Pantoea and *Xanthomonas* species associated with blight and die-back of *Eucalyptus*

by

Lorinda Swart

Submitted in partial fulfilment of the
requirements for the degree
Magister Scientiae

in

The Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

Supervisor: Prof T.A. Coutinho
Co-supervisor: Prof S.N. Venter

Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree **Magister Scientiae** to the University of Pretoria contains my own independent work. This work has not previously been submitted for any other degree at any other University

_____

Lorinda Swart

December 2009

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

- Firstly, I would like to give all the credit of this accomplishment to the Lord, Jesus Christ. He has given me gifts and talents which I used to the best of my capabilities. Without Jesus in my life this would not have been possible.

- To my parents Albert and Monica, who always encouraged me and always believed in me. Thank you for the opportunity to further my studies and enrich my life with these degrees. Also thank you for the financial support.

- To my husband, Cleon, for the encouragement and never letting me quit. You always supported my studies and my love for science and for this I will always be grateful.

- To my supervisors, Teresa and Fanus, thank you for giving me this splendid opportunity. Thank you for all your guidance and support in the completion of this study. I enjoyed working with you and hope we could do so in future again.

- To the only person whom without this dissertation and project would not have been possible, Carrie Brady, thank you for being by mentor. Thank you for all your help in the completion of this project/dissertation. From helping me to use Bionumerics to trouble shooting PCR's, you were always willing to give your advice and lend a hand.

- To all of my fellow students in the pathology lab who befriended me, Sonya, Carrie, Nico, Pieter and Ancel.

- UP, TPCP and NRF for financial support during the completion of this study

- To all persons involved in the collection of the isolates used in this study

- To all the staff and members at Stimuplant and DSC Lab, thank you for allowing me to complete this dissertation in work hours.

- I would like to dedicate this dissertation to my friend Bernet van der Merwe, who tragically passed away at the start of our MSc degree. We enrolled for this degree together and were supposed to finish it together. She had more willpower than I did, but I got there in the end. Thank you, my friend. This one is for you.

Chapter 1

General Introduction

General Introduction

The forestry industry is expanding globally due to increasing demand for pulp in the paper industry and for solid timber products. *Eucalyptus* species, hybrids and clones are grown as plantation species in many countries of Africa, Asia, South America and New Zealand and also in Australia in its native host range. It is estimated that this species covers a surface area of 18 million hectares in 90 different countries (Turnbull 1999, Carle *et al*. 2002, FAO 2000). In South Africa, *Eucalyptus* species, hybrids and clones are grown commercially and they account for more than 50% of newly afforested areas (Anonymous, 1996).

The rapid expansion of the forestry industry in South Africa into export markets over the last twenty years has demonstrated the international competitiveness of the industry. Annually 7.4 million tons of round wood is produced from *Eucalyptus* in South Africa (The South African Forestry and Forest Products Industry, 2002). The growing of *Eucalyptus* plantations has also become very popular since they grow rapidly and needs little maintenance. The fast growth of local plantations makes tree breeding an ideal possibility for genetic enhancement of the tree and the production of new genotypic material is relatively rapid. This has led to South Africa being considered as a world leader in selective breeding and cloning of *Eucalyptus* species.

Along with the increase in the breeding of new *Eucalyptus* clones in South Africa and other countries, numerous pathogens causing diseases on these trees has also emerged. One of these emerging pathogens of *Eucalyptus* seedlings in nurseries is the bacterial pathogen *Pantoea ananatis*. This pathogen was first discovered as the causal agent of bacterial blight and die-back of *Eucalyptus s*pecies, hybrids and clones in South Africa in 1998 by Coutinho and co-workers (2002). After this pathogen was discovered on *Eucalyptus* in South Africa, a disease causing similar disease symptoms to that of bacterial blight and die-back was found in other countries around the world. Some of these countries include Uruguay, Argentina, Colombia and Uganda (Brady 2005). These bacterial pathogens were subsequently identified as belonging to the genus *Pantoea*.

In this study similar disease symptoms to those of bacterial blight and die-back were observed in a number of other countries as well as from other outbreaks in countries

where *Pantoea* species had already been isolated and identified i.e. Argentina, Colombia, Rwanda, South Africa (2 locations), Thailand, Uganda and Uruguay. The bacterial isolates were investigated on a basis of phenotypic and genotypic techniques in order to accurately identify them. An accurate identification of these isolates will lead to a better understanding of the relationship between these two organisms and this will assist with the management and prevention of this disease on *Eucalyptus* trees.

In this study two different bacterial genera were isolated from the diseased *Eucalyptus* trees. The bacterial isolates obtained from diseased leaf material were shown to belong to the genera *Pantoea* and *Xanthomonas*. In chapter 2, a literature study was undertaken on the taxonomy and pathology of these two genera. The identification techniques used in this study are based on techniques that have been employed by other researchers in a polyphasic identification approach. The techniques used in this study include the phenotypic identification of isolates by gram determination and fermentation capabilities. Genotypic identification techniques included 16S rRNA gene sequencing, *GyrB* gene sequencing and AFLP analysis. In addition to these identification techniques pathogenicity tests were also conducted on the isolates in order to fulfil Koch's postulates. The use of the combination of these techniques proved to be successful in the identification of unknown *Pantoea* (Chapter 3) and *Xanthomonas* (Chapter 4) isolates. The use of a combination of these techniques also provides a more cost effective alternative to the use of DNA:DNA hybridization, which is considered the "gold standard" for accurate identification of unknown isolates.

By completing this study a better understanding of the bacteria involved in bacterial blight and die-back in various parts of the world was created. This study may potentially assist with the control of these bacterial pathogens in the near future in order to support the forestry industry. A better understanding of these pathogens can lead to the rapid identification of these pathogens in the field, and this will ensure that the correct control and management procedures be implemented early to minimize losses to important crops.

Chapter 2

The taxonomy and pathology of the Genera *Pantoea* and *Xanthomonas*

The taxonomy and pathology of the Genera *Pantoea* and *Xanthomonas*

1) INTRODUCTION

Pantoea and *Xanthomonas* species occur in close association with plants and are mostly encountered as pathogens, causing diseases on a wide variety of host (Table 1). Forestry and agricultural crops infected by species of these two genera can result in devastating losses and the diseases they cause are therefore, usually of economic importance. For example, citrus is one of the most important crops in third world countries and can be infected by a devastating disease, citrus canker, caused by *Xanthomonas campestris* pv. *citri*. In the mid-1990's the United States of America's government launched an eradication program of citrus canker and spent over $200 million dollars on destroying infected trees (Graham *et al.*, 2004). Despite this effort, citrus canker still continues to spread in that country.

Stewart's vascular wilt is a disease which causes severe devastation on maize and corn crops (Mergaert *et al*., 1993). The causal agent of this disease, *Pantoea stewartii* subsp. *stewartii* is a quarantine pathogen in many countries. This disease is spread through infected seed lots, and the risk of infection through seeds in other countries is considered such a great threat that the import of corn seed is prohibited in certain countries unless the seed is certified as being free of the pathogen (Coplin *et al.,* 2002).

Table 1: List of diseases caused by *Pantoea* and *Xanthomonas* spp. on economically important hosts

GENUS: *PANTOEA*

Host	Disease	Pathogen	Reference
Maize	Stewart's wilt	*P. stewartii* subsp. *stewartii*	Elliot, 1941
	Leaf-spot	*P. ananatis*	Paccola-Meirelles *et al*., 2001
	Brown stalk rot	*P. ananatis*	Goszczynska, 2007
Sugarcane	Soft rot	*P. ananatis*	Serrano, 1928

Eucalyptus trees	Bacterial blight and dieback	*P. ananatis*	Coutinho *et al*., 2002
Onion	leaf-blight, seed stalk rot and bulb decay Leaf and seed stalk necrosis	*P. ananatis* *P. agglomerans*	Gitaitis and Gay, 1997, Walcott *et al*., 2002 Hattingh and Walters, 1981
Rice	Palea browning Stem necrosis	*P. ananatis* *P. ananatis*	Azegami, 1983 Cother *et al.*, 2004
Cantaloupe fruit	post harvest disease	*P. ananatis*	Bruton *et al*., 1991
Sudangrass	necrotic leaf-blotch disease	*P. ananatis*	Azad *et al*., 2000
Honeydew melon	brown spot	*P. ananatis*	Wells *et al*., 1987
Gypsophila paniculata	Crown and root gall	*P. agglomerans*	Cooksey, 1986, Brown 1934
Human	dyspnea and endema	*P. ananatis*	De Beare *et al*., 2004
Cotton	Seed and boll rot	*P. agglomerans*	Medrano and Bell, 2006
Pineapple	Pink disease Bacterial fruitlet brown rot	*P. citrea* *P. ananatis*	Kageyama *et al.*, 1992 Cha *et al.*, 1997a Serrano, 1928
Beet	Tumorogenic root gall	*P. agglomerans*	Burr *et al.*, 1991
Netted melon	Internal fruit rot	*P. ananatis*	Kido *et al.,* 2008

GENUS: *XANTHOMONAS*

Host	Disease	Pathogen	Reference
Citrus	Citrus canker	*X. citri* subsp *citri*	Hasse, 1915
Rice	Bacterial blight and bacterial leaf streak	X. oryzae	Swings *et al*., 1990
Cotton	Bacterial blight	*X. citri* subsp. *malvasearum*	Atkinson, 1891
Eucalyptus trees	Dieback	*X. campestris* pv *eucalypti*	Truman, 1974
Beans	Bacterial blight	*X. axanopodis* pv. *phaseoli*	Smith, 1897
Mango	Bacterial black spot	*X. campestris* pv *mangiferaeindicae*	Patel, 1948
Poplar	Bacterial canker	*X. populi*	Koning, 1938
Sugarcane	Leaf scald	*X. albilineans*	Ricaud and Ryan, 1989
Walnut	Blight	*X. arboricola* pv. *juglandis*	Teviotdale and Scroth, 1998
Prunus spp.	Bacterial spot	*X. arboricola* pv. *prunus*	Various, 1993

The genus *Pantoea* was described by Gavini *et al*., in 1989. Species within the genus not only cause plant diseases but are also found in environmental samples (Sedkova *et al.*, 2005) and cause disease in humans (De Baere *et al*., 2004). Reports of plant diseases caused by *Pantoea* spp. are increasing and an accurate identification of the pathogen is necessary for managing these diseases. Even though pathogens of this genus are increasing in importance worldwide, identification techniques are still very basic, mostly employing phenotypic characteristics for identification and basic genotypic methods i.e. 16S rRNA sequencing which will in most cases only identify the isolates to genus level (Janda and Abbott, 2007).

The genus *Xanthomonas* was proposed by Dowson in 1939. Species within the genus cause a wide variety of diseases on different plants. Unlike *Pantoea* spp. *Xanthomonas* spp. are only associated with plants. Accurate identification of *Xanthomonas* spp. has

become more reliable since the taxonomy of the genus was revised in 1995 by Vauterin *et al.* 16S rRNA sequencing is mostly used for the identification of a member of the genus *Xanthomonas*, but techniques that give more resolution are required for species identification. Techniques that have been used for this purpose include AFLP, RFLP analyses and *GyrB* sequencing (Rademaker *et al.*, 2000, Simoes *et al.*, 2007 and Aritua *et al.*, 2008).

Understanding the taxonomy and pathogenicity of bacterial pathogens gives insight into the design of management strategies of the diseases they cause. When the identity of a pathogen has been confirmed it is possible to compare it with other pathogens which are similar, thus resulting in a better diagnosis and control of bacterial diseases. The aim of this review is to assess the various genotypic methods used in the identification of bacteria and to determine which of these methods could contribute towards a better understanding or resolve the taxonomy of the Genera *Pantoea* and *Xanthomonas*.

2) THE GENUS *PANTOEA*

2.1) Taxonomy of the genus *Pantoea*

The genus *Pantoea* forms part of the family *Enterobacteriaceae.* This family was formed to include members which are typically straight, rod-shaped bacteria. Members of this family are mostly mobile by means of a single peritrichous flagellum. Most of the members of this family are oxidase negative and catalase positive and grow well at 37 ºC (Grimont and Grimont, 2005). Members of the family *Enterobacteriaceae* are Gram negative organisms and facultatively anaerobic, fermenting D-glucose in the absence of oxygen. Bacteria placed within the family *Enterobacteriaceae* are mostly encountered in the human digestive tract (enteric bacteria) and diseases thereof i.e. *Shigella, Salmonella* and *Escherichia*. However, the family *Enterobacteriaceae* is also comprised of phytopathogenic bacteria, occurring and causing diseases on a wide variety of plant-hosts and these pathogens include species from genera such as *Erwinia, Pectobacterium* and *Pantoea.*

Pantoea species are ubiquitous and occupy diverse ecological niches. The members of the genus that are associated with plants are either pathogenic, saprophytic and/or

epiphytic in nature (Kwon *et al*., 1997). The name *Pantoea* comes from the Greek adjective “pantoios” which means “from all sorts and sources”, which is an appropriate name since the bacteria belonging to this genus are isolated from very diverse geographical and ecological environments.

The species formerly known as *Erwinia herbicola, E. milletiae*, *E. ananas*, *E. uredovora* and *E. stewartii*, which formed part of the *Erwinia herbicola-Enterobacter agglomerans* complex were later re-classified, some synonymised and placed in the genus *Pantoea* (Ewing and Fife, 1972). The species belonging to this complex showed extreme heterogeneity at both the phenotypic and genotypic levels (Mergaert *et al*., 1993). The species *Erwinia herbicola, E. milletiae* and *Enterobacter agglomerans* were found to be synonymous and re-classified as *P. agglomerans* which is the type species of the genus *Pantoea. E. ananas* and *E. uredovora* were found to be synonymous and moved to the genus *Pantoea* as a single species *P. ananatis*. *E. stewartii* was re-classified as *P. stewartii* subsp. *stewartii*. (Gavini *et al.,* 1989). This also led to the description of a new subspecies *Pantoea stewartii* subsp. *indologenes* (Mergaert *et al*., 1993). These species were included in the genus *Pantoea* based upon DNA:DNA hybridization results, phenotypic data and electrophoretic protein patterns (Gavini *et al*., 1989, Mergaert *et al*., 1993). A new species was also added to the genus at that time, *P. dispersa* (Gavini *et al.*, 1989). In 1992 another three new species were added to the genus, *P. citrea, P. terrea* and *P. punctata*. These new species were isolated from fruit and soil samples in Japan and are referred to as the Japanese species (Kageyama *et al*., 1992).

Originally, there were seven described *Pantoea* species, *P. agglomerans, P. ananatis, P. dispersa, P. stewartii* and the Japanese species *P. citrea, P. terrea and P. punctata.* The species *P. stewartii* is further sub-divided into two subspecies *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes.* Recently, four new species have been added to the genus. *P. vagans, P. eucalypti, P. deleyi*, isolated from *Eucalyptus* in various countries, and *P. anthophila* which was previously known as the Beji protein profiling group VII (Beji *et al.,* 1988, Brady *et al.,* 2009a)

When the genus *Pantoea* was established with the type species *P. agglomerans,* Lind and Ursing (1986) showed that the DNA groups suggested by Brenner *et al.* (1984) grouping the closest with this species were DNA hybridization groups II, III, IV, V and VI.

Three of these DNA hybridization groups were also recently classified as *Pantoea* species by Brady *et al.,* (2009b) (in press), along with the transfer of *Pectobacterium cypripedii* to the genus emended as *Pantoea cypripedii.* Brenner HG II was renamed *P. septica* and HG IV renamed as *P. eucrina*. Brenner HG V was found to comprise of two unique species and were renamed *P. brenneri* and *P. conspicua* respectively. DNA hybridization group I was also found to share a common trait to that of DNA hybridization groups II to VI. This suggests that the genus *Pantoea* may consist of DNA hybridization group I in addition to the species that have already been identified and the newly described species (Grimont and Grimont, 2005 and Brady *et al.,* 2009b in press).

The Japanese species that were isolated from fruit and soil in Japan were found to be phylogenitically distant to the other *Pantoea* species and more closely related to the genus *Tatumella*. These species were thus transferred from the genus *Pantoea* and re-classified as *T. citrea, T. punctata* and *T. terrea* (Brady *et al.,* 2009c in press). The emended genus of *Pantoea* is currently comprised of 13 species.

2.2) Pathogenicity of the genus *Pantoea*

2.2.1) Stewart's wilt caused by *Pantoea stewartii*

The most devastating and economically important disease caused by a *Pantoea* species is Stewart's wilt caused by *P. stewartii* subsp. *stewartii.* This species infects and causes a vascular wilt of sweet corn and maize. This disease causes substantial losses of maize each year in various countries of the world where it occur. Symptoms of the disease include water-soaked lesions and vascular wilting on sweet corn seedlings and leaf blight of mature maize plants (Coplin and Kado, 2001). Once infected the bacteria multiply in the xylem of the plant and then spreads through it. Systemic infection occurs when the bacteria spreads from the leaves into the vascular tissue (Block *et al*., 1999).

Pantoea stewartii subsp. *stewartii* is suspected to be transferred to new regions by seed transmission. It has, however, not been undisputable proven that the pathogen is indeed transmitted by infected seed lots (Block *et al*., 1998). Most countries, including South Africa, considers *P. stewartii* subsp. *stewartii* as a quarantine pathogen and the risk of infected maize seed is considered so important that the shipment of seed is prohibited

unless it is certified pathogen free (Coplin *et al*., 2002). It was first suspected that this pathogen was spread by infected seed lots when plants growing in the field or in greenhouses from seed obtained from infected plants would develop the disease. It was later discovered that two beetles (corn flea beetle and tooth flea beetle) served as vectors for this pathogen (Block *et al*., 1998).

2.2.2) Disease caused by *Pantoea ananatis*

The second most devastating agent of a plant disease caused by a member of the genus *Pantoea* is *P. ananatis.* This bacterium has been found to cause a large variety of disease symptoms on a wide range of hosts (See Table 1). For the purpose of this review, two plant diseases and one case where *Pantoea* was isolated from a human, will be considered.

Leaf spot of maize caused by *P. ananatis* was first described in Brazil in 1982 (Pacolla-Meirelles *et al*., 2001). The frequency and severity of the disease has increased and today this disease is encountered in nearly all maize producing areas of Brazil. Disease symptoms include dark-green water-soaked spots that progress to form necrotic lesions that are straw coloured. The size and the number of lesions are linked to the genotype of maize infected. When the disease is encountered under optimal conditions the plant suffers a reduced plant cycle and there is a large decrease in grain size and weight. Research showed that grain yield decreases with as much as 63% in susceptible cultivars. This is due to the fact that nitrogen is not effectively dispersed throughout the infected plants. It is also known that the severity of the disease increases after rain when there is a rise in temperature and high humidity. The identity of the causal organism of this disease was questioned.

Bacterial blight and dieback of *Eucalyptus* trees caused by *P. ananatis* was first described in 2002 (Coutinho *et al*., 2002). It was found within a single nursery in Kwa-Zulu Natal infecting *E. grandis* X *E. nitens* hybrids. It soon spread to other nurseries and plantations infecting other *Eucalyptus* species hybrids and clones. The symptoms of the disease include tip-dieback and leaf spots on young leaves. Initially the leaf-spots are water-soaked but later they coalesce to form larger lesions. In the advance stages of the disease the tree assumes a scorched appearance and following repeated infection it will

become stunted and die. A disease causing similar symptoms was also observed in other countries, for example, some in South America, Uganda and Thailand. This phytopathogen appears to spread from the leaf petiole into the main-vein and from there will infect adjacent tissues. This disease is very important from a forestry point of view, and causes large losses, particularly in nurseries, every year. This tree is used in the manufacturing of solid timber products but even more importantly it forms the basis of the international pulp and paper industry. Selective tree breeding of *Eucalyptus* species has temporarily solved outbreaks of the disease as resistant material has now been selected.

Pantoea ananatis has also been found to cause diseases in humans. In 2004, a 73 year old female was hospitalized with dyspnea and bilateral ankle edema (De Baere *et al*., 2004).The usual signs of fever and chills were not encountered until after a colonoscopy of the woman was performed. Blood samples were taken and a pure culture of the causal agent was obtained. This organism was found to be a Gram negative, facultative anaerobic rod. 16S rRNA gene sequencing was used for rapid identification of the bacterium, and this showed that the pathogen belongs to the genus *Pantoea.* Further investigation concluded that the causal agent was in fact *Pantoea ananatis.* This was confirmed with certainty using several biochemical tests, 16S rRNA sequencing and tRNA-PCR. *Pantoea* has previously been isolated from hospitals, but these isolates all belonged to *Pantoea agglomerans.* This was a first report of *Pantoea ananatis* being isolated from a clinical source.

2.2.3) Disease caused by *Pantoea agglomerans*

Pantoea agglomerans is the type species of the genus *Pantoea*. It has been found to cause diseases in the ornamental plant, *Gypsophila paniculata,* causing crown and root gall (Cooksey, 1986). Other hosts include pearl millet (Frederickson, 1997), onion (Hattingh and Walters, 1981) and cotton (Medrano and Bell, 2006). *P. agglomerans* was also isolated from various clinical isolates, for example, knee lacerations (Gavini *et al.*, 1989), nosocomial septicemia (Maki *et al.*, 1976), septic arthritis (Kratz *et al.*, 2003) and peritonis (Lim *et al.*, 2006).

Crown and root galls on the ornamental plant, *Gypsophila,* was first described in the 1930's. The causal agent of this disease was first described as *Bacterium gypsophilae* (Brown, 1932) until the organism was later identified as *Agrobacterium gypsophilae* (Brown, 1934). Finally, the causal agent was confirmed as *P. agglomerans* (Cooksey, 1986). Disease symptoms include soft, light brown galls that occur on the pruned ends of the plant, usually during spring and summer. These galls can develop to 10 cm in diameter and can also be accompanied by stem rot.

In 1991, Burr *et al.* identified a tumorous disease on table beets similar to bacterial pocket disease and crown gall on this plant. This disease was found to cause tumors on beets varying in size from 5 cm or greater in diameter. Tumours were encountered on the crown of infected beet roots. The causal agent of this disease was identified as *Erwinia herbicola*, which was later re-named as *P. agglomerans* (Burr *et al.,* 1991).

2.2.4) Disease caused by *Pantoea citrea*

Pantoea citrea was found to be the causal agent of pink disease of pineapple (Cha *et al.* 1997a). When pineapples are infected with *P. citrea*, the pathogen induces the production of the enzyme glucosedehydrogenase within the pineapple. This enzyme breaks down the sugar molecules in the pineapple resulting in the brown colour upon heating. The production of this compound will not affect the quality of the fresh fruit. Only upon a heat treatment do these compounds turn the fruit pink to reddish-brown. Cha *et al.*, (1997b) established that the compound responsible for the discolouration of pineapples when infected with *P. citrea* is glucose dehydrogenase (GDH). This was proven when a *P. citrea* mutant that is unable to produce GDH was used to infect pineapples and the organism could not elicit the disease (Cha *et al.,* 1997b). Pink disease of pineapple caused by *P. citrea* is different to the disease caused on pineapples by *P. ananatis*. The reason for this lies in the fact that infection of pineapples with *P. ananatis* can be observed prior to the heating of the fruit and it causes rot and brown marbling of the pineapple fruit (Serrano, 1928).

2.2.5) *Pantoea* as a biocontrol agent

Pantoea spp. does not only cause diseases on plants, some of the species, for example *P. agglomerans, P. ananatis* and *P. dispersa* are used as biocontrol agents. The genetically modified *P. agglomerans* strain C9-1 is used to control the disease, fire blight on apples and pears (Nunes *et al.,* 2001). This biocontrol agent produces an antibiotic which inhibits the growth of the causal agent of fire blight, *E. amylovora. P. agglomerans* does not only inhibit *E. amylovora* by producing an antibiotic, it also outcompetes the pathogen for colonization space and nutrients. *P. agglomerans* grows at the same optimal and beyond the optimal temperature of the fire blight pathogen, thus having an advantage over the fire blight pathogen for temperature range. One of the disadvantages of *P. agglomerans* as a biocontrol agent against fire blight is that the biocontrol agent needs to colonize the stigma of flowers of the host plant before colonization of the fire blight pathogen (Johnson and Stockwell, 1998).

Pantoea dispersa is used as a biocontrol agent against leaf scald disease of sugar cane (Zhang and Birch, 1997). Leaf scald disease is caused by the bacterial phytopathogen *Xanthomonas albilineans. X. albilineans* invades the xylem tissue of the sugar cane plant causing chlorosis of new sugar cane leaves. This pathogen is encountered in its natural reservoir, blady grass, and because of this natural reservoir eradication of the pathogen is difficult. *X. albilineans* produces an albicidin. This albicidin is responsible for the disease in sugarcane. The biocontrol agent, *P. dispersa,* is capable of producing an extracellular albicidin detoxifying compound. The albicidin detoxifying agent does not only remove the toxic albicidin from the sugarcane to protect the plant, but also out competes the pathogen for colonization space on the host plant (Zhang and Birch, 1997).

Pantoea ananatis is present on pome fruit trees i.e. pear and apple trees as an epiphyte. Strains of this bacterium were isolated from the fruits and leaves of these trees and were shown to have the ability to control the growth of the mold, *Penicillum expansum,* which is known to cause postharvest disease of these fruit. This *P. ananatis* strain has successfully been used as a biological control agent to control this disease under storage conditions (Torres *et al.,* 2005).

3) THE GENUS *XANTHOMONAS*

3.1) Taxonomy of the genus *Xanthomonas*

The genus *Xanthomonas* forms part of the family *Xanthomonadaceae.* Members of the genus *Xanthomonas* are Gram negative rods, which can also be slightly curved with rounded ends. The cells occur mostly alone or in pairs but chains and filaments are also observed. No spores or other resting structures have been reported to be formed by members belonging to this genus. Also no pili or fimbrae have been reported (Swings *et al.*, 1993). Most xanthomonads form convex, round, yellow mucoid colonies on agar three days after incubation. The optimal growth temperature for xanthomonads is 28 ºC and no xanthomonad can grow below 5 ºC or above 40 ºC. *Xanthomonas* is an obligate aerobic organism degrading D-glucose to form acid (Saddler and Bradbury, 2005).

The genus *Xanthomonas* was first proposed by Dowson in 1939. This genus was formed to include a complex group of bacterial species which were diverse in physiological and phytopathological characteristics. The first xanthomonad plant disease recorded, more than a 100 years ago, was 'yellow – disease' of hyacinth plants (Vauterin et al., 1995). In the original description of *Xanthomonas* in 1939, 60 species were included. At that time bacteria were only classified based on phenotypic, biochemical, morphological and pathogenicity characteristics (Simoes *et al*., 2007). This system has led to inaccurate and confusing classifications of species within the genus (Swings and Civerolo, 1993).

Originally the taxonomy of the genus *Xanthomonas* was based on a "new-host new-species" concept using the single phenotypic characteristic, host pathogenicity, as the only criterion for describing a new *Xanthomonas* species (Swings and Civerolo, 1993) This method of bacterial taxonomy gave rise to a complex genus containing more than 100 species. It was then decided, based upon bacterial nomenclature rules, that the single factor differentiating these species from each other is not sufficient for the differentiation between each of these species. Due to the complexity of the genus, all *Xanthomonas* species was then merged into a single species, *X. campestris* by Dye and Lelliot (Saddler and Bradbury, 2005). In 1978, Young *et al.* proposed the reclassification of the nomenspecies into a pathovar system as a temporary solution for the complex nomenclature until a more suitable classification could be established based on general

rules of bacterial nomenclature. The pathovar system states that pathovars are to be distinguished from one another based on host pathogenicity. Currently, there are more than a 140 pathovars recognized as part of the genus *Xanthomonas* (Vauterin *et al*., 2000) There are several shortcomings of the pathovar classification scheme (Vauterin and Swings, 1997) namely, i) It has not been confirmed that a pathovar of a certain host will not be able to induce disease on a different kind of host ii) The pathovars of different plant hosts shows a large amount of heterogeneity and iii) Xanthomonads that are non-pathogenic to plants cannot be characterized based on the pathovar system because they do not fulfil the criterion for classification in the pathovar scheme i.e. host pathogenicity. Even though these xanthomonads are not important from an agricultural point of view their existence cannot be neglected (Vauterin *et al*., 2000). Our knowledge of the host range of *Xanthomonas* spp. is incomplete due to the fact that wild plant species or plant species that are of no economic importance have rarely been examined to identify the *Xanthomonas* spp associated with them (Swings and Civerolo, 1993). As a results of the pathovar system: (Swings and Civerolo, 1993) i) Different organisms have been given the same pathovar name because they were isolated from the same host plant, and ii) New pathovars that are in fact the same organism, have been named because a previously identified organism was isolated from a different host plant. Only in a few cases a clear comparison was drawn between the genomic group and the host plant from which the organism was isolated.

The absence of discriminatory phenotypic characteristics was and still is a major problem in the taxonomy of *Xanthomonas*. Methods such as fatty acid analysis, protein electrophoresis and antibody typing are increasingly used in diagnostic methods to replace the classical phenotypic tests which gave little taxonomic clarity. Even though molecular methods are more generally used for species identification, phenotypic characteristics are still required so as to adhere to the standard principle of species identification (Swings and Civerolo, 1993).

In recent years with the advances in molecular biology the taxonomy of the genus *Xanthomonas* has successfully been re-evaluated using molecular techniques. In 1995, Vauterin *et al*. reclassified the genus *Xanthomonas* using a comprehensive DNA:DNA hybridization study of 183 strains. The revised classification was based on genomic relatedness rather than phenotypic and phytopathogenic data. This taxonomic scheme is

the most widely accepted. Based on their scheme the genus *Xanthomonas* today contains 20 species i.e. *X. fragariae, X. populi, X. oryzae, X. albilineans, X. sacchari, X. vesicatoria, X. axonopodis, X. vasicola, X. codiae, X. arboricola, X. hortorum, X. translucens, X. bromi, X. campestris, X. cassavae, X. cucurbitae, X. pisi, X. melonis, X. theicola and X. hyacinthi*. Since 1995, another 7 species have been added to the genus *Xanthomonas*. These validly published species include: *X. cynarae* causing bacterial bract spot of artichoke (Trebaol *et al.,* 2000), *X. euvesicatoria*, *X. perforans, X. gardneri* causing bacterial spot diseases of either tomato or pepper (Jones *et al.,* 2004), *X. citri, X. fuscans* and *X. alfalfae* causing disease of citrus fruit, *Medicago sativa* respectively (Schaad *et al.,* 2005).

Another characteristic feature of *Xanthomonas* spp., which do not form part of the xanthomonad taxonomy, are xanthomodins, and these are used as a taxonomic marker. Xanthomonadins are especially used to differentiate the *Xanthomonas* plant pathogens from other yellow-pigmented plant pathogens such as *Erwinia* and *Pantoea* spp. The function of the xanthomonadins has not completely been established but it is thought to be involved in the protection of the bacteria against photobiological damage (Swings *et al.,* 1993).

It is very important to have a proper classification system to ensure that bacteria belonging to this genus are accurately and rapidly identified to ensure effective disease diagnosis and disease management to protect agriculturally important crops (Vauterin *et al*., 2000). This new classification system also allows for the identification of the non-pathogenic xanthomonads which occur as epiphytes on plants.

3.2) Pathogenicity of the genus Xanthomonas

Xanthomonads are mainly phytopathogens and are rarely encountered in any other environments (Hayward, 1993). These bacteria are found infecting a wide host range of plants. To date infection of *Xanthomonas* on plant hosts has included 124 monocotyledonous and 268 dicotyledonous plants. Included in these plants are many economically important crops which make these bacteria a threat to agriculture worldwide. Some of the crops which are susceptible to *Xanthomonas* are cotton, beans, rice and crucifers to name only a few (Swings and Civerolo, 1993). For the purpose of

this review the most important diseases caused by xanthomonads infecting woody hosts will be discussed.

3.2.1) Bacterial dieback of *Eucalyptus* spp.

Xanthomonas was first reported to cause disease on *Eucalyptus* in Australia in 1963 (Truman, 1974). The symptoms of the disease included dieback followed by blackening of the twigs. This blackened area increases in size until the terminal buds and the leaves that are formed last, died. In the advance stages of the disease the tree became completely defoliated. This disease is very uncommon and was only considered as locally important thus no control method was implemented (Wardlaw *et al.,* 2000).

3.2.2) Bacterial black spot of mangoes

Mangoes are very important agriculturally and rank fifth in the world for production (Gagnevin and Pruvost, 2001). The disease of the woody host, mango trees (*Mangifera indica*) is caused by *Xanthomonas campestris* pv. *mangiferaendicae.* This disease has been reported in most tropical and sub-tropical countries where mangoes are cultivated. The first report of this disease was in India in 1948 (Patel *et al.*, 1948). The disease occurrence on mangoes is high in countries where a high rainfall is accompanied by high temperatures, for example, in the Kwa-Zulu Natal province in South Africa.

According to Gagnevin and Pruvost (2001), *X. campestris* pv. *mangiferaendicae* appears to infect all parts of the mango tree situated above the ground, with lesions on the leaves and the fruits being the most common. Lesions on the leaves and fruit start out as water-soaked lesions. The lesions on the leaves are angular. These lesions turn black with age and are raised, a chlorotic halo can also be observed. The lesions on the fruit will eventually crack open. The infection will cause the fruit to drop prematurely and will also severely affect the quality of the fruit.

Xanthomonas campestris pv. *mangiferaendicae* overwinters in mango trees. Leaf and stem lesions will serve as a source of inoculum. The causal agent survives as an epiphyte on various parts of the mango tree although humid conditions are required for survival (Gagnevin and Pruvost, 2001). It is believed that the pathogen infects the tree

through lesions on the stem and leaves and through natural openings e.g. stomata, wax and oil glands etc., and from there on spreads throughout the tree. The role of insects in the dispersion of the pathogen is not known although reports do suggest that it is possible (Gagnevin and Pruvost, 2001).

Mango bacterial black spot is a very difficult disease to control and chemical control of the disease is generally ineffective (Gagnevin and Pruvost, 2001). Hayward, (1993) stated that the incidence of the disease can be maintained by various factors: 1) The disease can be regulated by planting more resistant cultivars, although the planting of very resistant cultivars is not recommended. No cultivars exist that is completely resistant to the disease 2) Planting of wind-breaks around the mango orchard decreases the incidence of the disease, this is due to the fact that the pathogen can be dispersed by the wind. 3) Thorough orchard maintenance will also reduce the incidence of the disease by spraying copper compound in the summer and pruning the trees etc.

3.2.3) Walnut blight disease

Xanthomonas arboricola pv. juglandis is the causal agent of walnut blight, one of the most widespread and important diseases of walnut (*Juglans regia*) worldwide causing severe damage to twigs, leaves and nuts (Loreti *et al*., 2001). This disease has been known since the 19th century when it was first reported by Ferraris in 1927. This disease is widely spread in walnut growing areas and a major cause of reduction in fruit yield and loss of tree vigour (Loreti *et al*, 2001). The bacterium invades the plant through natural openings, for example, through stomata in the presence of free water or high humidity. The disease is mainly spread by rain and wind, thus the spread of the disease usually occurs in the rainy season (Loreti *et al.*, 2001).

Although susceptibility to the disease varies in different *Juglans* spp., no known natural resistance of the walnut tree to the pathogen exists (Tamponi and Donati, 1990). New succulent growth is very susceptible to the pathogen and the tree gains more resistance as it matures. Breeding programs do exist to select for a more resistant walnut hybrid against walnut blight (Loreti *et al*., 2001).

Control of the disease includes the use of copper sprays, which is the most widely accepted control measurement. Two problems exist with this method of control of the disease: i) Copper residues left after spraying are very toxic to the environment, and ii) The bacterium causing the disease may develop resistance to copper, thus rendering the copper ineffective (Mc Neil *et al*., 2001). This study was used to assess the efficacy of a bacteriophage as a possible control measurement of the disease. Lytic bacteriophages were found in abundance in the soil under the walnut trees, but the use of these bacteriophages as a biocontrol agent was found to be ineffective. The use of the antibiotic streptomycin as a possible control agent for the disease was tested by Polito *et al*. (2002). The use of streptomycin for the control of the disease is becoming a more popular control measurement in areas were copper resistant bacteria occur. The results showed that streptomycin application during the bloom period causes fruit drop. It is recommended that when streptomycin is to be used as a control measure that the application only occurs post bloom and pre bloom to prevent the loss of fruit (Mc Neil *et al*., 2001).

3.2.4) Bacterial spot of peach

Bacterial spot also known as ‘bacterial shot hole’ is a very common disease and can be found on various stone fruit trees. The causal agent of the disease, *X. campestris* pv *pruni* can cause disease on all *Prunus s*pp. The disease is frequently encountered on plum, peach and nectarine but can also be found on apricot and cherry varieties. The disease was first described in 1903 in the USA occurring on Japanese plum (Anonymous, 2006).

Xanthomonas arboricola pv. *pruni* overwinters in twigs that were infected late in the summer season (Springer *et al.*, 2000). These bacteria are then released from the twigs in the form of a bacterial ooze. The bacteria are dispersed through wind and rain. Infection occurs through the stomata or leticels when high moisture content is present. The disease affects the leaves, twigs and fruit. First signs of infection include water soaked spots on the leaves. The spots are concentrated near the tip of the leaf since this is where moisture is concentrated. The spots will enlarge and become necrotic eventually falling out hence the name ‘shot hole’. Heavily affected leaves may die and drop to the ground. Severe defoliation will affect the tree vigour and this leads to a

reduced size of the fruits. Symptoms on the fruit are similar to the symptoms on the leaves but the water soaked lesions on the fruit become sunken. As the fruit increases in size, cracks will appear around the spots. The disease poses more of a problem in areas with high rainfall and warmer summers. Losses due to bacterial spot disease are from the loss of tree vigour due to severe defoliation for future fruit seasons and the loss of fruit that cannot be marketed because it does not meet the standard.

There is no known resistance in *Prunus* spp. towards the disease but some cultivars possess more resistance than others (Hayward, 1993). Disease management practices should include the maintaining of good tree vigour by pruning, applying fertilizer and watering when necessary. Trees which are less vigorous show more susceptibility to the disease than trees that are more vigorous. Copper sprays are also applied but the same problems exist as described previously.

3.2.5) Bacterial canker of poplar

The disease bacterial canker of polar (*Populus* spp.) is the most devastating disease of poplar in Europe. The disease is caused by the bacterium *X. populi* and should not be mistaken with the causal agent of bark necrosis of poplar, *X. campestris* pv *populi* (Saddler, 2002). This disease has been known to occur on poplars for over 100 years. Poplar trees are of economical and social value. As a social value it is planted for the protection of soil and afforestation in polluted areas. Poplars are also valuable in maintaining the natural biodiversity of the ecosystem since it plays host to a number of endangered animals and insects (Vanden Broeck, 2003).

Xanthomonas populi enters the plant through wounds on the bark and leaves (Saddler, 2002). First signs of infections occur on the young branches of the previous year, just before ‘bud burst’. The tissue around the infected buds swells and cracks excreting a thick bacterial suspension. Generally the buds that were infected during the previous season die, but can also produce leaves which are blackened or discoloured due to leaf invasion by the bacteria. When the bark is stripped from the tree the cambium, phloem and parenchemous tissue appear glassy due to the internal progression of the bacteria through the tree. The infected twigs and buds die to form bacterial cankers. These active cankers cause degradation of the wood structure (Hayward, 1993).

Resistance of polar trees to the disease varies between species. The older poplar species exhibit a high susceptibility to the disease whereas newer clones are resistant to the disease. The bacteria are spread between trees through wind and rain action (Hayward, 1993). This disease is mainly managed by planting species and clones of polar that are resistant to the disease.

4) THE IDENTIFICATION OF *PANTOEA* AND *XANTHOMONAS* SPECIES

Rapid and reliable identification of plantpathogenic species are very important in effective disease management strategies. Due to the high phenotypic similarity between species of *Pantoea* and *Xanthomonas*, phenotypic techniques by themselves cannot accurately identify or distinguish between species. For a more reliable approach, molecular techniques are used.

4.1) 16S rRNA gene sequencing

Using the 16S rRNA gene sequence for determining phylogenetic relationships is a technique that has been used (Stackebrandt *et al.*, 2002). It is a very powerful tool and needs to be used in conjunction with other techniques in a polyphasic approach for bacterial identification (Stackebrandt and Goebel, 1994). The largest part of the 16S rRNA gene is highly conserved in bacteria since it plays a vital role in DNA replication. A smaller part of this gene is more variable amongst members of different genera. This part of the gene can be used for determining phylogenetic relatedness between organisms (Hauben *et al*., 1997). Comparing the 16S rRNA gene sequences of bacteria is a very efficient tool for the classification of bacteria to genus level but is often inefficient to classify many bacteria to the species and sub-species level.

The 16S gene sequence has been used in studies to identify members of the genera *Pantoea* and *Xanthomonas.* It was found that members of the genus *Pantoea* could be distinguished from other closely related *Enterobacteriaceae* when comparing 16S rRNA sequences but the taxonomy within the genus *Pantoea* could not be resolved based upon this technique alone (Brady, 2005). This technique was used in conjunction with another molecular technique and the taxonomy has now been effectively resolved.

As for members belonging to the genus *Xanthomonas,* Hauben et al. (1997) showed that from all the validly described *Xanthomonas* type strains, only 3 distinct groups from the 20 known species could accurately be identified using this technique. The species of the genus *Xanthomonas* showed a high degree of overall sequence similarity with the mean similarity value equal to 98.2%. The three groups formed with this analysis included Group I which included the species *X. albilineans, X. hyacinthi, X. theicola* and X. translucens. The second phylogenetic group, Group II were referred too as the *Xanthomonas* core group and this group included all the other *Xanthomonas* species except *X. sacchari* which formed the third phylogenetic group which groups separately from the other species. This technique alone can thus not be used for identification of *Xanthomonas* species but as shown by Hauben *et al*. (1997), it can be used in conjunction with DNA:DNA hybridization to resolve the complex taxonomy of *Xanthomonas.* As with determining relationships between *Pantoea* species, sequencing the 16S rRNA gene of *Xanthomonas* species is not sufficient for species identification due to sequence similarity.

4.2) Amplified Fragment Length Polymorphism (AFLP)

AFLP's is a very powerful determinative tool since it uses the entire bacterial genome to detect polymorphisms and not only a part of the genome or a single gene. Some of the applications of this technique include: i) The creation of genetic maps for new species, ii) Determination of relatedness amongst different isolates, and iii) Genetic diversity and molecular phylogeny studies (Vos *et al.*, 1995). This technique has been used in bacterial identification and typing (Brady *et al.*, 2007). The AFLP technique was designed by Vos *et al*. (1995). It includes the restriction enzyme digestion of the whole bacterial genome, but combines the power of restriction analysis with the selection flexibility of PCR. A subset of the restriction fragments are selected by two different PCR reactions. This technique is then concluded by separating the resulting subset of fragments on a polyacrylamide gel for visualization of the resulting pattern also called a DNA fingerprint.

The result of AFLP analysis of a specific isolate is a unique DNA fingerprint that can be compared to the DNA fingerprints of other isolates in a database in order to determine

the identity or taxonomic relationship. Specialized computer software has been designed for the rapid analysis of the fingerprint data such as AFLP fingerprint profiles. The computer software compares results using two basic similarity coefficients (Vauterin and Vauterin, 1992). The first being Dice similarity coefficient: The software scores the resulting bands of the DNA fingerprint as either being present or absent. Bands will be either present or absent depending on whether restriction enzyme cut-sites or the nucleotides adjacent to these cut-sites were affected. The second similarity coefficient used is Pearson. Bands are not scored as present or absent, thus band intensity will not affect the results, instead it uses a mathematical equation to analyse the total pattern formed by the fingerprint.

In the AFLP technique used for the study of *Pantoea,* the restriction enzymes *EcoRI* and *MseI* were used for the restriction enzyme digestion (Brady *et al.*, 2007). The objective by the studies done by Brady *et al.*, was to develop an AFLP based typing system for the genus *Pantoea.* In order for this AFLP typing system to be successful this method should be able to discriminate between the seven different *Pantoea* species, but even more importantly, it needed to distinguish between the two subspecies of the species *P. stewartii* i.e. *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes.* Brady *et al.* (2007) showed that the AFLP technique forms 15 unique clusters of which all of the six species falls into distinct separate clusters. *P. stewartii* clusters together in a distinct separate cluster with a clear distinction in the cluster between *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes.* It was concluded that AFLP analysis was a rapid, reliable technique for the typing of the genus *Pantoea.*

When using the AFLP technique for the determination of phylogenetic relationships among *Xanthomonas* spp. Rademaker *et al*. (2000) used the restriction enzymes Eco RI and *Taq* I. In this study, 80 *Xanthomonas* strains belonging to the 20 different described *Xanthomonas* species were used in an AFLP experiment to determine the effectiveness of the AFLP technique to distinguish between the different *Xanthomonas* species. The primary goal of this experiment was to determine whether the clusters formed with the AFLP genomic fingerprinting technique corresponds to the DNA:DNA hybridization clusters formed. Twenty-four clusters were formed in the AFLP experiment which corresponded to 19 of the clusters that was identified during DNA:DNA hybridization studies. The high correlation between DNA:DNA homology studies and the genomic

fingerprinting technique, AFLP, showed that it can be used as an alternative and can be used as a rapid highly discriminatory screening technique to determine the taxonomic diversity and phylogenetic structure of bacterial populations.

4.3) Multilocus Sequence Based Approaches

A multilocus sequence approach is based on the sequencing of a certain set of housekeeping genes, and comparison of the sequence data between genera and species. The housekeeping genes form part of the core genome. The core genes are present in all bacteria and can thus be compared between genera and are also suitable to compare strains belonging to different genera since they are not highly susceptible to horizontal gene transfer (HGT). The housekeeping genes are not often subjected to HGT due to the fact that they play a critical role in the biological processes that the bacteria need for survival or proper functionality (Gevers *et al.,* 2005). These genes should also evolve more rapidly than ancient more conserved genes e.g. 16S rRNA gene.

MLSA should make use of genes that are ubiquitous in the specific genus or genera being studied. These genes should be present in a single copy in the genome, and it should not be present in the vicinity of a site where recombination frequently occurs (Zeigler, 2003, Yamamoto and Harayama, 1996) When more than one core gene has been sequenced in a genus, concatenated trees could be constructed in order to identify specific groups of isolates (Brown *et al.,* 2001).

MLSA can be used for the identification and assigning of isolates to a specific genus with as much success as DNA:DNA hybridization (Wertz *et al.,* 2003). Using MLSA is more advantageous than DNA:DNA hybridization since sequencing has become a rapid, inexpensive technique with high output that can be automated. DNA:DNA hybridization results may also vary between different laboratories where as a database exists where sequencing data can be deposited of sequences of core genes readily available to every one. The computer software for the analysis of sequence data is often free and readily available.

In a MLSA study conducted by Brady and co-workers, four housekeeping genes i.e. *gyrB*, *rpoB*, *atpD* and *infB* was sequenced and used for the classification, identification and phylogenetic analyses of the genus *Pantoea*. This MLSA study distinguished between the seven validly published *Pantoea* species and identified 10 new potential species belonging to the genus. It was also showed that these housekeeping genes are reliable genetic markers with the *gyrB* gene producing the most consistent results. When the MLSA results were compared to DNA:DNA hybridization data, a high similarity was found between the two methods. MLSA was shown to be a rapid identification and classification technique as apposed to DNA:DNA hybridization which is time consuming and expensive (Brady *et al.,* 2008). Brady and co-workers also successfully used a MLSA approach to add eight new species to the *Pantoea* genus (Brady *et al.,* 2009a and Brady *et al.,* 2009b) and transferred the three Japanese isolates i.e. *P. citrea*, *P. terrea* and *P. punctata* to the genus *Tatumella* (Brady *et al.,* 2009c).

Young and co-workers conducted a MLSA study on all validly published *Xanthomonas* species, using the housekeeping genes: *dnaK*, *fyuA*, *gyrB* and *rpoD*. This analysis identified most of the species established by DNA:DNA hybridization. The study showed high congruence between the *gyrB* gene sequencing data and DNA:DNA hybridization data. MLSA also offered a simpler way to identify unknown strains as members of the *Xanthomonas* species or to identify new *Xanthomonas* species (Young *et al.,* 2008). A similar study used a MLSA approach (*gyrB* gene sequencing) to indicate the inter species relatedness and as a rapid and accurate species level identification technique of *Xanthomonas* species (Parkinson *et al.,* 2007) The resulting phylogenetic tree was supported by high bootstrap values and currently this technique is used for the routine diagnosis of xanthomonad species. The study showed that *gyrB* phylogeny could distinguish between 24 of the 27 validly published *Xanthomonas* species and had good correlation with DNA:DNA hybridization results.

4.4) DNA:DNA hybridization

This technique has been used since the 1960's to determine the relatedness between different bacteria, and is to date the most important criterion for the description of a bacterial species (Goris *et al*., 2007). Scientists believe that the taxonomic information of

an organism is incorporated in its entire genomic sequence and to accurately investigate the taxonomy of all organisms the entire genome should thus be assessed.

The aim of a DNA hybridization study is to determine the degree of genetic similarity between two organisms. The principle of the DNA:DNA hybridization technique is based on the denaturing of double stranded (ds) DNA of two different organisms. The denaturing step of the protocol is carried out at a very high temperature and the ds DNA will separate from its complement forming single stranded (ss) DNA. The ss DNA of the target organism is labelled and then allowed to re-hybridize 1) with its complement and 2) the organism to which it will be compared to. The first hybridization serves as the control, the hybridization value obtained should be equal to 100% since the two single stranded molecules are complementary to each other. The second hybridization value is that of the test organism. This value will indicate the genetic similarity between the two different strains and it can then be decided whether these organisms belong to the same or different species (Mehlen *et al*., 2004). In order for an unknown organism to belong to the same species a hybridization value of higher than 70% is required between the two organisms (Cho and Tiedje, 2001).

An advantageous characteristic of DNA:DNA hybridization is the fact that this technique surveys the level of total genomic homology, which is the most reliable method for the delineation of species within a genus (Swings and Civerolo, 1993). DNA:DNA hybridization thus surveys the total genomic DNA of an organism to determine relatedness and not only certain housekeeping genes of an organism that are very conserved amongst different bacteria.

Limitations regarding the DNA:DNA hybridization methods include that: i) It is not a sensitive enough technique to detect close relationships between strains and populations. Pair wise similarities are observed, but no information on the description of the individual strains is noted. ii) No database can be established with the purpose of identifying isolates based on DNA:DNA hybridization results (Vauterin *et al*., 2000). Because of these drawbacks of DNA:DNA hybridization researchers are constantly seeking for new techniques which can become the “golden standard” for bacterial taxonomy (Goris *et al*., 2007).

DNA:DNA hybridizations have been used for the reclassification of the genus *Xanthomonas*. Before 1995, no appropriate classification system for the genus *Xanthomonas* existed and their taxonomy was very confusing. In 1995, Vauterin *et al*. employed DNA:DNA hybridization to resolve the complex taxonomy of the genus *Xanthomonas*. The study was performed using 183 *Xanthomonas* trains including 62 pathovars. All of these strains formed 20 homology groups also referred to as genomic species. The 20 homology groups obtained from the results of the DNA:DNA hybridization study is to date considered the 20 species of the genus *Xanthomonas.* This technique was successfully employed to clarify the taxonomy of this genus, and this taxonomical classification is widely accepted (Vauterin *et al*., 1995).

Jones *et al*. (2004) used a DNA:DNA hybridization study to determine the relatedness of the causal agents of bacterial spot of tomato and pepper. Two different causal agents of the disease were isolated but due to the lack of molecular techniques for accurate identification, both of the isolates were classified as *X. campestris* pv. *vesicatoria.* Even though these two different isolates were classified as the same pathovar, they had different phenotypic characteristics. One of the two isolates was strongly amylotic whereas the other isolated was non-amylotic. Jones *et al*. (2004) used DNA:DNA hybridization to determine whether these two isolates did belong to the same species or even the same genus. It was concluded that one of the two isolates did not share 70% or more DNA similarity with the other isolate, with the type strain of *X. axonopodis* or with any of the other *Xanthomonas* type strains. It was suggested that this isolate be given species status.

In a DNA:DNA hybridization study done by Gavini *et al.* (1989) the genus *Pantoea* was established. Ten strains that belonged to the previously known "*Erwinia herbicola – Enterobacter agglomerans*" complex was included. These strains showed more than 75% homology to the type strain of *E. herbicola*, thus showing that these strains all belonged to the same species. The new genus, *Pantoea* was thus established, with *P. agglomerans* being the type species.

4.5) Polyphasic approach to identification

All of the above mentioned molecular identification techniques have their advantages and disadvantages. For an accurate approach to bacterial identification a polyphasic approach should best be followed. A polyphasic approach is the integration of various diagnostic methods for the identification and description of bacteria. The proposed polyphasic approach for the identification or description of bacteria should include phenotypic, biochemical and molecular characteristics. This is also a solution to the costly and time consuming, 'golden standard' technique DNA:DNA hybridization for the accurate identification and delineation of bacterial species. This will ensure the accurate identification of isolates in a limited amount of time. It will also ensure that certain techniques will prove better in combination with other techniques and these techniques can complement one another in terms of their short comings. A polyphasic approach to bacterial taxonomy will also allow several other benefits such as i) Developing of long term management practices, for example, deploying host resistance, ii) Integration of the diagnostic protocols into management programs, iii) Understanding the interrelationship among pathogenic variants and iv) most importantly the development of a stable nomenclature (Rademaker *et al*., 2005). It is not only important to be able to distinguish between different species but also to understand the diversity within each species e.g. the *Pantoea* subspecies level and *Xanthomonas* pathovar level.

The taxonomy of the genus *Xanthomonas* was refined and resolved by using a polyphasic approach. Three-hundred-and-forty *Xanthomonas* strains comprised of 80 pathovars representing the 20 described DNA hybridization groups were used. Rep-PCR was used in conjunction with the DNA:DNA hybridization data by Vauterin *et al*. (1995) to determine whether a polyphasic approach could be used for the rapid identification and the classification of new or unknown *Xanthomonas* strains that are pathogenic or associated with plants. The polyphasic approach was successful. It was found that 6 of the genospecies described by Vauterin *et al*. (1995) contained clearly defined subspecific groups within the clusters and also allowed for the classification of 166 xanthomonad strains that could not previously be classified (Rademaker *et al*., 2005). The use of a polyphasic approach has also proven that the previous pathovar notation was an artificial system and several new species within the genus was described (Vandamme *et al.,* 1996, Rademaker *et al.,* 2000).

5) DETERMINING THE PATHOGENECITY OF *PANTOEA* AND *XANTHOMONAS* SPECIES

The aim of a host pathogenicity test is to determine whether a suspected pathogen can cause disease on a plant host under laboratory conditions or not. A pathogenicity test is considered a standard test in phytobacteriology for the identification of plant pathogens. Every pathogenicity test is typically preceded by a Hypersensitivity reaction test to determine whether the plant pathogen induces a hypersensitive reaction (HR) in a nonhost. The HR is a good indication of whether the bacterium in question is a plant pathogen or not. Most bacterial plant pathogens induce a hypersensitive response when it is injected into a non-susceptible host or nonhost plant. Usually plants with soft, succulent leaves that have large intracellular spaces of the leaves are used for the hypersensitivity test e.g. tobacco and pepper plants. Tobacco are usually used for plant pathogens for the hypersensitivity reaction, but is a unreliable test plant for the determination of the HR of xanthomonads, since many *X. campestris* pathovars do not elicit a HR in tobacco. Tomato and pepper plants are used for the hypersensitivity test of *Xanthomonas* spp. (Lelliot and Stead, 1987). It is also stated by Coplin and Kado, 2001 that some *Pantoea* species i.e. *P. stewartii* and *P. agglomerans* pvs. *gypsophilae* and *betae* will not induce a HR on tobacco when the isolates were grown on rich media e.g., Luria Brutani agar, prior to inoculation. These isolates should be transferred from the rich media to IM liquid medium (Ivanoff's medium for *P. stewartii*) before being inoculated into the tobacco leaves for a HR.

A positive hypersensitive response is the rapid collapse of the inoculated tissue or the drying, light brown necrosis of the water soaked tissue within 3 days (Lelliot and Stead, 1987). When a positive HR is observed, the inoculated bacterium is a plant pathogen. When a positive HR is observed and it has been determined that the bacterium in question is a plant pathogen, a pathogenicity test is conducted by inoculating the bacterium in question into the host it was originally isolated from. In a pathogenicity test 3 different treatments should be inoculated into the host plant in order to determine the accurate pathogenicity of the bacterium in question towards the host plant. These treatments include: 1) The putative pathogenic bacterium within an aqueous suspension in a concentration that does not exceed the recommended concentration, 2) An aqueous

bacterial suspension of a known pathogen towards the host cell (positive control) and 3) Sterile water (negative control) (Lelliot and Stead, 1987).

Pathogenicity tests have a very important role in fulfilling Koch's postulates for bacterial diseases. Koch's postulates states that when a disease is observed, the causal agent must be present in all cases of the disease. The causal agent must be isolated from the diseased host and obtained as a pure culture. The original infection process should be recreated by inoculating the causal agent into the susceptible host. The same disease symptoms should be observed in the original host, and the same causal agent should be re-isolated from the diseased material (Grimes, 2006).

Various inoculation methods of introducing the pathogen into the host plant has been used, some of these inoculation methods are described below:

"Cut stump" and "whorl" methods

Coplin *et al.* (2002) determined the pathogenicity of *P. stewartii* subsp. *stewartii* towards maize by conducting pathogenicity trials. *P. stewartii* subsp. *stewartii* is the causal agent of Stewart's vascular wilt of maize and sweetcorn. Eight day old sweet corn seedlings were inoculated with the pathogen using two different inoculation methods. In the 'cut stump' method the bacterium was inoculated by pipetting the bacterial suspension onto the decapitated ends of the seedlings. With the 'whorl' inoculation method the bacterial suspension in a potassium phosphate buffer (pH 7) was placed on the whorls of the seedlings. The plants were checked for disease development after 10 days. Every pathogenicity trial was also preceded by a HR. It was found that the causal agent could induce disease on the 8-day old corn seedlings under laboratory conditions (Coplin *et al*., 2002).

"Spray" inoculation and direct method

Roumagnac *et al*. (2004) characterized the *Xanthomonas* species responsible for bacterial blight of onion by applying a polyphasic approach. One of the characterization techniques involved included pathogenicity trials. Bacterial suspensions were prepared containing the recommended bacterial concentration. Ten different *Allium* spp. plants including onion, garlic and Welsh onion were inoculated with the bacterial suspension by pricking the leaf with a syringe and spraying the wounds with the bacterial suspension.

Other inoculation methods were also used. After injuring the plant with the needle the bacterial suspension was applied directly to the wounds with a cloth, also the bacterial suspension was directly inoculated into the veins of the plants. Disease development was observed for 2 weeks. The results showed that all *Xanthomonas* strains isolated from the *Allium* spp. induced the typical symptoms of bacterial blight of onion (Roumagnac *et al*., 2004).

6) CONCLUSIONS

The taxonomy and distribution of *Pantoea* and *Xanthomonas* species are very complex. These organisms are plant associated and can occur as saprophyte, epiphytes or pathogens on the host plant. The devastating diseases caused by these organisms need to be controlled and this can only be done when the identity of these pathogens can rapidly and accurately be determined, the ecology of different strains are understood and when the pathogenicity of the pathogen towards a certain plant host is known. It also allows for the better understanding of the ecology of different strains of the same species.

Phenotypic techniques for the identification of these bacteria is inadequate since most of these organisms have very similar phenotypic characteristics. Several techniques exist to do this, but the best approach for the above mentioned process is to apply a polyphasic approach for the identification of these bacteria.

The use of the 16S rRNA gene for the identification of bacteria is not accurate. This gene can be used for the identification to genus level but cannot successfully be used for the identification of a bacterium to species level. The 16S rRNA gene is conserved amongst bacteria since this gene plays an important role in the formation of the RNA polymerase which is responsible for the replication of the bacterial genome. Thus the evolutionary clock of the 16S rRNA gene is very slow.

DNA:DNA hybridisation is thought to be the golden standard for the delineation of a bacterial species but other techniques e.g. 16S rRNA gene sequencing are still compulsory for the description of a new bacterial species (Stackebrandt *et al*., 2002). AFLP has also been proven to be a very effective technique for discriminating between

several species for the genus *Pantoea* (Brady *et al.*, 2007) and *Xanthomonas* (Rademaker *et al*., 2000).

The genus *Pantoea* is a fairly recent described genus and much confusion exists about its taxonomy and nomenclature. Even though the genus *Xanthomonas* has been known since the early 1930's much research has been done on clarifying the taxonomy and nomenclature of these bacteria. Constant research and keeping up with new technology regarding science will ensure that the taxonomy and pathogenicity of these organisms are kept up to date. The taxonomy of the genus *Xanthomonas* has been temporarily resolved but care should be taken when new species are described for this genus, employing the correct techniques which would result in an accurate and reliable identification.

7) REFERENCES

- Anonymous. 2006. Extract from European and Mediterranean Plant Protection Organization. *Xanthomonas arboricola* pv. *pruni*. OEPP/EPPO Bulletin 36: 129 – 133
- Aritua, V., Parkinson, N., Thwaites, R., Heeney, J.V, Jones, D.R., Tushemereirwe, W., Crozier, J., Reeder, R., Stead, D.E., and Smith, J. 2008. Characterization of the *Xanthomonas* sp. causing wilt of enset and banana and its proposed reclassification as a strain of *X. vasicola*. Plant Pathology 57: 170 – 177
- Atkinson. G.F. 1891. Black 'rot' of cotton. Alabama Agricultural Experiment Station Bulletin 27: 16
- Azad, H.R., Holmes, G.J., and Cooksey, D.A. 2000. A new leaf blotch disease of sudangrass caused by *Pantoea ananas* and *Pantoea stewartii.* Plant Disease 84: 973 – 979
- Azegami, K., Ozaki, K., and Matsuda, A. 1983. Bacterial palea browning, a new disease of rice caused by *Erwinia herbicola.* Bulletin for National Institute of Agricultural Science 39: 1 -12
- Beji, A., Mergaert, J., Gavini, F., Izard, D., Kersters, K., Leclerc, H., and de Ley, J. 1988. Subjective synonymy of *Erwinia herbicola, Erwinia milletiae,* and *Enterobacter agglomerans* and redefinition of the taxon by genotypic and phenotypic data. International Journal of Systematic Bacteriology 38: 77 – 88
- Block, C.C., Hill, J.H., and McGee, D.C. 1998. Seed transmission by *Pantoea stewartii* in field and sweet corn. Plant Disease 82: 755 – 780
- Block, C.C., Hill, J.H., and McGee, D.C. 1999. Relationship between late-season severity of Stewart's bacterial wilt and seed infection in maize. Plant Disease 83: 527 – 530
- Brady, C.L. 2005. Taxonomy of *Pantoea* associated with bacterial blight of *Eucalyptus.* Master thesis. The Faculty of Natural and Agricultural Sciences, Department of Microbiology and Plantpathology, University of Pretoria, Pretoria, South Africa.
- Brady, C.L., Venter, S., Cleenwerck, I., Vancanneyt, M., Swings, J., and Coutinho, T. 2007. A FAFLP system for the improved identification of plant-

pathogenic and plant-associated species of the genus *Pantoea*. Systematic and Applied Microbiology 30: 413 – 417

- Brady, C.L., Cleenwerck, I., Venter, S.N., Vancanneyt, M., Swings, J., and Coutinho, T. 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Systematic and Applied Microbiology 31: 447 – 460
- Brady, C.L., Venter, S.N., Cleenwerck, I., Engelbeen, K., Vancannyet, M., Swings, J., and Coutinho, T.A. 2009a. *Pantoea vagans* sp. nov., *Pantoea eucalypti* sp. nov., *Pantoea deleyi* sp. nov. and *Pantoea antophila* sp. nov. International Journal of Systematic and Evolutionary Microbiology 59: 2339 – 2345
- Brady, C.L., Cleenwerck, I., Venter, S.N., Engelbeen, K., de Vos, P., and Coutinho, T.A. 2009b. Emended description of the genus *Pantoea* and description of four novel species from human clinical samples, *Pantoea septica* sp. nov., *Pantoea eucrina* sp. nov., *Pantoea brenneri* sp. nov. and *Pantoea conspicua* sp nov., and transfer of *Pectobacterium cypripedii* (Hori 1911) Brenner *et al.* 1973 emend. Hauben *et al.* 1998 to the genus *Pantoea* emend. as *Pantoea cypripedii* comb. nov. (In Press)
- Brady, C.L., Venter, S.N., Cleenwerck, I., Vandemeulebroecke, K., de Vos, P., and Coutinho, T.A. 2009c. Transfer of *Pantoea citrea, Pantoea punctata* and *Pantoea terrea* to the genus *Tatumella* emend. as *Tatumella citrea* comb. nov., *Tatumella punctata* comb. nov., *Tatumella terrea* comb. nov. (Kageyama *et al.,* 1992) and description of *Tatumella morbirosei* sp. nov. (In Press)
- Brenner, D.J., Fanning, G.R., Leete Knutson, J.K., Steigerwalt, A.G., and Krichevsky, M.I. 1984. Attempts to classify Herbicola Group- *Enterobacter agglomerans* strains by deoxyribonucleic acid hybridization and phenotypic testing. International Journal of Systematic Bacteriology 34: 45 – 55
- Brown, N.A. 1932. Another gall-forming bacterium. Phytopathology 22: 924 – 925.
- Brown, N.A. 1934. A gall similar to crown gall, produced on *Gypsophila* by a new bacterium. Agricultural Research 48: 1099 – 1112
- Brown, J.R., Douady, C.J., Italia, M.J., Marshall, W.E., and Stanhope, M.J. 2001. Universal trees based on large combined protein sequence data sets. Nature Genetics 28: 281

- Bruton, B.D., Wells, J.M., Lester, G.E., and Patterson, C.L. 1991. Pathogenicity and characterization of *Erwinia ananas* causing postharvest disease on cantaloupe fruit. Plant Disease 75: 180 -183
- Burr, T.J., Katz, B.H., Abawi, G.S., Crosier, D.C. 1991. Comparison of tumorogenic strains of *Erwinia herbicola* isolated from table beet with *E. h. gypsophilae*. Plant Disease 75: 855 – 858
- Cha, J.-S., Pujol, C., Ducusin, A.R., Macion, E.A., Hubbard, C.H., and Kado, C.I. 1997a. Studies on *Pantoea citrea,* the causal agent of pink disease of pineapple. Phytopathology 145: 313 – 319
- Cha, J-S., Pujol, C., and Kado, C.I. 1997b. Identification and characterization of a *Pantoea citrea* gene encoding glucose dehydrogenase that is essential for causing pink disease of pineapple. Applied and Environmental Microbiology 63: 71 – 76
- Cho, J.C., and Tiedje, J.M. 2001. Bacterial species determination from DNA-DNA hybridization by using genome fragments and microarrays. Applied and Environmental Microbiology 67: 3677 – 3682
- Cooksey, D.A. 1986. Galls of *Gypsophila paniculata* caused by *Erwinia herbicola.* Plant Disease 70: 464 – 468
- Coplin, D.L., and Kado, C.I. 2001. Gram negative bacteria, *Pantoea*. In: Plant pathogenic bacteria 3rd edition. N.W. Schaad, J.B. Jones and W. Chun eds. The American Phytopathological Society (APS Press), St. Paul, Minnesota, p 73 – 83
- Coplin, D.L., Majerczak, D.R., Zhang, Y., Kim, W.S., Jock, S., and Geider, K. 2002. Identification of *Pantoea stewartii* subsp. *stewartii* by PCR and strain differentiation by PFGE. Plant Disease 86: 304 – 311
- Cother, E.J., Reinke, R., McKenzie, C., Lanoiselet, V.M., and Noble, D.H. 2004. An unusual stem necrosis of rice caused by *Pantoea ananas* and the first record of this pathogen on rice in Australia. Australian Plant Pathology 33: 494 – 503
- Coutinho, T.A., Preisig, O., Mergaert, J., Cnockaert, M.C., Riedel, K.-H., Swings, J., and Wingfield, M.J. 2002. Bacterial blight and dieback of *Eucalyptus* species, hybrids and clones in South Africa. Plant Disease 86: 20 – 25
- De Baere, T., Verhelst, R., Labit, C., Verschraegen, G., Wauters, G., Claeys, G., and Vaneechoutte, M. 2004. Bacteremic infection with *Pantoea ananatis.* Journal of Clinical Microbiology 42: 4393 – 4395
- Elliot, C. 1941. Bacterial wilt of dent corn inbreds. Phytopathology 32: 262 – 265

- Ewing, W.H., and Fife, M.A. 1972. *Enterobacter agglomerans* (Beijernick) comb. nov. (the herbicola – lathyri bacteria). International Journal of Systematic Bacteriology 22: 4 – 11
- Frederickson, D.E. 1997. A disease of pearl millet in Zimbabwe caused by *Pantoea agglomerans*. Plant Disease 81: 959
- Gagnevin, L., and Pruvost, O. 2001. Epidemiology and control of mango bacterial black spot. Plant Disease 85: 928 – 935
- Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K., and De Ley, J. 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and Description of *Pantoea dispersa* sp. nov. International Journal of Systematic Bacteriology 39: 337 – 345
- Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F.L., and Swings, J. 2005. Re-evaluating prokaryotic species. Nature Reviews 3: 733 – 739
- Gitaitis, R.D., and Gay, J.D. 1997. First report of leaf blight, seed stalk rot and bulb decay of onion by *Pantoea ananatis* in Georgia. Plant Disease 81: 1096
- Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., and Tiedje, J.M. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. International Journal of Systematic and Evolutionary Microbiology. 57: 81 – 91
- Goszczynska, T., and Botha, W.J. 2007. Isolation and identification of the causal agent of brown stalk rot, a new disease of maize in South Africa. Plant Disease 91: 711 – 718
- Graham, J.H., Gottwald, T.R., Cubero, J., and Achor, D.S. 2004. *Xanthomonas axonopodis* pv. *citri* : factors affecting successful eradication of citrus canker. Molecular Plant Pathology 5: 1 – 15
- Grimes, D.J. 2006. Koch's postulates – Then and Now. Microbe 1: 223 – 228
- Grimont, P.A.D., and Grimont, F. 2005. Genus XXIII. *Pantoea* Gavini, Mergaert, Beji, Mielcarek, Izarc, Kersters and De Ley 1989b, 343^{VP} emend. Mergaert, Verdonck and Kersters 1993, 172. Pages 713 – 721 in: Bergey's manual of systematic bacteriology, Volume two, Part B. D.J. Brenner, N.J. Krieg, J.T. Staley and G.M. Garrity, eds. Springer.

- Hasse, C.H. 1915. *Pseudomonas citri*, the cause of citrus canker. Journal of Agricultural Research 4: 97 – 100
- Hattingh M.J., and Walters, D.F. 1981. Stalk and leaf necrosis of onion caused by *Erwinia herbicola*. Plant Disease 65: 615 – 618
- Hauben, L., Vauterin, L., Swings, J., and Moore, E.R.B. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. International Journal of Systematic Bacteriology 47: 328 – 335
- Hayward, A.C. 1993. The hosts of *Xanthomonas*. *Xanthomonas.* J.G. Swings and E.L. Civerolo, eds. Chapman and Hall, London, UK. 1 – 119
- Janda, J.M., and Abbott, S.L. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. Journal of Clinical Microbiology 45: 2761 – 2764
- Johnson, K.B., and Stockwell, V.O. 1998. Management of Fireblight: A case study in microbial ecology. Annual Reviews of Phytopathology 36: 227 – 248
- Jones, J.B., Lacy, G.H., Bouzar, H., Stall, R.E., and Schaad, N.W. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Systematic and Applied Microbiology 27: 755 – 762
- Kageyama, B., Nakae, M., Yagi, S., and Sonoyama, T. 1992. *Pantoea punctata* sp. nov., *Pantoea citrea* sp. nov., and *Pantoea terrea* sp. nov. isolated from fruit and soil samples. International Journal of Systematic Bacteriology 42: 203 – 210
- Kido, K., Adachi, R., Hasegawa, M., Yano, K., Hikichi, Y., Takeuchi, S., Atsuchi, T., and Takikawa, Y. Internal fruit rot of netted melon caused by *Pantoea ananatis* (*Erwinia ananas*) in Japan. Journal General Plant Pathology 74: 302 – 312
- Koning, H.C. 1938. The bacterial canker of poplars. Mededelingen Phytopathologisch Laboratorium 'Willie Commelin Scholten' Baarn 14: 3 -47
- Kratz, A., Greenberg, D., Barki, Y., Cohen, E., Lifshitz, M., and Soroka, D. 2003. *Pantoea agglomerans* as a cause of septic arthritis at palm tree thorn injury; case report and literature review. Archives of disease in childhood 88: 542 – 544
- Kwon, S.-W., Go, S.-J., Kang, H.-W., Ryu, J.-C., and Jo, J.-K. 1997. Phylogenetic analysis of *Erwinia* species based on 16S rRNA gene sequences. International Journal of Systematic Bacteriology. 47: 1061 – 1067

- Lelliot, R.A., and Stead, D.E. 1987. Host tests. In: Methods for the diagnosis of bacterial diseases of plants. T.F Preece eds. Blackwell Scientific Publications, London, UK, 152 – 168
- Lim, P-S., Chen, S-L., Tsai, C-Y., Pai, M-A. 2006. *Pantoea* peritonis in a patient receiving chronic ambulatory peritoneal dialysis (Case report). Nephrology 11: 97 – 99
- Lind, E., and Ursing, J. 1986. Clinical strains of *Enterobacter agglomerans* (synonyms *Erwinia herbicola, Erwinia milletiae*) identified by DNA-DNA-Hybridization. Acta pathologica microbial and immunology scandinavica 94: 205 – 213
- Loreti, S., Gallelli, A., Belisario, A., Wajnberg, E., and Corazza, L. 2001. Investigation of genomic variability of *Xanthomonas arboricola* pv. *juglandis* by AFLP analysis. European Journal of Plant Pathology 107: 583 – 591
- Maki, D.G., Rhame, F.S., Mackel, D.C., and Bennet, J.V. 1976. Nationwide epidemic of septicemia caused by contaminated intravenous products. I. Epidemiologic and clinical features. American Journal of Medicine 60: 471 – 485
- McNeil, D.L., Romero, S., Kandula, J., Stark, C., Stewart, A., and Larsen, S. 2001. Bacteriophages: A potential biocontrol agent against walnut blight (*Xanthomonas campestris* pv. *juglandis*). New Zealand Plant Protection 54: 220 – 224
- Medrano, E.G., and Bell, A.A. 2006. Role of *Pantoea agglomerans* in opportunistic bacterial seed and boll rot of cotton (*Gossypium hirsutum*) grown in the field. Journal of Applied Microbiology. 102: 134 – 143
- Mehlen, A., Goeldner, M., Ried, S., Stindl, S., Ludwig, W., and Schleifer, K.H. 2004. Development of a fast DNA/DNA hybridization method based on melting profiles in microplate. Systematic and Applied Microbiology 27: 689 – 695
- Mergaert, J., Verdonck, L., and Kersters, K. 1993. Transfer of *Erwinia ananas* (synonym, *Erwinia uredovora*) and *Erwinia stewartii* to the Genus *Pantoea* emend. as *Pantoea ananas* (Serrano1928) comb. nov. and *Pantoea stewartii* (Smith 1898) comb. nov., Respectively, and Description of *Pantoea stewartii* subsp. *indologenes* subsp. nov. International Journal of Systematic Bacteriology 43: 162 – 173

- Nunes, C., Usall, J., Teixido, N., and Vinas, I. 2001. Biological control of postharvest pear diseases using a bacterium, *Pantoea agglomerans* CPA-2. International Journal of Food Microbiology 70: 53 – 61
- Paccola-Meirelles, L.D., Ferreir, A.S., Meirelles, W.F., Marriel, I.E., and Casela, C.R. 2001. Detection of a bacterium associated with a leaf spot disease of maize in Brazil. Phytopathology 149: 275 – 279
- Parkinson, N., Aritua, V., Heeney, J., Cowie, C., Bew, J., and Stead, D. 2007. Phylogenetic analysis of *Xanthomonas* species by comparison of partial gyrase B gene sequences. International Journal of Systematic and Evolutionary Microbiology 57: 2881 – 2887
- Patel, M.K., Kulkarni, Y.S., and Moniz, L. 1948. *Pseudomonas mangiferae – indicae* pathogenic on mango. Indian Phytopathology 1: 147 – 152
- Polito, V.S., Pinney, K., Buchner, R., and Olson, W. 2002. Streptomycin applications to control walnut blight disease can prevent fertilization and increase in fruit drop. Plant Pathology 37: 940 – 942
- Rademaker, J.L.W., Hoste, B., Louws, F.J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P., and de Bruijn, F.J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. International Journal of Systematic and Evolutionary Microbiology 50: 665 – 677
- Rademaker, J.L.W., Louws, F.J., Schultz, M.H., Rossbach, U., Vauterin, L., Swings, J., and de Bruijn, F.J. 2005. A comprehensive species to strain taxonomic framework for *Xanthomonas.* Phytopathology 95: 1098 – 1110
- Ricuad, C., and Ryan, C.C. 1989. Leaf scald, in Diseases of Sugarcane. Major Diseases (eds C. Ricuad, B.T. Egan, A.G. Gillaspie Jr and C.G. Hughes), Elsevier, Amsterdam, 39 – 58
- Roumagnac, P., Gagnevin, L., Gardan, L., Sutra, L., Manceau, C., Dickstein, E.R., Jones, J.B., Rott, P., and Pruvost, O. 2004. Polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species. International Journal of Systematic and Evolutionary Microbiology 54: 15 – 24
- Saddler, G.S. 2002. *Xanthomonas populi*. IMI descriptions of fungi and bacteria. No 1460.

- Saddler, S.S., and Bradbury, J.F. 2005. Genus I. *Xanthomonas* Dowson 1939, 187 AL emend. Vauterin, Hoste, Kersters and Swings 1995, 483. Pages 63 – 90 in Bergey's manual of systematic bacteriology, Volume two, Part B. D.J. Brenner, N.J. Krieg, J.T. Staley and G.M. Garrity, eds. Springer.
- Schaad, N.W., Postnikova, E., Lacy, G.H., Sechler, A., Agarkova, I., Stromberg, P.E., Stromberg, V.K., and Vidaver, A.K. 2005. Reclassification of *Xanthomonas campestris* pv. *citri* (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as *X. smithii* subsp. *citri* (ex Hasse) sp. nov. nom. rev. comb. nov., *X. fuscans* subsp. *aurantifolii* (ex Gabriel 1989) sp. nov. rev. comb. nov., and *X. alfalfae* subsp. *citumelo* (ex Riker and Jones) Gabriel *et al.,* 1989 sp. nov. rev. comb. nov.; *X. campestris* pv. *malvacearum* (ex Smith 1901) Dye 1978 as *X. smithii* subsp. *smithii* nov. comb. nov. nom. nov.; *X. campestris* pv. alfalfae (ex Riker and Jones, 1935) Dye 1978 as *X. alfalfae* subsp. alfalfae (ex Riker *et al.,* 1935) sp. nov. nom. rev.; and "var. fuscans" of *X. campestris* pv. *phaseoli* (ex Smith, 1987) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. Systematic and Applied Microbiology 28: 494 – 518
- Sedkova, N., Tao, L., Rouviere, P.E., and Cheng, Q. 2005. Diversity of carotenoid synthesis gene clusters from environmental *Enterobacteriaceae* strains. Applied and Environmental Microbiology 71: 8141 - 8146
- Serrano, F.B. 1928. Bacterial fruitlet brown rot of pineapples in the Phillippines. Philippine Journal of Science 36: 271 - 305
- Simoes, T.H.N., Goncalves, E.R., Rosato, Y.B., and Mehta, A. 2007. Differentiation of *Xanthomonas* species by PCR-RFLP of *rpf*B and *atp*D genes. FEMS Microbiology Letters 1 – 7
- Springer, T., van der Zwet, T., and Biggs, A.R. 2000. Bacterial spot, *Xanthomonas pruni*. Fruit pathology West Virginia university. 1 – 2
- Stackebrandt, E., and Goebel, B.M. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. International Journal of Systematic Bacteriology. 44: 846 - 849.
- Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kampfer, P., Maiden, M.C.J., Nesme, X., Rossello-Mora, R., Swings, J., Truper, H.G., Vauterin, L., Ward, A.C., and Whitman, W.B. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology.

International Journal of Systematic and Evolutionary Microbiology 52: 1043 – 1047

- Swings, J., and Van den Mooter, M., and Vauterin, L. 1990. Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris* pv. *oryzae*) and bacterial leaf streak (*Xanthomonas campestris* pv. *oryzicola*) of rice as pathovars of *Xanthomonas oryzae* (ex-Ishiyama 1922) sp. nov., nom. rev. International Journal of Systematic Bacteriology 40: 309 – 11
- Swings, J.G., and Civerolo, E.L. 1993. Taxonomy of the genus. *Xanthomonas*. J.G. Swings and E.L. Civerolo, eds. Chapman and Hall, London, UK. 157 – 192
- Swings, J.G., Vauterin, L., and Kersters, K. 1993. The bacterium *Xanthomonas. Xanthomonas.* J.G. Swings and E.L. Civerolo, eds. Chapman and Hall, London, UK. 121 - 156
- Tamponi, G., and Donati, G.P. 1990. Walnut cultivars susceptible to *Xanthomonas juglandis.* Acta Horticulturae 284: 301
- Torres, R., Teixido, N., Ussal, J., Abadias, M., and Vinas, I. 2005. Post-harvest control of *Penicillium expansum* on pome fruits by the bacteria *Pantoea ananatis* CPA-3. The Journal of Horticultural Science and Biotechnology 80: 75 – 81
- Trebaol, G., Gardan, L., Manceau, C., Tanguy, J-L., Tirilly, Y., and Boury, S. 2000. Genomic and phenotypic characterization of *Xanthomonas cynarae* sp. nov., a new species that cause bacterial bract spot of artichoke (*Cynara scolymus* L.) International Journal of Systematic and Evolutionary Microbiology 50: 1471 – 1478
- Truman, R. 1974. Die-back of *Eucalyptus citriodora* caused by *Xanthomonas eucalypti* sp. nov. Phytopathological Notes 64:143 – 144
- Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews 60: 407 – 438
- Vanden Broeck, A. 2003. EUFORGEN Technical guidelines for genetic conservation and use for European black polar: *Populus nigra.* International plant genetic institute, Rome, Italy. 6 pages
- Vauterin, L. and Vauterin, P. 1992. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. European Journal of Microbiology 1: 37 – 42

- Vauterin, L., Hoste, B. Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas.* International Journal of Systematic Bacteriology 45: 472 – 489
- Vauterin, L., and Swings, J. 1997. Are classification and phytopathological diversity compatible in *Xanthomonas*? Journal of industrial microbiology and biotechnology 19: 77 – 82
- Vauterin, L., Rademaker, J., and Swings, J. 2000. Synopsis on the taxonomy of the genus *Xanthomonas*. Phytopathology 90: 677 – 682
- Vos, P., Hogers, R., Bleeker, R., Reijans, M., van de Lee, T., Hornes, T., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407 – 4414
- Walcott, R.R., Gitaitis, R.D., Castro, A.C., Sanders Jr, F.H., and Diaz-Perez, J.C. 2002. Natural infestation of onion seed by *Pantoea ananatis*, causal agent of centre rot. Plant Disease 86: 106 – 111
- Wardlaw, T.J., Kile., G.A., and Dianese, J.C. 2000. Diseases of Eucalypts associated with viruses, phytoplasmas, bacteria and nematodes. P.J. Keane, G.A. Kile, F.D. Podger and B.N. Brown, eds. CSIRO Publishing, Collingwood, Australia. 339 – 352
- Wells, J.M., Sheug, W.S., Ceponis, M.J., and Chen, T.A. 1987. Isolation and characterization of strains of *Erwinia ananas* from honeydew melons. Phytopathology 77: 511 – 514
- Wertz, J.E., Goldstone, C., Gordon, D.M., and Riley, M.A. 2003. A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. Journal of Evolutionary Biology 16: 1236 – 1248
- Yamamoto, S., and Harayama, S. 1996. Phylogenetic analysis of *Acinetobacter* strains based on the nucleotide sequences of *GyrB* genes and on the amino acid sequences of their products. International Journal of Systematic and Evolutionary Microbiology 26: 87 – 95
- Young, J.M., Dye, D.W., Bradbury, J.F., Panagopoulos, C.G., and Robbs, C.F. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. New Zealand Journal of Agricultural Research 21: 153 – 177
- Young, J.M., Park, D.C., Shearman, J.M., and Fargier, E. 2008. A multilocus sequence analysis of the genus *Xanthomonas.* Systematic and Applied Microbiology 31: 366 – 377

- Zeigler, D.R. 2003. Gene sequences useful for predicting relatedness of whole genomes in bacteria. International Journal of Systematic and Evolutionary Microbiology 53: 1893 – 1900
- Zhang, L., and Birch, R.G. 1997. Mechanisms of biocontrol by *Pantoea dispersa* of sugarcane leaf scald disease caused by *Xanthomonas albilineans.* Journal of Applied Microbiology 82: 448 – 454

Chapter 3

Pantoea species associated with bacterial blight and dieback of *Eucalyptus* from various parts of the world

Pantoea species associated with bacterial blight and dieback of *Eucalyptus* from various parts of the world

ABSTRACT

Bacterial blight and die-back of *Eucalyptus* is caused by *Pantoea ananatis* in South Africa. In surveys undertaken in Argentina, Colombia, Indonesia, Uganda and Uruguay a disease resembling that occurring in South Africa was observed. Infected leaf samples were collected from plants in these six countries and from recent outbreaks of the disease in South Africa at two different locations i.e. Kwa Zulu Natal (Mtunzini) and Mpumalanga (White River). Bacteria were isolated from infected tissue and phenotypic analyses completed. 16S rRNA and *gyrB* gene sequencing was then performed on all isolated strains. F-AFLP's were also used and the resulting profiles compared to those of the type strains of all species within the genus. *Pantoea* species were consistently isolated from infected leaves collected from all countries. The most prominent of these species included: *P. ananatis*, *P. vagans*, *P. dispersa* and *P. eucalypti*. Also isolated from the infected leaves were members belonging to the family *Enterobacteriaceae* and potentially new species belonging to the genus *Pantoea*. Representative strains of the potentially new species and other unknown isolates were inoculated into leaves of a known susceptible *E. grandis* X *E. nitens* clone. Mostly the *P. ananatis* species were found to be virulent while the remaining species were only moderately virulent. The bacterial blight and die-back disease on *Eucalyptus*, considered in this study, appears to be caused by a complex of *Pantoea* species. These species are opportunistic and symptoms are expressed when host plants are susceptible and environmental conditions are favorable for infection.

1) INTRODUCTION

Eucalypts are versatile trees that grow in a diverse range of habitats (Doughty 2000, Turnbull 2000). They were initially introduced to the tropical and subtropical regions of the Southern Hemisphere for fuel wood, windbreaks and land reclamation purposes (Poynton 1979, Evans 1982, Potts & Pederick 2000). Over the years, eucalypts have become a major source of commercial forest products such as pulpwood, fiber board, sawn timber, poles, mine timber props, charcoal, honey and essential oils (Sedjo 1999,

Turnbull 1999). A combination of factors such as wood properties, high productivity and versatile ecological adaptation have made them the most widely planted hardwood trees in many parts of the world (Eldridge *et al.* 1994, Turnbull 2000). Today, eucalypts are grown as plantation species in several countries in Africa, Asia, South America and New Zealand as well as throughout their natural range in Australia (Eldridge *et al.* 1994). Eucalypt plantations are rapidly expanding and the total area is estimated to be at least 18 million hectares in 90 countries (Turnbull 1999, Carle *et al*. 2002, FAO 2000).

There are currently four known bacterial species that infect and cause disease of *Eucalyptus* spp. The first bacterial disease that was observed on this host was bacterial die-back caused by *Xanthomonas campestris* pv. *eucalypti*. This disease was first identified in Australia causing blackened areas on the distal part of the twigs and severely infected trees would become completely defoliated (Truman, 1974). The second recorded disease on *Eucalyptus* is bacterial wilt caused by the pathogen *Ralstonia solanacearum.* This disease was first identified in Brazil (Dianese *et al.,* 1990) but has subsequently been reported elsewhere (Coutinho *et al*., 2000). More recently a bacterial leaf spot and die-back disease caused by *Pantoea ananatis* was recorded in South Africa (Coutinho *et al*., 2002), and most recently bacterial leaf blight was identified on *Eucalyptus* in Brazil caused by *Xanthomonas axonopodis* (Goncalves *et al.,* 2008).

The genus *Pantoea* forms part of the family *Enterobacteriaceae* which consists of more than a 100 genera (Brenner and Farmer III, 2005). The bacteria belonging to this genus are non-spore forming, straight rods that are Gram negative and facultatively anaerobic fermenting sugars to form lactic acid. The optimum growth temperature for these bacteria ranges from 22 ºC to 37 ºC. They grow well on basic microbiological media and can use glucose as a carbon source although certain genera require the addition of amino acids and vitamins. The bacteria of this family are also oxidase negative and catalase positive and if motile this occurs with the help of peritrichous flagella with some exceptions e.g. *Tutamella* (Farmer III, 2005). Bacteria belonging to this family can be human, animal or plant associated bacteria mostly occurring internal to its host. Generally these bacteria can be found almost in all natural habitats worldwide (Brenner and Farmer III, 2005).

The genus *Pantoea* was first proposed in 1989 by Gavini *et al.* when the type species *Pantoea agglomerans* a member of the *Enterobacter agglomerans – Erwinia herbicola* complex was transferred to this new genus. Also included in this genus was a newly described species *Pantoea dispersa* (Gavini *et al.,* 1989). In 1992, three more *Pantoea* species was added to the genus, namely, *P. citrea, P. terrea* and *P. punctata* which were isolated from soil and fruit samples in Japan (Kageyama *et al.,* 1992). In 1993, two more species was added when Mergaert *et al.* (1993) proposed the transfer of *Erwinia ananas* and *Erwinia stewartii* to the genus as *Pantoea ananas* and *Pantoea stewartii,* respectively. In 2009, four more species were added to the genus: *P. vagans, P. eucalypti, P. deleyi* and *P. anthophila* (Brady *et al.,* 2009). Very recent work showed there are in fact thirteen species and two sub species that reside in this genus. Originally the genus *Pantoea* consisted of seven species and along with the four new species described by Brady *et al.,* (2009), these are the *Pantoea* species included in this study. The other *Pantoea* species will not be referred to in this study.

Pantoea species have been isolated from diverse ecological niches. These bacteria are occasionally associated with plants as epi- or endophytes, causing no disease on their host. They are also found mostly associated with plants as pathogens causing a wide range of symptoms. Infection of *Eucalyptus* trees by *Pantoea* species were first noted when leaf spots appeared on young leaves. These leaf spots were water soaked and coalesced to form larger necrotic lesions. The trees were multi stemmed and following repeated infection tree growth became stunted (Coutinho *et al.,* 2002).

Pantoea species have been identified as pathogens of humans. *P. ananatis* was isolated from a 73 year old women suffering from dyspnea and bilateral ankle edema (De Baere *et al.,* 2004). Other *Pantoea* spp. isolated from humans include *P. agglomerans* (Kratz *et al.,* 2003, Lim *et al.,* 2006) and *P. dispersa* (Schmid *et al*, 2003).

The aim of this study was to identify *Pantoea* spp. and other members of the *Enterobacteriaceae* associated with bacterial blight and dieback of eucalypts in Argentina, Colombia, Indonesia, Rwanda, South Africa, Thailand, Uganda and Uruguay. Bacteria were isolated from two regions in South Africa, White River (Mphumulanga) and Mtunzini (Kwa Zulu Natal). Their ability to cause disease symptoms in this host was also investigated.

2) MATERIALS AND METHODS

2.1) Disease survey

Diseased leaves and shoots were collected from either seedlings or cuttings in nurseries or from newly established eucalypt plantations in Argentina, Colombia, Indonesia, Rwanda, South Africa, Thailand, Uganda and Uruguay. In these countries the eucalypt species infected were *E. grandis* except in South Africa where the disease occurred on the *E. grandis* x *E. nitens* hybrid. Symptoms on diseased material from all countries were similar (Fig. 1). The initial symptom was the appearance of water-soaked lesions on the leaf which with time became necrotic. Lesions were often concentrated along the main vein of the leaf and it is suspected that this is due to the movement of the inoculum from the petiole into the vein. When young leaves were infected, die-back of the shoot occurred. With repeated infection and die-back of the shoot, the tree becomes multi-stemmed and stunted.

2.2) Bacterial isolations

Diseased leaf samples were surface sterilized by placing them for 1 minute in a 10% sodium hypochlorite solution followed by 70% ethanol for a further minute. Leaves were then rinsed in sterile water to remove excess ethanol. Diseased leaf material was crushed in 2 ml of sterile water using a sterile mortar and pestle. Bacterial suspensions were plated onto Nutrient Agar (NA) and incubated for 48 hours at 28 ºC. Bacterial colonies were then purified.

2.3) Phenotypic testing

Pure strains of all isolates were obtained from infected leaves collected from the different countries. All pure strains were subjected to Gram staining and the Hugh Leifson test using OF media supplied by Biolab (Biomerieux). Strains (27) that were Gram positive and those that were Gram negative, but not facultatively anaerobic, were excluded from the study. Strains included in the study amounted to 64 isolates.

2.4) DNA extraction

Isolates that belonged to the family *Enterobacteriaceae*, as determined by the phenotypic testing, was inoculated into nutrient broth (Biolab) and incubated overnight at 28 °C. DNA extractions were performed on all isolates using the Dneasy Blood and Tissue Kit (QIAGEN) according to instructions of the manufacturer. The DNA concentration of the extracted DNA was determined by loading the samples on a 1% agarose gel (Whitehead Scientific) along with known standards. The gel contained 1 µl ethidium bromide for every 10 ml of agarose gel, and run on the gel in 1 X TAE Buffer at 80 V and 400 mA for 40 minutes.

2.5) 16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene of all isolates were amplified using primer pair *pA and pH (Coennye *et al.,* 1999). Each 100 µL PCR reaction contained 1X Reaction buffer, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 100 pmol of each of the above mentioned primers (Inqaba Biotechnologies), 1 U Taq Gold (Roche) and 50 – 100 ng of genomic DNA. PCR amplification was carried out in an Mastercycler epgradient (Eppendorf). PCR cycles included an initial denaturation step at 94 ºC, 30 cycles of denaturation at 94 ºC for 1 min, annealing of the primers at 58 ºC for 1 min, primer extension at 72 ºC for 1 min. A final extension step was done at 72 ºC for 5 min. Reactions were stored at 4 ºC until used. Sterile water was used as a negative control to monitor contamination and DNA of an isolate that is known to have been successfully amplified previously using this protocol was used as a positive control. The amplification products were loaded on a 1% agarose gel (Whitehead Scientific), containing 1 µl Ethidium bromide for every 10 ml of the agarose gel, and were run in 1 X TAE Buffer at 80 V and 400 mA for 40 minutes. When viewing the gel under UV light, single bands for every isolate of the 16S rRNA gene was observed. The 16S rRNA gene was purified from the PCR reaction using the QIAquick® PCR Purification Kit (QIAGEN).

Initially, the 16S rRNA gene of all isolates was only partially sequenced in order to determine the identity of the genus. The forward primer, 16F536 (*pD) 5’ CAG CAG CCG CGG TAA TAC 3’ was used for this purpose (Coenye *et al.,* 1999). This primer sequences approximately 700 bp which is known to be variable amongst different

genera of bacteria. The partial sequences of the isolates were then submitted for BLAST analysis in the Genbank/EMBL database in a homology search (www.ncbi.nlm.nih).

The entire 16S rRNA gene of 64 isolates with a ≥ 97% sequence similarity to the plant pathogenic bacteria belonging to the genera *Pantoea*, *Enterobacter* and *Erwinia* was then sequenced. Three forward primers and four reverse primers were used for this purpose (Table 1).

Each 10 µl sequencing reaction contained 2 µl of Big Dye Sequencing Mix (Applied BioSystems), 1 X Sequencing Buffer, 3.2 pmol of the appropriate internal primer and 150 ng of purified 16S rRNA PCR product. PCR reactions were carried out in an Mastercycler epgradient (Eppendorf). Sequencing PCR cycles included an initial denaturation step at 96 ºC for 5 sec followed by 25 cycles of denaturation at 96 ºC for 10 sec, primer annealing at 55 ºC for 5 sec and primer extension at 60 ºC for 4 min. The reactions were held at 4 ºC until sequenced. Sequencing PCR products were precipitated from the PCR reaction using the sodium acetate precipitation method. The PCR products were sequenced on an ABI Prism™ 3100 Automated Sequencer (Applied BioSystems). After sequencing of the isolates using the different internal primers a consensus sequence of the full length 16S rRNA gene (± 1500 bp) was assembled manually, using the program Bio-edit Sequence Alignment Editor v 5.0.9. (Hall, 1999), based on overlapping sequences between the fragments generated by the different internal primers.

Consensus sequences of the different 16S rRNA genes were aligned along with the type strains (acquired from the Genbank/EMBL database) of different *Pantoea*, *Enterobacter* and *Erwinia* species using the program ClustalX (Thompson *et al.,* 1997) and the overhangs were trimmed. Modeltest3.7 (Posada and Crandall, 1998) was used to determine the best-fit evolutionary model to apply to the data set. Maximum-Likelihood and Neighbour-Joining analyses where performed using Phyml (Guindon and Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000), respectively, by applying the model selected by Modeltest 3.7. Only the Maximum-Likelihood phylogenetic trees are shown. A bootstrap analysis was run using a 1000 replicates to show branch support. The tree was rooted with the outgroup, *Shigella boydii*.

2.6) *GyrB* gene sequencing and phylogenetic analysis

The same isolates included for 16S rRNA gene sequencing was used for the sequencing of the housekeeping gene, *GyrB*. The *GyrB* gene was amplified using primers that were specifically designed for members of the family *Enterobacteriaceae* (Table 2) (Brady et al., 2008).

Each 50 µl PCR amplification reaction contained 5 µl of 10 X Buffer, 2 µl of $MgCl_2$, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 200 pmol of each of the amplification primers forward (5' TAARTTYGAYGAYAACTCYTAYAAAGT 3') and reverse (5' CMCCYTCCACCARGTAMAGTTC 3') amplification primers (Inqaba Biotechnologies), 1 U Taq (Southern Cross Biotechnologies) and 50 – 100 ng of genomic DNA. Amplification of the *GyrB* gene was carried out in an Eppendorf Mastercycler epgradient (Merck chemicals (Pty) Ltd.). Amplification cycles included a initial denaturation step at 95 ºC for 5 min, 3 cycles of denaturation at 95 ºC for 1 min, primer annealing at 55 ºC for 2 min 15 sec, primer extension at 72 ºC for 1 min 15 sec. These 3 cycles including a longer annealing time will allow the degenerate primers to anneal to the template DNA. There-after 30 cycles of denaturation at 95 ºC for 35 sec, primer annealing at 55 ºC for 1 min 15 sec, primer extension at 72 ºC for 1 min 15 sec and a final extension step at 72 ºC for 7 min. The reactions were stored at 4 ºC until further use. As previously described a positive and a negative control was also included in the reactions.

Amplification reactions were run on a 1% agarose under conditions as described above. Amplification of the *GyrB* gene with the above mentioned primers should result in a 1000 bp fragment of the *GyrB* gene. Visualization of the amplification products under a UV light showed different sizes of bands. PCR amplification products resulting in a clear single band at 1000 bp was cleaned using the QIAquick® PCR purification Kit as described before. For isolates showing more than one clear band, the band at a 1000 bp was excised from the gel and purified using the QIAquick® Gel Extraction kit (QIAGEN).
The *GyrB* gene was sequenced using the following primers: gyrB PF 5' TAARTTYGAYGAYAACTCYTAYAAAGT 3' and gyrB PF7 5' GTVCGTTTCTGGCCVAG 3' (Brady *et al.*, 2007) (Table 2). Each 10 µl sequencing reaction contained 2 µl of Big Dye sequencing mix, 1 X Sequencing Buffer, 6.4 pmol of the appropriate primer and 150

ng of *gyrB* PCR product. PCR reactions were carried out in an Eppendorf Mastercycler epgradient . Sequencing PCR cycles included an initial denaturation step at 96 ºC for 5 sec, 25 cycles of denaturation at 96 ºC for 10 sec, primer annealing at 55 ºC for 5 sec, primer extension at 56 ºC for 4 min 15 sec. Sequencing reactions were held at 4 ºC until sequenced. Sequencing PCR products were precipitated from the PCR reaction using the sodium acetate precipitation method. The PCR products were sequenced on an ABI Prism 3100 Automated Sequencer. The sequence data was used to obtain a consensus sequence of the partial *GyrB* gene was assembled manually, using the program Bio-edit Sequence Alignment Editor v 5.0.9., based on overlapping sequences between the fragments.

Consensus sequences of the different *GyrB* genes were aligned along with the type strains (acquired from the Genbank/EMBL database) of different *Pantoea*, *Enterobacter* and *Erwinia* species using the program ClustalX (Thompson *et al.,* 1997) and the overhangs were trimmed. Modeltest3.7 (Posada and Crandall, 1998) was used to determine the best-fit evolutionary model to apply to the data. Maximum-Likelihood and Neighbour-Joining analyses where performed using Phyml (Guindon and Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000), respectively, by applying the model selected by Modeltest 3.7. Only Maximum-Likelihood phylogenetic trees are shown. A bootstrap analysis was run using a 1000 replicates to show branch support. The tree was rooted with the outgroup, *Citrobacter freundii.*

2.7) AFLP (Amplified Fragment Length Polymorphism) analysis

Only possible *Pantoea* isolates were included for AFLP analysis, isolates identified as other plant pathogenic bacteria where excluded. Total genomic DNA was isolated from all isolates using the DNeasy® Blood and Tissue kit. The AFLP protocol consists of a Restriction enzyme digestion step, adaptor ligation step, two PCR amplification steps and the running of isolates on a polyacrylamide gel.

Total genomic DNA (50 – 100ng) was digested with 12 U *EcoRI* (Roche diagnostics) and 8 U *Tru91* (Roche diagnostics) in 5 X Restriction/ligation buffer (50 mM Tris HAc, 50 mM MgAc, 250 mM KAc, 25 mM DDT). The digestion reaction was incubated at 37 ºC for 2 hours. After digestion, the temperature was increased to 70 ºC and incubated for 15 min

to allow for the inactivation of the enzymes. Double stranded adaptors with sticky ends, complementary to the restriction enzyme cut sites of *EcoRI* and *Tru91* were added to the reaction. When these adaptors are ligated to the target fragments it ensures that the original restriction enzyme cut site is not restored on the target fragments (Zabeau and Vos, 1993). 5pmol *EcoRI* adaptor and 50pmol *Tru91* adaptor were added to the reaction along with 5 X Restriction and Ligation Buffer, 0.3 mM ATP and 1 U of T4 DNA ligase (Roche). The ATP produces the energy to ligate the adaptors to the fragments and T4 DNA ligase catalyzes the reaction. The ligation reaction was incubated at 20 ºC for 2 hours to allow for sufficient ligation. The reaction was then diluted 1:10 with Nuclease Free water (Promega).

The pre-amplification reaction contained: 1 X Reaction Buffer, 1.5 mM $MgCl_2$, 250 mM of each nucleotide (dATP, dCTP, dGTP, dTTP), 100 pmol of each primer Eco-00 (5' GAC TGC GTA CCA ATT C 3') and Tru91-00 (5' GAT GAG TCC TGA CTA A 3') (Inqaba Biotechnologies), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 2 µL of the diluted (1:10) restriction and ligation reaction. The reaction was made up to a final volume of 25 µL with Nuclease Free water (Promega). The amplification was carried out in a Eppendorf Mastercycler epgradient (Merck chemicals (Pty) Ltd.) and the amplification conditions included an initial denaturation step at 94 ºC for 3 minutes, 20 cycles of denaturation at 94 ºC for 30 seconds, primer annealing at 56 ºC for 1 minute and primer extension at 72 ºC for 1 minute, and extension at 72 ºC for another 5 minutes. The pre-amplification reaction was held at 4 ºC until further use. Each reaction was then diluted 1:50 with Nuclease Free water (Promega).

The selective amplification reaction contained 1 X Reaction Buffer, 1.5 mM $MgCl_2$, 250 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 0.5 pmol of the fluorescently labeled Eco-C primer (5' GAC TGC GTA CCA ATT CC 3') (LI-COR Biosciences) and 2.4 pmol of the primer Tru-GC (5' GAT GAG TCC TGA CTA AGC 3') (Inqaba Biotechnologies), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 5 µL diluted pre-amplification reaction. The reaction was made up to a final volume of 20 µL with Nuclease Free water (Promega). Amplification was carried out in a Eppendorf Mastercycler epgradient (Merck chemicals (Pty) Ltd.) and the amplification conditions included an initial denaturation step at 94 ºC for 5 minutes, 9 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 65 ºC for 30 sec and primer extension at 72 ºC for 1

minute, where the primer annealing temperature decreased 1 ºC per cycle until a final annealing temperature of 56 ºC was reached. This was followed by 23 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 56 ºC for 30 sec, and primer extension at 72 ºC for 1 min, and another primer extension step at 72 ºC for 5 min. The selective amplification reaction was stored at 4 ºC until further use.

LI-COR gels were prepared by using 20 ml Long Ranger gel solution (8% Long Ranger gel solution (LI-COR Biosciences), 7M Urea, 10 X TBE Buffer) and 150 µL 10% APS (Ammonium persulphate) and 15 µL TEMED for polymerisation. Gels were cast using the gel casting apparatus and allowed to polymerise for 1 hour. The LI-COR apparatus was set up according to manufacturers instructions and a pre-run was preformed at 1500 V and 35 W. The pre-run monitored the voltage and power and standardized the ions within the gel and the buffer. Samples were prepared for loading by mixing 1 µL of the selective amplification with 1 µL of loading buffer (95 % formamide, 20 mM EDTA, bromophenol blue). The sample mixture was heated to 90 ºC for 3 min and incubated on ice for 10 min before it was loaded onto the gel. Approximately 1 µL of sample mixture was loaded onto the gel and a molecular sizing standard marker, IRD-700, was loaded on each end of the gel for accurate gel analysis when normalizing the gel. The samples were run on the gel in a LI-COR IR^2 automated sequencer (LI-COR Biosciences) for 4.5 hours at 42 W in 0.8 M TBE Buffer.

When the gel had run to completion, the resulting fingerprint banding patterns was analysed using the software program Bionumerics. Gels were normalized using the IRD-700 standard. Bands between 50 – 700 bp were used in the analysis. AFLP profiles were compared by constructing an UPGMA tree, using the Pearson correlation coefficient.

2.8) Pathogenicity trials

Hypersensitivity and pathogenicity tests had previously been performed with strains isolated from diseased *Eucalyptus* in Thailand, Uganda and Uruguay (Greyling, 2007) and were not repeated in this study. These strains included *P. ananatis*, *P. dispersa*, *P. vagans* and *P. eucalypti*. The species *P. vagans* and *P. eucalypti* were shown in a MLSA study done by Brady *et al.,* (2009) to belong to the genus *Pantoea* and are thus included

in this study. In the study done by Greyling (2007) it was shown that, with the exception of *P. ananatis*, all other species were found to be moderately pathogenic.

Representative isolates of strains shown to belong to the genus *Pantoea* in this study, but not to any of the seven validly described *Pantoea* species, and also other unknown isolates were used in the pathogenicity tests undertaken. These strains included BCC 568 from Rwanda, BCC213 from Thailand, BCC581 and BCC 585 from Colombia and BCC 679, BCC 681, BCC 682 from South Africa. The inoculum was prepared by placing single colonies of each isolate into 50 ml of Nutrient Broth. The flasks were incubated overnight at 28 ºC. The concentration of the bacterial cells was then adjusted to approximately 10^8 CFU/ml in a sterile saline solution determined using a previously prepared growth curve.

In order to determine whether or not the isolates were pathogenic, the appearance (or lack thereof) of the hypersensitivity reaction (HR) in tobacco (*Nicotiana tabacum* cv samsun) was recorded. A bacterial suspension was injected into the leaves of the tobacco plants using a 1 ml insulin syringe. Two leaves were inoculated per isolate. The syringe was inserted into the main vein of the leaf and the leaf panels were flooded with the bacterial suspension. A negative control containing only sterile saline solution and a positive control containing the bacterial blight and die-back pathogen, *Pantoea ananatis*, that was isolated from South Africa (LMG20103), was also inoculated in this study. Tobacco plants were kept in a greenhouse at approximately 26 ºC with natural day and night light cycles. The plants were assessed after 24, 48 and 36 hours for the development of a HR. A positive HR response is observed as a complete and rapid collapse of the inoculated leaf tissue or the drying, light brown necrosis of the water soaked tissue within 36 hours of the inoculation (Lelliot and Stead, 1987). The test was repeated to confirm results.

Isolates that showed a positive HR response were inoculated into the leaves of a *Eucalyptus grandis* X *Eucalyptus nitens* (GN188) hybrid that is known to be susceptible to *Pantoea ananatis.* The *Eucalyptus* leaf panels were flooded with the bacterial suspension as described above. A positive and negative control was included as described above. Inoculated plants were covered with plastic bags in order to induce high humidity. Bags were removed after seven days and the inoculated leafs were

assessed for disease development every 24 hours for a period of 7 days. Plants were kept at 26 ºC with natural day/night light cycles.

Pathogen re-isolations were done from inoculated *Eucalyptus* leaves that have developed lesions during the pathogenicity trials. Isolations were done as described previously from infected leaf material. Pure-cultures were obtained for the re-extracted bacterial pathogen and DNA extractions were performed. The 16S rRNA gene of purified isolates was amplified using the method described above, and the 16S rRNA gene was partially sequenced using the method described above. The partial 16S rRNA gene sequences were submitted to BLAST analysis in Genbank for identification. This process was undertaken in order to fulfill Koch's postulates.

3) RESULTS

3.1) Bacterial isolations

A total of 125 bacterial isolates were obtained from infected leaves collected in the 8 countries. Two isolates from Argentina, 17 isolates from Colombia, 15 isolates from Indonesia, 5 isolates from Rwanda, 57 isolates from the two sites in South Africa (34 for Mtunzini and 23 for White River), 20 isolates from Thailand, 1 isolate from Uganda and 8 isolates from Uruguay.

3.2) Phenotypic testing

A total of 64 isolates were identified as being facultatively anaerobic rod shaped Gram negative bacteria. These results indicate that these isolates may belong to the family *Enterobacteriaceae.* The 27 isolates that did not produced these phenotypic results were excluded from the study after the phenotypic identification, since these isolates where not of importance to this specific study, these isolated bacteria were most probably randomly isolated endophytes. The isolates included in this study after phenotypic testing were from the following localities: Two from Argentina, 9 isolates from Colombia, 4 isolates from Rwanda, 31 isolates from the two sites in South Africa (14 for Mtunzini and 17 for White River), 13 isolates from Thailand, 1 isolate from Uganda and 4 isolates

from Uruguay. No isolates from the family *Enterobacteriaceae* were obtained from Indonesia.

3.3) DNA extractions

Genomic DNA was successfully extracted from all 64 isolates using the DNeasy Blood and Tissue Kit (Qiagen). A clear band of genomic DNA was visible on an agarose gel when stained with Ethidium bromide and viewed under an UV light

3.4) 16S rRNA gene sequencing and phylogenetic analysis

Partial sequences of approximately 700bp were obtained for the 64 facultative anaerobic Gram negative isolates. When a homology search was done by comparing these fragments against the EMBL/Genbank database using a BLAST search, the majority of isolates had between 97-100% similarity to a species belonging to the genus *Pantoea*. Sequencing the entire 16S rRNA gene of all isolates were then undertaken and a phylogenetic tree was constructed using the maximum likelihood parameter (Fig. 2). Modeltest3.7 was used to determine the evolutionary model most applicable to the dataset. The chosen model was GTR+I+G, I and G was also determined using Modeltest3.7. Included in this tree are the type strains of all the described *Pantoea* spp., newly described species by Brady *et al,* (2009) and of other closely related plant pathogenic members of the Enterobacteriaceae (*Erwinia* and *Enterobacter*). This was only a preliminary identification step and only a few representative strains of the plant pathogenic bacteria were included, especially those that were most closely related to the genus *Pantoea*.

The resulting Maximum Likelihood tree generally has low bootstrap support. The phylogenetic tree showed that distinct *Pantoea* clusters were formed. Isolates falling into these clusters can with 97% certainty be said to belong to the genus *Pantoea.* Some isolates did not group in known *Pantoea* species clusters but cluster with the type strains of other plant pathogenic bacteria. These included those clustering with species of *Enterobacter*, viz. (group 10) BCC378 and BCC288. Isolates grouping within groups 8, 9 and 11 may also belong to the genus *Enterobacter* since these isolates clusters in close proximity with the *Enterobacter* type strains and reference strains but remains

unresolved due to a lack of bootstrap support. Another isolate grouped with the representative strains from the genus *Erwinia*, and thus could accurately be identified as belonging to this genus viz. BCC 575. From this tree it can also be seen that members of the phytopathogenic bacteria share an extremely conserved 16S rRNA gene, due to the low bootstrap support for most clades. A distinct grouping (viz. group 5) fell within the core *Pantoea* species but did not cluster with a described *Pantoea* spp. These isolates were those from Rwanda, viz. BCC568, BCC569, BCC570 and BCC571, isolates from Colombia, BCC581, BCC582, BCC585, BCC588 and BCC589 and two isolates from South Africa, BCC679 and BCC681. These would appear to be isolates representing an undescribed species that may possibly belong to the genus *Pantoea*. According to the phylogenetic tree these isolates are the most closely related to the genus *Pantoea*.

According to Fig. 2, 19 isolates from South Africa (BCC700, BCC701, BCC695, BCC699, BCC698, BCC703, BCC028, BCC 027, BCC033, BCC030, BCC043, BCC029, BCC045, BCC049, BCC059, BCC056, BCC032, BCC047 and BCC060), 1 isolate from Uruguay (BCC583) and 1 isolate from Thailand (BCC367) grouped within the *Pantoea ananatis/Pantoea allii* cluster (group 1). One isolate from Uruguay (BCC771) and 3 isolates from South Africa (BCC683, BCC694 and BCC676) grouped with the type strain of *P. eucalypti*. One Isolate from Uganda (BCC691) and 3 isolates from Colombia (BCC580, BCC586 and BCC573) group with the type strain of *P. agglomerans/P. vagans* (group 2). The isolate, BCC684, from South Africa grouped with the type strain of *Pantoea stewartii* subsp. *indologenes* (group 4). Nine isolates from Thailand (BCC380, BCC379, BCC566, BCC210, BCCBCC212, BCC213, BCC211, BCC070 and BCC073), 1 isolate from Argentina (BCC016), 2 isolates from South Africa (BCC692, BCC682), grouped with the type strain of *Pantoea dispersa* (group 6).

3.5) *GyrB* sequencing and phylogenetic analysis

Sequencing of the *GyrB* gene was undertaken on all 64 isolates. Amplification of the partial *GyrB* gene using the primers described previously resulted in a fragment of approximately 1000 bp in size. This resulting fragment was sequenced using the previously described primers.

A Maximum Likelihood tree was constructed using Modeltests 3.7 to predict the tree that was most suited to the dataset (Fig. 3). The chosen model was GTR+I+G. In the resulting Maximum Likelihood tree all *Pantoea* type strains formed distinct clusters. High bootstrap values provide support for the identity of most of the isolates that grouped with the *Pantoea* type strains.

In a similar manner to what was found with the 16S rRNA gene sequencing results, some isolates did not fall into any of the known *Pantoea* species clusters but clustered closer with the type strains of other plant pathogenic bacteria. Isolates BCC017, BCC209, BCC288, BCC378, BCC678, BCC019, BCC014, BCC693, BCC677 and BCC044 all grouped within the genus *Enterobacter* (viz. groups 10 and 11).The isolates BCC017 and BCC209 grouped with the type strain of *Enterobacter cowanii* with a bootstrap support value of 99.7% and a 100% respectively, thus accurately identifying these two isolates as this species. The isolate BCC575 (viz. group5) groups nearest to the type strain of *Erwinia rhapontici*. This isolate is believed to belong to the genus *Erwinia*.

Isolates (viz group 9) included those from Rwanda, BCC568, BCC569, BCC570 and BCC571, isolates from Colombia, BCC581, BCC582, BCC585, BCC588 and BCC589 and two Isolates from South Africa, BCC679 and BCC681, do not cluster with type strains of a known *Pantoea* species. They represent one or more undescribed species that may possibly belong to the genus *Pantoea* since it is more closely related to this genus than other type strains included. This distinct cluster was also observed with 16S rRNA gene sequencing but with poor bootstrap support. However, *GyrB* gene sequencing provides stronger support for the phylogenetic position of this cluster.

The isolate BCC213 groups within the *Pantoea dispersa* cluster in the 16S rRNA gene tree. However, according to the Maximum Likelihood tree constructed using the *GyrB* gene data, the isolate grouped closely to the *Pantoea dispersa* cluster but with the reference strain of *Pectobacterium cypripedii* with a 100% bootstrap value to support this branch (viz group 7). Isolates BCC 292 and BCC682 (viz. group 8) grouped within the *P. dispersa* cluster with 16S rRNA gene sequencing, but when analised using the *GyrB* gene these isolates formed a distinct separate group (viz. group 8) that contains no reference strain. Thus these two isolates could not accurately be identified.

The results obtained from the *GyrB* sequencing supported the groupings of all other isolates as indicated by 16S rRNA gene sequencing. Unfortunately the GyrB gene could not be sequenced for 3 isolates, namely BCC771, BCC210 and BCC211. Their identification is thus based on 16S rRNA gene sequencing and the results obtained from AFLPs.

3.6) AFLP (Amplified Fragment Length Polymorphism) analysis

A DNA fingerprint requires a minimum of 30 – 50 bands for sufficient analysis (Janssen *et al.,* 1996). The fingerprints resulting from this AFLP study resulted in approximately 100 bands which is sufficient for analysis. Only isolates that grouped within the known *Pantoea* species clusters was included in the AFLP analysis, since a detailed database with fingerprints of type strains and representative strains of the different *Pantoea* species is available for analysis.

In the resulting UPGMA dendrogram (Figure 4) 9 distinct groups were formed that contained isolates of interest. Group 5 includes the type (LMG 2665^{T}), and representative strains of *Pantoea ananatis*. The UPGMA dendrogram (see Figure 4) produced from the AFLP analysis of the isolates confirmed the clustering observed for the 16S rRNA and GyrB gene sequencing. AFLP confirms the identity of the following isolates: Isolates belonging to *P. ananatis*: BCC 028, BCC 029, BCC 056, BCC 060, BCC 030, BCC 695, BCC 032, BCC 033, BCC 049, BCC 043, BCC 027, BCC 059, BCC 045, BCC 367, BCC 698, BCC 703, BCC 700, BCC 699, BCC 701, BCC 583 and BCC 047. Isolates belonging to *P. dispersa*: BCC 073, BCC 566, BCC 379, BCC 380, BCC 210, BCC 016 and BCC 070. Isolates belonging to the *P. eucalypti*: BCC 683, BCC 676, BCC 694, BCC 771. Isolates belonging to *P. vagans*: BCC 691 and BCC 573.

Sequencing of the 16S rRNA gene, and the *GyrB* gene of the isolate BCC586 resulted in a clustering with the type strains of *Pantoea vagans* whilst AFLP analysis shows that this isolate's identity is *P. dispersa*. Similarly with isolate BCC 580, where sequencing of the genes implicates the identity of this isolate as *P. vagans*, and AFLP analysis identifies this isolate as *P. eucalypti*. The conflicting identities of these isolates may be a result of the unevenness of the AFLP banding patterns. The intensity of the banding patterns for

the different isolates was not standardized for all the fingerprints. The bands at the start of the fingerprint were dark, but as the bands progressed the intensity of the bands faded. When looking at the resulting fingerprint for BCC 586 two thick, blotchy bands can be seen near the start of the fingerprint. These two bands could possibly have skewed the analysis and excluded this isolate from the *P. vagans* cluster because these two bands share more similarity to the DNA fingerprint of *P. dispersa*. This also proves that sequencing is a more reliable approach than AFLP for identification of unknown *Pantoea* isolates.

3.7) Pathogenicity trials

All isolates used in the pathogenicity tests elicited moderate to no hypersensitivity responses when inoculated into tobacco plants when compared to the positive control (LMG 20103) (results not showed). The hypersensitive response indicates that the bacterial isolates in question are indeed plant pathogens, and would be able to induce disease in *Eucalyptus* plants. No response was elicited by the negative control.

Isolates BCC568, BCC213, BCC679, BCC682 and BCC583 elicited a mild to strong hypersensitive response where symptoms ranged from yellowing of the leaves to complete necrosis of the inoculated leaf tissue. These isolates were found to be moderately pathogenic when compared to the positive control after inoculations of the susceptible *Eucalyptus* GN clones. Isolates BCC581, BCC585, BCC681 and BCC583 elicited a very mild hypersensitive response.

When the bacterial suspension was inoculated into healthy susceptible *Eucalyptus* plants, disease symptoms started to develop between 2 – 5 days depending on the isolate. All inoculations with the respective isolates showed signs of disease development. Disease symptoms initially appeared at the site of inoculation and spread along the main vein of the leaf. Symptoms on the *Eucalyptus* leaves can be seen in Figure 5. Severity of the symptoms ranged from moderately pathogenic to pathogenic. Isolates BCC679, BCC568, BCC682 and BCC213 showed less severe lesions on the leaves where as isolates BCC367, BCC583, BCC585, BCC681 and BCC581 showed more severe disease symptom development. All of the inoculated isolates were less

pathogenic than the positive control (LMG20103) when lesions were compared. The negative control produced no lesions.

When the pathogenicity results were recorded, the causal agent of the resulting lesions were re-isolated. Partial sequencing of the 16S rRNA gene and comparison to the partial sequence of the inoculated organism resulted in 100% similarity when aligned using Bio Edit, thus indicating that Koch's postulates were fulfilled. The bacterial organism associated with the disease symptoms was inoculated into healthy *Eucalyptus* leaf tissue, similar disease symptoms were induced and the causal organism was re-isolated from the diseased leaf material.

Further studies into the pathogenicity of these isolates should include the testing of these bacterial strains on different plant hosts than *Eucalyptus,* especially for the proposed new species.

4) DISCUSSION and CONCLUSIONS

The disease symptoms on *Eucalyptus* leaf material from Argentina, Colombia, Rwanda, South Africa, Thailand, Uganda, and Uruguay was similar. Isolates obtained were identified using a polyphasic approach. A combination of phenotypic (Gram stains and Hugh Leifson) and genotypic (16S rRNA gene sequencing, *GyrB* gene sequencing and AFLP analysis) were used in this study. Previous studies have shown the difficulty of identifying species of the genus *Pantoea* using only phenotypic techniques due to the high degree of similarities between species of the genus *Pantoea* and related members of the *Enterobacteriaceae* (Mergaert *et al.,* 1984; Verdonck *et al.,* 1987). Misidentification of these isolates would frequently occur due to phenotypic resemblance between these isolates when identification was based solely on colony morphology and physiological tests.

Results in this study showed that 16S rRNA gene sequencing can potentially be used to identify isolates belonging to the genus *Pantoea* and to investigate their taxonomic position. Unfortunately, this technique cannot accurately distinguish between the different *Pantoea* species. This is due to the fact that the 16S rRNA gene is highly conserved amongst members of the family Enterobacteriaceae (Stackebrandt *et al.,*

1999). Although this technique cannot accurately identify isolates to the species level, it is to date still a requirement of the description of a bacterial species (Stackebrandt *et al.,* 2002). The large database that exists of 16S rRNA sequence information also makes this technique attractive for initial identification of species. Since this technique does not identify the species of *Pantoea* accurately, other identification techniques were also employed. When investigating the Maximum Likelihood tree constructed from the 16S rRNA gene data, it can be seen that the representative isolates of *P. ananatis* and *P. allii* forms a single cluster, not differentiating between the two species. A MLSA study done by Brady and co-workers (results unpublished) shows that *P. allii* is a new species belonging to the genus *Pantoea,* clearly separated from its closest *Pantoea* relative *P. ananatis.* Thus indicating that 16S rRNA gene sequencing alone cannot be used for identification of *Pantoea* species. Also, when examining the 16S rRNA gene sequencing data, isolates BCC682 and BCC692 falls within the cluster that identifies these isolates as *P. dispersa.* When examining the *GyrB* gene sequencing data it can be seen that these two isolates do not fall within the *P. dispersa* cluster supporting the above mentioned statement.

GyrB gene sequencing allows for a more accurate identification of *Pantoea* isolates. This gene forms part of the replication machinery of the bacterial cell, and is thus a requirement for normal replication of the bacterial genome. This gene is conserved and is not highly subjected to horizontal gene transfer, which renders it a dependable gene for phylogenetic identification. Brady *et al.,* (2008) has shown that this gene has high discriminatory resolution between species of the genus *Pantoea*. The *GyrB* gene of isolates BCC771, BCC210 and BCC211 could not be amplified using the primers specified. Although, the *GyrB* gene of these isolates could not be amplified isolates BCC771 and BCC210 could still be accurately identified, given that the identity given by the sequencing of the 16S rRNA gene and AFLP analysis corresponded. Isolate BCC211 posed more of a challenge since 16S rRNA gene sequencing indicates that this isolate clusters within the *P. dispersa* cluster and AFLP analysis showed that this isolate does not cluster with a known *Pantoea* isolate. Thus, this isolate could not be identified.

AFLP analysis allows for the identification of bacterial isolates based on a whole genome approach. Where sequencing methods depends on the identification of isolates based on one specific gene. AFLP analysis has also previously been shown as a reliable and

reproducible method for typing of *Pantoea* species (Brady *et al.,* 2007). The AFLP technique was used to confirm the identity of isolates. AFLP analysis and the construction of a UPGMA dendrogram supported the identity of isolates that grouped in distinct clusters in *GyrB* gene sequencing as it formed the same clusters in AFLP analysis, with some exceptions. The isolate BCC580 was identified as *P. vagans* with 16S rRNA gene sequencing and *GyrB* gene sequencing by clustering with the representative strains of *P. vagans.* When comparing these results to those generated by the AFLP technique, they do not correspond. AFLP analysis identified this isolate as *P. eucalypti* since it clustered with the representative isolates of *P. eucalypti*. The same observation can be made for BCC586, where this isolate clusters with *P. vagans* using the sequencing techniques whilst AFLP analysis indicates that this isolate is in fact *P. dispersa.* This controversy might have arisen due to irregularities in the banding patterns itself or due to unevenness in the intensity of the banding patterns. When investigating the individual bands in the banding patterns, it can be seen that both of these banding patterns are unique. These isolates grouped in the above mentioned clusters due to one or two individual bands that are highly similar to bands observed in the type strains of *P. eucalypti* and *P. dispersa* respectively for BCC580 and BCC586. The software used for analysis of the banding patterns could not distinguish between these two isolates since it does share similar individual bands and on the basis of these individual similar bands with high intensity the isolates are thought to be similar. High congruence is not observed between these isolates to support the identities given with AFLP analysis. With exception to the above mentioned isolated incidences, *GyrB* gene sequencing and AFLP analysis are sufficient techniques for the identification of bacterial isolates belonging to the family *Enterobacteriaceae* up to species level.

Most isolates elicited a positive hypersensitivity response within 48 hours indicating that these isolates are plant pathogens. According to Coplin and Kado (2001) *P. stewartii* and *P. agglomerans* are said to possess a *hrp* secretion system, responsible for inducing a hypersensitive reaction in plants, but will not necessarily induce a positive HR when grown on rich media such as nutrient agar. All inoculated strains in this study did however elicit a positive HR. Pathogenicity tests revealed that the inoculated bacterium was the causal agent of blight and dieback since similar symptoms were observed on inoculated plants. This was further confirmed when the bacterium was re-isolated from the inoculated plants and their identity confirmed. The inoculation method used for

pathogenicity tests in this study, is a very stringent method of inoculation and future studies should include a less stringent inoculation method to compare results of the inoculation methods. As different *Pantoea* species were isolated from diseased *Eucalyptus* leaf material showing signs of bacterial blight and dieback, it is most likely that the disease is caused by a complex of bacterial species.

Certain isolates did not group into the distinct *Pantoea* clusters using *GyrB* gene sequencing analysis and AFLP analysis, but clustered outside the genus *Pantoea* with its closest plant pathogenic relatives, namely, *Enterobacter, Erwinia* and *Pectobacterium*. The identity of these isolates does not suggest that these bacteria are the causal agent of bacterial blight and dieback on *Eucalyptus*, but rather suggests that these bacteria may have been present within the leaf tissue as endophytes at the time of isolation. These bacteria are not thought to be epiphytes since the leaf material was surface sterilized before pathogen isolations occurred. In a study done by Kuklinsky-Sobral and co-workers (2004) they showed that the most dominant endophytic bacteria, isolated from two cultivars of soybean plants, belonged to the family *Enterobacteriaceae*. More specifically *Enterobacter* spp. and *Erwinia* spp. were isolated as endophytes from the soybean plant. *Enterobacter* spp. were also isolated as endophytes from other plants and crops e.g. strawberry (Tanprasert and Reed, 1997), red clover (Sturz *et al.,* 1998), citrus plants (Araujo *et al.,* 2001, Gardner *et al.,* 1982) and cotton (McInroy and Kloepper, 1995 and Musson *et al.,* 1995) to name a few. *Erwinia* spp. have also less frequently been isolated as endophytes from plants and crops such as cotton (Misaghi and Donndelinger, 1990), alfalfa (Gagne *et al.,* 1987) and sugar beet (Jacobs *et al.,* 1985).

All genotypic identification techniques identified a group of isolates that fall within the delineation of the genus *Pantoea* but do not cluster with a distinct *Pantoea* type strain or reference strain and could thus not be identified. Preliminary results suggest that these isolates may be new species or subspecies of the genus *Pantoea*. Further investigation will need to be implemented to identify these potentially new species. More samples will also need to be taken.

The polyphasic identification approach used in this study proved to be a rapid and reliable technique for the identification of unknown bacteria isolated from diseased

Eucalyptus leaf samples. The alternative technique and the golden standard in bacterial taxonomy is DNA:DNA hybridization. The disadvantages of this technique includes its high cost since the reagents and equipment used is expensive, the technique is not rapid and it cannot be standardised between different laboratories to compare results. The polyphasic approach used in this study thus provides an improved alternative with more advantages for the forestry industry when unknown bacteria causing disease needs to be identified. *Pantoea* species also infect and cause disease on a wide variety of agriculturally important hosts other than *Eucalyptus*. Accurately identifying these species may lead to the development of a method for controlling and managing this pathogen. The principles and techniques used in this study can also be used in future for the identification of other disease causing bacteria.

A summary on the species identified by this polyphasic identification study identified the following isolates from diseased *Eucalyptus* leaf material from the different countries. The results showed that the following species are associated with bacterial blight and dieback of *Eucalyptus* in the following geographical regions

- Species isolated from Africa (South Africa: Mtunzini, South Africa: White River, Rwanda and Uganda) included *P. ananatis*, *P. stewartii*, *P. vagans* and *P. eucalypti*
- Species isolated from South America (Argentina, Colombia and Uruguay) included *P. ananatis*, *P. dispersa* and *P. vagans*
- Species isolated from Asia (Thailand) included *P. ananatis* and *P. dispersa*
- No *P. agglomerans* isolates were isolated from any of the geographical regions
- New species probable belonging to the genus *Pantoea* was isolated from the following geographical regions: Africa (South Africa: White River and Rwanda) and South America (Colombia).

5) REFERENCES

- Araujo, W.L., Maccheroni, W., Aguilar-Vildosa, C.I., Barrosa, P.A.V., Saridakis, H.O., and Azevedo, J.L. 2001. Variability and interactions between endophytic bacteria and fungi isolated from leaf tissue of citrus rootstocks. Canadian Journal of Microbiology 47: 229 - 236
- Brady, C.L., Venter, S., Cleenwreck, I., Vancanneyt, M., Swings, J., and Coutinho, T. 2007. A FAFLP system for the improved identification of plant-pathogenic and plant-associated species of the genus *Pantoea*. Systematic and Applied Microbiology 30: 413 – 417
- Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., and Coutinho, T. 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Systematic and Applied Microbiology 31: 447 – 460
- Brady, C.L., Venter, S.N., Cleenwerck, I., Engelbeen, K., Vancannyet, M., Swings, J., and Coutinho, T.A. 2009. *Pantoea vagans* sp. nov., *Pantoea eucalypti* sp. nov., *Pantoea deleyi* sp. nov. and *Pantoea antophila* sp. nov. International Journal of Systematic and Evolutionary Microbiology 59: 2339 – 2345
- Brady, C.L., Cleenwerck, I., Venter, S.N., Engelbeen, K., de Vos, P., and Coutinho, T.A. 2009b. Emended description of the genus *Pantoea* and description of four novel species from human clinical samples, *Pantoea septica* sp. nov., *Pantoea eucrina* sp. nov., *Pantoea brenneri* sp. nov. and *Pantoea conspicua* sp nov., and transfer of *Pectobacterium cypripedii* (Hori 1911) Brenner *et al.* 1973 emend. Hauben *et al.* 1998 to the genus *Pantoea* emend. as *Pantoea cypripedii* comb. nov. (In Press)
- Brenner, D.J. and Farmer III, J.J. 2005. Family I. Enterobacteriaceae. Rahn 1937, Nom. Fam. Cons. Opin. 15, Jud. Comm. 1958a, 73; Ewing, Farmer, and Brenner 1980, 674; Judicial Commission 1981, 104. Pages 587 – 850 in Bergey's Manual of Systematic Bacteriology, Volume two, Part B. D.J. Brenner, N.J. Krieg, J.T. Staley and G.M. Garrity, eds. Springer.
- Carle, J., Vuorinen, P., and Lungo, D.A. 2002. Status and trends in global forest plantation development. Forest Products Journal 52: 13 – 23
- Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J.R.W., Kersters, K., and Vandamme, P. 1999. Classification of *Alcaligenes faecalis*-like isolates from

the environment and human clinical samples as *Ralstonia gilardii* sp. nov. International Journal of Systematic Bacteriology 49: 405 – 413

- Coplin, D.L., and Kado, C.I. 2001. II Gram-negative bacteria, B-3 Pantoea. Pages 73 – 82 in Laboratory Guide for identification of Plant Pathogenic Bacteria, Third edition. N.W. Schaad, J.B. Jones, and W. Chun, eds. American Phytopathology Society Press.
- Coutinho, T.A., Roux, J., Riedel, K.-H., Terblanche, J., and Wingfield, M.J. 2000. First report of bacterial wilt caused by *Ralstonia solanacearum* on eucalypts in South Africa. Forest Pathology 30: 205 – 210
- Coutinho, T.A., Preisig, O., Mergaert, J., Cnockaert, M.C., Riedel, K.-H., Swings, J., and Wingfield, M.J. 2002. Bacterial blight and dieback of *Eucalyptus* species, hybrids and clones in South Africa. Plant Disease 86 20 – 25
- De Baere, T., Verhelst., R., Labit, C., Verschraegen, G.W., Claeys, G., and Vaneechoutte, M. 2004. Bacteremic infection with *Pantoea ananatis*. Journal of Clinical Microbiology. 42 4393 – 4395
- Dianese, J.C., Dristig M.C.G., and Cruz, A.P. 1990. Susceptibility to wilt associated with *Pseudomonas solanacearum* among six species of *Eucalyptus* growing in equatorial Brazil. Australasian Plant Pathology 19: 71 – 76
- Doughty, R.W. 2000. The *Eucalyptus*: A natural and commercial history of the gum tree. Baltimore, The John Hopkins University Press.
- Eldridge, K., Davidson, J., Harwood, C., vsn Wyk, G. 1994. Eucalypt domestication and breeding. Oxford University Press, New York
- Evans, J. 1982. Plantation forestry in the tropics. Oxford science publications. Oxford: Claredon Press.
- Farmer III, J.J. 2005. Genus XXXVII. Tatumella. Hollis, Hickman and Fanning 1982, 267^{VP} (Effective publication: Hollis, Hickman, Fanning, Farmer, Weaver and Brenner 1981, 86) (Group EF-9 Hollis, Hickman, Fanning, Brenner and Weaver 1980) Pages 825 – 826 in Bergey's manual of systematic bacteriology, Volume two, Part B. D.J. Brenner, N.J. Krieg, J.T. Staley and G.M. Garrity, eds. Springer.
- FAO 2000. Global forest resources assessment 2000 – Main report: FAO Forestry paper http://www.fao.org/forestry/fo/fra/main/index.jsp
- Gagne, S., Richard, C., Rousseau, H., and Antoun, H. 1987. Xylem residing bacteria in alfalfa roots. Canadian Journal of Microbiology 33: 996 – 1000

- Gardner, J.M., Feldman, A.W., and Zablotowicz, M. 1982. Identity and behavior of xylem-residing bacteria in rough lemon roots of Florida citrus trees. Applied and Environmental Microbiology 43: 1335 – 1342
- Gavini, F., Mergaert, J., Beji, A., Mielcarek, D.I., Kersters, K., and De Ley, J. 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. International Journal of Systematic Bacteriology 39 337 – 345
- Greyling, I. 2007. *Pantoea* spp. associated with leaf and stem diseases of *Eucalyptus.* Msc thesis dissertation.
- Goncalves, R.C., Lau, D., Oliveira, J.R., Maffia, L.A., Cascardo, J.C.M., and Alfenas, A.C. 2008. Etiology of bacterial leaf blight of eucalypts in Brazil. Tropical Plant Pathology 33: 180 – 188
- Guindon, S. and Gascuel, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology. 52: 696 – 704
- Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95 – 98
- Jacobs, M.J., Bugbee, W.M., and Gabrielson, D.A. 1985. Enumeration, location and characterization of endophytic bacteria within sugar beet roots. Canadian Journal of Botany 63: 1262 – 1265
- Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M., and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142: 1881 – 1893
- Kageyama, B., Nakae, M., Yagi,.S., and Sonoyama, T. 1992. *Pantoea punctata* sp. nov., *Pantoea citrea* sp. nov., and *Pantoea terrea* sp. nov. isolated from fruit and soil samples. International Journal of Systematic Bacteriology 42 203 – 210
- Kratz, A., Greenberg, D., Barki, Y., Cohen, E., and Lifshitz, M. 2003. *Pantoea agglomerans* as a cause of septic arthritis after palm tree thorn injury; case report and literature review. Archives of Disease in Childhood. 88: 542 – 544
- Kuklinsky-Sabral., J., Araujo, W.L., Mendes, R., Geraldi, I.O., Pizzirani-Kleiner, A.A., and Azevedo, J.L. 2004. Isolation and characterization of soybean-

associated bacteria and their potential for plant growth promotion. Environmental Microbiology 6. 1244 – 1251

- Lelliot, R.A., and Stead, D.E. 1987. Host tests. Methods for the diagnosis of bacterial diseases of plants. T.F Preece eds. Blackwell Scientific Publications, London, UK, 152 – 168
- Lim, P., Chen, S., Tsai, C., and Pai, M. 2006. *Pantoea* peritonis in a patient receiving chronic ambulatory peritoneal dialysis. Nephrology 11: 97 – 90
- McInroy, J.A., and Kloepper, J.W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant and Soil 173: 337 – 342
- Mergaert, J., Verdonck, L., Kersters, K., Swings, J., Boeufgras, J., and De Ley, J. 1984. Numerical taxonomy of *Erwinia* species using API systems. Journal of General Microbiology 130: 1893 – 1810
- Mergaert, J., Verdonck, L., and Kersters, K. 1993. Transfer of *Erwinia ananas* (synonym, *Erwinia uredovora*) and *Erwinia stewartii* to the genus *Pantoea* emend. as *Pantoea ananas* (Serrano 1928) comb. nov. and *Pantoea stewartii* (Smith 1898) comb. nov., respectively, and description of *Pantoea stewartii* subsp. *indologenes* subsp. nov. International Journal of Systematic Bacteriology 43 162 – 173
- Misaghi, I.J., and Donndelinger, C.R. 1990. Endophytic bacteria in symptom free cotton plants. Phytopathology 80: 808 – 811
- Musson, G., McInroy, J.A., and Kloepper, J.W. 1995. Development of delivery systems for introducing endophytic bacteria into cotton. Biocontrol Science and Technology 4: 407 – 416
- Posada, D. and Crandall, K.A. 1998. Modeltests: testing the model of DNA substitution. Bioinformatics 14: 817 – 818
- Potts, B.M., and Pederick, L.A. 2000. Morphology, phylogeny, origin, distribution and genetic diversity of the eucalypts. In: Keane, P.J. Kile, G.A., Podger, F.D., Brown, B.N. (eds). Diseases and pathogens of eucalypts, CSIRO Publishing, Collingwood. 11 – 43
- Poynton R, 1979. Tree planting in southern Africa, Vol 2. The Eucalypts. Department of Forestry, Pretoria, South Africa.
- Schmid, H., Weber, C., and Bogner, J.R. 2003. Isolation of a *Pantoea dispersa* – like strain from a 71-year-old woman with acute myeloid leukemia and multiple myeloma. Infection 31: 66 – 67

- Sedjo, R.A. 1999. The potential of high-yield plantation forestry for meeting timber needs. New Forests 17: 339 – 359
- Stackebrandt, E., Tindall, B., Ludwig, W., and Goodfellow, M. 1999. Prokaryotic diversity and systematics. Biology of the prokaryotes. J.W. Lengeler, G. Drews, and H.G. Schlegel, eds. Blackwell Science. George Thieme Verlag, Stuttgart, Germany. 674 – 722
- Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D, Kampfer, P., Maiden, M.C.J., Nesme, Xavier, Rosselló-Mora, R., Swings, J., Trüper, H. G., Vauterin, L., Ward, A.C., and Whitman, W.B. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. International Journal of Systematic and Evolutionary Microbiology 52: 1043 – 1047
- Sturz, A.V., Christie, B.R., and Matheson, B.G. 1998. Association of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. Canadian Journal of Microbiology 44: 162 – 167
- Swofford, D.L. 2000. PAUP*. Phylogenetic analysis using parsimony and other methods (software). Sinauer Associates, Suderland, MA.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougnin, F., and Higgins, D.G. 1997. The ClustalX-Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876 – 4882
- Tanprasert, P., and Reed, B.M. 1997. Detection and identification of bacterial contaminants from strawberry runner explants. In Vitro Cellular Developmental Biology 33. 221 – 226
- Truman, R. 1974. Die-back of *Eucalyptus citriodora* caused by *Xanthomonas eucalypti* sp.n. Phytopathological Notes. 64 143 – 144
- Turnbull, J.W. 1999. Eucalypt plantations. New Forests 17: 37 – 52
- Turnbull, J.W. 2000. Economic and social importance of Eucalypts. In: Keane P.J., Kile, G.A., Podger, F.D., Brown, B.N. (eds). Diseases and pathogens of Eucalypts, CSIRO Publishining, Collingwood, 1 – 9
- Verdonck, L., Mergaert, J., Rijckaert, C., Swings, J., Kersters, K., and De Ley, J. 1987. The genus *Erwinia:* A numerical analysis of phenotypic features. International Journal of Systematic Bacteriology. 37: 4 – 18

- Zabeau, M., and Vos, P. 1993. Selective restriction fragment amplification: A general method for DNA fingerprinting. European Patent Office, publication 0 534 858 A1

Table 1: List of primers used for 16S rRNA amplification and 16S rRNA sequencing (Coenye *et al.,* 1999). Primers *pA and pH were used for amplification of the 16S rRNA gene, whilst all other primers were used during partial sequencing of this gene

Name	**Synonym**	**Sequence (5' → 3')**	**Position on 16S gene**
16F27[1]	*pA	AGA GTT TGA TCC TGG CTC AG	8 – 27
16R1522	pH	AAG GAG GTG ATC CAG CCG CA	1541 – 1522
16F536	*pD	CAG CAG CCG CGG TAA TAC	519 – 536
16F926	*O	AAC TCA AAG GAA TTG ACG G	908 – 926
16F1112	*3	AGT CCC GCA ACG AGC GCA AC	1093 – 1112
16R519	pD	GTA TTA CCG CGG CTG CTG	536 – 519
16R685		TCT ACG CAT TTC ACC GCT AC	704 – 685
16R1093	3	GTT GCG CTC GTT GCG GGA CT	1112 – 1093

* Indicates forward primers

Table 2: List of primers used for the amplification and sequencing of the *GyrB* gene for members of the family Enterobacteriaceae (Brady *et al.,* 2008).

Name	**Sequence (5' → 3')**	**Type of primer**
*gyrB PF	TAARTTYGAYGAYAACTCYTAYAAAGT	Amplification/Sequencing
gyrB 02R	CMCCYTCCACCARGTAMAGTTC	Amplification
*gyrB PF7	GTVCGTTTCTGGCCVAG	Sequencing

* Indicates forward primers

Figure 1: Symptoms of bacterial blight and dieback on *Eucalyptus grandis* and *E. grandis* X *E. nitens* hybrids in different countries: South Africa (A), Uganda (B) and Colombia (C).

Figure 2: [next page] 16S rRNA gene sequencing phylogenetic tree, using the Maximum-Likelihood parameter, of unidentified isolates from Argentina, Colombia, Indonesia, Rwanda, South Africa, Thailand, Uganda and Uruguay and selected members of the family *Enterobacteriaceae.* Species clusters and unknown clusters are indicated in different colours and labeled. Supporting bootstrap values are indicated above the branches.

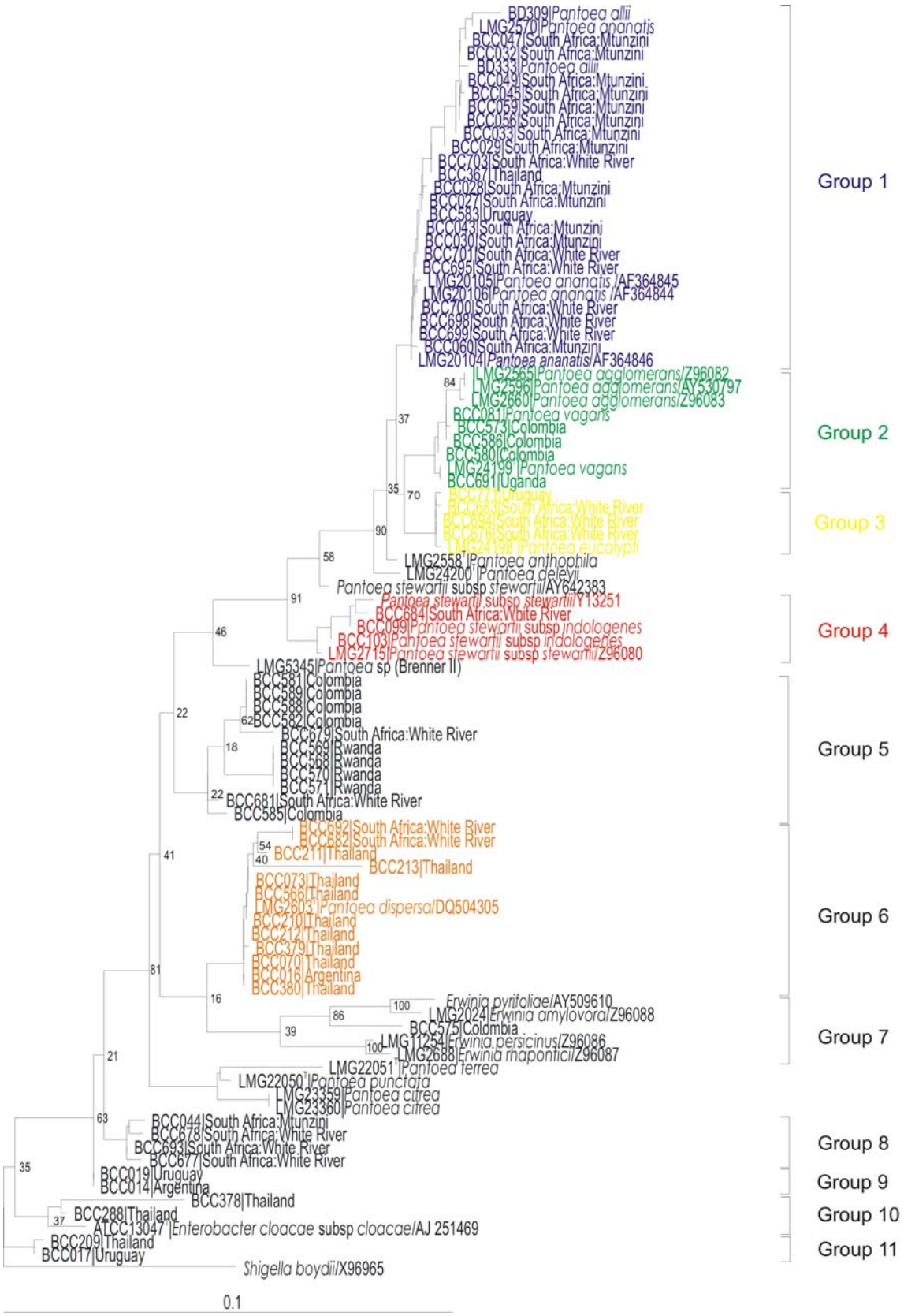

BD309|Pantoea allii
LMG2570|Pantoea ananatis
BCC047|South Africa:Mtunzini
BCC032|South Africa:Mtunzini
BD333|Pantoea allii
BCC049|South Africa:Mtunzini
BCC045|South Africa:Mtunzini
BCC059|South Africa:Mtunzini
BCC056|South Africa:Mtunzini
BCC033|South Africa:Mtunzini
BCC029|South Africa:Mtunzini
BCC703|South Africa:White River
BCC367|Thailand
BCC028|South Africa:Mtunzini
BCC027|South Africa:Mtunzini
BCC583|Uruguay
BCC043|South Africa:Mtunzini
BCC030|South Africa:Mtunzini
BCC701|South Africa:White River
BCC695|South Africa:White River
LMG20105|Pantoea ananatis /AF364845
LMG20106|Pantoea ananatis /AF364844
BCC700|South Africa:White River
BCC698|South Africa:White River
BCC699|South Africa:White River
BCC060|South Africa:Mtunzini
LMG20104|Pantoea ananatis/AF364846
LMG2565|Pantoea agglomerans/Z96082
LMG2596|Pantoea agglomerans/AY530797
LMG2660|Pantoea agglomerans/Z96083
BCC081|Pantoea vagans
BCC573|Colombia
BCC586|Colombia
BCC580|Colombia
LMG24199|Pantoea vagans
BCC691|Uganda
BCC571|Uruguay
BCC683|South Africa:White River
BCC694|South Africa:White River
BCC676|South Africa:White River
LMG24198|Pantoea eucalypti
LMG2558T|Pantoea anthophila
LMG24200T|Pantoea deleyi
Pantoea stewartii subsp stewartii/AY642383
Pantoea stewartii subsp stewartii/Y13251
BCC684|South Africa:White River
BCC099|Pantoea stewartii subsp indologenes
BCC103|Pantoea stewartii subsp indologenes
LMG2715|Pantoea stewartii subsp stewartii/Z96080
LMG5345|Pantoea sp (Brenner II)
BCC581|Colombia
BCC584|Colombia
BCC589|Colombia
BCC588|Colombia
BCC582|Colombia
BCC679|South Africa:White River
BCC568|Rwanda
BCC569|Rwanda
BCC570|Rwanda
BCC571|Rwanda
BCC681|South Africa:White River
BCC585|Colombia
BCC692|South Africa:White River
BCC682|South Africa:White River
BCC211|Thailand
BCC213|Thailand
BCC073|Thailand
BCC566|Thailand
LMG2603T|Pantoea dispersa/DQ504305
BCC210|Thailand
BCC212|Thailand
BCC379|Thailand
BCC070|Thailand
BCC016|Argentina
BCC380|Thailand
Erwinia pyrifoliae/AY509610
LMG2024|Erwinia amylovora/Z96088
BCC575|Colombia
LMG11254|Erwinia persicinus/Z96086
LMG2688|Erwinia rhapontici/Z96087
LMG22051T|Pantoea terrea
LMG22050T|Pantoea punctata
LMG23359|Pantoea citrea
LMG23360|Pantoea citrea
BCC044|South Africa:Mtunzini
BCC678|South Africa:White River
BCC693|South Africa:White River
BCC677|South Africa:White River
BCC019|Uruguay
BCC014|Argentina
BCC378|Thailand
BCC288|Thailand
ATCC13047T|Enterobacter cloacae subsp cloacae/AJ 251469
BCC209|Thailand
BCC017|Uruguay
Shigella boydii/X96965
0.1
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11

Figure 3: [next page] *GyrB* gene sequencing phylogenetic tree using the Maximum-Likelihood parameter, of unidentified isolates from Colombia, Uruguay, Rwanda, Argentina, Thailand and South Africa and selected members of the family *Enterobacteriaceae.* Species clusters and unknown clusters are indicated in different colours and labeled. Supporting bootstrap values are indicated above the branches.

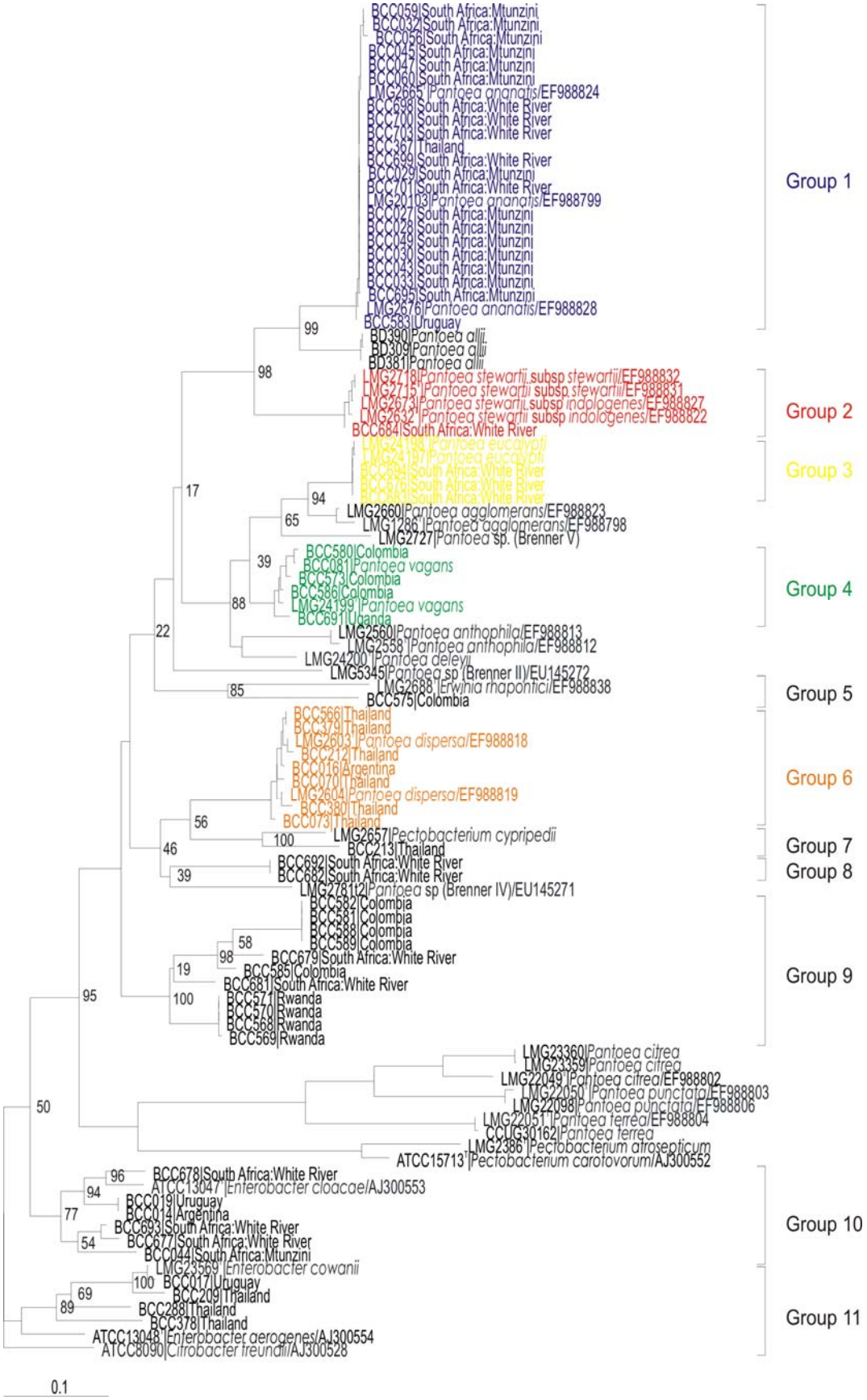

BCC059|South Africa:Mtunzini
BCC032|South Africa:Mtunzini
BCC056|South Africa:Mtunzini
BCC045|South Africa:Mtunzini
BCC047|South Africa:Mtunzini
BCC060|South Africa:Mtunzini
LMG2665|Pantoea ananatis/EF988824
BCC698|South Africa:White River
BCC700|South Africa:White River
BCC703|South Africa:White River
BCC367|Thailand
BCC699|South Africa:White River
BCC029|South Africa:Mtunzini
BCC701|South Africa:White River
LMG20103|Pantoea ananatis/EF988799
BCC027|South Africa:Mtunzini
BCC028|South Africa:Mtunzini
BCC049|South Africa:Mtunzini
BCC030|South Africa:Mtunzini
BCC043|South Africa:Mtunzini
BCC033|South Africa:Mtunzini
BCC695|South Africa:Mtunzini
LMG2676|Pantoea ananatis/EF988828
BCC583|Uruguay
BD390|Pantoea allii
BD309|Pantoea allii
BD381|Pantoea allii
LMG2718|Pantoea stewartii subsp stewartii/EF988832
LMG2715|Pantoea stewartii subsp stewartii/EF988831
LMG2673|Pantoea stewartii subsp indologenes/EF988827
LMG2632|Pantoea stewartii subsp indologenes/EF988822
BCC684|South Africa:White River
LMG24194|Pantoea eucalypti
LMG24197|Pantoea eucalypti
BCC694|South Africa:White River
BCC676|South Africa:White River
BCC681|South Africa:White River
LMG2660|Pantoea agglomerans/EF988823
LMG1286|Pantoea agglomerans/EF988798
LMG2727|Pantoea sp. (Brenner V)
BCC580|Colombia
BCC081|Pantoea vagans
BCC573|Colombia
BCC586|Colombia
LMG24199|Pantoea vagans
BCC691|Uganda
LMG2560|Pantoea anthophila/EF988813
LMG2558|Pantoea anthophila/EF988812
LMG24200|Pantoea deleyi
LMG5345|Pantoea sp (Brenner II)/EU145272
LMG2688|Erwinia rhapontici/EF988838
BCC575|Colombia
BCC566|Thailand
BCC379|Thailand
LMG2603|Pantoea dispersa/EF988818
BCC212|Thailand
BCC016|Argentina
BCC070|Thailand
LMG2604|Pantoea dispersa/EF988819
BCC380|Thailand
BCC073|Thailand
LMG2657|Pectobacterium cypripedii
BCC213|Thailand
BCC692|South Africa:White River
BCC682|South Africa:White River
LMG27812|Pantoea sp (Brenner IV)/EU145271
BCC582|Colombia
BCC581|Colombia
BCC588|Colombia
BCC589|Colombia
BCC679|South Africa:White River
BCC585|Colombia
BCC681|South Africa:White River
BCC571|Rwanda
BCC570|Rwanda
BCC568|Rwanda
BCC569|Rwanda
LMG23360|Pantoea citrea
LMG23359|Pantoea citrea
LMG22049|Pantoea citrea/EF988802
LMG22050|Pantoea punctata/EF988803
LMG22098|Pantoea punctata/EF988806
LMG22051|Pantoea terrea/EF988804
CCUG30162|Pantoea terrea
LMG2386|Pectobacterium atrosepticum
ATCC15713|Pectobacterium carotovorum/AJ300552
BCC678|South Africa:White River
ATCC13047|Enterobacter cloacae/AJ300553
BCC019|Uruguay
BCC014|Argentina
BCC693|South Africa:White River
BCC677|South Africa:White River
BCC044|South Africa:Mtunzini
LMG23569|Enterobacter cowanii
BCC017|Uruguay
BCC209|Thailand
BCC288|Thailand
BCC378|Thailand
ATCC13048|Enterobacter aerogenes/AJ300554
ATCC8090|Citrobacter freundii/AJ300528
0.1
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11

Figure 4: [next infold page]UPGMA tree constructed using the Pearson correlation coefficient based on AFLP analysis of unidentified isolates from diseased *Eucalyptus.* The seven known *Pantoea* species and newly classified *Pantoea* species are included in this tree.

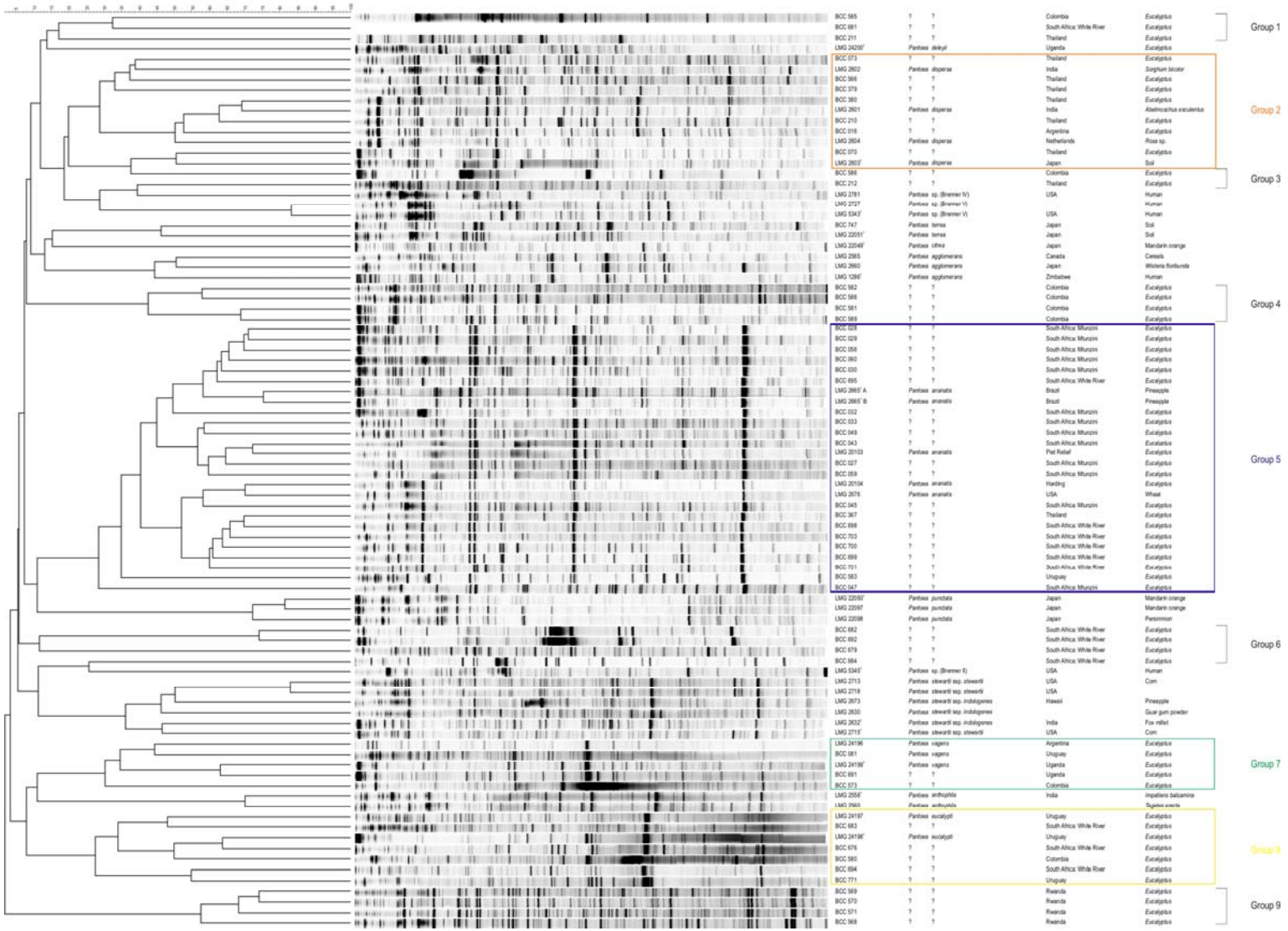

BCC 585 ? ? Colombia Eucalyptus
BCC 681 ? ? South Africa: White River Eucalyptus
BCC 211 ? ? Thailand Eucalyptus
LMG 24200T Pantoea deleyi Uganda Eucalyptus
BCC 073 ? ? Thailand Eucalyptus
LMG 2602 Pantoea dispersa India Sorghum bicolor
BCC 566 ? ? Thailand Eucalyptus
BCC 379 ? ? Thailand Eucalyptus
BCC 380 ? ? Thailand Eucalyptus
LMG 2601 Pantoea dispersa India Abelmoschus esculentus
BCC 210 ? ? Thailand Eucalyptus
BCC 016 ? ? Argentina Eucalyptus
LMG 2604 Pantoea dispersa Netherlands Rosa sp.
BCC 070 ? ? Thailand Eucalyptus
LMG 2603T Pantoea dispersa Japan Soil
BCC 584 ? ? Colombia Eucalyptus
BCC 212 ? ? Thailand Eucalyptus
LMG 2781 Pantoea sp. (Brenner IV) USA Human
LMG 2727 Pantoea sp. (Brenner V) Human
LMG 5343T Pantoea sp. (Brenner V) USA Human
BCC 747 Pantoea terrea Japan Soil
LMG 2255T Pantoea terrea Japan Soil
LMG 22049T Pantoea citrea Japan Mandarin orange
LMG 2565 Pantoea agglomerans Canada Cereals
LMG 2660 Pantoea agglomerans Japan Wisteria floribunda
LMG 1286T Pantoea agglomerans Zimbabwe Human
BCC 582 ? ? Colombia Eucalyptus
BCC 588 ? ? Colombia Eucalyptus
BCC 581 ? ? Colombia Eucalyptus
BCC 589 ? ? Colombia Eucalyptus
BCC 028 ? ? South Africa: Mpumalanga Eucalyptus
BCC 029 ? ? South Africa: Mpumalanga Eucalyptus
BCC 056 ? ? South Africa: Mpumalanga Eucalyptus
BCC 060 ? ? South Africa: Mpumalanga Eucalyptus
BCC 030 ? ? South Africa: Mpumalanga Eucalyptus
BCC 695 ? ? South Africa: White River Eucalyptus
LMG 2665T A Pantoea ananatis Brazil Pineapple
LMG 2665T B Pantoea ananatis Brazil Pineapple
BCC 032 ? ? South Africa: Mpumalanga Eucalyptus
BCC 033 ? ? South Africa: Mpumalanga Eucalyptus
BCC 049 ? ? South Africa: Mpumalanga Eucalyptus
BCC 043 ? ? South Africa: Mpumalanga Eucalyptus
LMG 20103 Pantoea ananatis Piet Retief Eucalyptus
BCC 027 ? ? South Africa: Mpumalanga Eucalyptus
BCC 059 ? ? South Africa: Mpumalanga Eucalyptus
LMG 20104 Pantoea ananatis Harding Eucalyptus
LMG 2676 Pantoea ananatis USA Wheat
BCC 045 ? ? South Africa: Mpumalanga Eucalyptus
BCC 367 ? ? Thailand Eucalyptus
BCC 698 ? ? South Africa: White River Eucalyptus
BCC 703 ? ? South Africa: White River Eucalyptus
BCC 700 ? ? South Africa: White River Eucalyptus
BCC 699 ? ? South Africa: White River Eucalyptus
BCC 701 ? ? South Africa: White River Eucalyptus
BCC 583 ? ? Uruguay Eucalyptus
BCC 047 ? ? South Africa: Mpumalanga Eucalyptus
LMG 22050T Pantoea punctata Japan Mandarin orange
LMG 22097 Pantoea punctata Japan Mandarin orange
LMG 22098 Pantoea punctata Japan Persimmon
BCC 682 ? ? South Africa: White River Eucalyptus
BCC 692 ? ? South Africa: White River Eucalyptus
BCC 679 ? ? South Africa: White River Eucalyptus
BCC 684 ? ? South Africa: White River Eucalyptus
LMG 5345T Pantoea sp. (Brenner II) USA Human
LMG 2713 Pantoea stewartii ssp. stewartii USA Corn
LMG 2718 Pantoea stewartii ssp. stewartii USA
LMG 2673 Pantoea stewartii ssp. indologenes Hawaii Pineapple
LMG 2630 Pantoea stewartii ssp. indologenes Guar gum powder
LMG 2632T Pantoea stewartii ssp. indologenes India Fox millet
LMG 2715T Pantoea stewartii ssp. stewartii USA Corn
LMG 24196 Pantoea vagans Argentina Eucalyptus
BCC 081 Pantoea vagans Uruguay Eucalyptus
LMG 24199T Pantoea vagans Uganda Eucalyptus
BCC 691 ? ? Uganda Eucalyptus
BCC 573 ? ? Colombia Eucalyptus
LMG 2558T Pantoea anthophila India Impatiens balsamina
LMG 2560 Pantoea anthophila Tagetes erecta
LMG 24197T Pantoea eucalypti Uruguay Eucalyptus
BCC 683 ? ? South Africa: White River Eucalyptus
LMG 24198T Pantoea eucalypti Uruguay Eucalyptus
BCC 676 ? ? South Africa: White River Eucalyptus
BCC 580 ? ? Colombia Eucalyptus
BCC 694 ? ? South Africa: White River Eucalyptus
BCC 771 ? ? Uruguay Eucalyptus
BCC 569 ? ? Rwanda Eucalyptus
BCC 570 ? ? Rwanda Eucalyptus
BCC 571 ? ? Rwanda Eucalyptus
BCC 568 ? ? Rwanda Eucalyptus
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9

Table 3: Summary of results obtained from 16S rRNA gene sequencing, *GyrB* gene sequencing and AFLP band pattern analysis with preliminary identifications in this study. Isolates that did not cluster in a distinct *Pantoea* cluster, was identified as the genus that was its closest relative

Isolate	**Origin**	**16S rRNA gene sequencing**	***GyrB* gene sequencing**	**AFLP analysis**	**Identity**
BCC014	Argentina	Unresolved	*Enterobacter* sp.	N/A	*Enterobacter* sp.
BCC016	Argentina	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa*
BCC017	Uruguay	Unresolved	*Enterobacter cowanii*	N/A	*Enterobacter* sp.
BCC019	Uruguay	Unresolved	*Enterobacter* sp.	N/A	*Enterobacter* sp.
BCC070	Thailand	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa*
BCC073	Thailand	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa*
BCC566	Thailand	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa* cluster	*P. dispersa*
BCC568	Rwanda	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC569	Rwanda	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC570	Rwanda	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC571	Rwanda	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC573	Colombia	*P. agglomerans/vagans* group	*P. vagans* cluster	*P. vagans* cluster	*P. vagans*
BCC575	Colombia	*Erwinia* sp.	*Erwinia* sp.	N/A	*Erwinia* sp.
BCC580	Colombia	*P. agglomerans/vagans* group	*P. vagans* group	*P. eucalypti* group	*P. vagans*
BCC581	Colombia	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC582	Colombia	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species

Table 3: Continued

Isolate	Origin	16S rRNA gene sequencing	*GyrB* gene sequencing	AFLP analysis	Identity
BCC585	Colombia	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC586	Colombia	*P. agglomerans/vagans* group	*P. vagans* group	*P. dispersa* group	*P. vagans*
BCC588	Colombia	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC589	Colombia	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Potential new species
BCC027	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC028	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC029	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC030	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC032	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC033	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC043	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC044	South Africa 1	Unresolved	Unresolved	N/A	Unresolved
BCC045	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC047	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC049	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC056	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC059	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC060	South Africa 1	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*

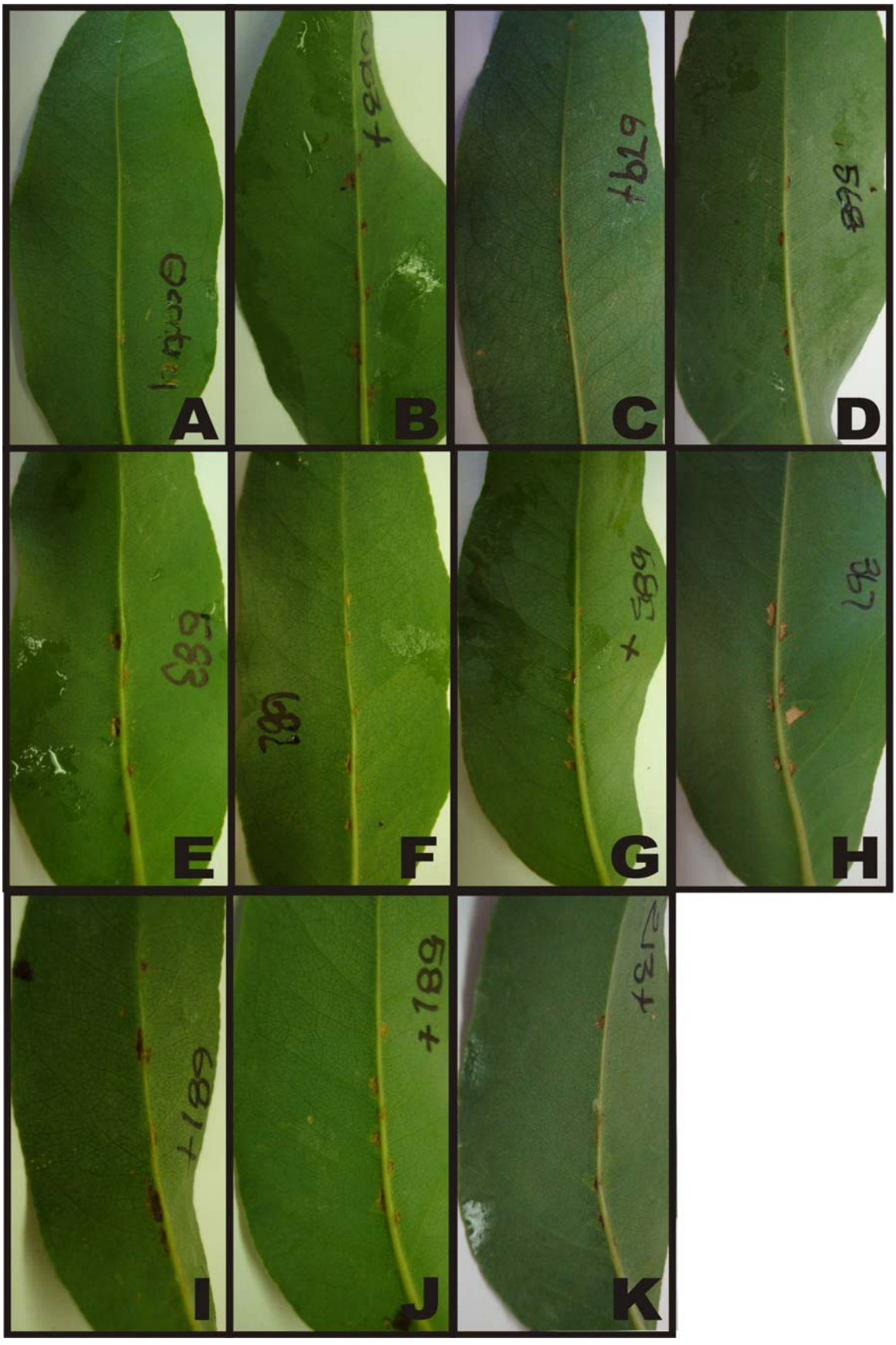
Table 3: Continued

Isolate	Origin	16S rRNA gene sequencing	*GyrB* gene sequencing	AFLP analysis	Identity
BCC676	South Africa 2	*P. eucalypti* group	*P. eucalypti* group	*P. eucalypti* group	*P. eucalypti*
BCC677	South Africa 2	Unresolved	Unresolved	N/A	Unresolved
BCC678	South Africa 2	Unresolved	*Enterobacter cloacae*	N/A	*Enterobacter* sp.
BCC679	South Africa 2	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Probable new species
BCC681	South Africa 2	Genus *Pantoea*	Genus *Pantoea*	Genus *Pantoea*	Probable new species
BCC682	South Africa 2	*P. dispersa* group	Outside *P. dispersa* group	Outside *Pantoea* cluster	Probable new species
BCC683	South Africa 2	*P. eucalypti* group	*P. eucalypti* group	*P. eucalypti* group	*P. eucalypti*
BCC684	South Africa 2	*P. stewartii* ssp. *indologenes* group	*P. stewartii* ssp. *indologenes* group	*P. stewartii* ssp. *indologenes* group	*P. stewartii* ssp. *indologenes*
BCC691	Uganda	*P. agglomerans/vagans* group	*P. vagans* group	*P. vagans* group	*P. vagans*
BCC692	South Africa 2	*P. dispersa* group	Outside *P. dispersa* group	Outside *Pantoea* cluster	Probable new species
BCC693	South Africa 2	Unresolved	Unresolved	N/A	Unresolved
BCC694	South Africa 2	*P. eucalypti* group	*P. eucalypti* group	*P. eucalypti* group	*P. eucalypti*
BCC695	South Africa 2	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC698	South Africa 2	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*

Table 3: Continued

Isolate	Origin	16S rRNA gene sequencing	*GyrB* gene sequencing	AFLP analysis	Identity
BCC699	South Africa 2	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC700	South Africa 2	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC701	South Africa 2	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC703	South Africa 2	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC583	Uruguay	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC771	Uruguay	*P. eucalypti* group	No amplification of *GyrB* gene	*P. eucalypti* group	*P. eucalypti*
BCC209	Thailand	Unresolved	*Enterobacter* sp.	N/A	*Enterobacter* sp.
BCC210	Thailand	*P. dispersa* group	No amplification of *GyrB* gene	*P. dispersa* group	*P. dispersa*
BCC211	Thailand	*P. dispersa* group	No amplification of *GyrB* gene	Inside *Pantoea* cluster	?
BCC212	Thailand	*P. dispersa* group	*P. dispersa* group	*P. dispersa* group	*P. dispersa*
BCC213	Thailand	*P. dispersa* group	*Pectobacterium cypripedi*	N/A	*Pectobacterium cypripedi*
BCC288	Thailand	*Enterobacter* sp.	*Enterobacter* sp.	N/A	*Enterobacter* sp.
BCC367	Thailand	*P. ananatis/allii* group	*P. ananatis* group	*P. ananatis* group	*P. ananatis*
BCC378	Thailand	*Enterobacter* sp.	*Enterobacter* sp.	N/A	*Enterobacter* sp.
BCC379	Thailand	*P. dispersa* group	*P. dispersa* group	*P. dispersa* group	*P. dispersa*
BCC380	Thailand	*P. dispersa* group	*P. dispersa* group	*P. dispersa* group	*P. dispersa*

Figure 5: [next page] Results of the pathogenicity trials. Isolates from Argentina, Colombia, Indonesia, Rwanda, South Africa, Thailand, Uganda and Uruguay was inoculated into the host *E. grandis* X *E. nitens* hybrid. **A:** Negative control (sterile saline solution) **B:** Positive control (*P. ananatis,* LMG 20103) **C:** BCC568 (Rwanda) **D:** BCC 213 (Thailand) **E:** BCC581 (Colombia) **F:** BCC 585 (Colombia) **G:** BCC 679 (South Africa) **H:** BCC 681 (South Africa) **I:** BCC 682 (South Africa) **J:** BCC 583 (Uruguay) **K:** BCC 367 (Thailand). Photographs taken 36 hours after inoculation.


A
B
C
D
E
F
G
H
I
J
K

Chapter 4

Xanthomonas species associated with bacterial blight and dieback of *Eucalyptus* in South Africa and Uruguay

Xanthomonas species associated with bacterial blight and dieback of *Eucalyptus* in South Africa and Uruguay

ABSTRACT

Three bacterial pathogens have been shown to cause blight and die-back of *Eucalyptus* species, namely *Pantoea ananatis, Xanthomonas campestris* pv. *eucalypti* and *X. axonopodis*. *P. ananatis* has only been reported infecting this host in South Africa while X.c. pv. *eucalypti* and X. *axonopodis* only occur in Australia and Brazil, respectively. In 2003 a newly established compartment of an *E. grandis* clone in the Mtunzini area of South Africa showed extensive leaf blight and die-back. Bacteria were commonly found exuding from the leaves and petioles. Bacterial isolations from diseased leaf material consistently yielded *P. ananatis* and a *Xanthomonas* sp., while isolations from the material collected at Uruguay yielded only a *Xanthomonas* species. 16S rRNA and *gyrB* gene sequencing was performed on all unknown strains once phenotypic tests were completed. F-AFLP's were also used and the resulting profiles were compared to those of the type strains of a number of species within the genus *Xanthomonas*. Pathogenicity tests were also undertaken using susceptible *Eucalyptus* seedlings and isolates were found to be pathogenic. Strains of *Xanthomonas* from the Mtunzini outbreak in South Africa all grouped with the type strain of *X. vasicola.* Isolates from Uruguay grouped with the type strain of *X. fuscans*. It seems to be closely related to *X. fuscans* subsp. *fuscans* which infects bean. Both of the species isolated from *Eucalyptus* in South Africa and Uruguay are first reports for both these species on *Eucalyptus*.

1) INTRODUCTION

Xanthomonas species are plant associated bacteria that can occur as epiphytes on the leaves or as pathogens causing a range of symptoms on a wide variety of economically important plants. These include crops and commercially important fruit and forest trees such as sugarcane (Ricaud and Ryan, 1989), rice (Swings *et al*., 1990), bananas (Yirgou and Bradbury, 1968) and cotton (Atkinson, 1891), citrus (Hasse, 1915), poplar (Koning, 1938) and *Eucalyptus* (Truman, 1974 and Goncalves *et al.,* 2008). *Xanthomonas* spp. are also encountered as endophytes on plants (Lodewyckx *et al.,* 2002). Due to the importance of the genus in agriculture and forestry, numerous studies have been

undertaken to provide an accurate and rapid means of identifying species in the genus which is often phenotypically very similar and difficult to identify (Vauterin *et al.,* 2000).

Prior to 1995, species in the genus *Xanthomonas* were assigned based on the "new host – new species" concept (Starr, 1981) which led to the identification of many new species on different hosts. The identification of an isolate was thus only based on a single phenotypic property i.e. host specificity. An unreasonable number of nomenspecies arose from this classification practice. In 1995, Vauterin *et al.* re-classified the genus based on a DNA:DNA hybridization study and 20 species were recognised. Currently, the genus *Xanthomonas* comprises 27 validly published species (Euzeby).

Hauben *et al.* (1997) revealed a low degree of genetic divergence in the 16S rRNA gene between different species of *Xanthomonas* and only three lineages were distinguished using this technique. Rademaker *et al.* (2000) using, Amplified Fragment Length Polymorphism (AFLPs), showed that it gave better resolution than 16S rRNA gene sequencing and was able to reveal the genotypic and phylogenetic relationship between 20 species of the genus as described by Vauterin *et al.* (1995). More recently, studies have been undertaken to sequence the protein encoding genes or core genes of bacteria to compliment 16S rRNA gene sequencing and to produce more accurate phylogenetic information (Zeigler, 2003; Naser *et al.,* 2005). Young *et al.* (2008), successfully used multilocus sequence analysis (MLSA) of species in the genus *Xanthomonas* to clearly differentiate the species that were established with the DNA:DNA reassociation technique by Vauterin *et al.* (1995). In two separate studies the *GyrB* gene was successfully used to resolve the phylogenetic position of the species *Xanthomonas.* In the first study, Parkinson *et al.,* (2007) could clearly indicate the inter-species relationship of *Xanthomonas* from the results. They also stated that this method can aid in rapid and accurate species identification. In the second study, Young *et al.,* (2008) undertook a MLSA study on 119 *Xanthomonas* strains, sequencing four core genes including the *GyrB* gene. The analysis clearly differentiated most of the species that have validly been established by DNA:DNA hybridization studies. MLSA is a simpler alternative to DNA:DNA hybridisations and can allocate isolates to a specific species. MLSA is also more advantageous since the data is transferable between different laboratories whereas DNA:DNA hybridization studies employs specialized equipment and only selected laboratories can perform this labour intensive technique.

Native Australian *Eucalyptus* trees or hybrids and clones are grown in South Africa as commercial forestry trees. These trees are also grown as plantation species in several countries in Africa, Asia, South America and New Zealand. *Eucalyptus* plantations are rapidly expanding and they are thus an attractive option for the production of pulp and paper. The fast growth of local South African plantations makes tree breeding a possibility. This has led to South Africa becoming a world leader in selective tree breeding and cloning of *Eucalyptus* species (Forestry South Africa, 2002)

Four bacterial pathogens have been identified to cause diseases of *Eucalyptus* trees. These pathogens are *Ralstonia solanecearum* causing bacterial wilt (Dianese *et al.,* 1990), *Xanthomonas eucalypti* (renamed: *X. campestris* pv. *eucalypti*i) causing bacterial dieback (Truman, 1974), *Pantoea ananatis* was identified as the causal agent of bacterial blight and dieback of *Eucalyptus* trees (Coutinho *et al.,* 2002) and most recently *Xanthomonas axonopodis* causing bacterial leaf blight (Goncalves *et al.,* 2008). In this study, unknown Gram negative, obligatory aerobic organisms were isolated from diseased *Eucalyptus* leaves showing typical signs of bacterial blight and dieback from Uruguay and South Africa. The objective of this study was to identify these strains using a polyphasic approach. Genotypic methods used included 16S rRNA and *GyrB* gene sequencing and AFLP analysis. When the identity of the isolates was confirmed, their ability to cause disease symptoms on *Eucalyptus* was determined.

2) MATERIALS AND METHODS

2.1) Disease symptoms

Eucalyptus grandis leaf and shoot material showing symptoms of blight and dieback were collected from KwaZulu Natal, South Africa and from plantations in Uruguay. The observed symptoms were similar in both countries. The initial symptom was the appearance of watersoaked lesions on the leaf, which became necrotic with time. Lesions often coalesced and were concentrated along the main vein. Infected young shoots died back and in some leaves it appeared as if the infection along the veins arose from infected petioles.

2.2) Bacterial isolations

Diseased leaf samples were obtained and were surface sterilized by placing them for 1 minute in a 10% sodium hypochlorite solution followed by 70% ethanol for a further minute. Leaves were then rinsed in sterile water to remove excess ethanol. Diseased leaf material was crushed in 2 ml of sterile water using a sterile mortar and pestle. Bacterial suspensions were plated onto Nutrient Agar (NA), and incubated for 48 hours at 28 ºC. Bacterial colonies were then selected and purified.

2.3) Phenotypic testing

Pure cultures of all isolates obtained from infected leaves collected from the two countries were subjected to Gram staining and the Hugh Leifson oxidative/fermentative test using media supplied by Biolab (Biomerieux). Only isolates that were Gram negative and obligatory aerobic were included. Strains that were Gram negative, facultatively anaerobic and fermentative were identified as *Pantoea* isolates (Chapter 3). These latter strains were only isolated from the leaf material collected in South Africa.

2.4) DNA extractions

Isolates that were Gram negative and aerobic, were inoculated into LB broth and incubated overnight at 28 °C. DNA extractions were performed on all isolates using the Dneasy Blood and Tissue Kit (QIAGEN) according to instructions of the manufacturer. The DNA concentration of the extracted DNA was determined by loading the samples on a 1% agarose gel (Whitehead Scientific), containing 1 µl ethidium bromide for every 10 ml of the agarose gel, and run on the gel in 1 X TAE Buffer at 100 V and 400 mA for 40 minutes.

2.5) 16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene was amplified using primer pair *pA and pH. Each 100 µL PCR reaction contained 1X Reaction buffer, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 100 pmol of each of the above mentioned primers (Inqaba Biotechnologies), 1 U Taq Gold (Roche) and 50 – 100 ng of genomic DNA. PCR amplification was carried out

in an Mastercycler epgradient (Eppendorf). PCR cycles included an initial denaturation step at 94 ºC, 30 cycles of denaturation at 94 ºC for 1 min, annealing of the primers at 58 ºC for 1 min, primer extension at 72 ºC for 1 min and a final extension step at 72 ºC for 5 min. Reactions were held at 4 ºC until used. Sterile water was used in a reaction as a negative control to monitor contamination and DNA of an isolate that is known to have been successfully amplified previously using this protocol was used as a positive control. The amplification products were loaded on a 1% agarose gel (Whitehead Scientific), containing 1 µl ethidium bromide for every 10 ml of the agarose gel, and run on the gel in 1 X TAE Buffer at 100 V and 400 mA for 40 minutes. Viewing the gel under UV light, single bands for every isolate of the 16S rRNA gene was observed. The 16S rRNA gene was purified from the PCR reaction using the QIAquick® PCR Purification Kit (QIAGEN).

Initially, the 16S rRNA gene of all isolates was only partially sequenced in order to determine the genus to which each isolate belonged. The forward primer, 16F536 (*pD) 5' CAG CAG CCG CGG TAA TAC 3' (Table 1) was used for this purpose. This primer amplifies a sequence of approximately 700 bp of the 16S rRNA gene which is known to be variable amongst different genera of bacteria. The partial sequences of the isolates were then BLASTed against Genbank/EMBL database in a homology search (www.ncbi.nlm.nih).

The entire 16S rRNA gene of the 20 isolates belonging to the *Xanthamonodaceae* with greater than 97% sequence similarity to this genus were then sequenced. Three forward primers and four reverse primers were used for this purpose (Table 1).

Each 10 µl sequencing reaction contained 2 µl of Big Dye Sequencing Mix (Applied Biosystems), 1 X Sequencing Buffer, 3.2 pmol of the appropriate internal primer and 150 ng of purified DNA (16S rRNA gene). PCR reactions were carried out in an Mastercycler epgradient (Eppendorf). Sequencing PCR cycles included an initial denaturation step at 96 ºC for 5 sec followed by 25 cycles of denaturation at 96 ºC for 10 sec, primer annealing at 55 ºC for 5 sec and primer extension at 60 ºC for 4 min. The reactions were held at 4 ºC until sequenced. Sequencing PCR products were precipitated from the PCR reaction using the sodium acetate precipitation method. The PCR products were sequenced on an ABI Prism™ 3100 Automated Sequencer (Applied Biosystems). After sequencing of the isolates using the different internal primers a consensus sequence of

the full length 16S rRNA gene was assembled manually, using the program Bio-edit Sequence Alignment Editor v 5.0.9., based on overlapping sequences between the fragments generated by the different internal primers.

Consensus sequences of the different 16S rRNA genes were aligned along with the type strains (acquired from the Genbank/EMBL database) of different *Xanthomonas* species using the program ClustalX (Thompson *et al.,* 1997). Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the evolutionary model best suited to the dataset. The chosen model was HKY+I. Pinvar was also determined using Modeltest3.7.Maximum-Likelihood and Neighbour-Joining analyses where performed using Phyml (Guindon and Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000), respectively, by applying the model selected by Modeltest 3.7. Only the Maximum-Likelihood phylogenetic trees are shown. A bootstrap analysis was run using a 1000 replicates to show branch support. Included in this tree are representatives of a number of described *Xanthomonas* spp. The tree was rooted with the outgroup *Xanthomonas albilineans*, which is a distantly related *Xanthomonas* species.

2.6) *GyrB* gene sequencing and phylogenetic analysis

The 20 isolates were used to sequence the housekeeping gene, *GyrB*. The *GyrB* gene was amplified using the primers described by Parkinson *et al.* (2007), listed in Table 2.

Each 50 µl PCR amplification reaction contained 3 µl of 10 X Buffer with added $MgCl_2$ at 15mM, 250 µM of each nucleotide (dATP, dCTP, dGTP, dTTP), 200 pmol of each of the amplification primers forward and reverse (Inqaba Biotechnologies), 1 U Taq (Southern Cross Biotechnologies) and 50 – 100 ng of genomic DNA. Amplification of the *GyrB* gene was carried out in an Eppendorf Mastercycler epgradient (Merck chemicals (Pty) Ltd.). Amplification cycles included an initial denaturation step at 94 ºC for 2.5 min, 34 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 50 ºC for 45 sec, primer extension at 68 ºC for 1 min and a final extension step at 68 ºC for 7 min. The reactions were held at 4 ºC until further use. As previously described a positive and a negative control was also included in the reactions.

Amplification reactions were run on a 1% agarose gel under conditions as described before. Amplification of the *GyrB* gene with the above mentioned primers results in a 830 bp fragment of the *GyrB* gene. When the amplification product was observed under a UV light to allow for visualization of the bands, a single band was observed at below 1000bp but above 750 bp. PCR amplification products resulting in a clear single band at the position mentioned above was cleaned using the QIAquick® PCR purification Kit as described previously.

The *GyrB* gene was sequenced using the following primers: X.gyr.fsp.s1 5' GGCAAGAGCGAGCTGTA 3' and X.gyr.rsp3 5' CTGGTCGGCGGCCAC 3' (Parkinson *et al.*, 2007). Each 10 µl sequencing reaction contained 2 µl of Big Dye sequencing mix, 1 X Sequencing Buffer, 3.2 pmol of the appropriate primer and 150 ng of the *gyrB* PCR product. PCR reactions were carried out in an Eppendorf Mastercycler epgradient (Merck chemicals (Pty) Ltd.). Sequencing PCR cycles included an initial denaturation step at 94 ºC for 2.5 min, 34 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 57 ºC for 45 sec, primer extension at 68 ºC for 1 min, and a final extension step at 68 ºC for 7 min. Sequencing reactions were held at 4 ºC until sequenced. Sequencing PCR products were precipitated from the PCR reaction using the sodium acetate precipitation method. The PCR products were sequenced on an ABI Prism 3100 Automated Sequencer (Perkin – Elmer). After sequencing of the isolates using these two primers, a consensus sequence of the partial *GyrB* gene was assembled manually, using the program Bio-edit Sequence Alignment Editor v 5.0.9. (Hall, 1999), based on overlapping sequences between the fragments.

Consensus sequences of the different *GyrB* genes were aligned along with the type strains (acquired from the Genbank/EMBL database) of different *Xanthomonas* species using the program ClustalX (Thompson *et al.,* 1997). Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the evolutionary model best suited to the dataset. The chosen model was GTR+I+G. Pinvar and gamma were also determined using Modeltest3.7. Maximum-Likelihood and Neighbour-Joining analyses where performed using Phyml (Guindon and Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000), respectively, by applying the model selected by Modeltest 3.7. Only the Maximum-Likelihood phylogenetic trees are shown. A bootstrap analysis was run using a 1000 replicates to show branch support. Included in this tree are representatives of a number

of the described *Xanthomonas* spp. The tree was rooted with the outgroup *Xanthomonas albineans*, which is a distantly related *Xanthomonas* species.

2.7) AFLP (Amplified Fragment Length Polymorphism) analysis

Total genomic DNA was isolated from all isolates using the DNeasy® Blood and Tissue kit. The AFLP protocol consists of a Restriction enzyme digestion step, adaptor ligation step, two PCR amplification steps and the running of isolates on a poly-acrylamide gel. Total genomic DNA (50 – 100ng) was digested with 12 U *EcoRI* (Roche diagnostics) and 8 U *Tru91* (Roche diagnostics) in 5 X Restriction/ligation buffer (50 mM Tris HAc, 50 mM MgAc, 250 mM KAc, 25 mM DDT). The digestion reaction was incubated at 37 ºC for 2 hours. After digestion, the temperature was increased to 70 ºC and incubated for 15 min to allow for the inactivation of the enzymes. Double strand adaptors with sticky ends, complementary to the restriction enzyme cut sites of *EcoRI* and *Tru91* were added to the reaction. When these adaptors were ligated to the target fragments it ensured that the original restriction enzyme cut site was not restored on the target fragments (Zabeau and Vos, 1993). 5pmol *EcoRI* adaptor and 50pmol *Tru91* adaptor were added to the reaction along with 5 X Restriction and Ligation Buffer, 0.3 mM ATP and 1 U of T4 DNA ligase (Roche). The ATP produced the energy to ligate the adaptors to the fragments and T4 DNA ligase catalyzes the reaction. The ligation reaction was incubated at 20 ºC for 2 hours to allow for sufficient ligation. The reaction was then diluted 1:10 with Nuclease Free water (Promega).

The pre-amplification reaction contained: 1 X Reaction Buffer, 1.5 mM $MgCl_2$, 250 mM of each nucleotide (dATP, dCTP, dGTP, dTTP), 100 pmol of each primer Eco-00 (5’ GAC TGC GTA CCA ATT C 3’) and Tru91-00 (5’ GAT GAG TCC TGA CTA A 3’) (Inqaba Biotechnologies), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 2 µL of the diluted (1:10) restriction and ligation reaction. The reaction was made up to a final volume of 25 µL with Nuclease Free water (Promega). The amplification was carried out in a Eppendorf Mastercycler epgradient (Merck chemicals (Pty) Ltd.) and the amplification conditions included an initial denaturation step at 94 ºC for 3 minutes, 20 cycles of denaturation at 94 ºC for 30 seconds, primer annealing at 56 ºC for 1 minute and primer extension at 72 ºC for 1 minute, and extension at 72 ºC for another 5

minutes. The pre-amplification reaction was held at 4 ºC until further use. Each reaction was then diluted 1:50 with Nuclease Free water (Promega).

The selective amplification reaction contained 1 X Reaction Buffer, 1.5 mM $MgCl_2$, 250 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 0.5 pmol of the fluorescently labeled Eco-C primer (5' GAC TGC GTA CCA ATT CC 3') (LI-COR Biosciences) and 2.4 pmol of the primer Tru-GC (5' GAT GAG TCC TGA CTA AGC 3') (Inqaba Biotechnologies), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 5 µL diluted pre-amplification reaction. The reaction was made up to a final volume of 20 µL with Nuclease Free water (Promega). Amplification was carried out in a Eppendorf Mastercycler epgradient (Merck chemicals (Pty) Ltd.) and the amplification conditions included an initial denaturation step at 94 ºC for 5 minutes, 9 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 65 ºC for 30 sec and primer extension at 72 ºC for 1 minute, where the primer annealing temperature decreased 1 ºC per cycle until a final annealing temperature of 56 ºC was reached. This was followed by 23 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 56 ºC for 30 sec, and primer extension at 72 ºC for 1 min, and another primer extension step at 72 ºC for 5 min. The selective amplification reaction was held at 4 ºC until further use.

LI-COR gels were prepared by using 20 ml Long Ranger gel solution (8% Long Ranger gel solution (LI-COR Biosciences), 7M Urea, 10 X TBE Buffer) 150 µL 10% APS (Ammonium persulphate) and 15 µL TEMED for polymerisation. Gels were cast using the gel casting apparatus and allowed to polymerise for 1 hour. The LI-COR IR^2 automated sequencer (LI COR Biosciences) was set up according to manufacturers instructions and a pre-run was preformed at 1500 V and 35 W for 30 minutes. The pre-run monitored the voltage and power and standardized the ions within the gel and the buffer. Samples were prepared for loading by mixing 1 µL of the selective amplification with 1 µL of loading buffer (95 % formamide, 20 mM EDTA, bromophenol blue). The sample mixture was heated to 90 ºC for 3 min and incubated on ice for 10 min before loading on the gel. Approximately 1 µL of sample mixture was loaded on the gel and a molecular sizing standard marker, IRD-700, was loaded on each end of the gel for accurate gel analysis when normalizing the gel. The samples were run for 4.5 hours and 42 W in 0.8 M TBE Buffer.

When the gel had run to completion, the resulting banding patterns was analysed using Bionumerics. Gels were normalized using the IRD-700 standard. The size of the bands included in the analysis was between 50 – 700 bp. Samples were compared by constructing an UPGMA tree, using the Pearson correlation coefficient.

2.8) Pathogenicity trials

Representative isolates of strains shown to belong to the genus *Xanthomonas,* previously isolated from South Africa and Uruguay were used in the pathogenicity tests. These strains included BCC 034, BCC 038 and BCC 062 from South Africa and BCC 765, BCC 766 and BCC 767 from Uruguay. The inoculum was prepared by placing single colonies of each isolate into 50 ml of Nutrient Broth. The flasks were incubated overnight at 28 ºC. The concentration of the bacterial cells was then adjusted to approximately 10^8 CFU/ml in a sterile saline solution.

In order to determine whether or not the isolates were pathogenic, the appearance (or lack thereof) of the hypersensitivity reaction (HR) in tobacco (*Nicotiana tabacum cv samsun*) was recorded. A bacterial suspension was injected into the leaves of the tobacco plants using a 1 ml insulin syringe. The syringe was inserted into the main vein of the leaf and the leaf panels were flooded with the bacterial suspension. A negative control containing only sterile saline solution and a positive control containing the bacterial blight and die-back pathogen, *Pantoea ananatis*, that was isolated from *Eucalyptus* trees in South Africa (LMG20103) (Coutinho *et al.,* 2002), was also inoculated in this study. Tobacco plants were kept in a greenhouse at approximately 26 ºC with natural day and night cycles. The plants were assessed after 24, 48 and 36 hours for the development of a HR. A positive HR response is observed as a complete and rapid collapse of the inoculated tissue or the drying, light brown necrosis of the water soaked tissue within 36 hours of the inoculation (Lelliot and Stead, 1987). The test was repeated to confirm results.

Isolates that showed a positive HR response were inoculated into the leaves of a *Eucalyptus grandis* X *Eucalyptus nitens* (GN188) hybrid that is known to be susceptible to *Pantoea ananatis.* The *Eucalyptus* leaf panels were flooded with the bacterial suspension as described above. A positive and negative control was included as

described above. Inoculated plants were covered with plastic bags in order to induce a high humidity. Bags were removed after seven days and the inoculated leafs were assessed for disease development every 24 hours for 7 days. Plants were kept at 26 ºC with natural day/night cycles.

Pathogen re-isolations were done from inoculated *Eucalyptus* leaves that have developed lesions during the pathogenicity trials. Isolations were done as described previously from infected leaf material. Pure-cultures were obtained for the re-extracted bacterial pathogen and DNA extractions were performed. The 16S rRNA gene of purified isolates was amplified using the method described above, and the 16S rRNA gene was partially sequenced using the method described above. The partial 16S rRNA gene sequences were submitted to BLAST analysis in Genbank for identification. This process was undertaken in order to fulfil Koch's postulates.

3) RESULTS

3.1) Bacterial isolations

A total of 20 isolates were obtained from infected leaves collected in the two countries. Sixteen isolates were obtained from South Africa and four isolates from Uruguay.

3.2) Phenotypic testing

All isolates were identified as being Gram negative and obligatory aerobic organisms. This indicates that the organisms could belong to any of a number Gram negative obligatory aerobic genera such as *Pseudomonas, Xanthomonas, Alcaligenes, Achromobacter, Brucella, Bordetella, Flavobacterium, Moraxella, Acinetobacter* or some still unnamed taxa.

3.3) DNA extractions

Genomic DNA was successfully extracted from all isolates since a clear band of genomic DNA was visible on the agarose gel when viewed under an UV light.

3.4) 16S rRNA gene sequencing and phylogenetic analysis

Partial sequences of approximately 700 bp was obtained for all 20 isolates. When a homology search was done by a BLAST search of these fragments against the EMBL/Genbank database, all isolates had between 97-100% similarity to species within the genus *Xanthomonas.* Sequencing of the full length 16S rRNA gene was then undertaken for all isolates. After aligning the sequences of representative *Xanthomonas* strains and the strains used in this study, the sequences were trimmed to 1380bp and evolutionary trees were drawn as described above.

In the resulting Maximum Likelihood tree (Fig. 1), isolates from South Africa and Uruguay formed clusters separate from each other. Isolates from South Africa grouped within a larger cluster that contained the type strains of *Xanthomonas arboricola* (LMG747^T) and *Xanthomonas hortorum* (LMG733^T) supported by a low bootstrap value. Also very closely related to the isolates from South Africa were the type strains of *X. theicola* (LMG8684^T), *X. populi* (LMG5743^T), *X. cucurbitae* (LMG690^T), *X. campestris* (568^T) and *X. vesicatoria* (LMG 911^T).

The isolates from Uruguay fell into a distinct cluster along with the following representative type species: *X. fuscans* subsp. *fuscans*, *X. fuscans* subsp. *aurantifolii* (LMG 9179) and *X. citri* (LMG9322). The Maximum Likelihood tree also shows that *X. citri* and *X. fuscans* share a high sequence similarity of the 16S rRNA gene and can in fact not be distinguished as separate species. The type strain of *Xanthomonas axonopodis* (LMG 538^T) clusters closely to the above mentioned species but are distinctly separate from these species.

3.5) *GyrB* sequencing and phylogenetic analysis

Amplification of the partial *GyrB* gene of all isolates used in this study with the primers described previously (Parkinson *et al.,* 2007) resulted in a fragment of approximately 830 bp.

A Maximum Likelihood tree was constructed using PAUP4.0* (Swofford, 2000) and Phyml (Guindon and Gascuel, 2003). Modeltests3.7 (Posada and Crandall, 1998) was

used to predict the evolutionary model that was most suited to the dataset (Fig. 2). A bootstrap analysis was performed with 1000 bootstraps to show branch support. The resulting Maximium Liklihood tree showed that all *Xanthomonas* species formed distinct clusters, except for the species *X. euvesicatoria* (NCPPB2968^T), *X. alfalfae* (LMG495^T) and *X. perforans* (NCPPB4321^T) which fell within one cluster with a 100% bootstrap support value. Upon closer investigation it was found that these three species share identical *GyrB* gene sequences. All isolates from South Africa grouped within one cluster along with the type strain of *Xanthomonas vasicola* (LMG 736^T) and a representative of *X. vasicola* pv *vasculorum* (LMG8711). All isolates from Uruguay grouped within one cluster along with the type strain of *Xanthomonas fuscans.* The *GyrB* gene of the *X. fuscans* subsp. *aurantifolii* was not successfully sequenced, and thus cannot be determined whether it will cluster in this group. All the *X. fuscans* subsp. f*uscans* type strains and representative strains are included in this group and clusters together.

3.6) AFLP (Amplified Fragment Length Polymorphisms) analysis

DNA fingerprints resulting from AFLPs of all isolates used in this study proved to be appropriate for analysis. Since no previous local database with fingerprint patterns for the genus *Xanthomonas* existed, all known *Xanthomonas* type strains of the different species were included for AFLP analysis in order to compare the patterns of the unknown isolates to those of the type strains.

The UPGMA dendogram (Figure 3) produced from AFLP banding patterns of the 20 isolates confirmed the grouping of the isolates from South Africa, whilst it raised some questions about the identification of the isolates from Uruguay. All isolates from South Africa formed a distinct cluster, labelled group 1. The *Xanthomonas* reference strain that is most closely related to these isolates is LMG 736, the type strain of *Xanthomonas vasicola* that was isolated from sorghum in New Zealand. These results confirm those obtained from 16S and *GyrB* gene sequencing. *Xanthomonas* isolates from Uruguay also formed a distinct cluster, labelled group 2. These isolates did not cluster with the type strain and representative isolates of *Xanthomonas fuscans* although these isolates were identified as *X. fuscans* with the two DNA sequencing techniques. In fact, the isolates from Uruguay formed a distinct cluster on their own.

3.7) Pathogenicity trials

Representative isolates from South Africa and Uruguay, chosen for pathogenicity testing elicited a hypersensitive response (HR) when inoculated into the tobacco leaves. When the HR elicited by the test organisms was compared to the HR elicited by the control organism (LMG 20103) it showed that the HR of some of the tests inoculations was more severe than that of the control inoculation. All positive HR was noted by a rapid and a complete necrosis of the inoculated leaf tissue. The positive HR indicates that inoculated isolates in question are indeed plant pathogens and would be able to induce disease symptoms in *Eucalyptus*. The negative control produced no HR. (Results not showed)

Inoculations into the *Eucalyptus* leaf tissue for the representative isolates of South Africa (BCC 034, BCC 038 and BCC 062) all produced lesions similar to the disease symptoms caused during infection with *Pantoea ananatis*. Initially symptoms were only observed at the site of inoculations but as time progressed the symptoms spread to adjacent tissues. When compared to the lesions produced by the positive control, lesions produced by the test inoculations were more severe (Fig. 4)

Inoculations into the *Eucalyptus* leaf tissue for the representative isolates from Uruguay (BCC 765, BCC 766 and BCC 767) all produced lesions more severe than the positive control inoculations. Initially symptoms were only concentrated in the area of inoculation but as time progressed the symptoms spread to adjacent tissues. The negative control produced no lesions (Fig. 4)

When the pathogenicity results were recorded, the causal agents of the resulting lesions were re-isolated. The 16S rRNA gene of the re-isolated organism from the diseased leaf material was partially sequenced and a comparison was made to the 16S rRNA sequence of the inoculated organism. It resulted in 100% similarity when aligned using Bio Edit, thus fulfilling Koch's postulates.

4. DISCUSSION and CONCLUSIONS

The disease seen on *Eucalyptus* trees, bacterial blight and dieback, was found to be caused by the organism *Pantoea ananatis* in South Africa (Coutinho e*t al.,* 2002). In this

thesis (Chapter 3) it was established that the same disease symptoms can be caused by different species of *Pantoea*. Similar symptoms to that of bacterial blight and dieback were observed on *Eucalyptus* trees from South Africa and Uruguay. The causal agent of these symptoms were isolated and a range of tests conducted in order to identify the unknown isolates.

A *Xanthomonas* species has been reported to cause disease on *Eucalyptus* previously. Truman (1974) identified *X. campestris* pv *eucalyptii* as the causal agent of die back of *E. citriodora* in Australia. In Brazil, *X. axonopodis* was found to be the most prevalent species causing bacterial leaf blight in *Eucalyptus* plantations (Goncalves *et al.,* 2008). Other bacterial species were also isolated in this study in association with the disease symptoms caused, i.e. *X. campestris, Pseudomonas syringae*, *P. putida*, *P. cichorii* and an *Erwinia* spp. When pathogenicity tests were preformed it was found that *X. axonopodis* was the most widespread species inducing the typical symptoms of the leaf blight and is thus considered pathogenic (Goncalves *et al.,* 2008)

In this study, *Eucalyptus* leaves that showed symptoms of bacterial blight and dieback were sampled and the causal organisms isolated. The bacteria isolated from the samples collected in South Africa were identified as *X. vasicola* while those collected from Uruguay were identified as *X. fuscans*. A polyphasic approach which included a combination of phenotypic (Gram stains and Hugh Leifson) and genotypic (16S rRNA gene sequencing, *GyrB* gene sequencing and AFLP analysis) identification techniques were used. Pathogenicity tests proved that these bacteria were the causal agent of the disease observed in the field.

Identification of these isolates using the genotypic technique, 16S rRNA sequencing, shows that this method cannot specifically distinguish between two *Xanthomonas* species, i.e. *X. fuscans* and *X. citri*. In a study conducted by Hauben *et al.* (1997) it was shown that the species of the genus *Xanthomonas* exhibited relatively high levels of sequence similarity within the 16S gene. The results showed that 16S rRNA gene sequencing is an inadequate method for identifying unknown *Xanthomonas* isolates. Results from the 16S rRNA gene sequencing showed that isolates from South Africa formed a cluster with the type strains of *X. arboricola* (LMG 747^T) and *X. hortorum* (LMG 733^T) with low bootstrap support. When results are compared to that obtained by

Hauben *et al.* (1997), results are in agreement. In their study, *Xanthomonas* species formed three lineages, where lineage 1 was the core group and consists of the following *Xanthomonas* species: *X. cassavae*, X. *axanopodis*, *X. cucurbitae*, *X. vasicola*, *X. codiae, X. bromi, X. vesicatoria, X. fragariae, X. pisi, X. oryzae, X. arboricola, X. populi, X. campestris* and *X. melonis.* The isolates from South Africa fell within this lineage. Lineage 2 was comprised of one species, *X. sacchari,* and Lineage 3 was comprised of the ancestral species *X. translucens, X. hyacinthi* and *X. albilineans*. The level of 16S rRNA sequence similarity within the core group of *Xanthomonas* was very high and ranged from 99 -100%. Although all of the above mentioned species was classified as a nomenspecies using DNA:DNA reassociation, it is not possible to identify the species in the core group using 16S rRNA gene sequencing due to the low divergence and the fact that it is highly conserved (Stackebrandt *et al.,* 1999). Results from the 16S rRNA gene sequencing showed that isolates from Uruguay formed a cluster (viz Group 2) with the type and reference strains of *Xanthomonas citri* and *Xanthomonas* fuscans but supported by a low bootstrap value. Both of the subspecies *X. fuscans* subsp. *fuscans* and *X. fuscans* subsp. *aurantifolii* are included in this group.

GyrB gene sequencing allows for a more accurate identification of these unknown *Xanthomonas* isolates (Young et al., 2008). This gene forms part of the replication machinery of the bacterial cell and is part of the “core-genes” of the bacterial DNA. This gene is conserved and is not frequently subjected to horizontal gene transfer, it is also present in single copy, which renders it a dependable gene for phylogenetic identification (Kasai *et al.,* 2000, Yamamoto and Harayama, 1995, Soler *et al.,* 2004, Kakinuma *et al.,* 2003, Hannula and Hanninen, 2007). This gene was used by Parkinson *et al.* (2007) in a phylogeneitc analysis of *Xanthomonas* species. All *Xanthomonas* species were clearly separated within that study except for the species *X. perforans, X. euvesicatoria* and *X. alfalfae*, which required further identification. Young *et al.,* (2008), confirmed the phylogenetic positions of the genus *Xanthomonas* with an MLSA study using four “core genes”. In this study, *GyrB* gene sequencing definitely proved to be a reliable technique for the species identification of the unknown isolates. The isolates from South Africa were clearly identified as *X. vasicola*, as all of the *X. vasicola* reference strains and type strain formed a distinct cluster with these unknown isolates. What was also noted from the phylogenetic tree constructed from the *GyrB* data, is that the unknown isolates from South Africa were more closely related to *X. vasicola* pv. *vasculorum* which was isolated

from sugarcane in South Africa than to the type strain of the species which was isolated from sorghum in New Zealand.

In the Parkinson study, the resulting MLSA phylogenetic tree indicated that three *Xanthomonas* species namely: *X. euvesicatoria*, *X. alfalfae* and *X. perforans* do not fall in separate clusters indicating separate species but instead formed one distinct cluster with a 100% bootstrap support value (Parkinson *et al.,* 2007). Upon closer investigation it was found that these three species share identical *GyrB* gene sequences. Following a recent analysis of the genus *Xanthomonas* using a MLSA technique, Young *et al.* (2008), proposed that *X. euvesicatoria* and *X perforans* formed a single group with high similarity and is better represented as a single subspecies of *X. axonopodis*. The same MLSA study also showed that *X. fuscans* and *X citri* share a high sequence similarity and it was proposed that these two species are in fact synonymous and should be reclassified as a single species or as a subspecies of *X. axonopodis*.

AFLP analysis allows for the identification of bacterial isolates based on a whole genome approach, where sequencing methods depends on the identification of isolates based on a specific gene. AFLP analysis was proven by Rademaker *et al.* (2000) to be a reliable method for typing and identifying members belonging to the genus *Xanthomonas*. In this study researchers concluded that a fingerprinting technique such as AFLP is a feasible technique to show the genetic relationships between *Xanthomonas* species. In the Rademaker *et al.,* (2000) study, this technique was shown to be able to distinguish between all 20 *Xanthomonas* species that had been described at that time. It was also stated that AFLP is a valuable alternative to DNA:DNA re-association studies which is impractical to use when analysing large numbers of environmental isolates. Fingerprinting techniques are less expensive and less time consuming when compared to DNA:DNA re-association studies. In the current study, AFLP's also proved to be a reliable technique for identification of unidentified isolates. Results showed that unidentified isolates from South Africa clustered with the type strain and reference strain of the species *X. vasicola.* Within this cluster several groupings could, however, still be seen. All reference strains of *X. vasicola* pv. *vasculorum* that were isolated from sugarcane in South Africa formed a grouping within the *X. vasicola* cluster, whereas unidentified isolates from South Africa appears to be more closely related to the type strain of *X. vasicola* pv *holcicola* that was isolated from sorghum in New Zealand. These

same results were observed when using *GyrB* gene sequencing. Isolates from *Eucalyptus* trees in Uruguay did not give a clear identification result when analyzed with AFLP fingerprinting. Although the *GyrB* gene sequencing results identified these isolates as *X. fuscans* with no distinction between the two *fuscans* subspecies, AFLP analysis did not provide the same results. *X. fuscans* type strains and reference strains of both subspecies formed a cluster away from the unidentified isolates from Uruguay. This result may occur due the fact that the fingerprints of these isolates were too different to that of *X.* fuscans. In this specific study AFLP was found not to be as a reliable technique for the typing of strains at the inter-species level of *Xanthomonas* since it cannot specifically group isolates within a species and some species may be split into different groups.

In this study, all unidentified *Xanthomonas* isolates elicited a positive hypersensitive response within 48 hours when they were inoculated into tobacco leaves. These results indicated that all of the unknown isolates are indeed pathogenic. Pathogenicity tests confirmed that the inoculated organism was the causal agent of the initial symptoms observed on the leaves since similar symptoms were noted on the inoculated plants during the pathogenicity trials. This was further confirmed when the bacterium inoculated was re-isolated from the plants in fulfilment of Koch's postulates. The symptoms found on the *Eucalyptus* leaves from South Africa and from Uruguay were indistinguishable from the symptoms that were caused by *Pantoea* isolates (Chapter 3) in countries such as Argentina, Colombia, Rwanda, South Africa, Thailand, Uganda and Uruguay. The interaction between these bacterial plant pathogens, if one exists, is unknown and further investigation is required.

From the data presented here it is clear that *Xanthomonas* spp. might also be involved in the disease symptoms observed for bacterial blight and die-back of *Eucalyptus*. Both *X. vasicola*, isolated from *Eucalyptus* in South Africa and *X. fuscans* isolated from *Eucalyptus* in Uruguay caused similar disease symptoms on its host, and these disease symptoms were similar to those seen on *Eucalyptus* in various countries where *Pantoea* spp. were identified as the causal organism. From the outbreak in South Africa both genera, *Xanthomonas* and *Pantoea,* were isolated as the causal agent of the disease symptoms (results unpublished). It would thus appear that the disease, bacterial blight

and die-back of *Eucalyptus*, is caused by a complex of species belonging to the genera *Pantoea* and *Xanthomonas*.

Xanthomonas species have not previously been found to cause disease symptoms on *Eucalyptus* in either South Africa or Uruguay. *X. campestris* pv. *eucalyptii* have been found to cause disease on *Eucalyptus* ornamentals in Australia in 1974 (Truman, 1974). The type strain and an additional strain (NCPPB 2338/BCC 205/BCC 206) of this specific organism was included in all the identification techniques (16S rRNA gene sequencing, *GyrB* gene sequencing, AFLP analysis) undertaken in this study. When studying the position of this specific isolate in the results generated it was found that this specific isolate certainly belongs to *X. campestris* but shows no similarity to the unknown *Xanthomonas* isolates that was isolated from *Eucalyptus* in South Africa and Uruguay. More sampling and further studies will confirm whether the isolation of these *Xanthomonas* species from *Eucalyptus* trees were an isolated anomaly or an emerging disease which will occur again. The possibility that these *Xanthomonas* isolates were present as epiphytes on the leaves is also eliminated since the leaves were surface sterilized before the causal agent was extracted.

All of the genotyping techniques used to identify the unknown *Xanthomonas* isolates from South Africa indicated that these isolates all belong to the same *Xanthomonas* species. The genotyping techniques indicated that these isolates are in fact *Xanthomonas vasicola.* The genotypic identification of the *Xanthomonas* isolates from Uruguay was not as straightforward. 16S rRNA gene sequencing and *GyrB* gene sequencing showed that these isolates are possible *X. fuscans* isolates. However, AFLP fingerprinting were not able to precisely identify these isolates.

The type strain of *Xanthomonas vasicola* was isolated from sorghum in New Zealand. It has subsequently also been found to cause disease symptoms in maize (*Zea mays*), sugarcane (*Saccharum officinarum)*, banana (*Musa* sp.) and others but has not previously been reported to infect and cause disease on *Eucalyptus* spp. *Xanthomonas fuscans* is a recently described species (Schaad *et al.,* 2005) and consists of two subspecies, *X. fuscans* subsp. *fuscans* which was isolated from bean in Canada (Schaad *et al.,* 2005) and *X. fuscans* subsp. *aurantifolii* which was isolated from citrus in Argentina (Schaad *et al.,* 2005). This is the first report of *Xanthomonas fuscans* being

associated with a disease of *Eucalyptus* trees, whilst *Xanthomonas axanopodis* was previously found to cause bacterial leaf blight on *Eucalyptus* trees from Brazil (Goncalves *et al.,* 2008).

Xanthomonas species occur on plants and can cause a variety of diseases on economically important plants, trees and crops. It is thus necessary to accurately and rapidly identify the isolates that are pathogenic to a specific host in order to efficiently manage the particular disease. In this study, the use of *GyrB* gene sequencing was confirmed as a reliable method to use for this purpose.

5) References

- Atkinson. G.F. 1891. Black 'rot' of cotton. Alabama Agricultural Experiment Station Bulletin 27: 16
- Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J.R.W., Kersters, K., and Vandamme, P. 1999. Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. International Journal of Systematic Bacteriology 49: 405 – 413
- Coutinho, T.A., Preisig, O., Mergaert, J., Cnockaert, M.C., Riedel, K.-H., Swings, J., and Wingfield, M.J. 2002. Bacterial blight and dieback of *Eucalyptus* species, hybrids and clones in South Africa. Plant Disease 86: 20 – 25
- Dianese, J.C., Dristig M.C.G., and Cruz, A.P. 1990. Susceptibility to wilt associated with *Pseudomonas solanacearum* among six species of *Eucalyptus* growing in equatorial Brazil. Australasian Plant Pathology 19: 71 – 76
- Euzeby, J.P. List of Prokaryotic Names with standing in nomenclature. http://www.bacterio.cict.fr
- Forestry South Africa. 2002. The South African Forestry and Forest Products Industry
- Goncalves, R.C., Lau, D., Oliveira, J.R., Maffia, L.A., Cascardo, J.C.M., and Alfenas, A.C. 2008. Etiology of bacterial leaf blight of *Eucalyptus* in Brazil. Tropical Plant Pathology 33: 180 – 188
- Guindon, S. and Gascuel, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52: 696 – 704
- Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41: 95 – 98
- Hannula, M., and Hanninen, M-L. 2007. Phylogenetic analysis of *Helicobacter* species based on partial *gyrB* sequences. International Journal of Systematic and Evolutionary Microbiology 57: 444 – 449
- Hasse, C.H. 1915. *Pseudomonas citri*, the cause of citrus canker. Journal of Agricultural Research 4: 97 – 100
- Hauben, L., Vauterin, L., Swings, J., and Moore, E.R.B. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. International Journal of Systematic Bacteriology 47: 328 – 335

- Kakinuma, K., Fukushima, M., and Kawaguchi, R. 2003. Detection and identification of *Escherichia coli*, *Shigella,* and *Salmonella* by micro-arrays using the *gyrB* gene. Biotechnology and Bioengineering 83: 721 – 728
- Kasai, H., Ezaki, T., and Harayama, S. 2000. Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. Journal of Clinical Microbiology 38: 301 – 308
- Koning, H.C. 1938. The bacterial canker of poplars. Mededelingen Phytopathologisch Laboratorium 'Willie Commelin Scholten' Baarn 14: 3 -47
- Lelliot, R.A., and Stead, D.E. 1987. Host tests. Methods for the diagnosis of bacterial diseases of plants. T.F Preece eds. Blackwell Scientific Publications, London, UK, 152 – 168
- Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M., and van der Lelie, D. 2002. Endophytic bacteria and their potential applications. Critical Reviews in Plant Sciences 21: 583 – 606
- Parkinson, N., Aritua, V., Heeney, J., Cowie, C., Bew, J., and Stead, D. 2007. Phylogenetic analysis of *Xanthomonas* species by comparison of partial gyrase B gene sequences. International Journal of Systematic and Evolutionary Microbiology 57: 2881 – 2887
- Posada, D. and Crandall, K.A. 1998. Modeltests: testing the model of DNA substitution. Bioinformatics 14: 817 – 818
- Rademaker, J.L.W., Hoste, B., Louws, F.J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P., and de Bruijn, F.J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. International Journal of Systematic and Evolutionary Microbiology 50: 665 – 677
- Ricuad, C., and Ryan, C.C. 1989. Leaf scald, in Diseases of Sugarcane. Major Diseases (eds C. Ricuad, B.T. Egan, A.G. Gillaspie Jr and C.G. Hughes), Elsevier, Amsterdam, 39 – 58
- Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., and Swings, J. 2005. Applications of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. Microbiology 151: 2141 – 2150
- Schaad, N.W., Postnikova, E., Lacy, G.H., Sechler, A., Agarkova, I., Stromberg, P.E., Stromberg, V.K., and Vidaver, A.K. 2005. Reclassification of *Xanthomonas*

campestris pv. *citri* (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as *X. smithii* subsp. *citri* (ex Hasse) sp. nov. nom. rev. comb. nov., *X. fuscans* subsp. *aurantifolii* (ex Gabriel 1989) sp. nov. rev. comb. nov., and *X. alfalfae* subsp. *citumelo* (ex Riker and Jones) Gabriel *et al.,* 1989 sp. nov. rev. comb. nov.; *X. campestris* pv. *malvacearum* (ex Smith 1901) Dye 1978 as *X. smithii* subsp. *smithii* nov. comb. nov. nom. nov.; *X. campestris* pv. alfalfae (ex Riker and Jones, 1935) Dye 1978 as *X. alfalfae* subsp. alfalfae (ex Riker *et al.,* 1935) sp. nov. nom. rev.; and "var. fuscans" of *X. campestris* pv. *phaseoli* (ex Smith, 1987) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. Systematic and Applied Microbiology 28: 494 – 518

- Soler, L., Yanez, M.A., Chacon, M.R., Aguilera-Arreola, M.G., Catalan, V., Figueras, M.J., Martinez-Murcia, A.J. 2004. Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes. International Journal of Systematic and Evolutionary Microbiology 54: 1511 – 1519
- Stackebrandt, E., Tindall, B., Ludwig, W., and Goodfellow, M. 1999. Prokaryotic diversity and systematics. Biology of the prokaryotes. J.W. Lengeler, G. Drews, and H.G. Schlegel, eds. Blackwell Science. George Thieme Verlag, Stuttgart, Germany. 674 – 722
- Starr, M.P. 1981. The genus *Xanthomonas*. The Prokaryotes. M.P. Starr, H. Stolp, H.G. Truper, A. Balows, and H.G. Schlegel, eds. Springer, Verlag, Berlin. 742 – 763
- Swofford, D.L. 2000. PAUP*. Phylogenetic analysis using parsimony and other methods (software). Sinauer Associates, Suderland, MA.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougnin, F., and Higgins, D.G. 1997. The ClustalX-Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25: 4876 – 4882
- Truman, R. 1974. Die-back of *Eucalyptus citriodora* caused by *Xanthomonas eucalypti* sp.n. Phytopathology 64: 143 – 144
- Vauterin, L., Hoste, B. Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas.* International Journal of Systematic Bacteriology 45: 472 – 489
- Vauterin, L., Rademaker, J., and Swings, J. 2000. Synopsis on the taxonomy of the genus *Xanthomonas*. Phytopathology 90: 677 – 682
- Yamamoto, S., and Harayama, S. 1995. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and

taxonomic analysis of *Pseudomonas putida* strains. Applied and Environmental Microbiology 61: 1104 – 1109

- Yirgou, D., and Bradbury, J.F. 1968. Bacterial wilt of enset (*Ensete ventricosum)* incited by *Xanthomonas musacearum* sp. n. Phytopathology 58: 111 – 112
- Young, J.M., Park, D.-C., Shearman, H.M., and Fargier, E. 2008. A multilocus sequence analysis of the genus *Xanthomonas*. Systematic and Apllied Microbiology 31: 366 - 377
- Zabeau, M., and Vos, P. 1993. Selective restriction fragment amplification: A general method for DNA fingerprinting. European Patent Office, publication 0 534 858 A1
- Zeigler, D.R. 2003. Gene sequences useful for predicting relatedness of whole genomes in bacteria. International Journal of Systematic and Evolutionary Microbiology 53: 1893 – 1900

Table 1: List of primers used for 16S rRNA amplification and 16S rRNA sequencing (Coenye *et al.,* 1999) Primers *pA and pH were used for amplification of the 16S rRNA gene, whilst all other primers were used during partial sequencing of this gene

Name	**Synonym**	**Sequence (5' → 3')**	**Position on 16S gene**
16F27[1]	*pA	AGA GTT TGA TCC TGG CTC AG	8 – 27
16R1522	pH	AAG GAG GTG ATC CAG CCG CA	1541 – 1522
16F536	*pD	CAG CAG CCG CGG TAA TAC	519 – 536
16F926	*O	AAC TCA AAG GAA TTG ACG G	908 – 926
16F1112	*3	AGT CCC GCA ACG AGC GCA AC	1093 – 1112
16R519	pD	GTA TTA CCG CGG CTG CTG	536 – 519
16R685		TCT ACG CAT TTC ACC GCT AC	704 – 685
16R1093	3	GTT GCG CTC GTT GCG GGA CT	1112 – 1093

* Indicates forward primers

Table 2: List of primers used for the amplification and sequencing of the *GyrB* gene for the genus *Xanthomonas*. (Parkinson *et al.,* 2007)

Name	**Sequence (5' → 3')**
X.gyr.PCR2F (forward)	AAGCAGGGCAAGAGCGAGCTGTA
X.gyr.rsp1 (reverse)	CAAGGTGCTGAAGATCTGGTC
X.gyr.fsp.s1	GGCAAGAGCGAGCTGTA
X.gyr.rsp3	CTGGTCGGCGGCCAC

Figure 1: [next page] 16S rRNA gene sequencing phylogenetic tree, using the Maximum Likelihood parameter of unidentified isolates from South Africa and Uruguay and representative strains of the genus *Xanthomonas.* Unknown clusters are indicated with brackets and labelled. Supporting bootstrap values are indicated above the branches.

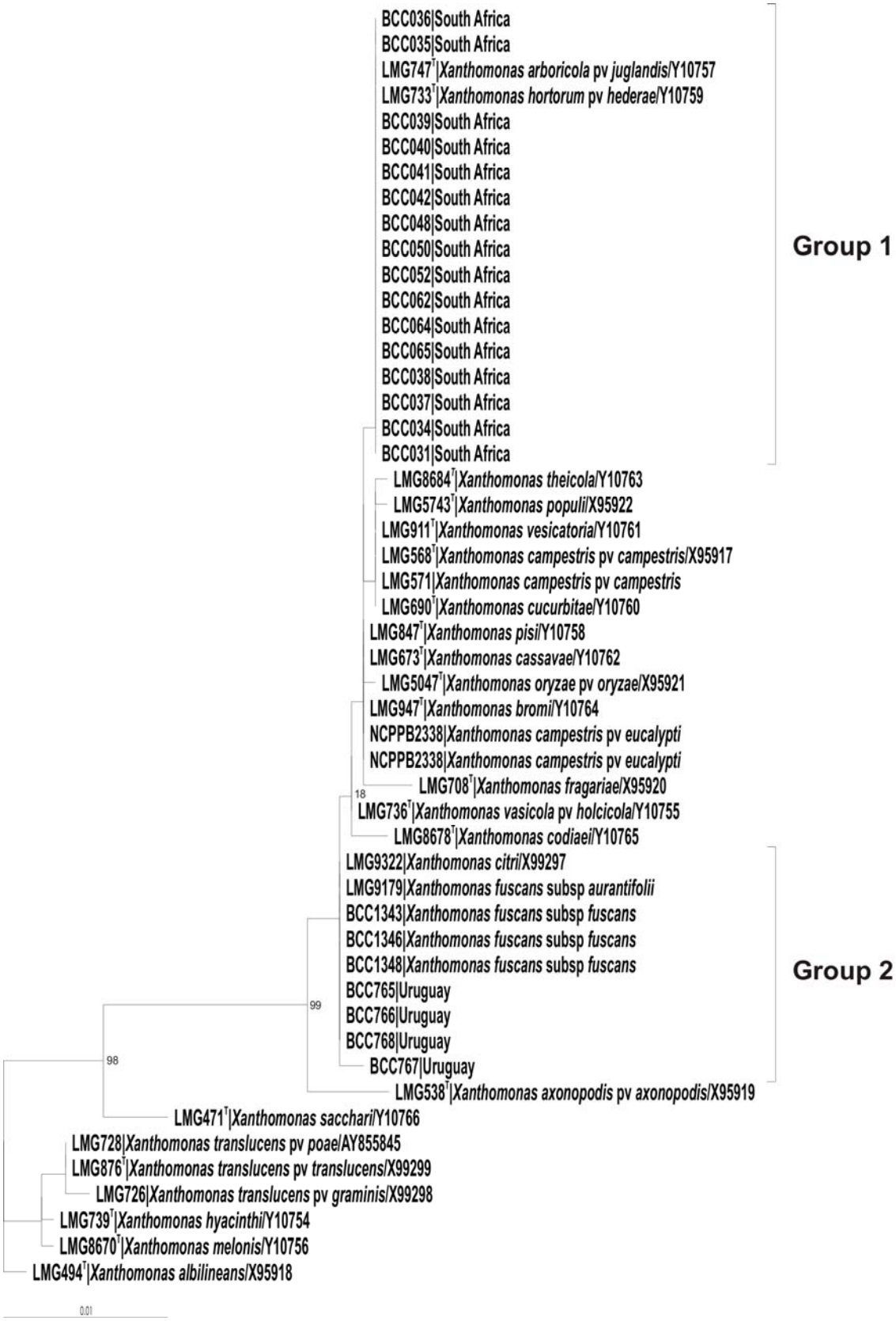

BCC036|South Africa
BCC035|South Africa
LMG747T|Xanthomonas arboricola pv juglandis/Y10757
LMG733T|Xanthomonas hortorum pv hederae/Y10759
BCC039|South Africa
BCC040|South Africa
BCC041|South Africa
BCC042|South Africa
BCC048|South Africa
BCC050|South Africa
BCC052|South Africa
BCC062|South Africa
BCC064|South Africa
BCC065|South Africa
BCC038|South Africa
BCC037|South Africa
BCC034|South Africa
BCC031|South Africa
Group 1
LMG8684T|Xanthomonas theicola/Y10763
LMG5743T|Xanthomonas populi/X95922
LMG911T|Xanthomonas vesicatoria/Y10761
LMG568T|Xanthomonas campestris pv campestris/X95917
LMG571|Xanthomonas campestris pv campestris
LMG690T|Xanthomonas cucurbitae/Y10760
LMG847T|Xanthomonas pisi/Y10758
LMG673T|Xanthomonas cassavae/Y10762
LMG5047T|Xanthomonas oryzae pv oryzae/X95921
LMG947T|Xanthomonas bromi/Y10764
NCPPB2338|Xanthomonas campestris pv eucalypti
NCPPB2338|Xanthomonas campestris pv eucalypti
LMG708T|Xanthomonas fragariae/X95920
18
LMG736T|Xanthomonas vasicola pv holcicola/Y10755
LMG8678T|Xanthomonas codiaei/Y10765
LMG9322|Xanthomonas citri/X99297
LMG9179|Xanthomonas fuscans subsp aurantifolii
BCC1343|Xanthomonas fuscans subsp fuscans
BCC1346|Xanthomonas fuscans subsp fuscans
BCC1348|Xanthomonas fuscans subsp fuscans
BCC765|Uruguay
BCC766|Uruguay
BCC768|Uruguay
BCC767|Uruguay
Group 2
99
98
LMG538T|Xanthomonas axonopodis pv axonopodis/X95919
LMG471T|Xanthomonas sacchari/Y10766
LMG728|Xanthomonas translucens pv poae/AY855845
LMG876T|Xanthomonas translucens pv translucens/X99299
LMG726|Xanthomonas translucens pv graminis/X99298
LMG739T|Xanthomonas hyacinthi/Y10754
LMG8670T|Xanthomonas melonis/Y10756
LMG494T|Xanthomonas albilineans/X95918
0.01

Figure 2: [next page] *GyrB* gene sequencing phylogenetic tree using the Maximum-Likelihood parameter, of unidentified isolates from South Africa and Uruguay and representative strains, including type strains of the genus *Xanthomonas.* Unknown clusters are indicated with brackets and labelled. Supporting bootstrap values are indicated above the branches.

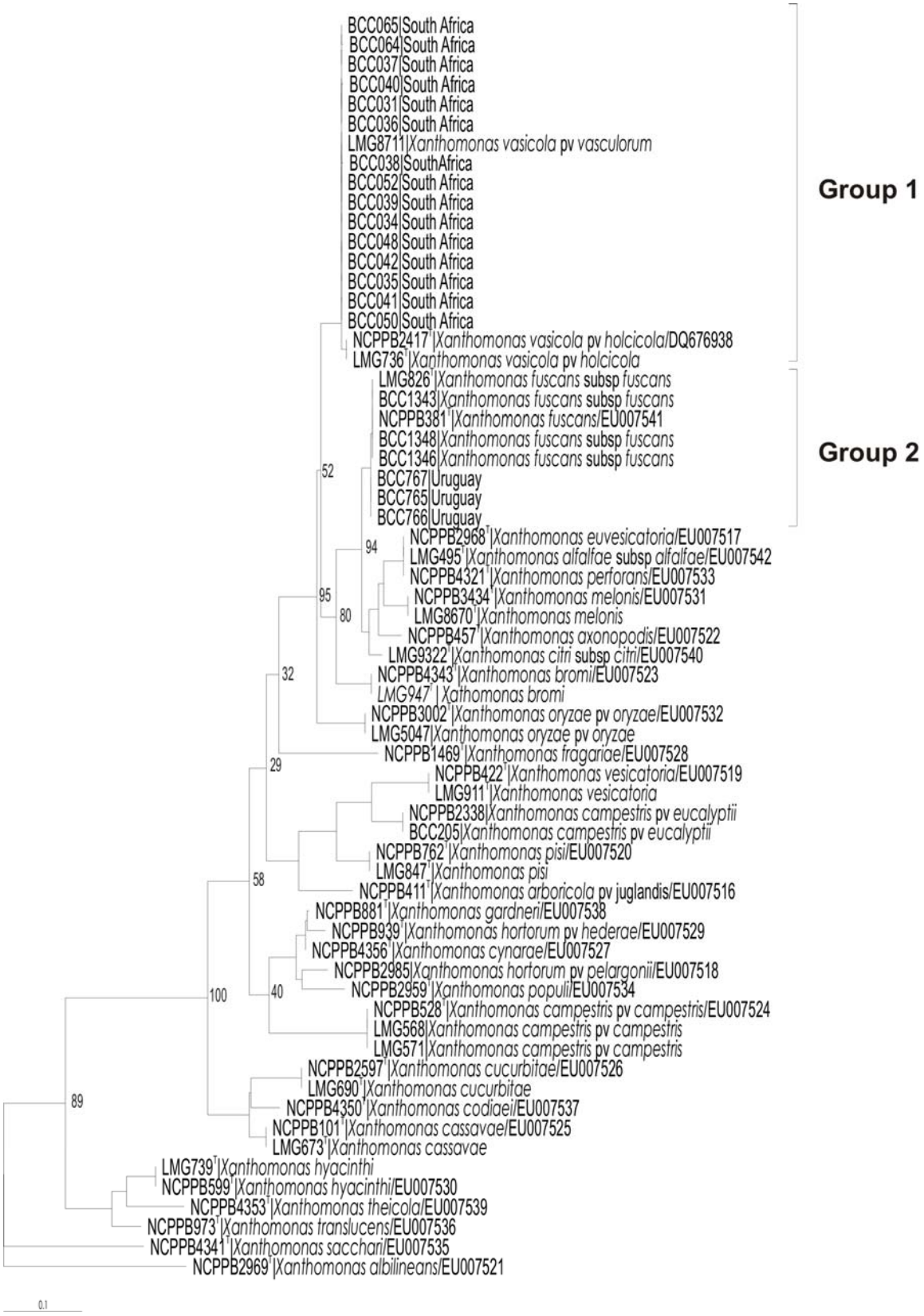

BCC065|South Africa
BCC064|South Africa
BCC037|South Africa
BCC040|South Africa
BCC031|South Africa
BCC036|South Africa
LMG8711|Xanthomonas vasicola pv vasculorum
BCC038|SouthAfrica
BCC052|South Africa
BCC039|South Africa
BCC034|South Africa
BCC048|South Africa
BCC042|South Africa
BCC035|South Africa
BCC041|South Africa
BCC050|South Africa
NCPPB2417T|Xanthomonas vasicola pv holcicola/DQ676938
LMG736T|Xanthomonas vasicola pv holcicola
Group 1
LMG826T|Xanthomonas fuscans subsp fuscans
BCC1343|Xanthomonas fuscans subsp fuscans
NCPPB381T|Xanthomonas fuscans/EU007541
BCC1348|Xanthomonas fuscans subsp fuscans
BCC1346|Xanthomonas fuscans subsp fuscans
BCC767|Uruguay
BCC765|Uruguay
BCC766|Uruguay
Group 2
NCPPB2968T|Xanthomonas euvesicatoria/EU007517
LMG495T|Xanthomonas alfalfae subsp alfalfae/EU007542
NCPPB4321T|Xanthomonas perforans/EU007533
NCPPB3434T|Xanthomonas melonis/EU007531
LMG8670T|Xanthomonas melonis
NCPPB457T|Xanthomonas axonopodis/EU007522
LMG9322T|Xanthomonas citri subsp citri/EU007540
NCPPB4343T|Xanthomonas bromi/EU007523
LMG947T|Xathomonas bromi
NCPPB3002T|Xanthomonas oryzae pv oryzae/EU007532
LMG5047|Xanthomonas oryzae pv oryzae
NCPPB1469T|Xanthomonas fragariae/EU007528
NCPPB422T|Xanthomonas vesicatoria/EU007519
LMG911T|Xanthomonas vesicatoria
NCPPB2338|Xanthomonas campestris pv eucalyptii
BCC205|Xanthomonas campestris pv eucalyptii
NCPPB762T|Xanthomonas pisi/EU007520
LMG847T|Xanthomonas pisi
NCPPB411T|Xanthomonas arboricola pv juglandis/EU007516
NCPPB881T|Xanthomonas gardneri/EU007538
NCPPB939T|Xanthomonas hortorum pv hederae/EU007529
NCPPB4356T|Xanthomonas cynarae/EU007527
NCPPB2985|Xanthomonas hortorum pv pelargonii/EU007518
NCPPB2959T|Xanthomonas populi/EU007534
NCPPB528T|Xanthomonas campestris pv campestris/EU007524
LMG568|Xanthomonas campestris pv campestris
LMG571|Xanthomonas campestris pv campestris
NCPPB2597T|Xanthomonas cucurbitae/EU007526
LMG690T|Xanthomonas cucurbitae
NCPPB4350T|Xanthomonas codiaei/EU007537
NCPPB101T|Xanthomonas cassavae/EU007525
LMG673T|Xanthomonas cassavae
LMG739T|Xanthomonas hyacinthi
NCPPB599T|Xanthomonas hyacinthi/EU007530
NCPPB4353T|Xanthomonas theicola/EU007539
NCPPB973T|Xanthomonas translucens/EU007536
NCPPB4341T|Xanthomonas sacchari/EU007535
NCPPB2969T|Xanthomonas albilineans/EU007521
52
94
95
80
32
29
58
100
40
89
0.1

Figure 3: [next infold page] UPGMA tree constructed using the Pearson correlation coefficient based on AFLP analysis of unidentified isolates from diseased *Eucalyptus.*

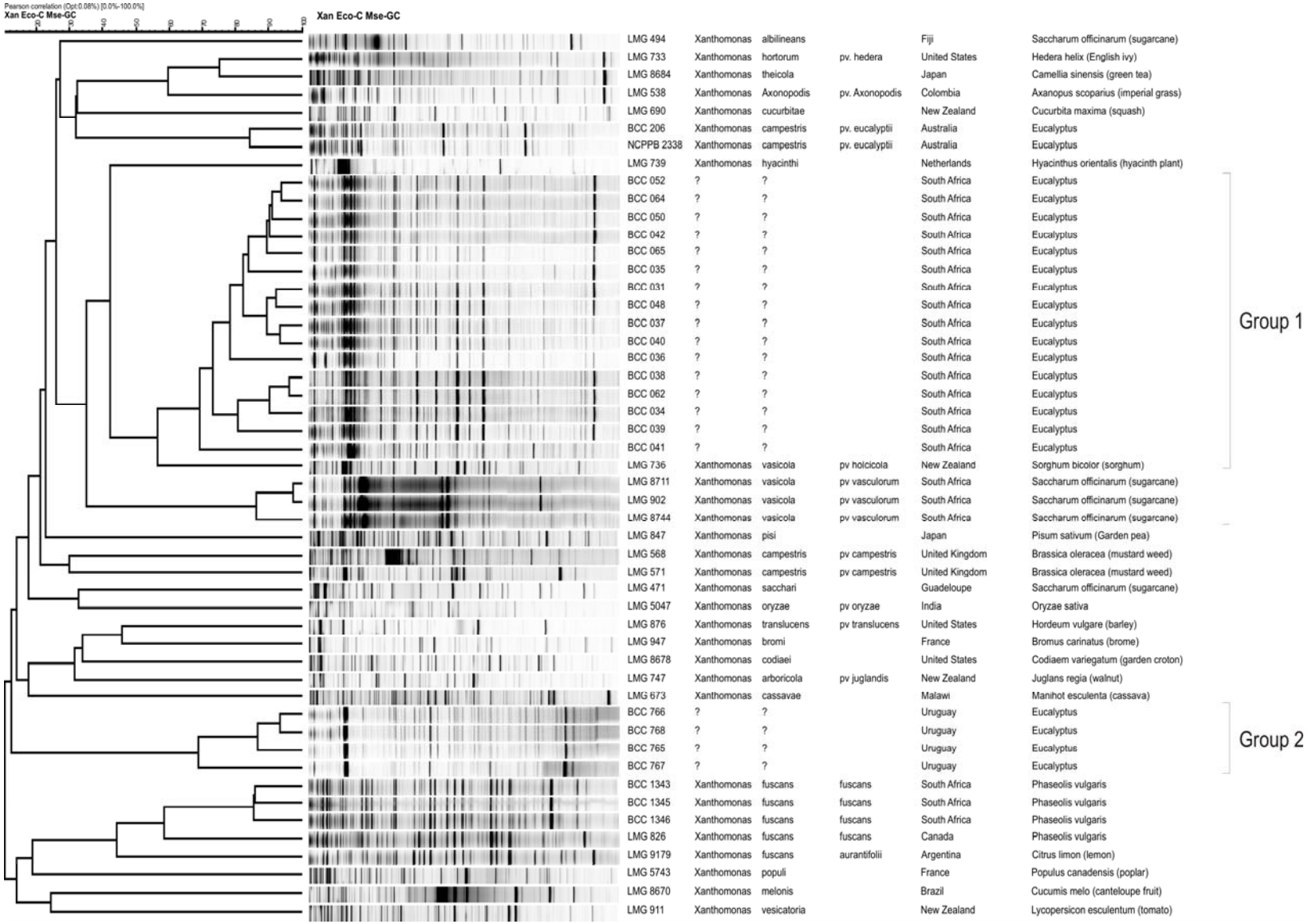
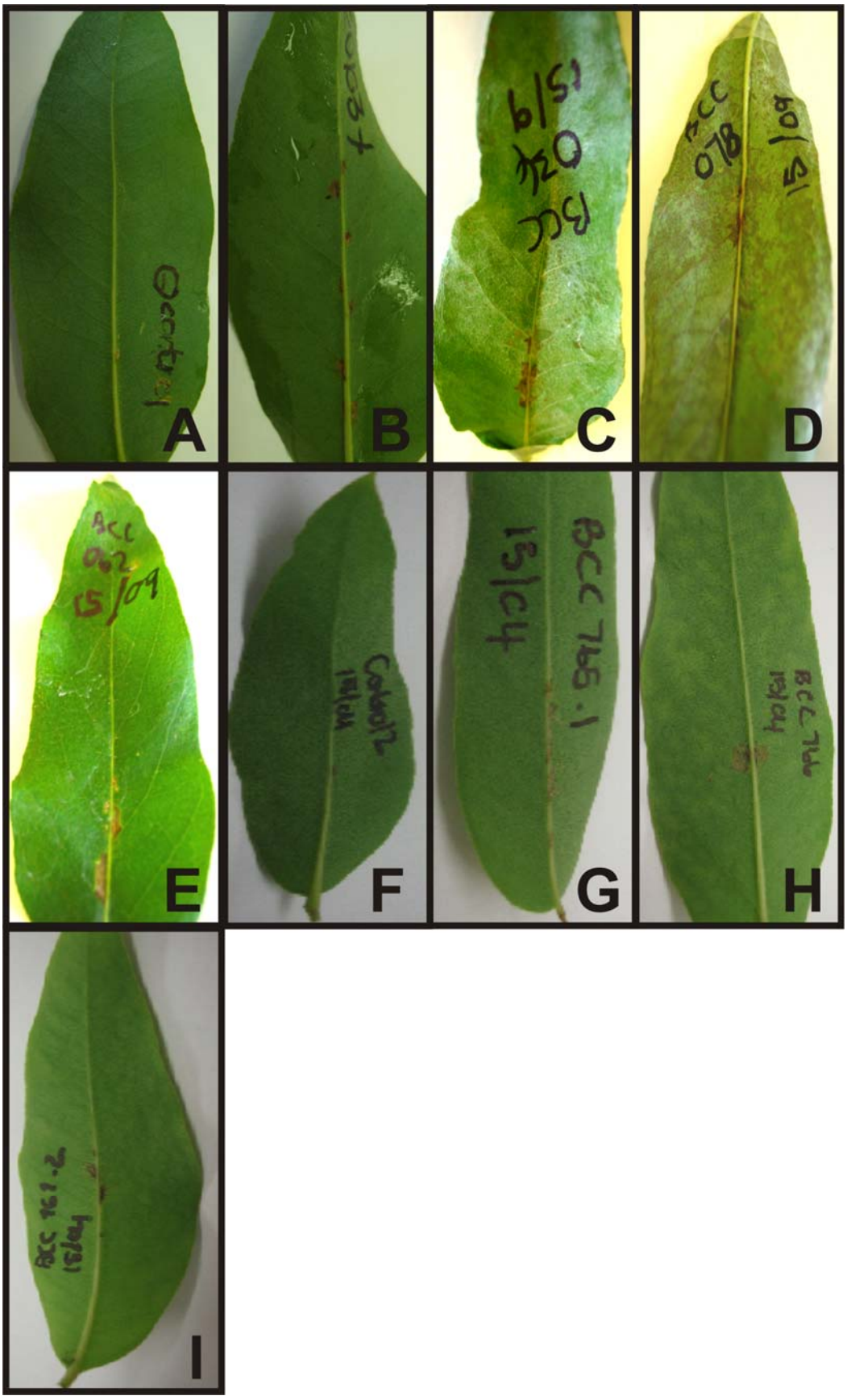

Pearson correlation (Opt:0.08%) [0.0%-100.0%]
Xan Eco-C Mse-GC
100 90 80 70 60 50 40 30 20 10 0
Xan Eco-C Mse-GC
LMG 494 Xanthomonas albilineans Fiji Saccharum officinarum (sugarcane)
LMG 733 Xanthomonas hortorum pv. hedera United States Hedera helix (English ivy)
LMG 8684 Xanthomonas theicola Japan Camellia sinensis (green tea)
LMG 538 Xanthomonas Axonopodis pv. Axonopodis Colombia Axanopus scoparius (imperial grass)
LMG 690 Xanthomonas cucurbitae New Zealand Cucurbita maxima (squash)
BCC 206 Xanthomonas campestris pv. eucalyptii Australia Eucalyptus
NCPPB 2338 Xanthomonas campestris pv. eucalyptii Australia Eucalyptus
LMG 739 Xanthomonas hyacinthi Netherlands Hyacinthus orientalis (hyacinth plant)
BCC 052 ? ? South Africa Eucalyptus
BCC 064 ? ? South Africa Eucalyptus
BCC 050 ? ? South Africa Eucalyptus
BCC 042 ? ? South Africa Eucalyptus
BCC 065 ? ? South Africa Eucalyptus
BCC 035 ? ? South Africa Eucalyptus
BCC 031 ? ? South Africa Eucalyptus
BCC 048 ? ? South Africa Eucalyptus
BCC 037 ? ? South Africa Eucalyptus
BCC 040 ? ? South Africa Eucalyptus
BCC 036 ? ? South Africa Eucalyptus
BCC 038 ? ? South Africa Eucalyptus
BCC 062 ? ? South Africa Eucalyptus
BCC 034 ? ? South Africa Eucalyptus
BCC 039 ? ? South Africa Eucalyptus
BCC 041 ? ? South Africa Eucalyptus
LMG 736 Xanthomonas vasicola pv holcicola New Zealand Sorghum bicolor (sorghum)
LMG 8711 Xanthomonas vasicola pv vasculorum South Africa Saccharum officinarum (sugarcane)
LMG 902 Xanthomonas vasicola pv vasculorum South Africa Saccharum officinarum (sugarcane)
LMG 8744 Xanthomonas vasicola pv vasculorum South Africa Saccharum officinarum (sugarcane)
LMG 847 Xanthomonas pisi Japan Pisum sativum (Garden pea)
LMG 568 Xanthomonas campestris pv campestris United Kingdom Brassica oleracea (mustard weed)
LMG 571 Xanthomonas campestris pv campestris United Kingdom Brassica oleracea (mustard weed)
LMG 471 Xanthomonas sacchari Guadeloupe Saccharum officinarum (sugarcane)
LMG 5047 Xanthomonas oryzae pv oryzae India Oryzae sativa
LMG 876 Xanthomonas translucens pv translucens United States Hordeum vulgare (barley)
LMG 947 Xanthomonas bromi France Bromus carinatus (brome)
LMG 8678 Xanthomonas codiaei United States Codiaem variegatum (garden croton)
LMG 747 Xanthomonas arboricola pv juglandis New Zealand Juglans regia (walnut)
LMG 673 Xanthomonas cassavae Malawi Manihot esculenta (cassava)
BCC 766 ? ? Uruguay Eucalyptus
BCC 768 ? ? Uruguay Eucalyptus
BCC 765 ? ? Uruguay Eucalyptus
BCC 767 ? ? Uruguay Eucalyptus
BCC 1343 Xanthomonas fuscans fuscans South Africa Phaseolis vulgaris
BCC 1345 Xanthomonas fuscans fuscans South Africa Phaseolis vulgaris
BCC 1346 Xanthomonas fuscans fuscans South Africa Phaseolis vulgaris
LMG 826 Xanthomonas fuscans fuscans Canada Phaseolis vulgaris
LMG 9179 Xanthomonas fuscans aurantifolii Argentina Citrus limon (lemon)
LMG 5743 Xanthomonas populi France Populus canadensis (poplar)
LMG 8670 Xanthomonas melonis Brazil Cucumis melo (canteloupe fruit)
LMG 911 Xanthomonas vesicatoria New Zealand Lycopersicon esculentum (tomato)
Group 1
Group 2

Figure 4 [next page]: *Xanthomonas* isolates from South Africa and Uruguay were inoculated into the host *E. grandis* X *E. nitens* hybrid. **A:** Negative control (sterile saline solution) **B:** Positive control (*P. ananatis,* LMG 20103) **C:** BCC 034 (South Africa) **D:** BCC 038 (South Africa) **E:** BCC 062 (South Africa) **F:** Negative control (sterile saline solution) **G:** BCC 765 (Uruguay) **H:** BCC 766 (Uruguay) **I:** BCC 767 (Uruguay)


A
B
C
D
E
F
G
H
I

Chapter 5

Summary

Summary

The pulp and paper industry is expanding world-wide to supply the needs and demands of the consumer. Due to this rapid expansion of commercial forests and our ever changing climate including the sporadic increase and decrease in rain and the increasing temperature caused by global warming, previously described and new pathogens are emerging which infect and cause diseases on commercial forest trees and agricultural crops. Research efforts are required to investigate mechanisms of disease control and eradication to prevent the propagation and rapid spread of these pathogens and ensure that there is limited economical loss of forestry and other agriculturally important plants and trees.

Since both *Xanthomonas* and *Pantoea* species are becoming increasing important as emerging bacterial pathogens, their rapid and accurate identification is crucially important. Little is known about bacterial pathogens on forestry trees since the most prominent diseases of these hosts are caused by fungi. The focus of this study was to investigate and identify the bacterial pathogens associated with *Eucalyptus.* However, as has been seen in various studies including this one, the identification of these pathogens is not always straightforward and often time consuming. In this study the use of polyphasic identification approach was used which employs a combination of phenotypic and genotypic identification techniques.

Both of the genera investigated in this study, namely *Pantoea* and *Xanthomonas,* have been found to infect a variety of agriculturally important plant hosts. *Pantoea* species have previously been isolated from *Eucalyptus* trees suffering from blight and dieback symptoms. The species isolated have included *P. eucalypti* from Uruguay, *P. vagans* isolated from Argentina, Colombia, Uganda and Uruguay and *P. deleyi* from Uganda. Since the first report of *Pantoea* on *Eucalyptus* trees from South Africa in 2002 it has spread locally causing sporadic outbreaks. This pathogen has also been isolated from *Eucalyptus* trees in other parts of the world including, Argentina, Colombia, Thailand, Uganda and Uruguay. X*anthomonas campestris* pv. *eucalypti* was previously found to cause disease on *Eucalyptus* trees in Australia. Since then, three other *Xanthomonas* species have been isolated from Eucalyptus, namely, *Xanthomonas* spp. from Brazil (Goncalves *et al.,* 2008), *X. vasicola* from South Africa and *X. fuscans* from Uruguay as seen in this study.

Phenotypic tests and 16S rRNA gene sequencing undertaken in this study provided an accurate indication of whether the isolates obtained belonged to the genera *Pantoea* or *Xanthomonas*. For a more accurate species identification, the use of a single gene approach (*GyrB* gene sequencing) and a whole genome typing approach (Amplified Fragment Length Polymorphism) was also used. Both of these techniques proved to be efficient in identification of *Pantoea* isolates but shortcomings were observed in using AFLP analysis for identification of unknown *Xanthomonas* isolates. Hypersensitivity response tests in tobacco leaves proved that the bacteria isolated from the diseased *Eucalyptus* leaf material are in fact plant pathogens. Inoculation of young *Eucalyptus* trees indicated that the isolated bacteria were indeed the causal agent of the disease symptoms on the trees thus fulfilling Koch's postulates. The pathogenicity trials also investigated the relationship between pathogen and host.

The ultimate conclusions from this study can briefly be summarized as followed:

- A polyphasic identification approach was reliable in identifying unknown species belonging to the family *Enterobacteriaceae*
- The 16S rRNA gene is conserved amongst members of the family Enterobacteriaceae and could only be used to identify isolates belonging to the Genus *Pantoea*
- *GyrB* gene sequencing and AFLP analysis identified unknown isolates to species level. Both of these techniques also indicated a set of isolates, possibly new species of the genus *Pantoea.* Further investigation i.e. DNA/DNA hybridization studies is needed to conclude this issue
- Koch's postulates was fulfilled and indicates that the causal agents of the observed disease were isolated
- A polyphasic identification approach for the rapid and reliable identification of unknown isolates including phenotypic techniques (Hugh Leifson testing, Gram determination) and genotypic tests (16S rRNA gene sequencing, *GyrB* gene sequencing and AFLP analysis). The latter techniques are more cost effective that the alternative golden standard DNA:DNA hybridization
- This polyphasic approach can also identify closely related plant pathogenic bacteria e.g. *Erwinia, Enterobacter*
- *Pantoea* species and possibly 5 new *Pantoea* species were isolated from *Eucalyptus* trees in the following countries across the globe: Argentina, Colombia, Rwanda, South Africa, Thailand, Uganda and Uruguay

- More than one *Pantoea* species is associated with bacterial blight and dieback of *Eucalyptus* trees in these countries
- This study shows that the distribution of the different *Pantoea* species in the continents is as follows:
    - **Africa**: *P. ananatis, P.dispersa, P. stewartii, P. vagans, P. eucalypti* and undescribed *Pantoea* species
    - **Asia:** *P. ananatis, P. dispersa*
    - **South America:** *P. ananatis, P.dispersa, P. vagans, P. eucalypti*

- Only three distinct lineages were observed when analyzing isolates belonging to the genus *Xanthomonas* with 16S rRNA gene sequencing. The 16S rRNA gene is highly conserved between species of *Xanthomonas*
- Rademaker *et al.,* (2000) and Parkinson *et al.,* (2008) used AFLP analysis and *GyrB* gene sequencing, respectively, for investigating the genetic relatedness between *Xanthomonas* species. The same methods were used in this study and also proved to be efficient in the identification of unknown *Xanthomonas* isolates
- *GyrB* gene sequencing proved to be the best identification method for *Xanthomonas* species. Unknown *Xanthomonas* species isolated from *Eucalyptus* trees in South Africa: Mtunzini was identified as *X. vasicola.* Unknown *Xanthomonas* isolates from diseased *Eucalyptus* in Uruguay was identified as *X. fuscans,* where no distinction could be made between the two *X. fuscans* subspecies
- Neither *X. fuscans* nor X. vasicola have previously been recorded to cause disease on *Eucalyptus* trees, this is thus a first report for both organisms
- Fulfilling Koch's postulates proved that isolated bacteria were indeed the causal agents of the disease symptoms and not a randomly isolated epi- or endophyte.

From the results it is also very clear that bacterial blight and dieback of *Eucalyptus* can be caused by different members of the genus *Pantoea*, since different *Pantoea* species were isolated from diseased leaves showing symptoms of bacterial blight and dieback. It is also possible that this disease is caused by a *Pantoea-Xanthomonas* complex in South Africa, since these isolates were isolated in combination with each other from South Africa: Mtunzini.