCRI 07-0526) specimen (Figure 4.7 d) from the Western Cape with no notable psylla-like breeding marks. However the sample tested negative for Laf or LafC DNA. The native “buchu” plant species which were assessed for the first time as potential host for greening disease were not only all asymptomatic, but also tested negative for Laf or LafC.

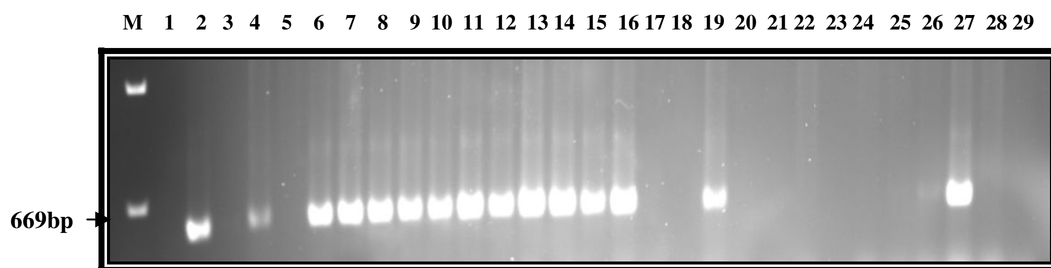


Figure 4.13 Agarose gel of amplified DNA from *Calodendrum capense* samples using PCR with primers A2/J5. No template (lane 1), positive control (lane 2), healthy control (lane 3), *Calodendrum capense* DNA extracts UPCRI 08-1003 - 1028 (lanes 4 – 29).

Table 4.3 *Calodendrum capense* plants that tested positive for Liberibacter DNA.

Natural location of *Calodendrum capense*	Accession no. (UPCRI)	Global positioning system (GPS) coordinates
Pretoria [a]	07 - 0508	S25° 46.598′ E28° 22.352′
	07 – 0509	S25° 46.591′ E28° 22.351′
	08 – 1003	S25° 44.915′ E28° 13.462′
	08 - 1005	S25° 43.550′ E28° 12.509′
	08 - 1006	S25° 40.760′ E28° 15.498′
	08 - 1007	S25° 40.764′ E28° 15.000′
	08 - 1008	S25° 40.766′ E28° 15.505′
	08 - 1009	S25° 40.769′ E28° 15.508′
	08 - 1010	S25° 40.769′ E28° 15.511′
	08 - 1011	S25° 40.770′ E28° 15.517′
	08 – 1012	S25° 40.777′ E28° 15.522′
	08 – 1013	S25° 40.779′ E28° 15.526′
	08 - 1014	S25° 40.783′ E28° 15.528′
	08 – 1015	S25° 40.783′ E28° 15.533′
	08 – 1018	S25° 47.387′ E28° 14.304′
	08 - 1026	S25° 47.229′ E28° 16.524′
Nelspruit [b]	07 - 0608	S25° 26.600′ E30° 58.228′
Limpopo [c]		

[a] Gauteng Province
[b] Mpumalanga Province
[c] Limpopo Province GPS reading not available

DNA sequencing and alignment

Direct sequencing of the seventeen positive *C. capense* extracts that yielded sequences of 669bp in length when using forward and reverse primers A2 or J5. The sequences were analyzed for nucleotide similarities with other known sequences from publicly available databases by using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST/). The BLAST analyses revealed the sequences to be similar to that of LafC (GenBank accession no. AF248498) at 92% and 98% similarity. Raw sequences were edited and cognate regions aligned with LafC sequence available on GenBank as shown in Figure 4.14. BLAST analysis of the edited sequences revealed 97% similarity to that of the subspecies sequence reference on GenBank. Sequence alignment revealed the presence of a 24bp insertion, present in LafC genome but not in Laf (Garnier *et al*., 2000). Additional nucleotides, which were not identified in the reference sequence, were identified at position 191, 310, 311, 326-328, 355-359 while nucleotide differences are also highlighted at positions 193, 194, 248, 307 and 309 in the intergenic region of *rplJ* gene as highlighted in Figure 4.14 and depicted in Table 4.4.

Figure 4.14 Alignment of sequences of LafC obtained from *Calodendrum capense* plants and LafC reference sequence (AF248498).

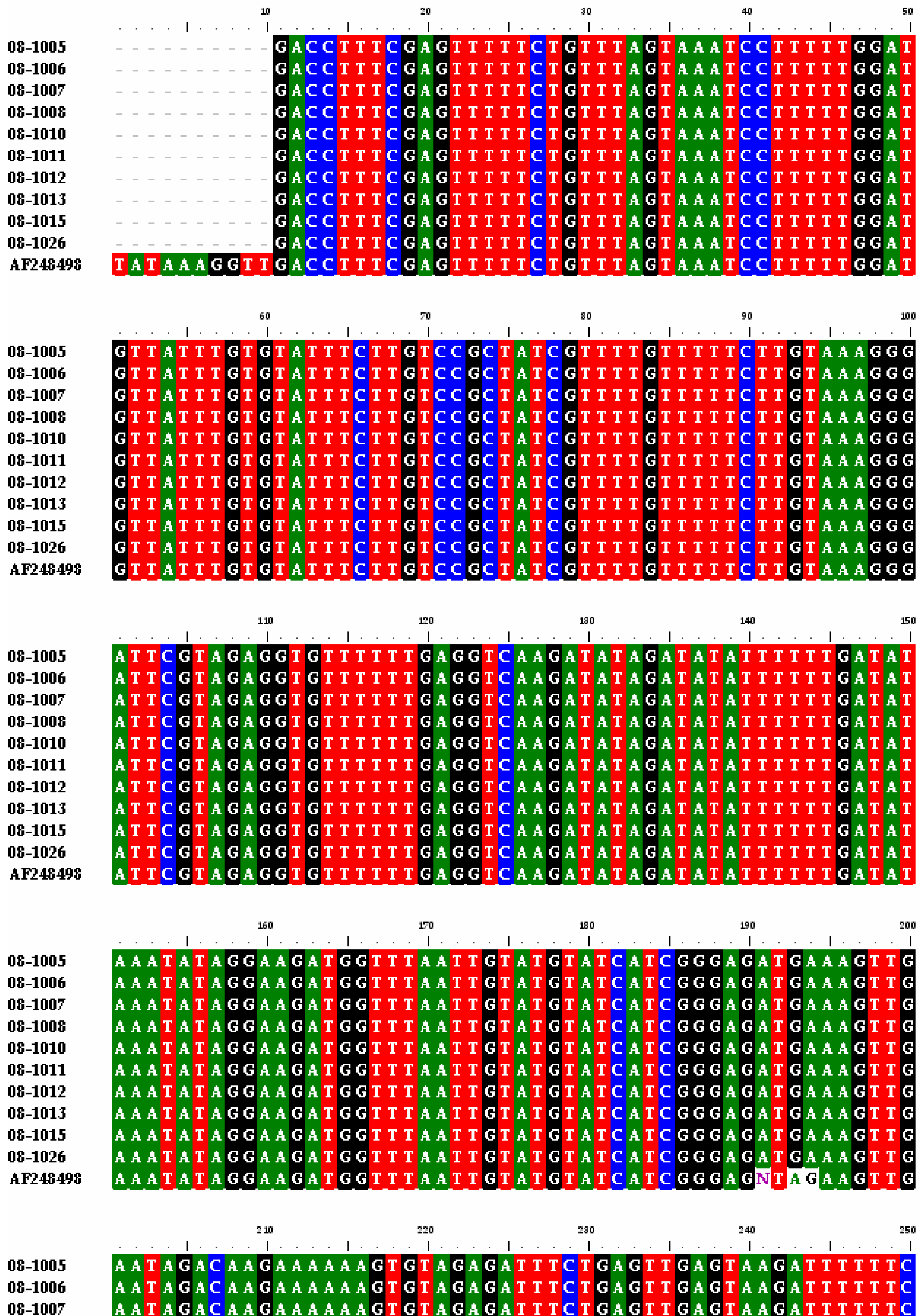


Figure 4.14 Continued

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08-1008    AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTTTC
08-1010    AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTTTC
08-1011    AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTTTC
08-1012    AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTTTC
08-1013    AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTTTC
08-1015    AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTTTC
08-1026    AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTTTC
AF248498   AATAGACAAGAAAAAAGTGTAGAGATTTCTGAGTTGAGTAAGATTTTGTC

                    260       270       280       290       300
08-1005    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1006    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1007    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1008    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1010    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1011    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1012    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1013    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1015    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
08-1026    TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC
AF248498   TTCTTCTGGGTCAGTTGTCGTTGCGCACTATAAAGGAATTAGTGTTGCAC

                    310       320       330       340       350
08-1005    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1006    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1007    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1008    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1010    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1011    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1012    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1013    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1015    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
08-1026    AGATCAAGGATCTTCGAAAGAAGGTGCG--AGAAGCTGGTGGAGGTGTGA
AF248498   AGATCAGGA--CTTCGAAAGAAGGTNNNCGAGAAGCTGGTGGAGGTGTGA

                    360       370       380       390       400
08-1005    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1006    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1007    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1008    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1010    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1011    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1012    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1013    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1015    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
08-1026    AGGTTGCGAAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT
AF248498   AGGTNNNNNAAAATCGCCTTGTTAAGATTGCCGTTCGTGATACTAGCGTT

                    410       420       430       440       450
08-1005    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1006    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1007    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1008    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1010    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1011    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1012    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1013    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
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Figure 4.14 Continued

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08-1015    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
08-1026    AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT
AF248498   AAGGAAGTTTCTGACCTTTTTGTTGGGCAATCACTGATTGTTTATTCAGT

                    460       470       480       490       500
           ....|....|....|....|....|....|....|....|....|....|
08-1005    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1006    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1007    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1008    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1010    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1011    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1012    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1013    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1015    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
08-1026    TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA
AF248498   TGATCCTATTGTTGCTCCTAAGATTTCTGTAAACTTCGCAAATGATAACA

                    510       520       530       540       550
           ....|....|....|....|....|....|....|....|....|....|
08-1005    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1006    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1007    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1008    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1010    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1011    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1012    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1013    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1015    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
08-1026    AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA
AF248498   AGCAATTTGTAGTTCTTGGTGGAGTTTTAGAAAATGATGTTCTTGATCAA

                    560       570       580       590       600
           ....|....|....|....|....|....|....|....|....|....|
08-1005    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1006    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1007    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1008    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1010    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1011    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1012    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1013    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1015    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
08-1026    TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC
AF248498   TGTTCTATAAAACAAATTGCTTCTTTGCCAAATATTGATGGTATCCGTGC

                    610       620       630       640       650
           ....|....|....|....|....|....|....|....|....|....|
08-1005    TATTATCATAAGTGCTATTC------------------------------
08-1006    TATTATCATAAGTGCTATTC------------------------------
08-1007    TATTATCATAAGTGCTATTC------------------------------
08-1008    TATTATCATAAGTGCTATTC------------------------------
08-1010    TATTATCATAAGTGCTATTC------------------------------
08-1011    TATTATCATAAGTGCTATTC------------------------------
08-1012    TATTATCATAAGTGCTATTC------------------------------
08-1013    TATTATCATAAGTGCTATTC------------------------------
08-1015    TATTATCATAAGTGCTATTC------------------------------
08-1026    TATTATCATAAGTGCTATTC------------------------------
AF248498   TATTATCATAAGTGCTATTCAATTTAATGCGACTAAATTGCTCAGGCTTC
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Table 4.4 Nucleotide differences obtained between the LafC reference sequence (AF248498) and sequences of this study.

Nucleotide position	Nucleotide(s) this study	Nucleotide(s) LafC reference sequence (AF248498)
191	A	-
310	A	-
311	T	-
326-328	GCG	-
355 - 359	TGCGA	-
193	G	A
194	A	G
248	T	G
307	A	G
309	G	A

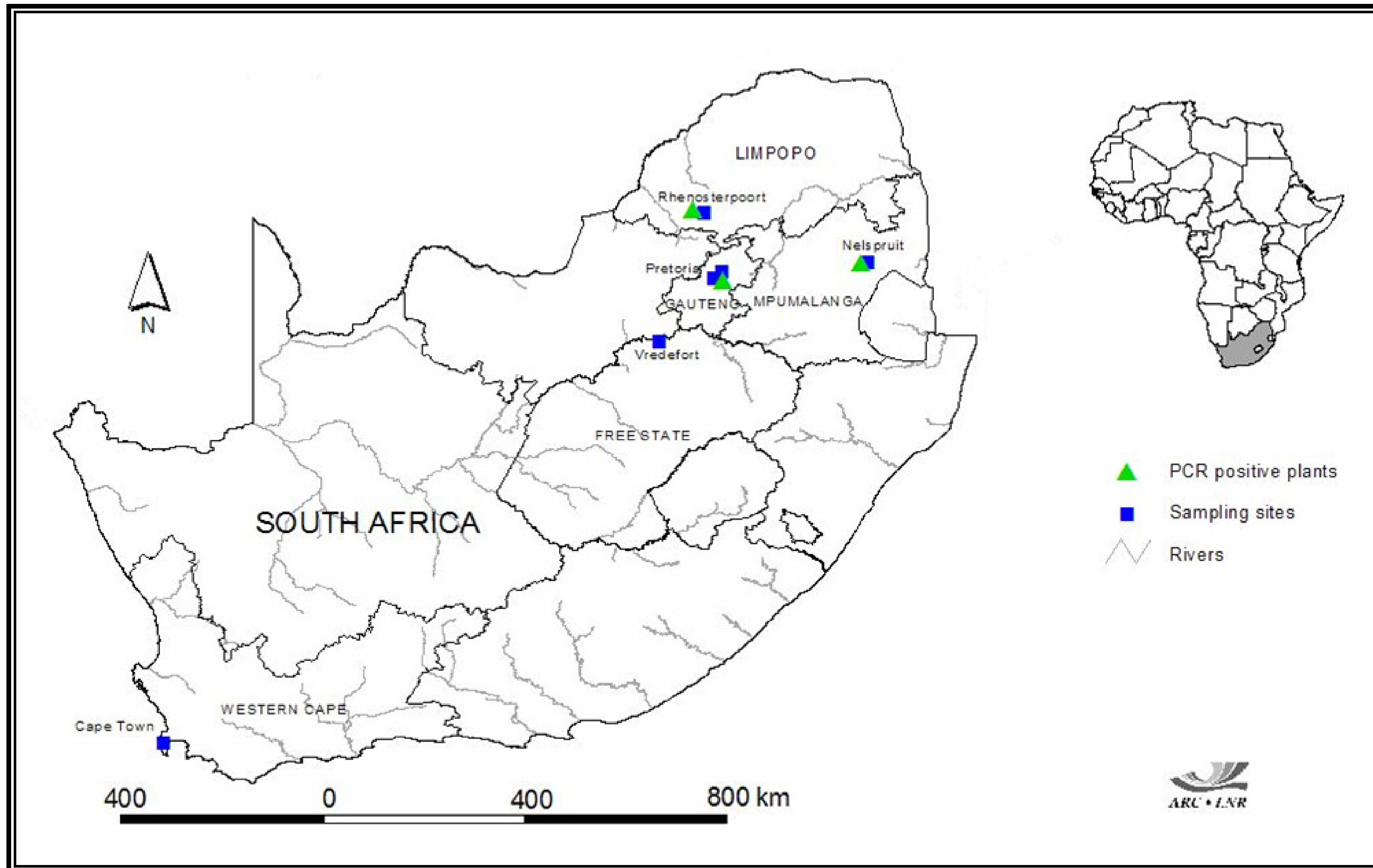

Figure 4.15 Map of South Africa indicating the sampling sites for Rutaceae plants used in this study.

4.5 Discussion

Unique genetic information is required for a microorganism to colonize plant tissue to establish a parasitic or symbiotic relationship thus allowing it to infect and replicate in the host as well as to avoid plant defence mechanisms (Keen & Staskawicz, 1988). Pathogenicity and host range are determined by several positive bacterial functions and the general failure of plants to recognize the bacteria and invoke an active plant defence mechanism (Keen & Staskawicz, 1988).

It is generally accepted that the citrus-infecting Liberibacter species can infect all citrus species regardless of rootstocks (Bové, 2006). LafC and Laf have been previously detected, using DNA techniques, from two naturally infected rutaceous plants, indigenous to South Africa. PCR successfully detected Liberibacter DNA from a *C. capense* ornamental tree (Garnier *et al*., 2000) while DNA Hybridization probe In 1.7 was used to detect the pathogen from a *Vepris lanceolata* plant previously cited as *Toddalia lanceolata* (Garnier *et al*., 2000; Korsten *et al*., 1996). Side-grafting technique was used to transmit Laf from a naturally infected *Clausena anisata* to a sweet orange indicator plant which later developed symptoms. Thin layer chromatography was used to confirm the presence of greening in the indicator plant (van der Berg *et al*., 1991 - 1992). What remains unknown is the host range of Laf or any other Liberibacter species.

A wide range of plants species from the Rutaceae family that displayed greening-like symptoms and psylla-like breeding marks were sampled for this study. These were tested for natural infection by the Laf bacterium. Rutaceous plants were targeted because, as in theory, Laf would most probably be able to infect plants that are genetically most closely related to its known *Citrus* host. Support of *T. erytreae* by plants

unrelated to citrus however, has previously led to speculations as to whether these plants might serve as sources of the bacterium (van den Berg & Deacon, 1989). The detection of newly identified Liberibacter species in plants unrelated to citrus (Duan *et al*., 2008; Liefting *et al*., 2008 a; 2008 b; Munyaneza *et al*., 2007) further indicates the possibility of Liberibacters having a broader host range than is currently known.

The use of a PCR targeting amplification of conserved 16S rDNA gene sequences have improved and simplified the detection and differentiation methods for Liberibacter species (Halbert & Manjunath, 2004). Conventional uniplex PCR and the multiplex PCR developed in this study, with primers targeting the conserved ribosomal protein genes *rpl*KAJL-*rpo*BC operon, were used to detect Liberibacter DNA within the extracted total DNA of each sample. One characteristic that all rutaceous species share is the presence of secretory oil glands which produce rich essential oils used for medicinal or fragrance purposes (Palmer & Pitman, 1972; van Wyk & van Wyk, 1997).

Non-rutaceous plants, also sampled, produce polysaccharides and phenolic compounds. These compounds have the ability to interfere with the amplification procedure by inhibiting the Taq polymerase activity. Host DNA specific primer reactions served as an important indicator for the presence of extracted DNA and the non-inhibition of the Taq polymerase as shown by the amplification of the 179bp band of the internal control DNA on the 1% agarose gel after electrophoresis. Rubisco DNA being a basic ubiquitous metabolic constituent of plants could be amplified consistently with varying intensities in nucleic acid extracts of the collected plant samples. The use of the conventional PCR, as an option to multiplex PCR, was regarded as critical because the PCR system eliminates competition for the utilization of the dNTP's and

polymerase enzyme by the different primer sets as it is an influential factor in multiplex PCR (Henegariu *et al*., 1997).

PCR amplification revealed amplicons suggestive of Liberibacter DNA presence within 17 *C. capense* plants. Generation and comparisons between ribosomal proteins gene sequences of the products obtained, revealed high similarities relative to the single sequence of LafC available on GenBank (accession no. AF248498). These sequences also revealed the presence of a 25bp sequence distinctive for LafC as identified by Garnier *et al* (2000). The results suggest that in South Africa, the natural occurrence of LafC appears to be restricted to only *C. capense* as none of the other indigenous rutaceous or non-rutaceous species tested, were positive for LafC. Conversely the commonly occurring Laf in citrus was not detected in any of the sampled indigenous plants species including *C. capense*. Real-time PCR could detect low concentration of Laf DNA from *C. capense* obtained from Limpopo province.

Nucleotide similarities between sequences obtained during this study and that of reference sequence AF248498 were based on the *rpl*A-*rpl*J fragment of the β operon ribosomal genes. The partial nucleotide differences observed in this gene fragment, between samples of this study and that of the reference sequence, may suggest additional variability in other parts of the genome which may indicate occurrence of variants of LafC. MAbs and *omp* based PCR-restriction fragment length polymorphism approaches were successfully used to react against and identify different variants of Las, even from isolates obtained in the same geographic region (Garnier *et al*., 1991; Gao *et al*., 1993; Garnier *et al*., 1993; Bastianel *et al*., 2005). Isolate variability analysis requires generation of more sequence data and validation of variability using more isolates in future.

The seventeen *C. capense* plants infected with LafC are located in three different provinces namely Gauteng, Limpopo and Mpumalanga, where it has not previously been reported. This study showed the unexpected and widespread incidence and distribution of LafC in *C. capense* found in South Africa. While blotchy mottle symptoms were obtained on samples from Gauteng and Mpumalanga, the Limpopo and some Gauteng samples did not show any foliar symptoms. Samples from Gauteng and Mpumalanga were mainly collected during the cooler months of March, May, June and July. Symptomatic samples were obtained during the May, June and July winter months. Asymptomatic but LafC infected samples indicate that the bacterium can occur within the native rutaceous plants without showing symptoms. When investigating alternate hosts, symptomless potential hosts should nevertheless still be tested.

The appearance of pronounced typical symptoms associated with citrus greening, during the cooler months, and the detection of LafC from the *C. capense* correlates with reports that pronounced symptoms expression on foliar of infected citrus are observed during cooler temperatures of 22 – 24^{0}C (Bové *et al*., 1974). Even though blotchy mottling was noted on some *C. capense* no other symptoms associated with the disease, i.e. die-back of shoots, were observed on plants from which the leaf samples were taken.

The long distance between the Western Cape where the LafC was first detected and the Limpopo, Gauteng and Mpumalanga Provinces in which LafC was found during this study, suggests that the bacterium was not recently introduced into the latter provinces but rather that the *C. capense* might be a preferred host for LafC. The lack of knowledge on possible vector(s) of LafC makes it difficult to speculate on how LafC was introduced in *C. capense* plants found in Limpopo, Gauteng and

Mpumalanga Provinces. It is possible that the LafC occurs naturally in Cape chestnut plants and that the unknown vector(s) occurs in all provinces. A similar situation is found in the Eastern Cape Province where *T. erytreae* occurs but there is no greening due to the absence of the Laf organism. Further studies are required to investigate whether LafC can be hosted by widely distributed indigenous rutaceous plants, including citrus, of South Africa and other African countries.

In this chapter it was shown that Liberibacter species could only be detected from several *C. capense* plants that were naturally infected with the bacterium. The other non-rutaceous and rutaceous plants failed to give the amplification of Liberibacter DNA from the extracts tested, therefore the samples were not sources of the bacterium. Although negative results were obtained it does not prove that a plant species cannot host a pathogen but that at the time of sampling and subsequent testing the material contained none or non-detectable levels of the pathogen. These plants could also contain phytoalexins that are low molecular weight antimicrobial compounds that accumulate in plants as a result of infection or stress and are associated with resistance in plants to diseases caused by bacteria or fungi (Kuć, 1995). These plants could also have other resistance mechanisms, requiring avirulence (*avr*) and corresponding resistance (*R*) genes or plant compounds that regulate plant-pathogen interactions (Dangl & Jones, 2001) that would inhibit infection of the plant by the Laf bacterium.

Rutaceous species, *M. paniculata*, *V. lanceolata* and *C. anisata* previously showed natural infection to Las and Laf respectively when using molecular techniques and side-grafting (Korsten *et al*., 1996; Lopes *et al*., 2006) and samples of each species were surveyed in this study. In this study, *M. paniculata* and *V. lanceolata* plants that were sampled showed no citrus greening-like symptoms and subsequently tested negative for

Liberibacter DNA. Some *C. anisata* leaf samples showed mottling-like symptom and psylla-like breeding marks but tested negative for Laf (Figure 4.7).

The introduction and adaptation of the LafC into *Citrus* plants could lead to detrimental effects, if it was to illicit a pathogenic behaviour in infected citrus as is the case with Laf. This could hold true for large citrus production areas such as Mpumalanga and Limpopo (refer to literature review 2.3) where the bacterium was shown to occur. Previous speculation includes the possibility of unknown native Liberibacter species spread by native psyllid to plants unrelated to citrus, and for these Liberibacters to be unexpectedly introduced to citrus (Nassuth & Manjunath, 2004). South Africa hosts two *Diaphorina* species, *Diaphorina turneri* sp. n. but its host plant is unknown and *Diaphorina amoena* Caper hosted by the plant *Strychnos innocua* Delile (Hollis, 1987) however neither was associated with citrus or its relatives in South Africa. The possible LafC vector(s) could show specific and preferential feeding and/or oviposition and nymphal development on *C. capense* and not on citrus leaves. *T. erytreae* was shown to have feeding, ovipositing and nymphal development preferences on citrus (*C. limon*) than on indigenous hosts which included *Z. capense* previously referred to as *Fagara capense* Thunb. (Moran, 1968 a; 1968 b; Moran & Buchan, 1975).

Beattie *et al* (2008) proposed that greening or Huanglongbing might have originated in Africa, before the separation of Gondwana, as it is the only continent where an asymptomatic rutaceous plant *V. lanceolata* has been associated with a Liberibacter pathogen, Laf, and its vector *T. erytreae*, therefore implicating the plant as a possible original natural host. It is speculated to have spread to other continents by anthropogenetical movement of infected plant material, insect vectors or grafting and other

practices (Beattie *et al*., 2008). It is also speculated that Liberibacter pathogens could be insect-borne pathogens transmitted to plants through feeding (Garnier et al., 1996). In this study both asymptomatic and symptomatic *C. capense* were shown to be natural hosts of the LafC bacterium. Its ability to host this bacterium might implicate the plant as one of the ancestral hosts of the bacterium which may have evolved as a non-pathogenic bacterium within the rutaceous plant. It is not known whether LafC may have evolved to an infectious species to citrus, Laf, which may have been transmitted to citrus by the psyllid vector.

The implication of detecting the LafC in *C. capense* plants from various widespread localities is of concern to commercial citrus producers. Further studies on this bacterium need to be conducted and include: (1) determination of host pathogen interactions between LafC and host; (2) determine if *Citrus* species can host LafC, if it to cause a disease in citrus and if so, how widespread it may already occur in citrus; (3) identify other hosts, other than the Cape chestnut, of LafC; (4) identification of putative vector(s) of the bacterium using insect transmission experiments starting with *T. erytreae,* as it is able to transmit Laf, the closest related Liberibacter to LafC. Insect and other arthropod associated bacteria are largely comprised of alpha and gamma-3 subclass of *Proteobacteria* (Zreik *et al*., 1998). Phloem-restricted plant pathogens belong to these subclasses, including LafC which belongs to alpha subclass, and are generally associated with psyllid vectors. Zreik *et al* (1998) predicted difficulties in detecting "*Ca*. Phloemobacter" from insects as they are known to carry uncharacterized symbiotic and/or parasitic bacteria. Similar to LafC, "*Ca*. Phloemobacter" belongs to *Proteobacteria* and detection thereof from insects might prove to be difficult due to interference from other bacteria; (5) role of the vector and host(s) in transmission of the bacterium.

The use of disease-free planting material, pruning or total removal of infected plants and psylla control by insecticide applications are the current customary methods for control and management of citrus greening in South Africa. The detection of the LafC from the *C. capense* led to several Liberibacter-affected citrus trees being tested for the LafC using its specific primers, but all samples testing negative (Garnier *et al*., 2000). A subsequent survey for Liberibacter species in citrus of South Africa was done in 2006 and sequencing results revealed that only Laf could be detected from infected citrus samples amplicons (Schwerdtfeger & Pietersen, 2007; Pietersen *et al*., 2008; Pietersen *et al*., 2010). It is therefore still not known if the LafC can be hosted by any *Citrus* species and if it can exist within citrus without causing apparent disease in the plant, however this would have to be validated by surveying more citrus plants in future or by transmitting LafC from *C. capense* to Citrus using inoculation techniques.

Until a common epidemiology of LafC in either its natural host *C. capense* and/or citrus is proven, it cannot be recommended that *C. capense* be included with the integrated management and control strategies of the disease in citrus producing countries where the plant is known to occur.

Chapter 5

Inoculation of rutaceous plant species with citrus greening-infected material

5.1 Introduction

Graft transmission has often been used to study the host range of viral and bacterial diseases of various plants, including *Citrus*. Some plants in the Rutaceae family have been investigated for their ability to host Las and Laf bacterium and it was found that various citrus relatives could be experimentally infected with the pathogen and exhibit typical symptoms following certain incubation periods (Hung *et al*., 2000).

In South Africa transmission experiments have mostly been done on citrus with relatively little done on rutaceous plants. A single report of the experimental transmission from a rutaceous plant is on a side-grafted sweet orange to a naturally growing *C. anisata*. The sweet orange that eventually exhibited greening-like symptoms 6 months after it was transferred to a temperature controlled glasshouse, and tested positive for Laf when thin layer chromatography was used as a detection technique (van den Berg *et al*., 1992).

Various members of the Rutaceae family are known to be distributed in South Africa but most have not been shown, experimentally or naturally, to host Laf. Rutaceous trees that are found in South Africa are *C. capense*, *C. anisata*, *Fagaropsis angolensis* (Engl.) H.M. Gardner, *Oricia bachmannii* (Engl.) Verdoorn, *Teclea gerrardii* Verdoorn, *T. natalensis*, *T. bremekampii, V. lanceolata, V. reflexa, Vepris carringtoniana* Mendonça *Z. capense, Z. davyi* and *Zanthoxylum leprieurii* Guill. et Perr (Palmer & Pitman, 1972; van Wyk & van Wyk, 1997). The rutaceous plants of South Africa further includes numerous small, aromatic shrubs called "buchu".

Graft transmissibility of Laf depends on various factors. The graft transmissibility of Laf has been reported as being variably influenced by the type of tissue used for inoculation, the amount of tissue and specific

pathogen isolate (McClean, 1970 a; van Vuuren, 1993). Liberibacter isolates from India, China, Philippine and South Africa were transmitted to periwinkle (*C. roseus*) a reservoir were the organism can multiply in high titers, via dodder (*C. campestris*) (Garnier & Bové, 1983). Hung *et al* (2000) graft-inoculated *D. citri* hosts *M. paniculata*, *M. euchrestifolia*, *L. acidissima* and *S. buxifolia* with Las infected citrus scions to test for their ability to host Las and only *L. acidissima* and *S. buxifolia* were able to host Las. *C. campestris* has also been used to transmit Lam to *N. tabacum* (Franscischini *et al*., 2007). These reports indicate that graft transmission is useful for determining experimental hosts of Liberibacter species.

In this study selected indigenous rutaceous species were assessed as potential hosts for Laf using the graft-inoculation procedure. Healthy seedlings of the rutaceous plants were inoculated with bark patches and/or buds of inoculum sources that were previously shown, by PCR, to harbour the Laf pathogen. Symptoms expressed on the grafted seedlings were monitored and recorded on a regular basis. PCR tests were performed to monitor the multiplication of Laf within the various inoculated seedlings.

5.2 Materials and methods

Plant material

Seed propagated seedlings of *C. capense*, *C. anisata*, *V. lanceolata* and *Z. capense* were obtained from a nursery in the Western Cape and used as receptor plants for the experiment. Three sweet orange (*Citrus sinensis* cv. Bahainina navel) plants on Carizzo citrange (*Poncirus trifoliate* (L.) Raf. X *Citrus sinensis* (L.) Osbeck)rootstock previously graft-inoculated with a Laf infected source were confirmed to be infected with the

pathogen by subjecting nucleic acid extracts, as extracted using CTAB buffer method (Doyle & Doyle, 1990) in annexure A1.2, as template in conventional PCR with primers A2/J5 (Hocquellet et al., 1999) (annexure A2.1). The Bahainina navel plants (accession numbers UPCRI 06-0150, UPCRI 06-0195, UPCRI 06-0280) were used as inoculum sources for the inoculation experiments to the various rutaceous species.

Graft-inoculation procedure

Three bark patches (1 ½ - 2cm X 1cm) and/or buds were cut from the Laf infected citrus plants that served as inoculum sources for the experiment. A scalpel was used to cut out the bark strip/patches from the inoculum source citrus plants and the recipient rutaceous seedling. The scalpel was sterilized by immersing in a 70% bleach solution in between the cuts. These strip/patches were inserted within an incision, on the rutaceous seedlings, of about the same length and width to ensure complete contact of the cambium on both the vertical and horizontal sides of the cut. While inoculation with buds was preferred to patches, insufficient buds were available therefore a combination of buds and patches was used for all inoculations. Three healthy Bahainina navel plants were inoculated with the same infected material to serve as a positive control for the experiment.

A single seedling of each rutaceous species and a Bahainina navel was not inoculated, and served as negative controls for the experiment. Strips of parafilm were used to seal the area around the inoculation wounds for the healing duration. Inoculation was considered successful when the inserted patch/strip remained viable and when a union was formed with the recipient plant.

Growing conditions after inoculation

All the seedlings were kept in a temperature controlled glasshouse at the experimental farm of the University of Pretoria to allow sufficient growth. The glasshouse temperature varied between 22°C and 30°C during the seasons with natural day length hours.

Sample preparation for conventional PCR and real-time PCR

All inoculated plants were tested using PCR. Petioles and/or midribs of leaves showing greening-like or mineral deficiency symptoms and asymptomatic leaves on plants were used for total DNA extraction procedure. Total DNA extraction using CTAB buffer (Doyle and Doyle, 1990) as described in Annexure A1.2, was used during this experiment. Total DNA extracts were used as template for conventional (Hocquellet *et al.*, 1999), multiplex and real-time PCR (Li *et al.*, 2006) with a modification that excluded the cytochrome oxidase based primer-probe set (Annexure A2.1 and A2.4) to test for successful transmission of Laf DNA to the indigenous rutaceous seedlings. For the real-time PCR, templates were prepared by diluting total DNA extracts to 1:10 with molecular grade water (SIGMA, Missouri, USA) and the LightCycler® Taqman® master (Roche Diagnostics, Mannheim, Germany) kit was used to perform the reactions.

Figure 5.1 Photo illustrating Rutaceae seedlings used for the transmission experiment. Rows from left to right are of *Vepris lanceolata, Clausena anisata, Calodendrum capense* and *Zanthoxylum capense*.

5.3 Results

Inoculation compatibility

Survival of the buds and/or patches confirms compatibility and successful grafting onto the indicator plant. Buds and patches that remained green were considered viable. Total numbers of viable buds and patches per plant were counted 12 weeks post inoculation (Table 5.1). Percentage of grafts taken per group of plant genus ($^{\text{total no. of grafts taken per genus x 100}}/_{\text{no. of grafts per seedling x total no. of plants per genus}}$), was as follows: *C. anisata* 83.3%, *Z. capense* 16.7%, *V. lanceolata* 13.3% and *C. capense* 3.3%. *C. capense* had 4 dead seedlings and 1 for *V. lanceolata*.

Symptom monitoring

Inoculated plants were regularly monitored for symptoms typically associated with citrus greening disease. During week 4 post inoculation (PI), yellowing symptoms associated mainly with mineral deficiencies were observed on one of ten inoculated seedlings of *Z. capense*, *C. anisata* and *C. capense* but PCR results did not indicate the presence of the bacterium at that time. By the 8th week PI the same *C. anisata* and *Z. capensis* seedlings and four other *C. anisata* seedlings still showed the yellowing symptoms. A seedling of *C. capense* (UPCRI 07 – 0662) lost its leaves by the 6th week. A second seedling of *C. capense* (UPCRI 07 – 0668) died by the 13th week of the experiment. No clear greening-like symptoms were observed on the rutaceous seedlings 1 year PI. Citrus plants used as positive controls showed moderate yellowing around the midrib veins only and hardening of leaves after 24 weeks. Two years PI typical leaf mottling or mineral deficiency symptoms induced by iron, zinc or manganese remained absent from the plants inoculated and

infected with Laf. Notably during this duration another seedling of *C. capense* (UPCRI 06-0662) died and leaves of the citrus positive control plants remained yellow around the hardened midrib. The uninoculated seedlings used as negative control remained healthy without symptoms for the duration of the experiment.

Determination of host range by PCR

During the fourth and eighth week PI both conventional and multiplex PCR were used to evaluate the inoculated seedlings total DNA extracts for the presence of the inoculated Laf. No amplification could be obtained from any of the inoculated seedlings but was obtained from Laf positive DNA extract used as positive control. Due to the slow replication of Laf bacterium, PCR tests were repeated on newly extracted total DNA twelve months PI. These however still tested negative for Laf.

Detection of Laf using the sensitive TaqMan real-time PCR was conducted two years PI using newly extracted total DNA of the inoculated rutaceous seedlings. The primer-probe set HLBafpr specific for Laf (Li *et al*., 2006), was used to detect Laf DNA from inoculated seedlings which resulted in positive cycle threshold values for certain samples. The results showed low Ct values for one *C. capense* seedling (27.75). High Ct values were obtained for one *Z. capense* (35.53) and two *C. anisata* (35.87 and 35.73). A Bahainina navel positive control plant had a Ct value of 29.94. Each real-time PCR run contained a negative (no template), positive (UPCRI 06-0293), and healthy control. Low Ct values implied the presence of high bacterial concentrations while higher Ct values are indicative of low bacterial concentrations within inoculated plants. Primer-probe combination HLBafpr was used

without inclusion of internal control COX-based primer-probe COXfpr (Li *et al*., 2006).

Conventional PCR using primers A2/J5 was used to test DNA extracts of *C. capense* (UPCRI 07-0667) and Bahainina navel positive control (UPCRI 07-0721) that showed low Ct values two years PI. An expected DNA amplicon of 669bp was observed on a 1% agarose gel after electrophoresis (Figure 5.2). The distribution of alternative experimental hosts of Laf, as determined in this study, in South Africa is summarized in Table 5.4.

Despite the low Ct values and thus high Laf concentration in *C. capense* (UPCRI 07-0668), the typical symptoms of leaf mottling, associated with Laf in citrus was not observed on the Laf infected *C. capense* plant. The Bahainina navel positive control plant showed yellowing around the hardened leaf midribs.

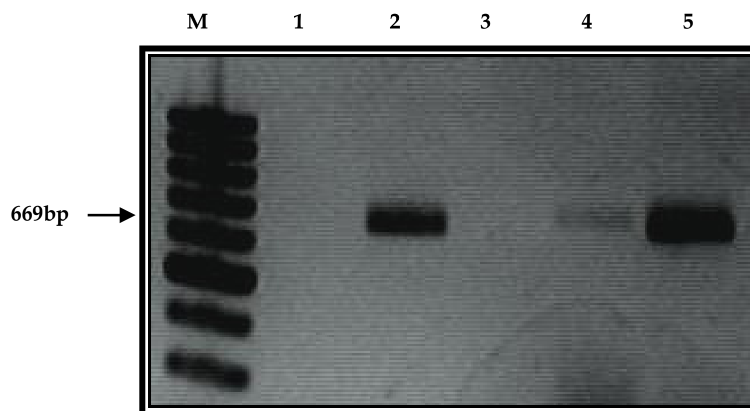

Figure 5.2 PCR amplification of Laf DNA from DNA extracts of inoculated plants. DNA marker (lane M) (Fermentas, Vilnius, Lithuania), no template (lane 1), positive control (UPCRI 07-0595), healthy control (UPCRI 06-0046), inoculated *Calodendrum capense* (UPCRI 07-0667) and Bahainina navel inoculated positive control (UPCRI 07-0721).

Table 5.1 Number of viable buds and/or patches per inoculated plant 12 weeks PI.

Inoculated plant species	Accession no. (UPCRI)	Viable inoculum per seedling
Zanthoxylum capense	07 - 0641	0
	07 - 0642	0
	07 - 0643	0
	07 - 0644	0
	07 - 0645	1
	07 - 0646	0
	07 - 0647	0
	07 - 0648	2
	07 - 0649	2
	07 - 0650	0
Calodendrum capense	07 - 0661	0
	07 - 0662	0
	07 - 0663	0
	07 - 0664	0
	07 - 0665	0
	07 - 0666	1
	07 - 0667	0
	07 - 0668	0
	07 - 0669	0
	07 - 0670	0
Vepris lanceolata	07 - 0681	0
	07 - 0682	1
	07 - 0683	0
	07 - 0684	1
	07 - 0685	2
	07 - 0686	0
	07 - 0687	0
	07 - 0688	0
	07 - 0689	0
	07 - 0690	0
Clausena anisata	07 - 0701	3
	07 - 0702	3
	07 - 0703	2
	07 - 0704	3
	07 - 0705	2
	07 - 0706	3
	07 - 0707	2
	07 - 0708	1
	07 - 0709	3
	07 - 0710	3
Citrus sinensis	07 - 0721	3
	07 - 0722	3
	07 - 0723	3
	07 – 0724	0

Table 5.2 Symptoms observed on grafted seedlings.

Inoculated plant species	Accession no. (UPCRI)	Symptoms observed week(s) PI					
		1	**4**	**8**	**12**	**16**	**20**
Zanthoxylum capense	07 - 0641	-	-	-	-	-	-
	07 - 0642	-	-	-	-	-	-
	07 - 0643	-	*	*	-	-	-
	07 - 0644	-	-	-	-	-	-
	07 - 0645	-	-	-	-	-	-
	07 - 0646	-	-	-	-	-	-
	07 - 0647	-	-	-	-	-	-
	07 - 0648	-	-	-	-	-	-
	07 - 0649	-	-	-	-	-	-
	07 - 0650	-	-	-	-	-	-
Calodendrum capense	07 - 0661	-	-	-	-	-	-
	07 - 0662	-	-	-	×	×	×
	07 - 0663	-	-	-	-	-	-
	07 - 0664	-	-	-	-	-	-
	07 - 0665	-	-	-	-	-	-
	07 - 0666	-	*	-	-	-	-
	07 - 0667	-	-	-	-	-	-
	07 - 0668	-	-	-	-	-	×
	07 - 0669	-	-	-	-	-	-
	07 - 0670	-	-	-	-	-	-
Vepris lanceolata	07 - 0681	-	-	-	-	-	-
	07 - 0682	-	-	-	-	-	-
	07 - 0683	-	-	-	-	-	-
	07 - 0684	-	-	-	-	-	*

Table 5.2 Continued

Inoculated plant species	Accession no. (UPCRI)	Symptoms observed					
		Week no. 1	**4**	**8**	**12**	**16**	**20**
Vepris lanceolata	07 - 0687	-	-	-	-	-	-
	07-0688	-	-	-	-	-	-
	07 - 0689	-	-	-	-	-	-
	07 - 0690	-	-	-	-	-	-
Clausena anisata	07 - 0701	-	-	-	-	-	-
	07 - 0702	-	-	-	-	-	-
	07 - 0703	-	-	-	-	-	-
	07 - 0704	-	-	*	-	-	-
	07 - 0705	-	-	*	-	-	-
	07 - 0706	-	-	*	-	-	-
	07 - 0707	-	-	*	-	-	-
	07 - 0708	-	*	*	-	-	-
	07 - 0709	-	-	-	-	-	-
	07 - 0710	-	-	-	-	-	-
Citrus sinnsis	07 - 0721	-	-	-	-	-	-
	07 - 0722	-	-	-	-	-	-
	07 - 0723	-	-	-	-	-	-
	07 – 0724	-	-	-	-	-	-

- = No typical citrus greening symptoms
× = Dead
* = Mottling-like symptoms

Table 5.3 Ct values of inoculated seedlings as obtained with real-time PCR.

Inoculated plant species	Accession no. (UPCRI)	Ct values with Laf specific primer-probe set HLBafpr 2 years PI
Zanthoxylum capense	07 - 0641	0
	07 - 0642	0
	07 - 0643	0
	07 - 0644	0
	07 - 0645	0
	07 - 0646	>35.99
	07 - 0647	>35.99
	07 - 0648	>35.99
	07 - 0649	0
	07 - 0650	35.53
Calodendrum capense	07 - 0661	0
	07 - 0662	0
	07 - 0663	0
	07 - 0664	33.73
	07 - 0665	0
	07 - 0666	0
	07 - 0667	27.75
	07 - 0668	0
	07 - 0669	0
	07 - 0670	
Vepris lanceolata	07 - 0681	0
	07 - 0682	0
	07 - 0683	0
	07 - 0684	0
	07 - 0685	0
	07 - 0686	0
	07 - 0687	0
	07 - 0688	0
	07 - 0689	>35.99
	07 - 0690	0
Clausena anisata	07 - 0701	35.81
	07 - 0702	>35.99
	07 - 0703	>35.99
	07 - 0704	35.85
	07 - 0705	0
	07 - 0706	0
	07 - 0707	>35.99
	07 - 0708	>35.99
	07 - 0709	0
	07 - 0710	>35.99
Citrus sinensis	07 - 0721	29.94
	07 - 0722	0
	07 - 0723	0
	07 – 0724	0

Ct values; 0 = negative, between 0 & 35.99 = positive and above 35.99 = negative

Table 5.4 A list of Rutaceae species that experimentally hosted Laf and their distribution in provinces of South Africa (van Wyk & van Wyk, 1997).

Plant species	Distribution in South Africa
Calodendrum capense	Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North West and Western Cape
Clausena anisata	Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga and Western Cape
Vepris lanceolata	Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North West and Western Cape
Zanthoxylum capense	Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga and Western Cape

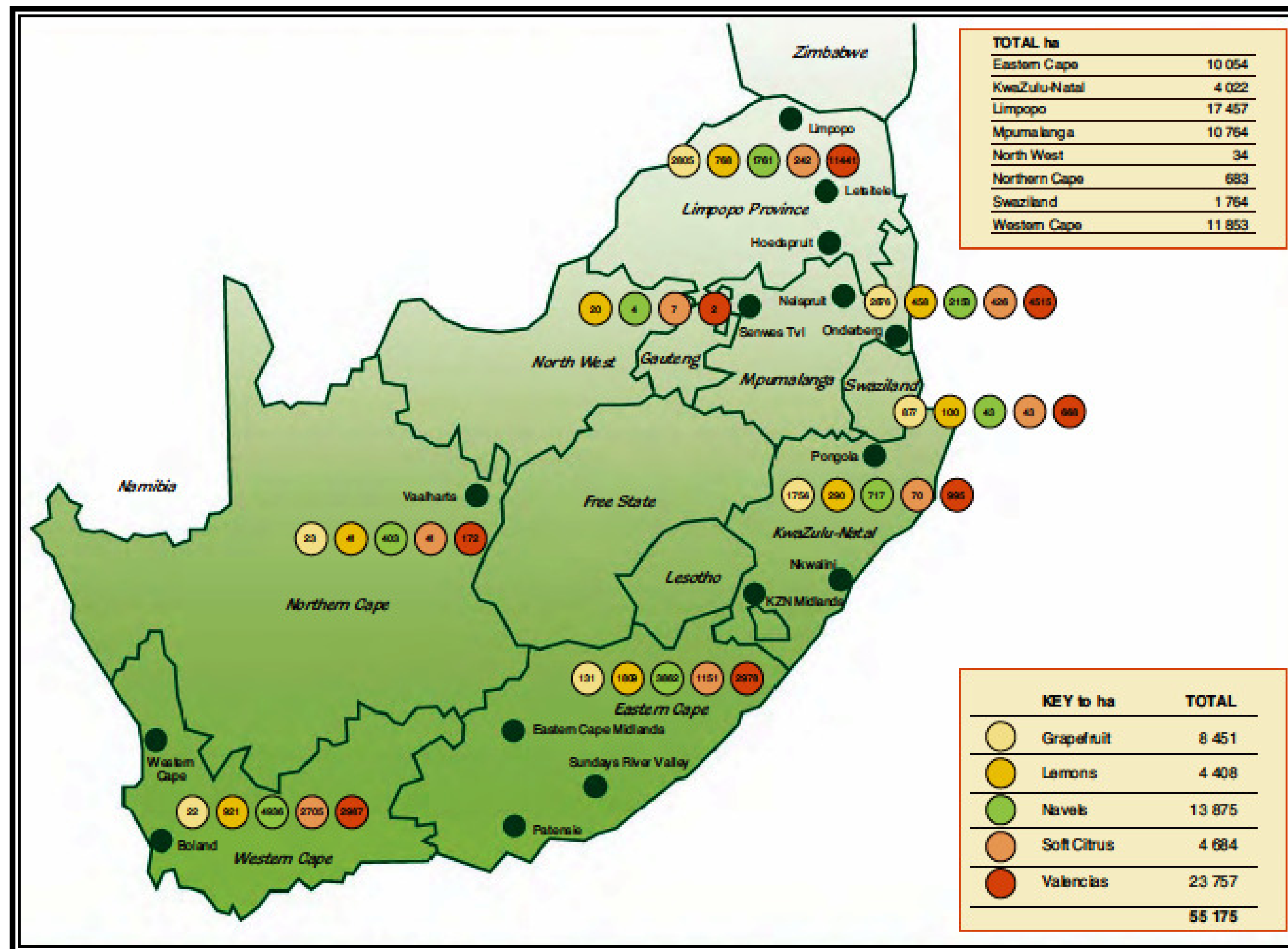

Figure 5.3 Map of Citrus producing areas of Southern Africa as illustrated in the Citrus Growers Association annual report 2008.

5.4 Discussion

Symptoms were previously used to identify a plant as a host of citrus greening disease, following graft-transmission or natural infection by vectors (Miyakawa & Yuan, 1990; Halbert & Manjunath, 2004). Hung *et al* (2000) also suggested that expression of symptoms associated with citrus greening on alternate plants to citrus, might be correlated to infection and replication of the bacterium within the plant. Transmission studies using inoculation methods have been utilised to assess only a few citrus relatives for their capability to host citrus infecting Liberibacter species. In the case of Laf, a field grown *C. anisata* source side-grafted with a healthy sweet orange, produced greening symptoms on the sweet orange (van den Berg *et al*., 1991-1992). In the case of Las, it was successfully transmitted to *S. buxifolia* and *L. acidissima* from infected citrus scions (Hung *et al*., 2005).

Native rutaceous plants namely *C. capense, C. anisata*, *V. lanceolata* and *Z. capense* are known to be natural hosts to *T. erytreae* psyllid in South Africa (Moran, 1968 b). In some instances, *T. erytreae* has been observed to reach large populations on *V. lanceolata* similar to citrus but to a lesser extend on other indigenous plants (Moran & Buchan, 1975). As hosts of the efficient vector of Laf, these plants could potentially acquire and host the bacterium during feeding periods of the vector. This motivated the selection of these plants for the inoculation tests.

Graft compatibility between citrus and the experimental rutaceous hosts often determines the successful transmission of Las/Laf (Hung *et al*., 2000). In the current study *Z. capense* showed poor graft compatibility to the citrus bark patches/strips. Poor incompatibility may have resulted in poor transmission of Laf since the cambium of both the receptor plant and inoculum source may have not been in contact. Seedlings of *C.*

capense species died as early as the third month and two years PI it is not known whether the death of these inoculated seedlings could be attributed to Laf as conventional and multiplex PCR results were negative for Laf when seedlings were still alive and DNA could be extracted from their respective leaves.

Neither conventional nor multiplex PCR were able to detect Laf in the inoculated citrus and rutaceous seedlings 1 year PI. This might be due to the inefficient transmission of Laf thus plants not being infected with Laf or the low concentrations and uneven distribution of the bacterium as indicated by the high Ct values obtained when using the more sensitive real-time PCR. Low concentrations of the bacterium could be difficult to detect using conventional PCR which has detection limitations. Li *et al* (2006) compared Ct values obtained for Las and Lam infected plants and found that Ct values for Las were generally higher than those infected with the Lam species and proposed that the difference in Ct values obtained with the primer-probe sets HLBaspr and HLBafpr could be used to differentiate Las from Laf (Li *et al*., 2006). It was also reported that some citrus samples tested positive for Liberibacter when using real-time PCR but not with conventional PCR, nor did they show any symptoms.

Preliminary results indicated that *C. capense*, *C. anisata* and *Z. capense* may be potential hosts of Laf. *C. capense* has been previously shown to host LafC (Garnier *et al*., 2000) but no report exists of it hosting Laf, which commonly infects citrus. This is the first report on the graft-inoculation and molecular detection of Laf from *C. capense, C. anisata* and *Z. capense* using TaqMan assay. An internal control was not included in this assay therefore the influence of inhibitors or DNA quality on the Ct values obtained is unknown.

Probe HLBp by Li *et al* (2006) was initially designed to target three Liberibacter species namely Laf, Las and Lam. Subsequently the probe together with primer LsoF and HLBr (Lsopr) were shown to target the '*Candidatus* Liberibacter solanacearum' (Lso) associated with tomato, capsicum and potato plants (Li *et al*., 2009). Specificity of primer-probe combination of Las, Laf, Lam and Los was proven as all four combinations targeted the specific Liberibacter concerned (Li *et al*., 2006; 2009). The possibility of these primer-probe combinations amplifying other unknown Liberibacter species that may exist in citrus or indigenous plants remains unknown.

Inoculation of some inoculated seedlings may have failed due to a number of factors which may include:

(1) The uneven distribution of the bacteria within the inoculum source tissue to be used for grafting and hence the possibility of inefficient transmission of Laf to the inoculated seedlings. It is known that the citrus greening bacterium is not evenly distributed throughout infected plants and this can influence the experimental transmission thereof to other plants.

(2) Type of tissue and amount used as inoculum. Buds and/or bark patches were used for inoculation in this experiment whereas buds would be preferential source to use, as possible source of high concentrations of the bacterium and as a source of meristematic cells that initiate the growth of new cells of shoots. Number of inoculum sources can be increased to increase the chances of efficient transmission of Laf to the inoculated seedlings.

(3) Death of the bark patches or buds inoculum prior to efficient transmission of the bacterium. Death of the inoculum source might be due to poor contact with cambium of the receptor plant and subsequent callus formation around the wound.

(4) Incompatibility of inoculum source and indicator plants, due to their genetic structure resulting in the lack of contact between the cambium cells of the two plants grafted together (Matthews, 1991; van Vuuren, 1993).

(5) High content of phenolic compounds and rich oils found in rutaceous plants may prevent or delay the infection of receptor plants with Laf.

(6) Pathogens can harbour avirulence (*avr*) genes which can lead to a hypersensitive reaction by a plant which harbour a corresponding resistance (*R*) gene which restricts the pathogen to cause disease and thus limiting its host range (Keen & Staskawicz, 1988; Dangl & Jones, 2001). An explanatory model by Dangl and Jones (2001) states that the interaction requires *R* products to recognise *avr*-dependent signals and the triggering of the signal-transduction events that culminates in activation of defence mechanisms and an arrest of plant growth. Hypersensitivity may allow a plant to be resistant to entire pathogen species (general resistance) or to certain genotypes of the species (specific resistance) (Keen & Staskawicz, 1988). Additional genes that may extend host range of pathogen or restrict the host range to fewer plant species or genotypes are known (Keen & Staskawicz, 1988). These include virulence (*vir*) and T-DNA genes which were shown to have pathogenicity effects, since deletion or mutations in their genes eliminated pathogenicity and reduced susceptibility in host range.

Lopes *et al* (2009) mentioned that from a comparative evolutionary perspective, the presence of high cell titers of Las in citrus and the association with lack of intense symptom expression may be indicative of Las being more adaptive than Lam to live in the phloem cells of citrus as a result of longer host-pathogen co-evolution. Graft inoculation tests in this study have shown that Laf could replicate in higher concentration in *C. capense* and lower concentration in *C. anisata* and *Z. capense* without symptom expression for two years PI. It is also possible that Laf might exist in *C. capense* plants without showing symptoms therefore illustrating possible adaptation and co-evolutionary relationship with the indigenous rutaceous plant.

C. capense, *C. anisata* and Z. *capense* were shown potentially to be hosts of Laf under experimental conditions. This is the first report on the ability, under experimental conditions, of *C. capense* and Z. *capense* to host Laf. Back-inoculation tests to healthy rutaceous species plants and DNA sequencing of Laf DNA are required to fulfil Koch's postulate and confirm presence of Laf in the infected *C. capense*, *C. anisata* and *Z. capense* inoculated plants. Furthermore, future studies are required to analyse the biological interactions between the bacterium and host and probable pathogenic behaviour in the pathogen elicits in the indigenous hosts. In addition to *C. anisata which* was previously found to be a host of Laf (van der Berg *et al*., 1991-1992), it is recommended that the *C. capense* and Z. *capense* plant species be included within the integrated management and control strategies of the disease.

C. capense, *C. anisata* and *Z. capense* are well distributed in all major areas where citrus is produced in South Africa (Figure 5.3 and Table 5.4). The Northern Cape Province is the only citrus producing area where these potential hosts of Laf have not been located. Coincidentally, this province is considered a citrus greening-free area as the disease is not

known to occur in the area (Pretorius & van Vuuren, 2006). The absence of Laf and experimental alternate hosts in the Northern Cape could further implicate that these rutaceous plants to play a role in the transmission hence epidemiology of Laf in affected areas where both Laf and the experimental hosts are known to occur.

South Africa hosts several rutaceous species in the form of trees (refer to section 5.1) and shrubs with the Cape flora consisting of 259 rutaceous species (Goldblatt, 1997). In this study only a few species were used for graft transmission purposes due to the difficulties in commercial availability or natural collection of either the seed for propagation or seedlings required for the experiment. A study such as this also requires locating rutaceous plants, therefore requires constant assistance from individuals who are familiar with the names and locations of rutaceous species. Expansion of host range studies for Laf should include other rutaceous species that are known to occur locally.

Appendices

Appendix A1

Protocols for total DNA extraction from plants tissue

A1.1 Total DNA extraction (Doyle & Doyle 1990) with modifications by J Brown, University of Arizona

- Up to 1g of weighed tissue was macerated in $N_{(1)}$ using a mortar and pestle.
- The powder of each sample was placed in a 50ml plastic blue cap screw-cap tubes. Recommended 25ml of 65°C CTAB containing 0.2ml per 100ml 2-ME was added to the tube.
- The sap was shake-incubated at 60^{0}C for 30 minutes and was left to cool.
- 1 volume of chloroform: isoamyl alcohol (24:1) was added then mixed by inverting.
- Tube contents were evenly divided between 2 labelled centrifuge tubes.
- The tubes were centrifuged at 9000rpm for 10 minutes at 10°C in a JA 20 rotor.
- Upper aqueous phase was placed in new tube (c20ml) and 2/3 volume of cold iso-propanol was added and mixed gently.
- The extract was incubated overnight at RT.
- The tubes were centrifuged at 9000rpm for 10 minutes at 4°C in a JA 20 rotor.
- The supernatant was poured off with caution not to remove loose pellet.
- 1ml cold wash buffer was added to the pellet.
- Contents were incubated for 20 minutes at RT.
- The tubes were centrifuged at 9000rpm for 5 minutes at 4°C in a JA 20 rotor.

- The supernatant was poured off and tubes were place in a 37°C incubator for 30-60 minutes or air-dried at RT.
- Pellet was re-suspended in TE buffer (1ul buffer per 1mg initial leaf material) and stored at -20°C.

A1.2 Total DNA extract (Doyle & Doyle 1990) with modifications by Fundecitrus

- Up to 500mg of petiole and midrib tissue was weighed and chopped finely with a scalpel blade and transferred to maceration bags.
- Tissue was macerated in 5ml CTAB containing 0.2ml per 100ml 2-ME using Homex 6 (BIOREBA AG, Reinach, Switzerland) semi-automated machine.
- 2ml of macerate was transferred to 2ml eppendorf tubes.
- Sap was placed in a shake incubator at 60°C incubator for 30 minutes.
- The tubes were centrifuged at 3000rpm for 5 minutes at 4°C.
- 900µl of supernatant was recovered and place in a new tube.
- 900µl (1 volume) of chloroform: isoamyl alcohol (24:1) was added and inverted to mix.
- The tubes were centrifuge at 14000rpm for 5 minutes at 10°C.
- 800µl of upper aqueous phase was placed in a new tube and 2/3 volume (480µl) of ice cold iso-propanol was added.
- The tubes were incubated at -80°C for 20 minutes.
- The contents were centrifuged at 14000rpm for 10 minutes at 4°C.
- The supernatant was poured off with caution to avoid discarding the loose pellet.
- 1ml cold wash buffer was added to the pellet and swirled.
- The tubes were centrifuged at 14000rpm for 10 minutes at 4°C.
- The supernatant was again poured off with caution.
- 1ml cold wash buffer was added to the pellet to rewash the pellet.
- The tubes were centrifuge at 14000rpm for 10 minutes at 4°C.
- Pellet was dried in "speedy-vac" for 3 minutes.
- The pellet was re-suspended in 100µl TE buffer.
- Place in labelled eppendorf tubes and stored at -20°C.

Annexure A2

Molecular techniques

A2.1 Laf conventional PCR protocol

1. 2% Triton X-100 (0.1)
2. 10X Bioline NH_4 buffer (Bioline, London, UK)
3. 2mM dNTP mix (200uM each)
4. Primer A2 (10µM) (Hocquellete *et al*., 1999)
5. Primer J5 (10µM) (Hocquellete *et al*., 1999)
6. 0.05M $MgCl_2$ (2mM)
7. 0.10M 2-ME (10mM)
8. 2000ug/ml (2ng/ml) BSA (10ug)
9. Molecular grade H_20
10. Biotaq™ DNA polymerase (5u/ul) (Bioline, London, UK)

A PCR master mix was prepared for each reaction using the above reagents. The final reaction volume for each tube was either 25 µl or 50µl. The reaction was performed in a thermocycler GenAmp® PCR System 2700 (Applied Biosystems, California, USA); Eppendorf MasterCycler Gradient (Eppendorf, Hamburg, Germany) and T-Cy (Creacon, Emmen, Netherlands) using the following steps: denaturing at 92°C for 20s, annealing at 62°C for 20s and extension at 72°C for 45s for 35 cycles. Aliquots of all working solutions were used and stored at -20°C.

A2.2 Laf, LafC and Las Multiplex PCR protocol

1. 2% Triton X-100 (0.1)
2. 10X Bioline NH_4 buffer (Bioline, London, UK)
3. 2mM dNTP's mix (100uM each)
4. Primer RBCL-H535 (2µM) (Nassuth *et al*., 2000)
5. Primer RBCL-C705 (2µM) (Nassuth *et al*., 2000)
6. Primer A2 (10µM) (Hocquellet *et al*., 1999)
7. Primer J5 (10µM) (Hocquellet *et al*., 1999)
8. 0.05M $MgCl_2$ (2mM)
9. 0.10M 2-ME (10mM)
10. 2000ug/ml (2ng/ml) BSA (10ug)
11. Molecular grade H_20
12. Biotaq™ DNA polymerase (5u/ul) (Bioline, London, UK)

A PCR master mix was prepared for each reaction using the above reagents. The final reaction volume for each tube was either 25 µl or 50µl. The reaction was performed in a thermocycler GenAmp® PCR System 2700 (Applied Biosystems, Carlifirnia, USA); Epperdorf Mastercycler gradient (Eppendorf, Hamburg, Germany) and T-Cy (Creacon, Emmen, Netherlands) using the following steps: denaturing at 92°C for 20s, annealing at 62°C for 20s and extension at 72°C for 45s for 35 cycles. Aliquots of all working solutions were used and stored at -20°C.

A2.3 LafC PCR protocol

1. 2% Triton X-100 (0.1)
2. 10X Bioline NH_4 buffer (Bioline, London, UK)
3. 2mM dNTP mix (200µM each)
4. Primer CAL1 (10µM) (Garnier *et al*., 2000 b)
5. Primer J5 (10µM) (Hocquellet *et al*., 1999)
6. 0.05M $MgCl_2$ (2mM)
7. 0.10M 2-ME (10mM)
8. 2000ug/ml (2ng/ml) BSA (10ug)
9. Molecular grade H_20
10. Biotaq™ DNA polymerase (5u/ul) (Bioline, London, UK)

A PCR master mix was prepared for each reaction using the above reagents. The final reaction volume for each tube was either 25 µl or 50µl. The reactions were performed in a thermocycler GenAmp® PCR System 2700 (Applied Biosystems, California, USA); Eppendorf MasterCycler Gradient (Eppendorf, Hamburg, Germany) and T-cy (Creacon Technologies, Emmen, Netherlands) using the following steps: denaturing at 92°C for 20s, annealing at 62°C for 20s and extension at 72°C for 45s for 35 cycles. Aliquots of all working solutions were used and stored at -20°C.

A2.4 Real-time PCR protocol using LightCycler® Taqman® Master

1. 5X Taqman Universal PCR master mix (Roche Diagnostics, Mannheim, Germany)
2. 5µM Primer HLBaf (250nM) (Li *et al*., 2006)
3. 5µM Primer HLBr (250nM) (Li *et al*., 2006)
4. 5µM Probe HLBp (150nM) (Li *et al*., 2006)
5. Taqman PCR grade H_20 (Roche Diagnostics, Mannheim, Germany)

Taqman Universal PCR master mix was prepared by pipetting 10 µl of Taqman master enzyme (Roche Diagnostics, Mannheim, Germany) into 1 vial of Taqman master reaction mix (Roche Diagnostics, Mannheim, Germany). PCR master mix was prepared with all the reagents in one tube. The final reaction volume for each capillary was 10 µl. The reaction was performed in a Roche LightCycler® 1.5 (Roche Diagnostics, Mannheim, Germany) using the following steps: 95°C for 20s followed by 45 cycles of 95°C for 1s, 58°C for 40s and 40°C for 40sec for cooling. Aliquots of all working solutions were used and stored at -20°C.

A2.5 DNA sequencing protocol

Sequencing reaction:

1. 2µl 2.5X Big Dye v3.1 (Applied Biosystems, Warrington, UK)
2. 1µl 5X Sequencing buffer (Applied Biosystems, Warrington, UK)
3. 2µl 3.2pmol primer
4. 5µl template

A sequencing reaction mix was prepared with the above mentioned reagents to the final volume of 10µl. The tubes where incubated in a thermocycler GenAmp® PCR System 2700 (Applied Biosystems, California, USA). The sequencing cycles consisted of 94°C for 1min, 94°C for 10s denaturation, 50°C for 5s annealing, 60°C for 4min and 4°C for holding.

Precipatation using the Ethylenediaminetetraacetic acid disodium salt/Sodium acetate/ethanol (EDTA/NaOAc/EtOH) method:

1. 1µl 125mM EDTA was added to the tube.
2. 1µl 3M NaOAc was added.
3. 25µl 100% EtOH was added.
4. Mixture was vortexed and incubated for 15 minutes at room temperature.
5. The tubes were centrifuged at 13000rpm for 15 minutes at 4°C.
6. Supernatant was removed carefully using a pipette.
7. 100µl of 70% EtOH was added and tubes were centrifuged at 13000rpm for 15 minutes at 4°C.
8. Supernatant was removed carefully using a pipette and samples dried at 94°C using a heating block for 1 minute or vacuumed using a freeze dryer.

Annexure A3

Buffer solutions and Chemicals utilized

Formula used to determine the grams (g) to be used to obtain a specific molar (M) in a solution: g = Mr x M x vol

Chloroform : isoamyl alcohol [24:1; 25ml]

Chloroform	24ml
Isoamyl alcohol	1ml

The chloroform was carefully added to the isoamyl alcohol and the container was wrapped with foil and stored away from light.

2% CTAB buffer [pH 8.0; 50ml]

NaCl (1.4M)	40.91g
EDTA (20mM)	3.72g
Tris base (mW 121.1:100mM)	6.05g
CTAB	10.00g
Distilled water	450ml
2-Mercaptoethanol	1ml

CTAB was added after all the reagents were heated and dissolved in 450ml distilled water. The pH was adjusted to 8.0 using HCl. The buffer was autoclaved before storage. 0.2ml per 100ml of 2-ME was added per 25ml CTAB buffer.

CTAB wash buffer

Ethanol (Absolute)	70ml
Distilled water	30ml

Reagents were mixed and stored at 4°C.

Ethidium bromide (5mg/ml)

Ethidium bromide	5mg
Deionized water	1ml

Reagents were mixed and stored at 4°C or room temperature when used.

Loading dye

Sucrose	4g
Bromophenol blue	0.025g

Final volume was made up to 10ml with distilled water.

50X TAE (Tris-acetate EDTA) buffer [pH 8.5; 1000ml]

Tris base	242g
Glacial acetic acid	57.1ml
EDTA (0.5M)	100ml
Deionized water	750ml

Tris base was dissolved in 750ml deionized water. The glacial acetic acid and the EDTA were added last and the solution was mixed thoroughly. The pH was adjusted to 8.5.

1X TAE (Tris-acetate EDTA) buffer [1000ml]

TAE buffer (50X)	20ml
Deionized water	980ml

Add the TAE buffer to the deionized water and mix thoroughly.

TE (Tris-EDTA) buffer [pH 8.0; 50ml]

Tris-HCl (10mM)	0.06g
EDTA (1mM)	0.02g
Distilled water	45ml

All the reagents were dissolved in the distilled water. The pH was adjusted with HCl and the final volume was made up by adding remaining volume of water. The buffer was sterilized by autoclaving it.

20X SB (Sodium boric acid) buffer [pH 8.0; 1000ml]

Boric acid	45g
NaOH	8g
Distilled water	800ml

The pH was adjusted to 8 by using boric acid and the final volume was reached by adding remaining volume of water. The buffer was sterilized by autoclaving it.

1X SB (Sodium boric acid) buffer [1000ml]

SB buffer (50X)	50ml
Distilled water	950ml

Add the SB buffer to the deionized water and mix thoroughly.

Index of scientific and common plant names

Acmadenia heterophylla	Buchu
Acmadenia macropetala	Buchu
Acmadenia mundiana	Buchu
Acmadenia obtusata	Duine buchu
Adenandra fragrans	
Adenandra obtusata	China flower
Adenandra uniflora	
Aeglopsis chevalieri	
Agathosma apiculata	
Agathosma capensis	Buchu
Agathosma cerefolium	Anysboegoe
Agathosma ciliaris	
Agathosma collina	
Agothosma crenulata	Buchu
Agathosma dielsiana	
Agathosma glabrata	
Agathosma gonaquensis	
Agathosma imbricate	Sand buchu
Agathosma lanceolata	
Agathosma mucrunulata	
Agathosma ovata	
Agathosma serpyllacea	
Atalantia missionis	
Balsamocitrus dawei	
Calodendrum capense	Cape chestnut
Casimiroa edulis	White sapote
Catharanthus roseus	Periwinkle
Citrus amblycarpa	
Citrus aurantifolia	Lime

Citrus aurantium	
Citrus depressa	
Citrus grandis	
Citrus hassaku	
Citrus hystrix	
Citrus ichangensis	
Citrus indica	
Citrus jambhiri	Rough lemon
Citrus junos	
Citrus kabuchi	
Citrus limetta	
Citrus limon	Lemon
Citrus x limonia	
Citrus medica	Citron
Citrus macroptera	
Citrus x nobilis	
Citrus paradisi	Grapefruit
Citrus reticulata	Mandarin
Citrus sinensis	Sweet orange
Citrus sunki	
Citrus unshiu	
X *Citroncirus webberi*	
Clausena anisata	Horsewood
Clausena anisum	
Clausena indica	
Clausena lansium	Wampee
Coleonema album	Cape may
Coleonema pulchellum	Confetti bush
Cuscuta australis	
Cuscata campestris	Dodder
Cuscata pentagona	Dodder
Diosma hirsute	Rooi buchu

Diosma prama	
Euchaetis meridionales	
Fagaropsis angolensis	Fagaropsis
Fortunella	Kumquats
Limonia acidissima	Wood apple
Lycopersicon esculentum	
Murraya euchrestifolia	
Murraya koenigii	Curry leaf plant
Murraya paniculata	Common jasmine orange
Myosis arvensis	Field forget-me-not
Nicotiana tabacum	Tobacco
Ochna serrulata	Mickey mouse plant
Oricia bachmannii	Twin-berry tree
Poncirus trifoliata	Trifoliate orange
Severinia buxifolia	Chinese box orange
Solanum lycopersicon	
Solanum tuberosum	
Sterculia murex	Lowveld chestnut
Strychnosa madagascariensis	Black monkey orange
Strychnosa spinosa	Green monkey orange
Swinglea glutinosa	
Teclea gerrardii	Zulu cherry-orange
Teclea natalensis	Natal cherry-orange
Toddalia asiatica	Orange climber
Toddaliopsis bremekampii	Wild mandarin
Trichilia emetica	Natal Mahogany
Triphasia trifolia	Lime berry
Vepris carringtoniana	Coastal white ironwood
Vepris lanceolata	White ironwood
Vepris reflexa	Bushveld white ironwood
Zanthoxylum capense	Small knobwood

Zanthoxylum chalybeum	Knobwood
Zanthoxylum davyi	Forest knobwood
Zanthoxylum leprieurii	Sand knobwood

(Palmer & Pitman, 1972; Manning & Goldblatt, 1996; Moriarty, 1997; Mustart *et al*., 1997; van Wyk & van Wyk, 1997).

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