

***Pantoea* spp. associated with leaf and stem diseases of
*Eucalyptus***

by

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This thesis is dedicated to my parents, Hendrik and Louise, and my brother,
De Waal – Thank you

“On the road that I have taken, one day, walking, I awaken, amazed to see where
I have come, where I’m going, where I’m from. This is not the path I thought.
This is not the place I sought. This not the dream I bought, just a fever of fate
I’ve caught. I’ll change highways in a while, at the crossroads, one more mile.
My path is lit by my own fire. I’m going only where I desire.”

Dean Koontz – The book of counted sorrows



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 hitherto not been submitted for any degree at any other University.

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Preface

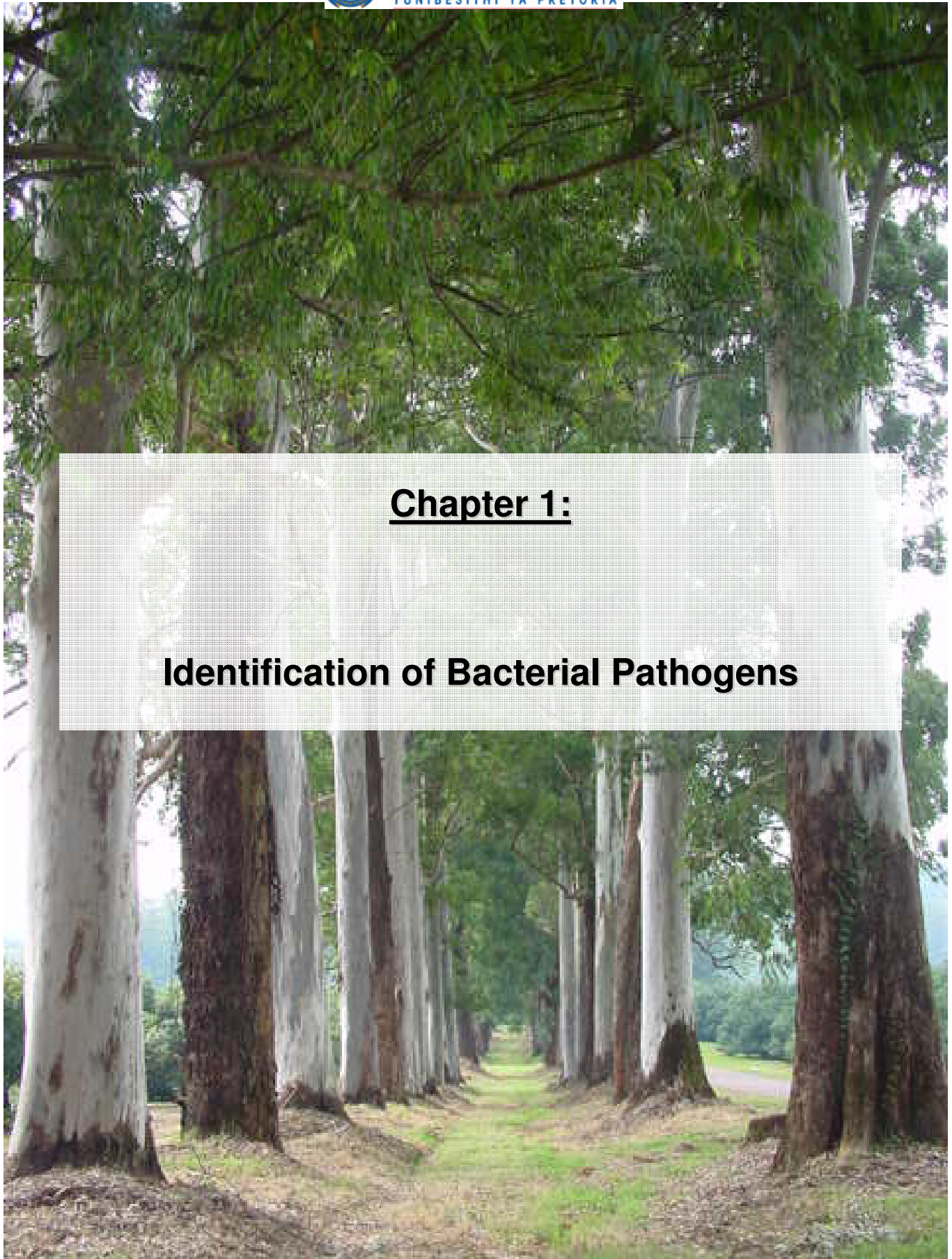
Global plantation forestry represents an expanding industry, mainly due to the increased awareness of the long-term sustainability of this resource and the reduced dependence on native forests for timber supplies. *Eucalyptus* species are among the most common tree species planted. The increase in global trade of *Eucalyptus* timber and timber products has increased the risk of introduced pathogens causing major losses in clonal as well as seedling plantation forestry. Comprehensive knowledge of these pathogens including studies of their origin, spread, virulence and interaction with other pathogens is needed to reduce their impact on plantation industries.

This thesis includes studies on bacterial species belonging to the genus *Pantoea*. Members of this genus have been reported from diverse sources, as pathogens, endo- and epiphytes. The taxonomy of this genus has been a point of great contention in the past. As techniques to identify and classify bacterial pathogens have evolved, we have developed a greater understanding of the relationship between members of the genus.

The first chapter represents a review of current methods employed in the identification and classification of bacterial plant pathogens. It also deals with the complexity of bacterial systematics and the challenges faced by modern bacteriologists. These challenges arise because multiple approaches need to be employed to identify and classify bacteria with no one single approach available. Techniques agreed upon by the global bacteriology community are often expensive and time consuming and not readily available to all. The need for expert knowledge on bacterial systematics in both the local and global forestry industry is also addressed.

The experimental sections of the thesis focus on bacterial pathogens belonging to the genus *Pantoea*. The potential interaction between two *Pantoea* species and the fungal pathogen, *Colletogloeopsis zuluense* is considered in chapter two. The identities of the two bacterial species are confirmed using phenotypic and genotypic characteristics. Pathogenicity trials were performed to consider the occurrence and nature of the purported interaction between these organisms.

In Chapter three, bacterial species isolated from diseased *Eucalyptus* tissue were identified. Their identification was made based on phenotypic characteristics as well as DNA-based identification methods. Pathogenicity screenings were also performed to determine the role of these isolates in the observed disease symptoms.



Chapter 1:

Identification of Bacterial Pathogens

1. Introduction

Forestry is one of the most important industries in South Africa, contributing substantially to foreign exchange and employment. Plantations, sustaining this industry, comprise approximately 1.5 million hectares (Anon 2005) distributed throughout Mpumalanga, Northern Province, KwaZulu Natal and the Eastern Cape. These plantations supply wood for pulp and paper, building timber, utility poles, support poles and various other wood and wood derived products. This constitutes a multimillion Rand industry dependent on wood for future growth and profit.

One of the three major tree species planted in South Africa is *Eucalyptus*. *Eucalyptus* (L'Heritier) species are adapted to grow in a wide range of climates and choice of species planted is dependant on climatic and other factors such as intended use of the trees i.e. pulp or saw timber etc. Factors such as their strong coppicing ability, the speed at which they recover from various growth impediments and their ability to survive adverse conditions contribute to the success of various *Eucalyptus* species in commercial forestry operations (Poynton 1979). Various other parts of the tree can be utilized in small scale industries such as tannins extracted from the bark and oils extracted from leaves used for medicinal, industrial or perfume purposes.

Various fungal pathogens of *Eucalyptus* have been found over the past 20 years but little is known about bacterial pathogens. *Colletogloeopsis zuluense* (syn. *Coniothyrium zuluense*) is a serious fungal pathogen causing cankers on *Eucalyptus* (Wingfield *et al.* 1996). In 1999, Van Zyl reported on the possible interaction between *C. zuluense* and two *Pantoea* species. The interaction between this fungus and the bacteria appeared to be synergistic where both microorganisms appeared to benefit from the interaction. Results showed that trees infected with *C. zuluense* and the two *Pantoea* species showed significant increase in lesion length compared to trees infected with *C. zuluense* alone (Van Zyl 1999). Interactions between bacteria and fungi resulting in increased virulence of either component have rarely been reported.

There have not been many reports of bacterial pathogens in forestry. Coutinho *et al.* (2002) described a bacterial leaf blight pathogen occurring on *Eucalyptus* causing severe damage in a single nursery in KwaZulu Natal. The pathogen has been identified as *Pantoea ananatis*, a member of the *Enterobacteriaceae*. This pathogen has subsequently spread to other major forestry regions in South Africa. The appearance of a disease similar to that caused by *P. ananatis* has been observed on *Eucalyptus* in Uganda, Uruguay and Thailand. The causal agent is unknown but it is believed to reside in the genus *Pantoea*.

This review serves as a background to studies in this thesis that deal with the interaction of bacteria and a stem canker disease caused by *Colletogloeopsis zuluense* (Van Zyl 1999) and the identification of the causal agent of the bacterial diseases observed in Thailand and Uruguay. The basis of the work relates to the taxonomy of these bacteria and the literature review focuses on this topic. The review is divided into two sections. The first of these deals with current methods used to distinguish between species in the Family *Enterobacteriaceae*. The second section deals with the historical developments in the classification of the various plant-associated bacteria in this Family, with a focus on *Pantoea*. The purpose of this review is to provide an overview of the evolution of taxonomy in the Genus *Pantoea* as identification techniques evolved to emphasize the use of a polyphasic approach to ensure a comprehensive taxonomic study of the genus in future.

2. Identification of plant associated Bacteria

Reliable identification of plant pathogenic bacteria is complex often requiring a suite of tests for conclusive classification and/or identification (Alvarez 2004). Bacteria are generally identified and classified based on two types of information or tests, genotypic information, which is derived from analyses based on the DNA or RNA of organisms, and phenotypic information which is derived from other sources e.g. function and structures of proteins, expression products of proteins and other metabolic and physiological characteristics (Stackebrandt *et al.* 1999).

Various API systems (bioMerieux SA, La balme-les-Grottes, France) are available for the identification of Gram Negative and Gram positive organisms. These use the utilization of various substrates to make appropriate identifications. Systems are available for identification based on presence or absence of certain enzymes. A disadvantage of the API systems is that additional tests are often needed before final identifications can be made (API, bioMerieux). Mergaert *et al.* (1984) and Verdonck *et al.* (1987) used various API systems to investigate and reclassify members of the genus *Erwinia*.

The Biolog Microlog MicroPlate system (Biolog, Inc., Hayward, USA) is available for Gram negative, Gram positive and Environmental cultures. This system consists of 96 dehydrated carbon sources on an ELISA plate that, when rehydrated with a fluid - containing the test organism - yields a colour change dependent on the Carbon utilization of the organism. In most cases no additional tests are required for identification except Gram stain properties (Microlog, Biolog). Klinger *et al.* (1992) tested the Biolog system with reference strains from the American Type Culture Collection (ATCC) and various water samples and found that the system could identify 98% of the ATCC strains and 93 % of the water isolates correctly. They did, however, find that identification of members of the genera *Enterobacter*, *Klebsiella* and *Serratia* was unreliable, but they could solve this problem by the inclusion of other key tests e.g. oxidase test.

When the two systems (API and Biolog) were compared, the results showed that API 20NE and Biolog GN identifications were in agreement at the genus level for environmental strains (Truu *et al.* 1999). There was, however, low consensus when results were compared on species level. More reliable results were obtained with the Biolog system because of the higher number of substrates used (Truu *et al.* 1999).

2.1.2. Fatty Acid Methyl Ester (FAME) Analysis

Fatty acids are the major building blocks of all lipids and lipopolysaccharides present in cells (Campbell 1999). Variations in chain length and positions of

double bonds can be used to distinguish between bacteria based on the different profiles created by different fatty acid compositions present in bacteria (Busse *et al.* 1996). Heyrman *et al.* (1999) and Peltroche-Llacsahuanga *et al.* (2000) stated the importance of highly standardized growth conditions needed for accurate analyses. In addition, Peltroche-Llacsahuanga *et al.* (2000) found that the amount of cell mass used for profiling has a significant influence on the results. This confirms the importance of growth conditions. FAME's have been used to identify bacteria from various sources including the roots of field-grown plants (Siciliano and Germida 1999). FAME's are useful as a third or fourth level identification technique but other techniques are needed to make a correct and comprehensive identification (Heyrman *et al.* 1999).

2.1.3. SDS-PAGE of whole cell proteins

The degree of similarity between protein patterns of different organisms can be used to identify bacteria to genus and species level using Polyacrylimide Gel Electrophoresis (PAGE) (Busse *et al.* 1996). It is, however, important that parameters like running conditions and weight markers used, be highly standardized (Vandamme *et al.* 1996). Beji *et al.* (1988) used SDS-PAGE of whole cell proteins to further study the relatedness of various *Erwinia* species. The PAGE analysis revealed seven electrophoretic groups with characteristic protein patterns. This led to the proposal of the subjective synonymy of strains received as *Enterobacter agglomerans*, *Erwinia herbicola* and *Erwinia milletiae*. The type strains of *Erwinia ananas* and *Erwinia uredovora* were, however, distinctly different based on protein patterns and DNA:DNA hybridization results (Beji *et al.* 1988).

2.2. Genotypic methods

Genotypic methods often present a rapid and reliable alternative to the classification and identification of particularly large amount of strains compared to phenotypic methods (Stackebrandt *et al.* 1999). Increased development of new techniques and improvement over old techniques have been prevalent in the last 30 years. This is subsequent to Woese (1987) showing that the 16S rRNA gene can be used to distinguish between *Archaea*, *Bacteria* and *Eucarya*. Methods have been described to investigate

the variation within genes or gene sequences (e.g. 16S and 23S rRNA genes) as well as methods for comparing whole genomes. Some of these methods will be discussed further in the following sections.

2.2.1. Methods based on whole genome analyses and comparisons

2.2.1.1. DNA:DNA Hybridization

The Ad Hoc Committee on the Reconciliation of Approaches to Bacterial Systematics defined the species concept of bacteria based on DNA Hybridization (Wayne *et al.* 1987). Here, those strains that show 70% or more DNA relatedness and have less than 5°C difference in melting temperature of DNA (T_m) represent distinct taxa (Wayne *et al.* 1987). The committee, however, felt strongly that DNA:DNA hybridization must be substantiated by phenotypic data. DNA:DNA hybridization studies, which are defined as the “indirect parameter of sequence similarity between two entire genomes” (Vandamme *et al.* 1996) have been used to classify or reclassify bacteria (Gavini *et al.* 1989; Kageyama *et al.* 1992; Mergaert *et al.* 1993; Rademaker *et al.* 2000; Jones *et al.* 2004). A disadvantage of DNA:DNA hybridization is the fact that the results are not always reproducible between laboratories, in addition, different hybridization methods yield different results even within the same laboratories (Vandamme *et al.* 1996). This is a labour intensive technique, needing highly standardized apparatus and only laboratories “highly specialized in bacterial systematics” have the infrastructure to perform the analyses and produce reliable results (Busse *et al.* 1996; Stackebrandt *et al.* 1999).

2.2.1.2. Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphisms (RFLP) is a DNA-based typing method commonly used in the identification of bacteria (Vandamme *et al.* 1996). Initially the whole genome of an organism was digested with one or a combination of restriction enzymes to yield a specific banding pattern (Vandamme *et al.* 1996). With the advent of the Polymerase Chain Reaction (PCR) so called PCR-RFLP’s have been used to amplify specific genes and detect variation within those genes (Watanabe & Sato 1998). The techniques have been used to identify members of *Erwinia* (Toth *et al.* 2001). Waleron *et*

al. (2002) used PCR-RFLP's of the *recA* gene fragment to identify and distinguish between members of the genus *Erwinia*. Pulsed-Field Gel Electrophoresis (PFGE) is another variation of the RFLP technique. It can be modified to distinguish between members of a specific genus or strains of a species (Zhang & Geider 1997).

2.2.1.3. Ribotyping

Ribotyping is a variation on the RFLP technique where labelled rRNA or rDNA fragments are used as universal probes to combine RFLP with the Southern Blot technique. The resolution of the banding patterns obtained using this technique depends on the species being studied and the choice of restriction enzyme (Stackebrandt *et al.* 1999; Lefresne *et al.* 2004). This technique has been automated (Grif *et al.* 2003) and can also be adapted to probe specific gene fragments utilizing the polymerase chain reaction (PCR) to amplify the gene of choice (Meays *et al.* 2004).

2.2.1.4. Amplified Fragment Length Polymorphism (AFLP)

The Polymerase Chain Reaction (PCR) has also been used as a basis for techniques based on whole genome analysis. Amplified Fragment Length Polymorphisms (AFLP) and variations of the technique have also become a popular method for the study of bacteria. Mueller & Wolfenbarger (1999) defined AFLP's as "...PCR-based markers for the rapid screening of genetic diversity". This is a useful classification technique that can be used for identification purposes once a comprehensive database, of suitable profiles, has been compiled. AFLP's have been used successfully to distinguish between *Erwinia carotovora* and *Erwinia chrysanthemi*, two closely related species (Avrova *et al.* 2002), identify pathogenic bacteria (Velappan *et al.* 2001; Jonas *et al.* 2004; Gzyl *et al.* 2005) and, using cDNA-AFLP's, to elucidate pathogenicity regulating genes in *Xanthomonas campestris* pv. *vesicatoria* (Noël *et al.* 2001).

2.2.2. Single or Multiple Gene methods

2.2.2.1. PCR based methods

Some bacteria can be identified based on the presence of unique genes or parts of genes. Audy *et al.* (1996) used primers specific for toxins of *Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola*, causal agents of common and halo blight of beans respectively. The primers were used separately and in combination to identify these organisms in seed, thus eliminating laborious culturing and phenotypic testing. The fireblight pathogen, *Erwinia amylovora*, has been identified from diseased tissue using the *amsB* gene which encodes for the synthesis of the capsular exopolysaccharide amylovoran (Bereswill *et al.* 1995). The *syrD* gene is necessary for the production of lipodepsipeptide toxins. The coding region of this gene was used to identify *Pseudomonas syringae* pathovars from diseased plant tissue (Bultreys and Gheysen 1999).

The 16S rDNA has also been used extensively for bacterial taxonomy. Restriction analysis of the amplified 16S gene with a unique restriction enzyme to yield restriction patterns (ARDRA – Amplified Ribosomal DNA Restriction Analysis) has been used to identify various marine bacteria (Caccamo *et al.* 1999). Bereswill *et al.* (1995) digested the amplified 16S gene with four different restriction enzymes and found that *haeIII* produced a unique restriction pattern that could be used to identify *Erwinia amylovora*.

Differences in number of rRNA operons and restriction sites located in these operons give rise to variations in length of the 16S-23S internal transcribed spacer regions between bacteria. The numbers of copies of the rRNA operons contained within bacterial species differ and the number and composition of the tRNA genes contained in the genome varies which contributes to length variations among bacteria. These variations make the 16S-23S spacer region ideal for identifying and typing bacteria without the need for direct sequencing (Gürtler *et al.* 1996). Zavaleta *et al.* (1996) used the amplified 16S-23S ITS region in a RFLP study to distinguish between *Leuconostoc oenos* and other related species included in the study.

Multiple genes can also be used in a multiplex PCR reaction to identify species using one PCR reaction. Multiplex PCR identification has been used to identify *Campylobacter jejuni* and *Campylobacter coli* using various virulence genes (Nayak *et al.* 2005), various enterotoxigenic *Staphylococcus aureus* (Cremonesi *et al.* 2005), and for the simultaneous genus- and species-specific identification of various Enterococci (Jackson *et al.* 2004). Metherell *et al.* (1997) used single and multiplex PCR to identify the host organisms using primers specific for type II restriction enzyme sequences. In a variation of this technique, Lee *et al.* (2004) used a multiplex PCR with primers specific for the 16S rDNA gene producing two bands. The larger of the two was then digested with a restriction enzyme to identify *Lactobacillus* species from kimchi.

2.2.2.2. Sequence analyses

Ribosomal RNA homology studies have been used extensively since Woese (1987) showed that the 16S rRNA gene could be used to distinguish between Eubacteria and Archaeobacteria and to distinguish between various members of the Eubacteria. The fact that rRNA is present in all bacteria, is composed of constant and variable regions (Vandamme *et al.* 1996), is functionally constant, is large and the amount of domains differ between bacteria (Woese 1987) makes them ideal molecules to use in determining the amount of relatedness of bacteria to one another.

The 16S rRNA gene is more widely used than the 23S rRNA gene for bacterial identification, mainly due to the size difference between these two genes. More 16S rRNA gene sequences are available for comparison studies as it is easier to amplify and sequence, as it is smaller (about 1.5 kb) than the 23S gene which is about 3 kb in size (Gürtler & Stanisich 1996). 16S rRNA gene sequences have been used in studies to determine relatedness and phylogenetic positions of members of various bacterial families and genera e.g. *Erwinia* (Kwon *et al.* 1997), *Xanthomonas* (Hauben *et al.* 1997), family *Enterobacteriaceae* (Hauben *et al.* 1998) and *Mycobacterium* (Turenne *et al.* 2001).

Sequencing of the 16S rRNA gene has proven useful in studies where bacteria could not be cultured, whether they were unculturable or too dangerous and pathogenic to culture. Weisburg *et al.* (1991) proved that lyophilised ampules of known pathogenic bacteria could be used to amplify full length 16S rDNA gene sequences without culturing these pathogens. This made microbial diversity studies in habitats such as activated sludge (Snaird *et al.* 1997), soils (Dunbar *et al.* 1999) and glaciers (Miteva *et al.* 2004) possible. Sequences obtained can be used in basic local alignment searches (BLAST) to identify homologues from online databases like Genbank.

Although the 16S rDNA has proved to be very useful in the identification of various previously unidentifiable bacteria (Drancourt *et al.* 2000) and new species (Wise *et al.* 1997), various authors have noted that the inability of 16S rRNA to distinguish between closely related species of the same genera is a major drawback (Lawrence *et al.* 1991; Stackebrandt and Goebel 1994). Stackebrandt and Ludwig (1994) discussed the importance of outgroup usage and reference strain inclusions in the outcome of 16S rRNA sequence analysis and state that this can have a profound effect on results and conclusions made in different analyses.

The 16S-23S internal transcribed spacer sequence has emerged seemingly to be the solution to resolving taxa where the 16S gene has failed to do so. Leblond-Bourget *et al.* (1996) found that they could successfully distinguish and reclassify members of *Bifidobacterium* based on 16-23S ITS sequences. They determined that the evolutionary rate of the 16S-23S ITS region is much higher than that of the 16S rRNA gene.

Other gene sequences have been used in the classification and identification of bacteria. Brown *et al.* (2000) used the Glyceraldehyde-3-phosphate dehydrogenase gene sequences to determine relatedness of *Erwinia* and *Brenneria* species. Lawrence *et al.* (1991) used the Glyceraldehyde-3-phosphate dehydrogenase and outer membrane protein 3A gene fragments to analyze and determine molecular relationships of certain enteric bacteria. Chan *et al.* (2003) developed a *recA* based PCR test to identify *Burkholderia fungorum*. Duaga (2002) used the *gyrB* and 16S rDNA to compare members

of the *Enterobacteriaceae* and found that *gyrB* was more effective for comparing closely related species while the 16S was more suitable for distantly related *Enterobacteriaceae*. This was confirmed by Van Houdt *et al.* (2005) when using *gyrB* to identify a biofilm-forming *Serratia* species.

It is clear that every technique, despite its apparent advantages, has inherent disadvantages to disqualify it as a stand-alone approach. It is apparent that, for authoritative taxonomy, a combination of approaches will need to be employed to reach reliable species classification and identification.

3. Taxonomy of *Pantoea*

The systematic approach to the classification of plant pathogenic *Pantoea* is perpetually evolving. This is due to the high level of relatedness between species, making exact classification tedious and complicated. To understand how this genus and other *Enterobacteriaceae* are classified, the context in which the family is placed systematically will be elaborated upon. Thus the following section describes the evolution of contemporary methodology in *Pantoea* classification.

3.1. Genus *Erwinia* – In the beginning...

The genus *Erwinia* was first proposed by Winslow *et al.* (1917) for all “Gram negative, non spore-forming, peritrichous, fermentative, rod-shaped bacteria that are plant-associated either as pathogens, saprophytes or epiphytes.” The genus was classified in the family *Enterobacteriaceae*. It was described to accommodate mostly plant-associated bacteria (Kwon *et al.* 1997). Yet the family has become a repository for bacteria characterized by yellow colonies found to be plant pathogenic or plant-associated.

In 1968 and 1969, Dye attempted to regroup members of the genus *Erwinia* into four distinct groups based on certain characteristics shared by some members of the genus. The “Amylovora” group contained all the so-called fireblight organisms, which produce dry necrotic lesions (Dye 1968). The “Carotovora” group contained all pectolytic organisms of the genus causing soft rot symptoms (Dye 1969a). The “Herbicola” group contained all those organisms that produced yellow pigmented colonies (Dye 1969b). Included in

this group was the pathogen *E. ananas*, first described by Serrano in 1928 as the causal agent of brown fruitlet rot of pineapple. Dye (1969b) found *E. ananas* to be closely related but sufficiently different to be classified as a variety of *E. herbicola* known as *E. herbicola* var. *ananas* (Serrano) comb.nov. 1969, with the synonym *E. ananas* Serrano 1928. *Erwinia stewartii* was also retained as a separate species in the group with *E. herbicola* var. *herbicola* as type species. Dye (1969b) hypothesized that *E. herbicola* is a saprophyte common to a wide variety of plants and due to inadequate pathogenicity tests it has been given an inordinate number of names. It has also been incorrectly identified as the causal agent of many diseases, because it is a common rapidly-growing organism that simply overgrew the actual pathogen (Dye 1969b). The last group contained all the “Atypical” *Erwinias*. These include *E. dissolvens*, *E. nimipressuralis* and *E. proteamaculans* (Dye 1969c).

Starr & Mandel (1969) found that the groups proposed by Dye (1968, 1969a, 1969b, 1969c) are composed of organisms with statistically different GC contents but he did not propose any nomenclatural changes, preferring to take an “agnostic” stance on *Erwinia* taxonomy. Twenty-two different species and pathovars were proposed for the genus *Erwinia* by Young *et al.* in 1978. *Erwinia herbicola* was mentioned as a non-pathogen found commonly associated with pathogens in diseased tissue. Pathogenic species belonging to the *herbicola* group included *E. ananas* pv. *ananas*, *E. ananas* pv. *uredovora*, and *E. stewartii* (Young *et al.* 1978).

The taxonomy of the genus *Erwinia* was again investigated by Dye in 1981 and he found that none of the analyses he used in this study supported his earlier suggestion that the genus be divided into the four groups. He found that *E. stewartii* was more constantly grouped with *E. amylovora* and proposed that *E. amylovora*, *E. stewartii*, *E. salicis* and *E. tracheiphila* be regarded as distinct species. Because *E. ananas* and *E. uredovora* consistently grouped apart from *E. herbicola*, Dye (1981) proposed that they also be regarded as distinct species, with *E. uredovora* as a pathovar of *E. ananas* due to its distinctive pathogenicity.

Using phenotypic and protein electrophoretic data obtained from strains of *Enterobacter agglomerans*, *E. herbicola* and *E. milletiae*, Mergaert *et al.* (1983) found a distinct overlap between these species and their type strains. They also found no distinct phenotypic characteristic that could be used to distinguish between strains belonging to these species. They proposed that a more general approach is needed to distinguish between members of the *Enterobacter agglomerans* – *Erwinia herbicola* complex. Attempts by Brenner *et al.* (1984) to distinguish between members of this group failed due to inconclusive results. Mergaert *et al.* (1984) confirmed the heterogeneity of this group when both the type strains of *Enterobacter agglomerans*, *E. herbicola* and *E. milletiae* grouped together in the same “subphenon”. Beji *et al.* (1988) concluded that *Enterobacter agglomerans*, *E. herbicola* and *E. milletiae* were synonyms.

Mergaert *et al.* (1984) attempted to clarify the taxonomic chaos that existed in the genus *Erwinia* by using phenotypic characteristics (API Systems). Their analyses revealed 12 phenons with 6 definite sub-phenons which corresponded to established *Erwinia* species. They could, however, not make a clear distinction between the amylovora, carotovora and herbicola groups. They found that *E. stewartii* grouped phenotypically more closely to the amylovora species than any of the herbicola species. They proposed the retention of *E. uredovora* as a pathovar of *E. ananas* due to their close proximity grouping. These results were confirmed by Verdonck *et al.* (1987).

3.2. Genus *Pantoea*

A new genus, *Pantoea* gen.nov. was proposed by Gavini *et al.* in 1989. They proposed the transfer, based on DNA hybridization results and phenotypic and genotypic data, of *Enterobacter agglomerans* (Beijerinck 1888) Ewing & Fife 1972 to the new genus as *Pantoea agglomerans* (Beijerinck 1888) comb.nov., type strain of the new genus. The name *Pantoea* is from Greek “*pantioios*” that refers to many “sorts and sources”. They also described *Pantoea dispersa* as a new species in the genus.

Three new species of *Pantoea* isolated from soil and fruit in Japan were described as *P. citrea*, *P. punctata* and *P. terrea*. DNA hybridization and

phenotypic results showed they differed significantly from *P. agglomerans* and warrant new species descriptions (Kageyama *et al.* 1992). Using DNA hybridization results and other phenotypic and genotypic data Mergaert *et al.* (1993) proposed the transfer of *Erwinia ananas* and *Erwinia stewartii* to the genus *Pantoea* as *Pantoea ananas* (Serrano 1928) comb.nov. and *Pantoea stewartii* (Smith 1898) comb.nov. They found that *E. ananas* and *E. uredovora* were genotypically highly related and proposed that they are subjective synonyms rather than pathovars of each other with *ananas* having “nomenclatural priority”. They also found that one of the hybridization groups was subdivided into two subgroups with distinct differences in biochemical characteristics as well as fatty acid composition. This led to the proposal that they be classified as sub-species namely, *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes*. These are the causal agents of Stewart’s wilt in maize and suspected causal agent of leaf spot on fox tail and pearl millet and rot of *Ananas comosus* (pineapple) respectively. Species classified in the genus *Pantoea* at present are *P. agglomerans* (type species), *P. ananatis*, *P. citrea*, *P. dispersa*, *P. punctata*, *P. stewartii* subsp. *stewartii*, *P. stewartii* subsp. *indologenes* and *P. terrea* (Brenner *et al.* 2005).

3.3. Genus *Pantoea* – separate but related to *Erwinia*

Based on 16S rRNA gene sequences, the members of the genus *Erwinia* and related genera were investigated (Kwon *et al.* 1997). Four clusters intermixed with other members of the *Enterobacteriaceae* such as *E. coli*, *Klebsiella* and *Serratia* were found. Cluster one contained the type strains of *E. ananas*, *E. uredovora*, *E. herbicola*, *E. milletiae* and *E. stewartii*, species that have already been transferred to the Genus *Pantoea*. The authors could, however, not fully support the reclassification of these strains in the genus *Pantoea* (Kwon *et al.* 1997). In contrast Hauben *et al.* (1998), using almost complete 16S rDNA sequences, divided the *Erwinia* species into three phylogenetic groups with the genus *Pantoea* and species contained within this genus forming a monophyletic unit closely related to *Erwinia*.

With the emphasis of taxonomy shifting more to molecular techniques Waleron *et al.* (2002) used PCR-RFLP’s of a *recA* gene fragment to identify and distinguish members of the genus *Erwinia* and closely related genera.

They found that the species reclassified by Hauben *et al.* (1998) showed five RFLP patterns, some common to more than one species. *P. stewartii* belonged in a group of its own whilst *P. ananatis* strains were divided into two groups, one of which contained *E. uredovora*. They found that those strains of *P. ananatis* and *E. uredovora* displaying ice-nucleating activity were grouped in one group whilst those strains without this activity fell into the other group. Species reclassified into the new genus *Brenneria* also formed a RFLP group of their own. The authors could make very preliminary correlations between RFLP groups per species to host-range and specificity as well as to geographical distributions (Waleron *et al.* 2002).

Although much work has been done on the taxonomic position of the various plant pathogens within the *Enterobacteriaceae*, confusion still exists. The herbicola-agglomerans group has not been fully resolved, even with the transfer of some of the species to a new genus. It is often difficult to identify these bacteria based on phenotype alone as they are so similar and one often has to rely on a range of techniques to correctly identify them.

4. Plant pathogenic bacteria associated with *Eucalyptus*

The first report of a bacterial pathogen causing disease on *Eucalyptus* in South Africa was in 2000 (Coutinho *et al.*). Bacterial wilt of *Eucalyptus*, caused by the pathogen *Ralstonia solanacearum* was first noticed on a *Eucalyptus grandis* x *Eucalyptus camaldulensis* (GC) hybrid in Zululand, KwaZulu Natal in 1997. The disease is characterized by symptoms such as wilting of growth tips, brown discoloration in the sapwood of stems and rapid death of trees usually within 6 months of infection. This pathogen was first reported from *Eucalyptus* in Brazil in 1983 (Sudo *et al.* 1983). This disease has subsequently been reported from various continents including Africa (Roux *et al.* 2000; Roux *et al.* 2001), Australia (Pegg *et al.* 2003) and South America (Alfenas *et al.* 2006).

In 2002 a bacterial blight disease was reported from KwaZulu Natal on *Eucalyptus grandis* x *Eucalyptus nitens* (GN) hybrids in a single nursery (Coutinho *et al.* 2002). The causal agent was identified, using pathogenicity screenings, Biolog tests, fatty acid profiles, %G+C content, 16S rDNA gene

analysis and DNA:DNA hybridization, as *Pantoea ananatis*. Symptoms include tip die-back leading to the formation of epicormic shoots, giving plants a decidedly stunted appearance. Watersoaked leaf spots that become corky with age are also characteristic of the disease. This disease has subsequently spread to various regions and can infect a multitude of *Eucalyptus* hybrids and clones (Coutinho *et al.* 2002).

Some bacterial pathogens, associated with *Eucalyptus*, have been reported from other countries. Truman (1974) reported on a bacterial pathogen causing die-back symptoms on *Eucalyptus citriodora*. The bacterium was identified as *Xanthomonas eucalypti*. Another *Xanthomonas* sp., *Xanthomonas axonopodis*, was reported as causing disease on various *Eucalyptus* spp. in South America (Alfnas *et al.* 2004). The disease is also characterized by necrotic lesions on leaves, death of young shoots and defoliation of young trees. None of these pathogens have been reported from South Africa to date. A disease with similar symptoms was reported from Uganda and the causal agent was provisionally identified as *Pantoea ananatis* based on phenotypic data and 16S rDNA gene sequence analysis (Nakabonge 2002). DNA:DNA hybridization analyses, however, showed that the associated bacteria were not *Pantoea ananatis* (Coutinho unpublished). Similar diseases have also been noticed in Uruguay and Thailand, and although the causal agent has not been conclusively identified, it is believed that they reside in the genus *Pantoea*.

5. Concluding Remarks

As technology has developed and advanced, a number of techniques have become available for the identification and classification of bacteria. Yet, there are problems that existed with the identification and taxonomy of some genera 15 years ago, that still persist. There is clearly no one definitive technique that can be used by all bacteriologists and taxonomists to identify bacteria. This needs to be recognized and multiple approaches are recommended.

Even with the development of sophisticated genotyping methods, the need for phenotypic data in bacterial taxonomy remains great, albeit only to provide

useful information to the scientific community (Busse *et al.* 1996; Vandamme *et al.* 1996; Alvarez 2004). Therefore, a polyphasic approach to the taxonomic study of bacteria is needed, incorporating both phenotypic and genotypic data to give the best possible results. Vandamme *et al.* (1996) stated that “Polyphasic taxonomy is not hindered by any conceptual prejudice except that the more information that can be integrated on a group of organisms, the better the outcome might reflect its biological reality.” This is especially true when dealing with the genus *Pantoea* and related genera and we support this view strongly.

Comprehensive systematics of pathogens in forestry is not only a priority but a necessity. We need to know what pathogens occur and where and how they function and spread (i.e. biology), not only to prevent the spread of these pathogens to non-infected areas but also to identify potential threats and react appropriately. The rapid spread of bacterial blight from a single nursery to other forestry regions in South Africa is testament to the need for, at the very least, basic systematic knowledge. The impact of this disease has been relatively minor when compared to other major pests and diseases, but maybe we have just been lucky thus far. Comprehensive knowledge of the causal agent is the only way to ensure future preparedness. Increased international trade of wood and wood products have compounded the problem of introduced pests and diseases and exacerbated the need for at least basic knowledge of bacterial and fungal systematics (Rossman & Miller 1996).

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Chapter 2:

Possible interactions between two *Pantoea* species and the *Eucalyptus* canker pathogen, *Colletogloeopsis zuluense*

Abstract

In 1999, *Pantoea ananatis* was reported as being one of two *Pantoea* species involved in a synergistic interaction with *Colletogloeopsis zuluense*, a known *Eucalyptus* pathogen, increasing the pathogenicity of the fungus. The second *Pantoea* member of the proposed interaction was preliminary identified as being closely related to *P. stewartii* subsp. *stewartii*. In this study the identity of the two *Pantoea* species were confirmed using the API identification system and other phenotypic characters as well as 16S rDNA gene sequence data analyses. Pathogenicity trials were also performed to reproduce the interaction found between the fungus and two bacterial species. The identity of the *P. ananatis* strain was confirmed using phenotypic and 16S data. The strain thought to be closely related to *P. stewartii* subsp. *stewartii* was identified as *P. stewartii* subsp. *indologenes* based on phenotypic and genotypic data. Pathogenicity tests to confirm the interaction between the three organisms failed, as no significant increase in lesion length was obtained when *C. zuluense* was inoculated in conjunction with the two bacteria compared to inoculations with the fungus alone.

Introduction

Non-native *Eucalyptus* species make up approximately 87% of hardwood species planted in South African forestry plantations (Chamberlain *et al.* 2005b). Approximately 53% of these plantations are situated in the Kwazulu Natal Province (Chamberlain *et al.* 2005b). The majority of *Eucalyptus* timber planted in South Africa is used for pulp and paper purposes whilst the remainder is used for mining timber, pole production and various other small industry uses (Poynton 1979; Chamberlain *et al.* 2005b).

The South African forestry and related industries contribute approximately R12 billion to the Gross Domestic Product (GDP) (Chamberlain *et al.* 2005a). Plantation forestry alone, contributed an estimated R3 billion in 2003 (Chamberlain *et al.* 2005a). Therefore, *Eucalyptus* pathogens like *Chrysosporthe austroafricana* Gryzenh, M.J. Wingf. (Gryzenhout *et al.* 2006), and *Colletogloeopsis zuluense* (M.J. Wingf., Crous & T.A. Cout.) M-N. Cortinas, M.J. Wingf. & Crous (Cortinas *et al.* 2006a), and the impact these native and introduced pathogens can have, is of great concern, not only to the forestry sector, but the general economy of South Africa (Wingfield 2003).

Colletogloeopsis zuluense was first reported as *Coniothyrium zuluense* Wingfield, Crous & Coutinho, infecting a susceptible *Eucalyptus* clone, ZG14 (Wingfield *et al.* 1996). This pathogen has subsequently been found infecting a variety of other *Eucalyptus* hybrids and clones (Van Zyl *et al.* 2002b). The pathogen was reclassified as *Colletogloeopsis zuluense* based on morphology and DNA sequence analyses (Cortinas *et al.* 2006a).

The canker disease caused by *C. zuluense* is characterised by the formation of necrotic lesions, usually on young stem tissue. Kino exudation can occur if the infections are severe, and the lesions can girdle the stem and young tissue leading to the formation of epicormic shoots or “feathering”. Typically these lesions are found on and in the bark but when infections are severe they spread into the wood fibre which can lead to stunted growth and top die-back (Wingfield *et al.* 1996).

It has subsequently been shown that this pathogen has a wide geographical distribution. The disease has been reported from countries like Thailand (Van Zyl *et al.* 2002a), Mexico (Roux *et al.* 2002), Hawaii (Cortinas *et al.* 2004), Ethiopia (Gezahgne *et al.* 2003, 2005) and China (Cortinas *et al.* 2006a). A disease with similar symptoms was reported from Uruguay, South America, but it has been shown that the pathogen responsible is a different species belonging to the genus *Colletogloeopsis*, *Colletogloeopsis gauchensis* M.-N Cortinas, Crous & M.J. Wingf (Cortinas *et al.* 2006b)

In his study of *C. zuluense*, Van Zyl (1999) consistently isolated two bacterial species associated with *C. zuluense* from cankers exuding copious amounts of kino. He identified these bacteria as *P. ananatis* and a species closely related to *P. stewartii* subsp. *stewartii*. Preliminary results from a later study by Brady (2005) indicated that the latter species was *P. stewartii* subsp. *indologenes*. Van Zyl (1999) proposed, based on pathogenicity results, that the relationship between the two bacteria and *C. zuluense* was synergistic in nature.

The bacteria reportedly involved in the above mentioned interaction, have been reported as phytopathogens in their own right. *P. ananatis* has been found to be the causal agent of bacterial blight on *Eucalyptus* in South Africa (Coutinho *et al.* 2002). *P. stewartii* subsp. *indologenes* is believed to be the causal agent of leaf spot of fox and pearl millet and rot of pineapple (*Ananas comosus*) (Mergaert *et al.* 1993). The latter pathogen has not been reported from *Eucalyptus*.

The inoculation studies done by Van Zyl (1999) showed that inoculations with the two bacteria, *P. ananatis* and *P. stewartii* subsp. *indologenes*, and *C. zuluense*, produced a significant increase in lesion size when compared to inoculations with *C. zuluense* alone. A slight, although not significant, increase was observed when *C. zuluense* was inoculated with *P. ananatis* when compared with inoculations of *C. zuluense* with *P. stewartii* subsp. *indologenes*. The bacteria inoculated alone or in combination produced no lesions.

Interactions between bacteria and fungi such as the one suggested for *C. zuluense* and the *Pantoea* spp., have previously been described. For example, Dewey *et al.* (1999) found a number of bacterial strains associated with the fungal pathogen, *Stagonospora (Septoria) nodorum* (Berk.) Castellani, E.G. Germano = *Septoria nodorum* (Berk.) Berk. Teleomorph: *Phaeosphaeria nodorum* (E. Muller) Hedjaroude = *Leptosphaeria nodorum* E. Muller, the causal agent of glume blotch of wheat. In pathogenicity trials they found that *Xanthomonas maltophila* and *Sphingobacterium multivorme* enhanced the speed and size of developing lesions. No lesions were formed by either bacteria alone. They also found that each of the isolates of *S. nodorum* from artificially and naturally infected material was associated with only one species of bacterium. Likewise, DaPeng *et al.* (1999) found that ice nucleation active (INA) bacteria, which included *P. ananatis*, associated with *Dothiorella gregaria* infections, increased the incidence of *Dothiorella* infections as well as the size of lesions.

The severity of stem cankers caused by *C. zuluense* has been largely negated with the selection and breeding of resistant *Eucalyptus* clones. The disease still occurs but is not as serious in South Africa as it was when first described. The effect that an interaction between *Pantoea* spp. and the fungus such as that described by Van Zyl (1999), could have on the disease situation is unknown. The aim of this study therefore was to confirm the identity of the *Pantoea* species and to repeat the pathogenicity trials with the fungus and the bacteria.

Materials and Methods

Surveys

In the period between 2003 and 2005, surveys were undertaken at Venters plantation in the Zululand forestry region in Kwazulu Natal. The surveys were conducted to specifically determine whether the *Pantoea* species remain a common feature of infections of *Eucalyptus* stems by *C. zuluense*. Samples were taken from twenty *Eucalyptus* (clone ZG14) trees showing typical symptoms of those induced by *C. zuluense*. These samples were taken from the infected *Eucalyptus* compartment previously identified by Van Zyl (1999)

as a site where the bacteria had previously been isolated. Surveys were conducted during spring, summer and autumn months. Lesions produced by *C. zuluense* were inspected under a dissection microscope to identify pycnidia of the fungus bearing spores. Masses of conidia from ten lesion were transferred to ten 2 % Malt Extract Agar (20g Malt Extract Broth [(Merck, Germany), 20 g Agar in 1L distilled H₂O) plates per lesion. Duplicate samples were placed on ten Nutrient agar (16g Nutrient Broth [Biolab, Biolab Diagnostics, Merck], 15 g Agar in 1L distilled H₂O) plates per lesion to obtain bacterial isolates. Fungal material was identified as *C. zuluense* based on spore morphology.

Bacterial Identification

Bacterial strains, isolated by Van Zyl (1999), were obtained from the bacterial culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. Pure cultures of the isolates, previously identified as *Pantoea ananatis* (BCC 110) and the tentatively identified *Pantoea* species (BCC 118), were streaked onto Nutrient Agar (16g Nutrient Broth [Biolab, Biolab Diagnostics, Merck], 15 g Agar in 1L distilled H₂O). These cultures were used in subsequent phenotypic and DNA sequence-based characterisation as well as in pathogenicity trials.

Phenotypic Characterisation

Gram stain characteristics were determined and oxidation fermentation tests (OF Basal medium [Difco] in 1L distilled H₂O; 10 ml of 10% Glucose [Sigma] solution filter sterilized and added after autoclaving) performed according to manufacturer's instructions. Further identification was done using API 20E strips (bioMérieux). Strips were inoculated with the test isolates, in 5 ml sterile distilled H₂O, according to manufacturer's instructions and profiles were identified using Analytical Profile Index (ApiLab) identification software.

DNA Sequence Comparisons

DNA Extraction

Single bacterial colonies were grown overnight in Nutrient broth in a shake incubator at 25 °C. DNA extractions were performed using the DNeasy

Classification and subsequent identification of bacteria is typically based on various levels of screening with each level requiring more in depth testing than the previous. Initial screening would typically include simple phenotypic tests such as the Gram stain and Oxidation-Fermentation tests. Commercial and Automated Identification systems would comprise the next level of screening and from there one could move into genotypic screening. The focus of this section of the review is on different techniques used in a polyphasic approach to classify plant pathogenic bacteria in general with special emphasis on members of the *Enterobacteriaceae*.

2.1. Phenotypic and Chemotaxonomic Methods

Phenotypic analyses form the basis for the formal classification from sub-species level all the way up to family level of bacteria (Vandamme *et al.* 1996). Classic phenotypic data include morphological descriptions of the cell (shape, Gram stain, flagellation, encapsulation, etc) and the colony (dimensions, form, margin, elevation etc.). Physiological and biochemical data (growth factor requirement, metabolism of certain substances, growth at different temperature ranges, growth in presence of antibiotics etc.) are also needed for a complete description of the organism (Alvarez 2004; http://www.rlc.dcccd.edu/mathsci/reynolds/micro/lab_manual/colony_morph.html 2005).

2.1.1. Automated Identification Systems

Automated systems have been developed to aid in the rapid identification of bacteria. These consist mainly of dehydrated substances that are rehydrated with the test strain. Based on the metabolism of the test bacteria colour changes in substrate are observed or induced with the addition of suitable reagents after a prescribed incubation period. Profiles that are created are read into a database suitable for the system used. Based on the profile, identification is made using comparisons to known profiles in the database (API Manual; Biolog Manual). The two most widely used systems are the bioMerieux API systems and the Biolog Microlog system. Other identification systems include the PhenePlate system (BioSys inova, Stockholm, Sweden), the BBL Minitek system (Becton Dickinson Microbiological Systems) and the Vitek system (bioMerieux Vitek, Inc., Hazelwood, USA).

Tissue Extraction Kit (Qiagen) according to the DNA extraction protocol for Gram Negative Bacteria supplied. DNA concentrations were estimated on a 1.5% agarose gel containing ethidium bromide exposed to a UV light.

16S rDNA gene amplification

The 16S rDNA gene region was amplified using the Polymerase Chain Reaction (PCR). PCR reactions were done in a total volume of 50 µl containing 2 U taq DNA Polymerase (Supertherm, Southern Cross Biotechnology), 1 µM of each primer with forward primer 16F27 / PA (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer 16R1522 / PH (5' AAG GAG GTG ATC CAG CCG CA 3') (10 pmol), 200 µM of each dNTP, 10X Reaction buffer (Supertherm, Southern Cross Biotechnology), 62.5 mM MgCl₂ (Supertherm, Southern Cross Biotechnology) and 25 ng DNA template. PCR mixtures were subjected to an initial denaturation step of 96 °C for 2 minutes. This was followed with 20 cycles of denaturation at 96 °C for 1 minute, primer annealing at 54 °C for 30s and fragment elongation at 72 °C for 90s. Ten cycles consisting of denaturation at 96 °C for 1 minute, primer annealing at 54 °C for 30s and elongation at 72 °C for 91s increasing the time with 1s per cycle followed. Final elongation at 72 °C for 5 minutes followed. The PCR products were visualised under UV light after electrophoresis on a 1.5% Agarose gel containing Ethidium bromide.

16S rDNA gene sequencing

DNA fragments were sequenced using an ABI Prism™ 3100 Sequencer. Purified PCR products of the 16S rDNA gene were sequenced using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase (Applied Biosystems, UK). DNA strands were sequenced using the forward (PA), reverse (PH) and internal forward or reverse primers (Table 1).

16S rDNA sequence analysis

Complete 16S rDNA sequences were manually assembled from the sequences obtained with the different internal primers. Sequences were aligned using the MAFFT 5.8 alignment program (Katoh *et al.* 2002; Katoh *et al.* 2005). Maximum parsimony analysis using the heuristic search option,

was performed on the aligned sequences using MEGA 3.1 (Molecular Evolutionary Genetics Analysis) (Kumar *et al.* 2004) software to produce phylogenetic trees. Branch support was determined with 1000 bootstrap replicates (Felsenstein 1985). The outgroup taxa used to root the tree were *Serratia marcescens*, *Enterobacter cloacae* and *Klebsiella pneumonia*, monophyletic sister groups to the other taxa.

Pathogenicity Trials

Glasshouse inoculation

A susceptible, 6- month- old, *Eucalyptus grandis* clone (ZG14) was inoculated with the two *Pantoea* species and a *Colletogloeopsis zuluense* isolate (CMW 2100), found to be most pathogenic by Van Zyl (1999), in various combinations. For each treatment, 15 trees were inoculated. *C. zuluense* isolates were grown on 2 % Malt extract Agar for 2 weeks. The bacteria were streaked onto Nutrient Agar and incubated overnight at 25 °C. Bacterial suspensions of both *Pantoea ananatis* and the tentatively identified *Pantoea* sp. were made by suspending bacterial growth in a sterile saline solution and adjusting the concentration to $\pm 10^8$ CFU/ml. Inoculation points were made by removing a 7 mm bark disc from young, green stem tissue. Control trees were inoculated with 10 μ l of the sterile saline solution and the bark replaced with a sterile agar disc. Wounds were covered with laboratory film (Parafilm, USA) to prevent desiccation of the inoculum. Similarly, 15 trees each were inoculated with each of the bacterial species as well as with both bacterial species by adding 10 μ l of the bacterial suspensions to the wounds, replacing the bark with a sterile agar disc and wrapping the wounds in laboratory film (Parafilm, USA). Agar discs containing fungal growth were used to inoculate 15 trees with the *C. zuluense* isolate alone. The *C. zuluense* isolate (CMW 2100) was then inoculated with each of the bacterial isolates alone as well as both bacterial isolates together by adding 10 μ l of the bacterial suspension, replacing the bark with an agar disc containing fungal growth and wrapping the wounds in laboratory film. Trees were kept in a greenhouse at a constant temperature of 30 °C throughout an artificial 12 hour day/night cycle. Lesion lengths (mm) were measured 6 weeks after inoculation. Statistical analyses

were performed using Bonferroni's Least Significant Differences (ANOVA analysis, NCSS97).

Field inoculation

Pathogenicity tests were conducted on 3- month- old ZG14 coppice stems in the Zululand area, KwaZulu Natal. Bacteria were streaked onto nutrient agar and incubated overnight at 25 °C. Suspensions were prepared by resuspending bacterial growth in sterile saline solution and adjusting the concentration of the suspensions to $\pm 10^8$ CFU/ml. Fungal isolates were prepared by growing *C. zuluense* isolates on 2% Malt Extract Agar for two weeks. Twenty stems were used per treatment. Stems were wounded, by removing a 7mm disc of bark to expose the cambium. Control trees were inoculated with 10 μ l of a sterile saline solution and the bark replaced with a sterile agar disc. Wounds were inoculated with bacteria by adding 10 μ l of the previously prepared bacterial suspension, either alone or in combination, and replacing the bark with a sterile agar disc. *C. zuluense* was inoculated by replacing the bark with a 7mm agar disc containing fungal growth. The two bacteria in combination with the *C. zuluense* was inoculated by adding 10 μ l of the bacterial suspension, either alone or in combination, to the wounds and replacing the bark with an agar disc containing fungal growth. All wounds were wrapped in laboratory film and cling wrap to prevent desiccation of the inoculum. Lesion lengths (mm) were measured after 6 weeks and statistical analyses were performed using Bonferroni's Least Significant Differences (ANOVA analysis, NCSS97).

Results

Surveys

We were unsuccessful in isolating any bacteria in association with *C. zuluense*. Isolations from diseased material, from the site where bacteria had previously been isolated by Van Zyl (1999), failed to produce any bacteria associated with the fungus. Due to the unsuccessful isolation of bacterial strains associated with *C. zuluense*, it was decided to include a fungal isolate previously identified by Van Zyl (1999) as *C. zuluense*. The chosen isolate

used in this study was found to be one of the most pathogenic strains of *C. zuluense* associated with the two bacterial species by Van Zyl (1999).

Bacterial Identification

Phenotypic characterization

Bacterial strains were Gram negative, fermentative, straight rods. All isolates belonged to the family *Enterobacteriaceae*. The isolate previously identified by Van Zyl (1999) as *P. ananatis* (BCC 110) was identified, using the API 20E system as *Pantoea* sp. 2 with 92.5% identity significance. The isolate (BCC 118), believed by Van Zyl (1999) to be closely related to *P. stewartii* subsp. *stewartii*, was also identified as *Pantoea* sp. 2 with a 96.3% identity.

DNA Sequence Comparisons

DNA sequence analysis

A \pm 1500 bp fragment was amplified from both isolates and these fragments were successfully sequenced using internal primer combinations (Table 1). A total fragment length of 1518 bp for BCC 110 and 1523 bp for BCC 118 was sequenced. A BLAST search revealed that BCC 110 was most similar to *P. ananatis* (Z96081) with a 99% similarity. The *P. stewartii* related isolate, BCC 118, was revealed to be most similar to *P. stewartii* subsp. *stewartii* (Z96080) with a 99% similarity, although a number of other *Enterobacteriaceae* were also found with sequence similarities ranging between 97-98 %. The sequences obtained were aligned with related members of the *Enterobacteriaceae* obtained from Genbank (<http://www.ncbi.nlm.gov> 2005) and the BCCM/LMG culture collection (University of Ghent, Belgium). MAFFT alignment resulted in a total of 1557 bp for final analysis.

Phylogenetic analysis of the aligned sequences resulted in 167 parsimony informative characters and 15 most parsimonious trees were generated. One of the 15 most parsimonious trees is represented in Fig 1. In all trees BCC 110 group consistently with the *P. ananatis* sequences, including the type strain, obtained from Genbank with high bootstrap support. BCC 118 grouped closely with the type strains of *P. stewartii* subsp. *indologenes*, also with high

bootstrap (73%) support. Although *P. stewartii* subsp. *stewartii* sequences grouped closely with the group containing the sequence of the type strain of *P. stewartii* subsp. *indologenes*, the clades were separate, with high bootstrap (98%) support.

Pathogenicity Trials

Greenhouse and Field trials

The *C. zuluense* isolate used was able to produce lesions on the susceptible clones, in both the greenhouse and field trials, when inoculated alone (Figs. 2 & 3). Neither *Pantoea* species were able to produce lesions when inoculated alone or in combination (Figs. 2 & 3). There was no significant difference between mean lesion lengths of the controls and any of the combination of treatments using the two *Pantoea* species. Based on the statistical analysis, no significant differences were observed between the inoculations with *C. zuluense* alone and *C. zuluense* in the various combinations with the two *Pantoea* species. Although there was no significant difference between the combinations of *C. zuluense* inoculations, there was a significant difference in lesion length between trees inoculated with *C. zuluense* and those serving as controls.

Discussion

Results of this study showed that bacterial isolates included in this study are members of the *Enterobacteriaceae*. In a previous study, Brady (2005) found that, based on AFLP data, isolate BCC 118 grouped closely with the type strain of *P. stewartii* subsp. *indologenes*. We believe the unknown *Pantoea* species to be *P. stewartii* subsp. *indologenes*, supported by phenotypic characteristics, 16S rDNA sequence analysis and AFLP data as presented by Brady (2005). Results of this study also confirmed the identity of isolate BCC 110 as *P. ananatis*, supporting results obtained by Van Zyl (1999).

In this study it was not possible to repeat the pathogenicity results obtained by Van Zyl (1999). Pathogenicity trials, in both greenhouse and field conditions, failed to show any synergy between *C. zuluense* and the two bacterial strains studied by Van Zyl (1999). Interactions between populations are never

simple, and a number of factors contribute to the successful interaction between any two or more populations. These interactions are interconnected, usually dependant on the biology of the organisms, the size of the populations and subject to changes in the environment (Atlas & Bartha 1998).

The biology of the organisms involved in the interaction would presumably be the determinative factor in the success of any interaction. This would include the ability of organisms to colonise the host plant and cause disease. When lesion lengths produced by *C. zuluense* alone, in both greenhouse and field conditions, were compared to lesions lengths obtained by Van Zyl (1999), a decrease in mean lesion length was observed. This would suggest that some attenuation of the pathogenicity of *C. zuluense* has occurred. The conclusion would be that the long term storage of the fungus might have affected both its ability to cause disease and its ability to interact with its bacterial partners.

Disparity in pathogenicity has also been reported for various *Pantoea* species (Hatting & Walters 1981; Bruton *et al.* 1991; Azad *et al.* 2000; Gitaitis *et al.* 2002). Authors have commented on the sporadic nature of infections and differences observed in disease severity (Azad *et al.* 2000; Gitaitis *et al.* 2002). *P. ananatis*, a member of the purported interaction between the bacteria and *C. zuluense*, has also been reported as an epiphyte on rice (Watanabe *et al.* 1996). This reported variability in pathogenicity casts doubt on the ability of at least one of the chosen bacterial isolates to cause disease and/or interact with a fungal partner.

Environmental factors such as temperature and relative humidity also appear to play a part in the sporadic nature and severity of infections. This effect is believed to be greater on the bacteria than the fungus because no significant difference was observed between lesions lengths of inoculations with *C. zuluense* alone between greenhouse and field trials. Azad *et al.* (2000) determined that, in pathogenicity screenings with *P. ananatis* and *P. stewartii*, symptoms were more severe in greenhouses with higher temperatures and relative humidity. These differences in pathogenicity, the ability to occur epiphytically and the ability of changing environmental factors to induce disease, would imply that, *P. ananatis* at least, is an opportunistic pathogen.

The variability, in terms of pathogenicity, of some members of the genus *Pantoea*, notably *P. ananatis*, makes it difficult to determine or even estimate the effect that an interaction between *C. zuluense* and the two bacterial species, *P. ananatis* and *P. stewartii* subsp. *indologenes*, might have on *Eucalyptus* forestry in South Africa. *C. zuluense* is a pathogen, whose effect on the industry has been largely negated by selection and breeding of more resistant *Eucalyptus* clones. *Pantoea* species are known for their ability to occur on a wide variety of hosts, both as epiphyte and pathogen (Gitaitis *et al.* 2002). The interaction such as that described by Van Zyl (1999) could have, if conditions become favourable, an impact on *Eucalyptus* clonal forestry in South Africa, the extent of which is impossible to determine without further study.

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Table 1: Primers used in the amplification and sequencing of the 16 rDNA gene region.

Primer	Sequence (5' – 3')
16F27 / PA	AGA GTT TGA TCC TGG CTC AG
16F358 / *Gamma	CTC CTA CGG GAG GCA GCA GT
16F536 / *PD	CAG CAG CCG CGG TAA TAC
16F926 / *O	AAC TCA AAG GAA TTG ACG G
16F1112 / *3	AGT CCC GCA ACG AGC GCA AC
16R339 / Gamma	ACT GCT GCC TCC CGT AGG AG
16R519 / PD	GTA TTA CCG CGG CTG CTG
16R685	TCT ACG CAT TTC ACC GCT
16R1093 / 3	GTT GCG CTC GTT GCG GGA CT
16R1485 / MH2	TAC CTT GTT ACG ACT TCA CCC CA
16R1522 / PH	AAG GAG GTG ATC CAG CCG CA

Figure 1: Tree 1 of 15 most parsimonious trees generated with aligned 16S rDNA gene sequences for the type strains of the seven *Pantoea* species (indicated in red), related members from the family *Enterobacteriaceae* and the bacterial isolates associated with *C. zuluense* (indicated in blue). *Serratia marcescens*, *Enterobacter cloacae* and *Klebsiella pneumonia* are used as monophyletic sister outgroups to root the tree. Total tree length = 776 steps, CI = 0.42, RI = 0.69. Bootstrap values are indicated above the branches.

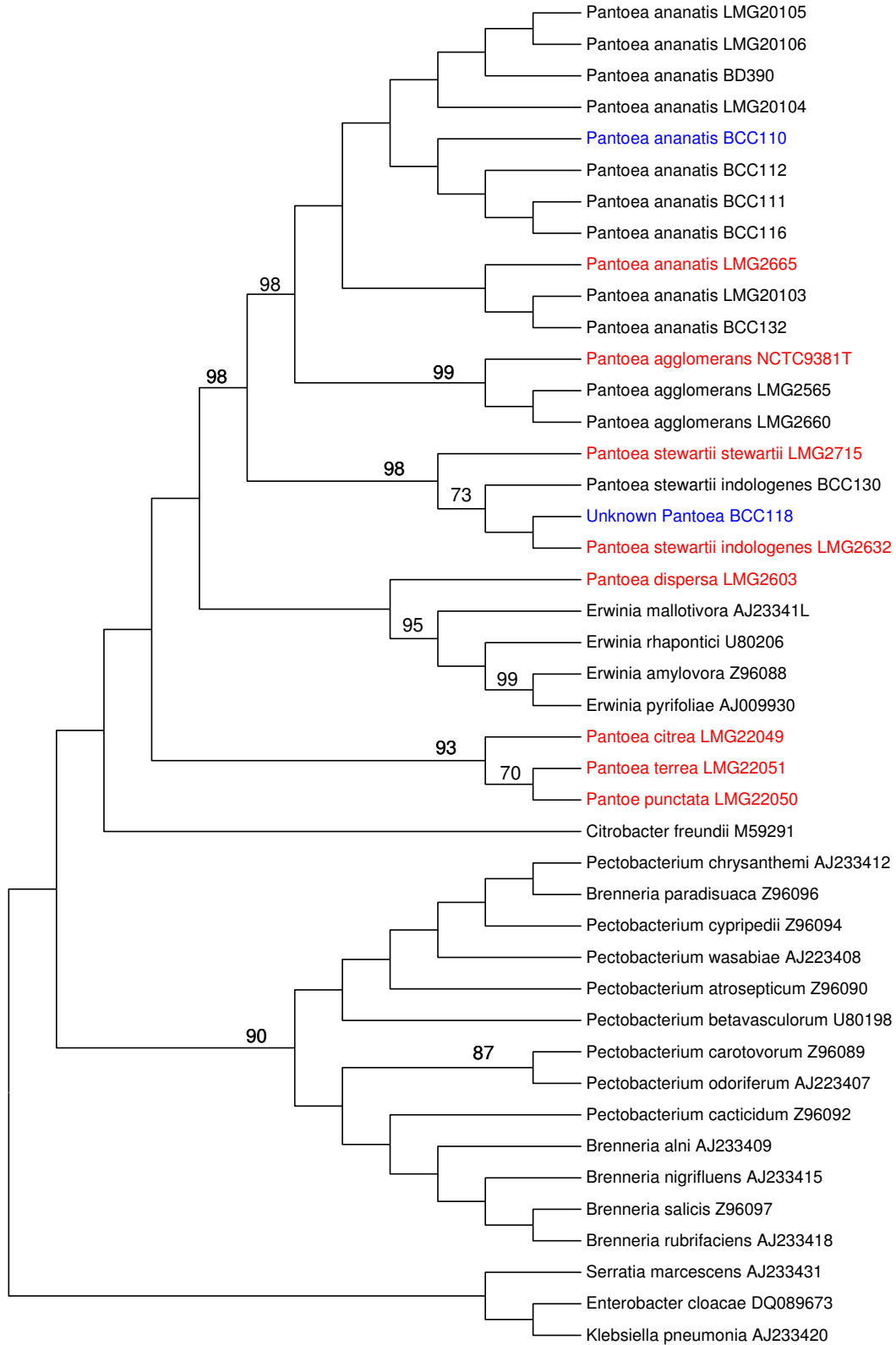


Figure 2: Average lesion lengths (mm) obtained with the different treatments, under greenhouse conditions. Bars represent 95% confidence limit for each treatment. **A:** Control, **B:** BCC 110 – *P. ananatis*, **C:** BCC 118 – Tentatively identified *Pantoea* species, **D:** BCC 110 + BCC 118, **E:** CMW 2100 - *Colletogloeopsis zuluense*, **F:** CMW 2100 + BCC 110, **G:** CMW 2100 + BCC 118, **H:** CMW 2100 + BCC 110 + BCC 118. The greenhouse was kept at constant 30 °C throughout an artificial 12 hour day/night cycle.

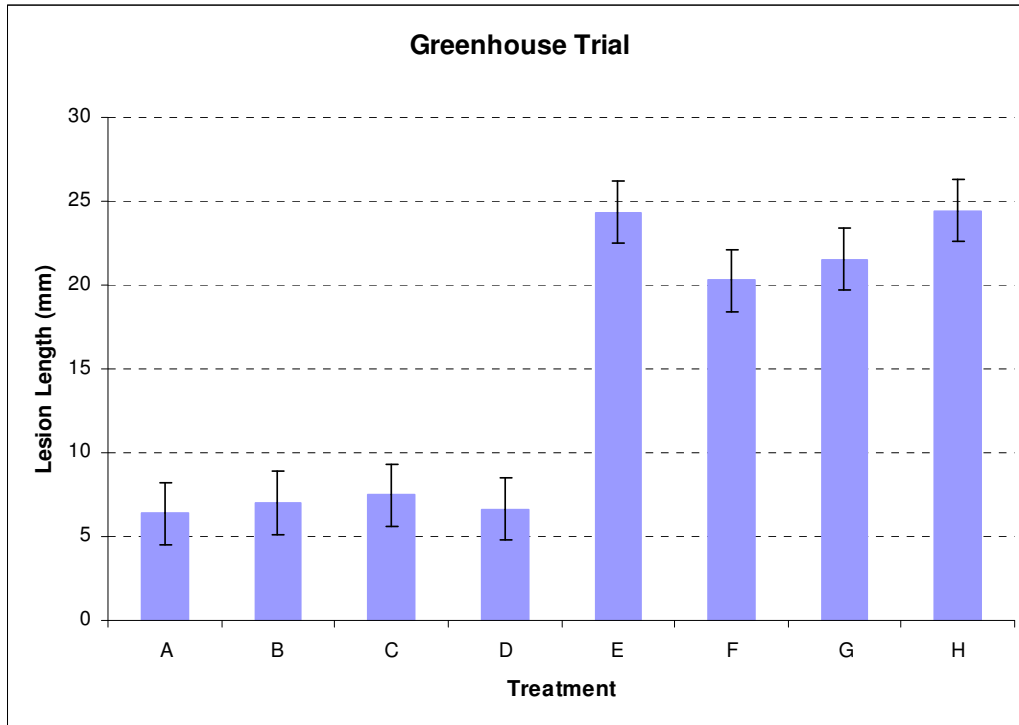
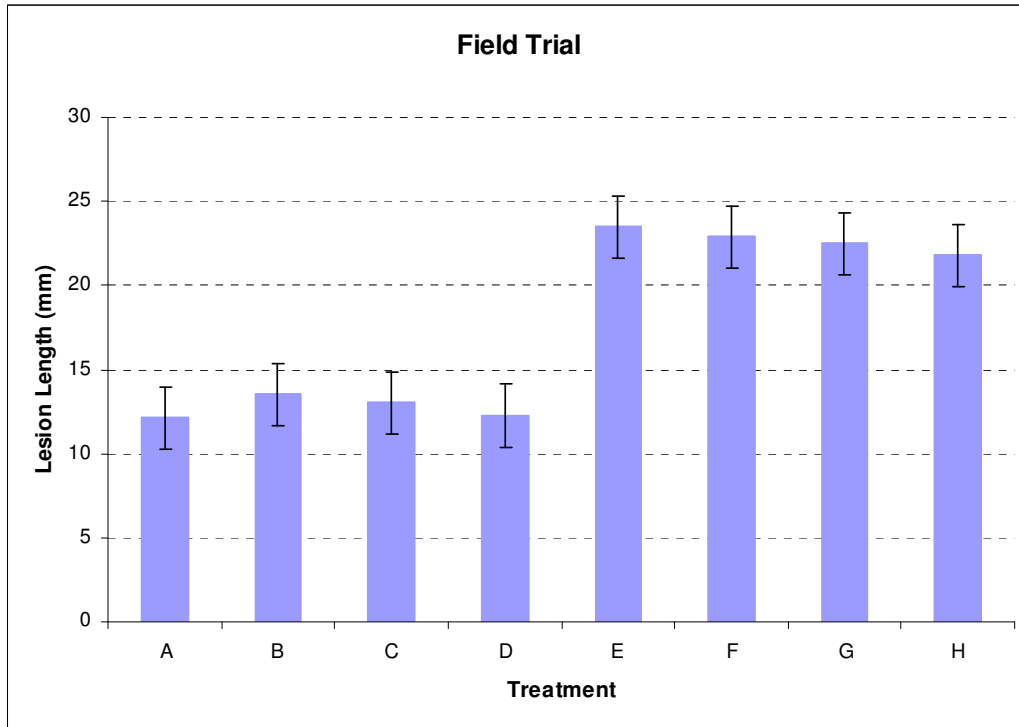


Figure 3: Average lesion lengths (mm) obtained with different treatments under field conditions. Bars represent 95% confidence limit for each treatment. **A:** Control, **B:** BCC 110 – *P. ananatis*, **C:** BCC 118 – Tentatively identified *Pantoea* species, **D:** BCC 110 + BCC 118, **E:** CMW 2100 - *Colletogloeopsis zuluense*, **F:** CMW 2100 + BCC 110, **G:** CMW 2100 + BCC 118, **H:** CMW 2100 + BCC 110 + BCC 118. Susceptible ZG 14 clones were inoculated in the Kwambonambi area in the Zululand forestry region, Kwazulu Natal.



Figures 4: Lesions associated with the inoculation of susceptible ZG 14 clones under greenhouse conditions. **A:** Control, **B:** BCC 110 – *P. ananatis*, **C:** BCC 118 – Tentatively identified *Pantoea* species, **D:** BCC 110 + BCC 118, **E:** CMW 2100 - *Colletogloeopsis zuluense*, **F:** CMW 2100 + BCC 110, **G:** CMW 2100 + BCC 118, **H:** CMW 2100 + BCC 110 + BCC 118





Chapter 3:

**Identification of *Pantoea* species associated
with bacterial blight in Uganda, Thailand and
Uruguay**

Abstract

Bacterial blight, caused by *Pantoea ananatis*, occurring on *Eucalyptus* was first reported in South Africa in 2002. Similar disease symptoms have subsequently been noted in Uganda, Thailand and Uruguay. In this study, bacteria found associated with bacterial blight symptoms in these three countries were characterized phenotypically using the API and Biolog identification systems. They were also examined genotypically using 16S rDNA and AFLP fingerprinting data. The majority of isolates from the three countries were found to belong to the genus *Pantoea*. Isolates from Uganda and Uruguay were found distributed throughout the larger *Pantoea ananatis* – *Pantoea agglomerans* grouping based on both phenotypic, 16S and AFLP data. Thailand isolates were consistently found associated with the type strain of *Pantoea dispersa*, LMG 2603^T. Isolates from the three countries were found to be moderately to non-pathogenic. Based on results obtained, we conclude that some isolates from Uganda and Uruguay represent new species of *Pantoea*. Thailand isolates appear to be closely related to *Pantoea dispersa* and this represents the first report of the bacterium from *Eucalyptus*. It also appears that a complex of *Pantoea* species are associated with bacterial blight symptoms in Uganda and Uruguay.

Introduction

In 2002, a blight disease with symptoms reminiscent of bacterial infection was reported on *Eucalyptus* leaves and shoots in a nursery in the Zululand forestry region of South Africa. The causal agent was identified as *Pantoea ananatis* using various techniques including 16S rDNA gene sequence analysis, DNA hybridization studies and pathogenicity screening (Coutinho *et al.* 2002). The disease is characterized by water-soaked necrotic spots on the leaves. These spots coalesce to form larger necrotic lesions that become corky with age. Bacteria could sometimes be seen oozing from the lesions when infections were recent and the humidity was high. Severe infection resulted in tip die-back and the formation of epicormic shoots, causing stunted growth or occasionally death. This disease was first noted occurring in a single nursery on *Eucalyptus grandis* x *Eucalyptus nitens* (GN) hybrids. It has subsequently appeared in other forestry regions, infecting different *Eucalyptus* hybrids and clones (Coutinho *et al.* 2002).

A disease with symptoms similar to those caused by *P. ananatis* in South Africa was reported from Uganda (Nakabonge 2002). The causal agent was provisionally identified as *P. ananatis*. Later, DNA hybridization studies showed that the associated bacterium was not *P. ananatis* (Coutinho unpublished). Diseases with similar symptoms have been noted in Uruguay and Thailand (Wingfield unpublished) but the causal agents have not been conclusively identified. It is, however, believed that they reside in the genus *Pantoea*.

The identification of plant pathogenic and associated bacteria is complex, with no single method available for comprehensive identification (Vandamme 1996; Alvarez 2004). Instead, bacteriologists need to apply multiple techniques, with increasing levels of complexity, to reliably identify bacteria. This is particularly true for members of the genus *Pantoea*.

The genus *Pantoea* was first proposed by Gavini *et al.* (1989) based on DNA hybridization, phenotypic and genotypic data. *Enterobacter agglomerans* (Beijerinck 1888) Ewing Fife 1972 was transferred to the new genus as

Pantoea agglomerans (Beijerinck 1888), type strain of the genus (Gavini *et al.* 1989). Mergaert *et al.* (1993) proposed the transfer of *Erwinia ananas* and *Erwinia stewartii* to the genus as *Pantoea ananas* (Serrano 1928) and *Pantoea stewartii* (Smith 1898), respectively. Currently, *Pantoea* includes *Pantoea agglomerans*, *P. dispersa*, *P. citrea*, *P. punctata*, *P. terrea*, *P. ananatis*, *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes* (Brenner *et al.* 2005).

The increase in trade and movement of wood and wood products globally, has increased the threat of pathogens being introduced to new environments (Wingfield *et al.* 2001; Wingfield 2003). This has emphasized the need for increased knowledge regarding pathogens of trees used to establish plantations. Knowing where pathogens occur and having knowledge of their biology is essential, not only for quarantine purposes, but also for the early evaluation of threats and to ensure appropriate responses. The rapid spread of bacterial blight disease from a single nursery to other forestry regions in South Africa (Coutinho *et al.* 2002) provides support for the view that these measures should be firmly in place. The aim of this study was, therefore, to identify the bacteria associated with bacterial blight in Uganda, Uruguay and Thailand using a polyphasic approach.

Materials and Methods

Bacterial isolates

Isolates from Uruguay were obtained from diseased leaf material. For isolations, leaves were surface sterilized by dipping them into 70% ethanol, followed by a wash in sterile water and then crushing the leaf material in 1.5 ml sterile saline solution using a mortar and pestle. Dilution series were made by transferring 100 µl of the pulp to 900 µl of sterile saline solution. The dilutions were then plated onto Nutrient Agar (16g Nutrient Broth [Biolab, Biolab Diagnostics, Merck], 15 g Agar in 1L distilled H₂O). Seventeen isolates from Uruguay were used in this study (Table 1).

Pure cultures of isolates from Uganda and Thailand were obtained from the bacterial culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. Six isolates from Uganda and eight isolates from Thailand were used in this study (Table 1). All isolates are maintained in the bacterial culture collection at FABI.

Phenotypic Characterization

The Gram reaction for all isolates from Uganda, Thailand and Uruguay were determined using Gram's Crystal Violet, Iodine and Safranin solutions (Fluka Biochemika, Sigma-Aldrich). Oxidation fermentation tests (OF Basal medium [Difco] in 1L distilled H₂O; 10 ml of 10% glucose [Sigma] solution filter sterilized and added after autoclaving) were performed following the manufacturer's instructions.

API 20E strips (Biomerieux) were used for phenotypic identification of isolates. Strips were inoculated with the test isolates in sterile distilled water according to the manufacturer's instructions. Additional tests to determine motility (20g Bacto Motility Test Medium [Difco] in 1L distilled H₂O) and oxidase (NNN'-Tetramethyl-p-phenylenediamine dihydrochloride [BDH Laboratory Supplies]) activity were also performed. Profiles were identified using the Analytical Profile Index (ApiLab) Identification software.

Biolog microplates for the identification of Gram negative bacteria (GN2 Microplates™, Biolog) were used for identification purposes. Pure cultures were grown overnight on the Biolog Universal Growth medium (Biolog) at 25 °C. Inoculation fluid (Biolog) was prepared with test strains as prescribed and microplates were inoculated according to instructions. Results were read as negative, borderline and positive after 4, 24 and 48 hours of incubation at 30 °C. Profiles were identified using Biolog's Microlog™ Identification software (Biolog).

Molecular Characterisation

DNA Extraction

Single bacterial colonies of the bacterial isolates were transferred to Nutrient Broth and incubated for 24 hours in a shake incubator at 25 °C. DNA extractions were performed using the DNeasy Tissue Extraction Kit (Qiagen) according to the supplied protocol for DNA extraction from Gram negative bacteria. DNA concentrations were estimated after electrophoresis on a 1.5% agarose gel containing ethidium bromide exposed to UV light.

16S rDNA Analysis

16S rDNA gene amplification

The 16S rDNA gene region was amplified in a total PCR reaction volume of 50 µl containing 2 U taq DNA Polymerase (Supertherm, Southern Cross Biotechnology), 10 µM of each primer with forward primer 16F27 / PA (5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer 16R1522 / PH (5' AAG GAG GTG ATC CAG CCG CA 3'), 200 µM of each dNTP (dATP, dGTP, dCTP, dTTP), 10X Reaction buffer (Supertherm, Southern Cross Biotechnology), 2 mM MgCl₂ (Supertherm, Southern Cross Biotechnology) and 25 ng DNA template. PCR mixtures were subjected to an initial denaturation step of 96 °C for 2 minutes. This was followed with 20 cycles of denaturation at 96 °C for 1 minute, primer annealing at 54 °C for 30s and fragment elongation at 72 °C for 90s. Ten cycles consisting of denaturation at 96 °C for 1 minute, primer annealing at 54 °C for 30s and elongation at 72 °C for 91s increasing the time with 1s per cycle, followed. Final elongation at 72 °C for 5 minutes followed. PCR results were visualized by exposing a 1.5 % Agarose gel containing ethidium bromide to UV light after electrophoresis.

16S rDNA gene sequencing

16S rDNA fragments were sequenced using an ABI Prism™ 3100 Sequencer. Purified PCR products of the gene were sequenced using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA

polymerase (Applied Biosystems). DNA strands were sequenced using the forward (PA), reverse (PH) and internal forward or reverse primers 16F358 (5' CTC CTA CGG GAG GCA GCA GT 3'), 16F536 (5' CAG CAG CCG CGG TAA TAC 3'), 16F926 (5' AAC TCA AAG GAA TTG ACG G 3'), 16F1112 (5' AGT CCC GCA ACG AGC GCA AC 3'), 16R339 (5' ACT GCT GCC TCC CGT AGG AG 3'), 16R519 (5' GTA TTA CCG CGG CTG CTG 3'), 16R685 (5' TCT ACG CAT TTC ACC GCT 3'), 16R1093 (5' GTT GCG CTC GTT GCG GGA CT 3'), 16R1485 (5' TAC CTT GTT ACG ACT TCA CCC CA 3') and 16R1522 (5' AAG GAG GTG ATC CAG CCG CA 3').

16S rDNA Gene Analysis

Consensus 16S rDNA gene sequences were obtained by manually assembling sequences obtained from the internal primers using MEGA 3.1 (Molecular Evolutionary Genetics Analysis) (Kumar *et al.* 2004) software. Consensus sequences were used in homology searches, using BLASTN, against sequences in the Genbank/EMBL database.

A preliminary phylogenetic tree was constructed by obtaining representative sequences for members of the *Enterobacteriaceae* from the Genbank/EMBL database. 16S sequences for the seven type strains of *Pantoea* were obtained from the Genbank/EMBL database and the BCCM/LMG culture collection (University of Ghent, Belgium). All sequences were aligned using the MAFFT 5.8 alignment program (Kato *et al.* 2002; Kato *et al.* 2005). Distance analyses were performed using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and other Methods) software (Swofford 2003) to construct a neighbour-joining tree. Support for branches was determined with a 1000 Bootstrap (Felsenstein 1985) replicates using the neighbour-joining search option.

A refined phylogenetic tree was constructed based on groupings obtained within the preliminary *Enterobacteriaceae* tree. Sequences were selected, re-aligned using MAFFT, re-analysed and a neighbour-joining tree was constructed to further elucidate relationships. 1000 Bootstrap replicates, using the neighbour-joining search option, were performed to determine

branch support. *Citrobacter rodentium* (AF025363), *Citrobacter sedlakii* (AF025364) and *Citrobacter diversus* (AF025371) were used as monophyletic sister groups to root the tree.

Amplified Fragment Length Polymorphism (AFLP) Analysis

DNA restriction digestion, ligation and amplification

Restriction enzyme digestions were performed in a total reaction volume of 15 μ l. DNA template (50-100 ng) was digested using 12U EcoRI (Fermentas Lifescience) and 8U MseI (New England Biolabs) in 1 X Restriction/Ligation Buffer (50 mM TrisHAc, 50 mM MgAc, 250 mM KAc, 25 mM DTT). Digests were incubated at 37 °C for 2 hours followed by a heating step of 15 minutes at 70 °C. EcoRI (5 pmol) and MseI (50 pmol) double stranded adaptors, 0.5 mM ATP and 2 U T4 DNA ligase (Roche) was added to the digestion reactions and incubated at 25 °C for 2 hours and diluted 1:10 using Sabax water (Adcock Ingram Ltd.).

Pre-amplification reactions were performed in a total reaction volume of 25 μ l containing 10X Reaction buffer (Supertherm, Southern Cross Biotechnology), 2 mM MgCl₂ (Supertherm, Southern Cross Biotechnology), 200 μ M of each dNTP (dATP, dGTP, dCTP, dTTP), 100 pmol of each Eco-00 (5' GAC TGC GTA CCA ATT C 3') and Mse-00 (5' GAT GAG TCC TGA CTA A 3'), 1 U Taq polymerase (Supertherm, Southern Cross Biotechnology) and 2 μ l of the diluted ligation reaction mixture. Pre-amplification mixtures were subjected to an initial denaturation step at 94 °C for 3 minutes, followed by 20 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 56 °C for 1 minute and elongation at 72 °C for 1 minute. Final elongation was performed at 72 °C for 5 minutes after which pre-amplification mixtures were diluted 1:50 using Sabax (Adcock Ingram Ltd.) water.

Selective amplification reactions were performed in a total reaction volume of 20 μ l containing 10X Reaction Buffer (Supertherm, Southern Cross Biotechnology), 1.5 mM MgCl₂ (Supertherm, Southern Cross Biotechnology), 200 μ M of each dNTP, 0.5 pmol fluorescently labelled Eco-C (5' GAC TGC

GTA CCA ATT CC 3') and 2.4 pmol Mse-GC (5' GAT GAG TCC TGA CTA AGC 3') primers, 1 U Taq polymerase (Supertherm, Southern Cross Biotechnology) and 5 µl of the diluted pre-amplification reactions. Reaction mixtures were subjected to an initial denaturation step at 94 °C for 5 minutes. This was followed by 9 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 65 °C for 30 seconds with annealing temperature decreasing by 1 °C per cycle until 56 °C is reached and elongation at 72 °C for 1 minutes. Twenty three cycles of denaturation at 94 °C for 30 seconds, primer annealing at 56 °C for 30 seconds and elongation at 72 °C for 1 minute with a final elongation step at 72 °C for 5 minutes followed. Prior to running selective amplifications on a LI-COR sequencing gel, samples were mixed with an equal volume of formamide loading buffer (95 % formamide, 20 mM EDTA, bromophenol blue), heated at 90 °C for 3 minutes and cooled on ice for 10 minutes.

LI-COR Gel Analysis

LI-COR gels were prepared using 20 ml Long Ranger Gel stock solution (8% Long ranger gel solution [LI-COR Biosciences], 7 M Urea, 10X TBE Buffer) with 150 µl 10% Ammonium persulphate and 15 µl TEMED added for gel polymerization. Gels were poured using LI-COR casting apparatus and polymerized for 45 minutes. A pre-run step was performed at 1500 V and 35W to equilibrate the ions in the gel and running buffer. Approximately 1 µl of each of the previously prepared selective amplification reactions were loaded onto the sequencing gels. A IRD-700 labelled sizing standard was also loaded onto the gel. Gels were run on a LI-COR IR² automated sequencer (LI-COR Biosciences) for 4 hours at 1500 V and 42 W with 0.8X TBE running buffer. Band patterns between 50 and 700 bp were analysed using Bionumerics 4.5 (Applied Maths, Kortrijk, Belgium) software. Gels were normalized using the size standard and a UPGMA dendrogram was constructed using the Pearson correlation coefficient.

Pathogenicity Screening

Bacterial isolates, used in both hypersensitivity reaction tests in tobacco and pathogenicity screenings on *Eucalyptus*, were grown on Nutrient Agar (16g

Nutrient Broth [Biolab, Biolab Diagnostics, Merck], 15 g Agar in 1L distilled H₂O) for 24 hours at 25 °C. Bacteria were then suspended in sterile saline solution and the concentration was adjusted to approximately 10⁸ CFU/ml. A *Pantoea ananatis* strain (LMG 20103), identified as the causal agent of bacterial blight in South Africa (Coutinho *et al.* 2002) and found to be most pathogenic, was used as a positive control. Trials were performed in a contained greenhouse at constant 26 °C with natural day/night cycles.

Hypersensitive reaction (HR)

Five-month-old Tobacco plants were used to determine whether or not a HR response was induced by the bacterial isolates from Uganda, Thailand and Uruguay used in this study (Table 1). Tobacco plants were inoculated using 1 ml insulin syringes (Lifeline). Plants were inoculated by inserting the syringe containing the bacterial suspension ($\pm 10^8$ CFU/ml) into the main leaf vein and flooding individual leaf panels with the bacterial suspension. Approximately 100–200 μ l of bacterial suspension was needed to flood each leaf panel. Two leaf panels were flooded per isolate. Sterile saline solution was used as negative controls. A pathogenic strain of *P. ananatis* (LMG 20103) was used as the positive control. Tobacco plants were kept in a confined greenhouse at 26 °C with natural day/night cycles. HR response development was assessed after 24 and 48 hours. The severity of the HR responses produced in the leaf panels were assessed as either necrotic (positive), yellow (moderate) or green (negative).

Pathogenicity on *Eucalyptus*

Three-month-old plants of the *Eucalyptus grandis* x *Eucalyptus nitens* (GN188) clone were used in pathogenicity screening. Leaves were inoculated by placing a 5 μ l drop of the bacterial suspension ($\pm 10^8$ CFU/ml) of the selected isolates from Uganda, Thailand and Uruguay (Table 1) and inserting the needle point through the suspension drop into the leaf. Four wounds were made per leaf and two leaves were inoculated per bacterial isolate (Table 1). Control plants were inoculated with a sterile saline solution. LMG 20103, a pathogenic *P. ananatis* isolate was used as a positive control. After inoculation, plants were covered with plastic bags for 7 days after which the

bags were removed. Inoculated plants were maintained for an additional week after which final symptom development was assessed. Symptom development was assessed as mild i.e. comparable to the negative control, moderate or pathogenic i.e. comparable to the positive control. Plants were kept in a confined greenhouse at constant 26 °C with natural day/night cycles.

Results

Phenotypic Characterisation

One of the major disadvantages of identifying *Pantoea* spp. using API 20E systems is the fact that the ApiLab software identifies profiles generated by *Pantoea* spp. as *Pantoea* sp. 1, 2, 3 or 4. Goszczynska *et al.* (2007) determined that the profiles generated for the type strains of *P. ananatis* (LMG 2665^T) and *P. agglomerans* (LMG 1286^T) were identified as *Pantoea* sp. 2 and *Pantoea* sp. 3, respectively, by the ApiLab identification software. We, therefore, assume that all isolates identified as either *Pantoea* sp. 2 or 3 are closely related to *P. ananatis* or *P. agglomerans*, respectively.

Six isolates from Uganda, eight isolates from Thailand and seventeen isolates from Uruguay were found to be Gram negative straight rods. Glucose was degraded fermentatively. The majority of isolates from Uganda, Thailand and Uruguay were identified, according to the ApiLab identification software, as either *Pantoea* sp. 2 or *Pantoea* sp. 3 (Table 2). The Biolog system identified the majority isolates from Uganda and Uruguay as *P. agglomerans*, while the majority of isolates from Thailand were identified as *P. dispersa* (Table 2).

Uganda isolates, with the exception of BCC 207, gave acceptable profiles according to the ApiLab identification software (Table 2). Isolates BCC 105, BCC 107 and BCC 109 were identified as *Pantoea* sp. 3 with 95.5 % identity significance. Isolates BCC 208 and BCC 691, also from Uganda, were identified as *Pantoea* sp. 3 with 82.6 % and 98.9 % identity significance, respectively.

Identification of Thailand isolates varied using API 20E strips (Table 2). Isolates BCC 210, BCC 212, BCC 379 and BCC 380 were identified as *Pantoea* sp. 2 with between 62 and 64.5% identity significance. Isolates BCC 211, BCC 213 and BCC 379 all produced unacceptable identification profiles on the basis of the trisodium citrate, D-melibiose and NO₂ production reactions, while isolate BCC 209 was identified as a *Serratia* sp.

The majority of Uruguay isolates produced doubtful identification profiles using the API system, with no identity significance (Table 2). The majority of isolates from Uruguay were identified as either *Pantoea* sp. 2 or *Pantoea* sp. 3. BCC 383 was identified as an *Aeromonas* sp. with 95.4% identity significance. The only other isolate that was identified with a high identity significance was BCC 759 which was identified as *Pantoea* sp. 3 with 95.5% identity significance.

Using the Biolog GN2 system, Uganda isolates (BCC 105, BCC 107, BCC 109, BCC 208 and BCC 691), with the exception of BCC 207, were identified as *P. agglomerans* with high percentage probability i.e. with a similarity index higher than 0.5 (Table 2). Isolates BCC 105, BCC 109, BCC 208 and BCC 691 were identified with a 99% probability as *P. agglomerans*. Isolate BCC 107 was identified as *P. agglomerans* with a similarity index of 0.449. Isolate BCC 207 was identified as *Escherichia vulneris* with a 96% probability.

The Biolog system showed that the majority of Thailand isolates represented *P. dispersa* with variable percentage probability (Table 2). Isolates BCC 210, BCC 212 and BCC 380 were identified with a 99% probability. BCC 378, which produced an unacceptable profile with the API 20E strips, was identified as *P. agglomerans* with a similarity index of 0.406. Isolate BCC 209, an outlier in all other analyses, was identified as a *Vibrio* sp. with 99% probability. Isolate BCC 211, also producing an unacceptable profile with the API 20E strips, was identified as *Enterobacter aerogenes* with a 92% probability.

Biolog results indicated that all Uruguay isolates were *P. agglomerans* with varying percentage probabilities and similarity indices (Table 2). Isolates BCC

756, BCC 758, BCC 759, BCC 760, BCC 763 and BCC 764 were thus identified as *P. agglomerans* with high percentages of probability (Table 2). Likewise, isolates BCC 757, BCC 761, BCC 762, BCC 381, BCC 382, BCC 383, BCC 574, BCC 583, BCC 611, BCC 916 and BCC 754 were identified as *P. agglomerans* with varying similarity indices (Table 2).

Molecular Characterisation

16S rDNA Analysis

A \pm 1500 bp fragment was amplified from all isolates from Uganda, Thailand and Uruguay. BLAST homology search results for all isolates showed that all the sequences were most homologous to members of the *Enterobacteriaceae*. The majority of isolates from Uganda, Thailand and Uruguay were found to be most homologous to members of the genus *Pantoea*. Percentage homologies ranged from 97-99% (Table 2).

MAFFT alignment of sequences of members of the *Enterobacteriaceae*, type strains of *Pantoea* and isolates from Uganda, Thailand and Uruguay resulted in a total of 1695 bp for preliminary analyses. These analyses showed that all isolates grouped within the family *Enterobacteriaceae* based on 16S rDNA sequence data. *Pantoea* isolates grouped closely together within the *Enterobacteriaceae* with the majority of Uganda, Thailand and Uruguay isolates grouping within the larger *Pantoea* clade. Isolate BCC 207 grouped away from the *Pantoea* clade and distant from the other Uganda isolates, grouping with an *Enterobacter kobei* isolate. Isolates BCC 209 and BCC 379 also grouped well apart from the large *Pantoea* clade. Based on 16S rDNA and AFLP data, isolates BCC 207, BCC 209 and BCC 379 were excluded from further study.

In subsequent 16S rDNA analyses with selected sequences, MAFFT alignment resulted in a total of 1556 bp for final analyses. Analyses showed that, within the *Pantoea* clade, the *Pantoea* species grouped separately from each other (Fig. 1). *P. stewartii* sequences grouped separately from *P. ananatis* sequences with high bootstrap support (100%). Distinction between

P. ananatis and *P. agglomerans* sequences was supported with 86% bootstrap value. *P. dispersa* and *P. terreus*, *P. citrea* and *P. punctata* grouped away from the other *Pantoea* species with high bootstrap support (79% and 96% respectively).

The Uganda isolates were distributed throughout the major *Pantoea ananatis*-*Pantoea agglomerans* clade (Fig. 1). Isolates BCC 105 and BCC 107 grouped on their own between the *P. ananatis* and *P. agglomerans* sequences with bootstrap support of 91%. Isolate BCC 109 also grouped on its own supported with high bootstrap (100%). Isolates BCC 208 and BCC 691 grouped within the *P. agglomerans* clade but separate from the known *P. agglomerans* isolates.

The majority of Thailand isolates grouped within the *P. dispersa* clade with high bootstrap support (92%) (Fig. 1). Isolate BCC 211, identified as a *Enterobacter* sp. using Biolog data, grouped basal to the *P. dispersa* clade. Isolate BCC 213, identified as *P. dispersa* with low similarity index using Biolog data, formed a group, also basal to the *P. dispersa* clade, with *Erwinia cypripedii* with 79% bootstrap support.

Analysis of the 16S rDNA gene sequence data for the bacterial strains under consideration showed that isolates from Uruguay grouped in three distinct clades within the *P. ananatis* - *P. agglomerans* grouping. The majority of isolates grouped close to the bacterial blight pathogen from South Africa (LMG 20103). Five of the Uruguay isolates (BCC 756, BCC 757, BCC 760, BCC 763 and BCC 764) grouped with a reference strain BCC 077. This reference strain (BCC 077) from Uruguay, has been provisionally identified as the new species, *Pantoea eucalypti* prov.nom., closely related to *P. agglomerans* (Venter unpublished) based on DNA hybridization results. The remaining Uruguay isolates grouped within the *P. agglomerans* clade, with isolate BCC 383 grouping with *P. agglomerans* (LMG 2565), known to be *P. agglomerans* based on DNA hybridization results (Venter unpublished), with 88% bootstrap support. Isolates BCC 758 and BCC 759 grouped basal to the two Uganda isolates BCC 208 and 691 with 99% bootstrap support.

Amplified Fragment Length Polymorphism (AFLP) Analysis

In the dendrogram constructed using band patterns obtained with AFLP analysis (Fig. 2), 16 different clusters could be seen. Cluster 5 contained the *P. agglomerans* isolates including the type strain (LMG 1286^T). *P. ananatis* isolates, including the type strain (LMG 2665^T), grouped in cluster 8. The type strains of *P. citrea*, *P. terrea* and *P. punctata* fell in to single groups, clusters 9, 10 and 13, respectively. Cluster 15 contained the type stain of *P. dispersa* (LMG 2603^T). The two *P. stewartii* sub-species formed a cluster separate from the other *Pantoea* species (cluster 16).

Uganda isolates grouped in two of the clusters obtained in the UPGMA dendrogram obtained after AFLP band pattern analysis (Fig. 2). Isolate BCC 109 and BCC 207 clustered apart from the other Uganda isolates in cluster 1. Cluster 2 contained BCC 105, BCC 107, BCC 208 and BCC 691 that grouped together with some of the isolates from Uruguay with a similarity value of 50%. The difference in groupings of BCC 109 and BCC 207 and the rest of the Uganda isolates correlated well with the 16S rDNA data (Fig. 1).

The majority of Thailand isolates grouped with the type strain of *P. dispersa* (Fig. 2) in cluster 15. Isolates BCC 210, BCC 212, BCC 378 and BCC 380 clustered with the type strain of *P. dispersa*. The isolate BCC 211 clustered with the two Uganda outlier isolates, BCC 109 and BCC 207, in cluster 1, grouping with BCC 207 with a 62% similarity value. BCC 213 formed a single cluster (cluster 12), grouping close to the type strains of *P. citrea*, *P. punctata* and *P. terrea*, corresponding to 16S rDNA results.

AFLP data showed that the Uruguay isolates fall into three major clusters (Fig. 2). BCC 383 clustered with the *P. agglomerans* strains in cluster 5. Three Uruguay isolates (BCC 756, BCC 757 and BCC 760) grouped with the reference strain (BCC 077) from Uruguay which represents the proposed new species *P. eucalypti* prov.nom., with a similarity value of 52%. The remaining isolates were split between clusters 2, containing Uganda isolates, and cluster

8 representing the *P. ananatis* cluster, with similarity values of approximately 50% for each grouping.

Results obtained from API, Biolog and molecular characterisations are summarised in table 3.

Pathogenicity Screening

Hypersensitive reaction (HR)

All isolates from Uganda, Thailand and Uruguay elicited moderate to no hypersensitive responses when compared to the positive control isolate LMG 20103. BCC 105, BCC 380 and BCC 574 elicited the strongest responses (Fig. 3) when compared to other isolates from the same country. No response was elicited by the negative control.

Pathogenicity screening

Uganda, Thailand and Uruguay isolates were found to be moderately pathogenic when compared to the positive control after inoculations of the susceptible *Eucalyptus* GN clones (Fig. 4). Isolate BCC 105 was found to be the most pathogenic of all Uganda isolates used. Thailand isolates produced similar results with no single isolate found to be more pathogenic than the other isolates. Isolates representing three of the *Pantoea* spp. from Uruguay, *P. ananatis* (BCC 382, BCC 574), *P. vagens* (BCC 761, BCC 763) and *P. eucalyptii* (BCC 756, BCC 760), were found to be moderately pathogenic (Fig. 4). These isolates were equally pathogenic but were found to be more pathogenic than the *P. agglomerans* isolate (BCC 383) from Uruguay. The negative control produced no lesions.

Discussion

In this study, bacteria isolated from diseased *Eucalyptus* leaf material from Uganda, Thailand and Uruguay were identified using phenotypic (API, Biolog) and genotypic (16S rDNA gene sequence, AFLP) data. Results showed that the bacteria isolated belonged to different species of the genus *Pantoea*. Bacterial isolates from Thailand were found to represent a known species

within the genus *Pantoea*. The isolates from Uganda represented a proposed new *Pantoea* species. Isolates from Uruguay were found to represent four *Pantoea* species, two known and two new proposed species. Weak hypersensitivity reactions were elicited by the isolates suggesting that they are not plant pathogens. It is, however, known that not all pathogens are able to elicit hypersensitivity reactions in tobacco (Alvarez 2004) and these results should thus be seen as preliminary.

The majority of isolates from Thailand were identified as *P. dispersa*. The type strain of this species was isolated from soil in Japan (Gavini *et al.* 1989). It has subsequently also been isolated from diverse hosts such as Okra (*Abelmoschus esculentus*), Grain sorghum (*Sorghum bicolor*), a *Rosa* sp. as well as from humans (<http://bccm.belspo.be/index.php> 2007, <http://www.ncppb.com/> 2007). This is the first report of *P. dispersa* strains found associated with disease symptoms on *Eucalyptus*. *P. dispersa* isolates from Thailand were found to be moderately pathogenic. Their mild pathogenicity does not, however, account for the severity of symptoms observed in field.

The majority of Uganda isolates were identified as a new species, *Pantoea vagens* prov.nom., closely related to *P. agglomerans*. This close relationship with *P. agglomerans* would explain why Uganda isolates were identified as *P. agglomerans* using both the API and Biolog systems. The inability of the API 20E system to distinguish between closely related species has been reported previously (Butler *et al.* 1975; Gavini *et al.* 1989; Mergaert *et al.* 1993) and is one of the major disadvantages of this system. Toth *et al.* (1999) reported on the variability of results obtained with the Biolog system and the inability of this system to distinguish between closely related *Erwinia* species. This emphasizes the need for DNA-based systems for comprehensive identification of plant pathogenic bacteria.

The isolates from Uruguay were found to represent four *Pantoea* spp. The majority of the isolates were identified as *P. ananatis* and *P. vagens* prov.nom. Smaller numbers of the isolates represented the proposed new

species *Pantoea eucalyptii* prov.nom. Isolates representing *P. vagens*, *P. ananatis* and *P. eucalyptii* were found to be moderately pathogenic with no single species being more pathogenic than the other. These isolates were, however, more pathogenic than the single isolate identified as *P. agglomerans*.

Isolates from all three countries were found to be moderately to non-pathogenic. Variability in pathogenicity was observed between species from the same region and species from different regions. Various studies have reported similar variability in pathogenicity and disease severity of members of the genus *Pantoea* (Azad *et al.* 2000; Gitaitis *et al.* 2002). Two of the major factors found to influence pathogenicity were temperature and relative humidity (Azad *et al.* 2000). In the present study the greenhouse was kept at 26°C but high humidity could not be induced. Hot, humid conditions with some free standing water were often the prevailing field conditions (Wingfield pers. comm.). We believe that the inability to replicate the hot and humid conditions, in part, explains why symptoms obtained with inoculated isolates on *Eucalyptus* were less severe than symptoms noted in field. This is particularly true for the isolates from Thailand.

The consistent isolation of *P. vagens* prov.nom. and *P. ananatis* from diseased leaves and shoot in Uruguay indicates that these bacteria could be responsible for similar disease symptom development on the same host. Similar findings have been reported by Stall *et al.* (1994) and Bouzar *et al.* (1999), both reporting on two closely related species of *Xanthomonas* causing similar disease symptoms on the same hosts. Goszczyńska *et al.* (2007) identified two *Pantoea* spp., one of which was *P. ananatis*, causing similar symptoms when the pathogens were injected into the stems of young maize plants. Goszczyńska *et al.* (2006) also reported on the isolation of two different *Pantoea* spp. from onion seed in South Africa, one of which was a new species, *P. allii*.

The isolation of multiple *Pantoea* species from diseased *Eucalyptus* tissue suggests that these bacteria have the ability to actively enter into different

types of interactions. *Pantoea agglomerans* is an example of this phenomenon, as it is known as a pathogen (Goszczyńska *et al.* 2006), an epiphyte (Sabaratnam & Beattie 2003) and an antagonist (Wright *et al.* 2001; Marchi *et al.* 2006). These interactions are not only limited to *Pantoea* spp., but can include other bacterial species such as *Erwinia amylovora* (Wright *et al.* 2001) and various *Pseudomonas* spp. (Sabaratnam & Beattie 2003; Marchi *et al.* 2006).

The variability in pathogenicity, the ability to occur epiphytically (Watanabe *et al.* 1996) or endophytically (Loiret *et al.* 2004) and the apparent influence of environmental conditions on disease development (Azad *et al.* 2000) implies that some members of the genus are opportunistic pathogens. Their ability to have various types of interactions with both phytopathogens and their host plants supports this view. Very little is known as to how or why these *Pantoea* species interact with each other and/or induce disease. Further studies are needed on the interactions between these bacteria, as well as conditions conducive to disease development to determine the risk that these apparently opportunistic pathogens pose to global forestry.

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Internet Sources

<http://bccm.belspo.be/index.php> 2007

<http://www.ncppb.com/> 2007

Table 1: Bacterial isolates used in this study with their sources and Genbank accession numbers

Isolate	Origin	Isolated from:	Received from:	Genbank accession number
BCC 105	Uganda	<i>Eucalyptus</i>	G. Nakabonge	-
BCC 107	"	"	"	-
BCC 109	"	"	"	-
BCC 207	"	"	"	-
BCC 208	"	"	"	-
BCC 691	"	"	"	-
BCC 209	Thailand	"	M.J. Wingfield	-
BCC 210	"	"	"	-
BCC 211	"	"	"	-
BCC 212	"	"	"	-
BCC 213	"	"	"	-
BCC 378	"	"	"	-
BCC 379	"	"	"	-
BCC 380	"	"	"	-
BCC 381	Uruguay	"	"	-
BCC 382	"	"	"	-
BCC 383	"	"	"	-
BCC 574	"	"	"	-
BCC 583	"	"	"	-
BCC 611	"	"	"	-
BCC 754	"	"	"	-
BCC 756	"	"	"	-
BCC 757	"	"	"	-
BCC 758	"	"	"	-
BCC 759	"	"	"	-
BCC 760	"	"	"	-
BCC 761	"	"	"	-
BCC 762	"	"	"	-
BCC 763	"	"	"	-
BCC 764	"	"	"	-
BCC 916	"	"	"	-

Table 1: Bacterial isolates used in this study with their sources and Genbank accession numbers (continued)

Isolate	Origin	Isolated from:	Received from:	Genbank accession number
Reference isolates:				
LMG 20103 <i>P. ananatis</i>	South Africa	<i>Eucalyptus</i>	BCC Culture Collection	AF364847
LMG 1286 ^T <i>P. agglomerans</i>	Zimbabwe	Human	LMG Culture Collection	AJ233423
LMG 2665 ^T <i>P. ananatis</i>	Brazil	<i>Ananas comosus</i> (Pineapple)	"	Z96081
LMG 2603 ^T <i>P. dispersa</i>	Japan	Soil	"	_1
LMG 2715 ^T <i>P. stewartii</i> subsp. <i>stewartii</i>	USA	<i>Zea Mays</i> (Sweet Corn)	"	Z96080
LMG 2632 ^T <i>P. stewartii</i> subsp. <i>indologenes</i>	India	<i>Setaria italica</i> (Foxtail Millet)	"	Y13251
LMG 22049 ^T <i>P. citrea</i>	Japan	<i>Citrus reticulata</i> (Mandarin)	"	_1
LMG 22050 ^T <i>P. punctata</i>	"	"	"	_1
LMG 22051 ^T <i>P. terrea</i>	"	Soil	"	_1

¹ 16S rDNA Sequences for the type species were obtained from the BCCM/LMG culture collection (University of Ghent, Belgium)



Table 2: Results obtained with API 20E and Biolog Identification Systems as well BLAST homology searches using the Genbank/EMBL database with percentage identity significance, similarity indices and percentage homology, respectively

Isolate	Country of Origin	API 20E		Biolog			BLAST Homology Results	
		Identity	% Significance	Identity	% Probability ^a	Similarity Index	Result	% Homology
BCC 105	Uganda	<i>Pantoea</i> sp. 3	95.5	<i>P. agglomerans</i>	99	0.560	<i>P. agglomerans</i>	99
BCC 107	“	“	95.5	“		0.449	“	99
BCC 109	“	“	95.5	“	99	0.536	“	99
BCC 207	“	Unacceptable Profile		<i>Escherichia vulneris</i>	96	0.505	<i>P. dispersa</i>	98
BCC 208	“	<i>Pantoea</i> sp. 3	82.6	<i>P. agglomerans</i>	99	0.590	<i>P. agglomerans</i>	99
BCC 691	“	“	98.9	“	99	0.548	“	99
BCC 209	Thailand	<i>Serratia</i> sp.	97.2	<i>Vibrio</i> sp.	99	0.640	<i>Escherichia coli</i>	99
BCC 210	“	<i>Pantoea</i> sp. 2	64.5	<i>P. dispersa</i>	99	0.673	<i>P. dispersa</i>	99
BCC 211	“	Unacceptable Profile		<i>Enterobacter aerogenes</i>	92	0.562	“	99
BCC 212	“	<i>Pantoea</i> sp. 2	62.3	<i>P. dispersa</i>	99	0.586	“	99
BCC 213	“	Unacceptable Profile		“		0.304	<i>Erwinia cypripedii</i>	98
BCC 378	“	Unacceptable Profile		<i>P. agglomerans</i>		0.406	<i>P. dispersa</i>	99
BCC 379	“	<i>Pantoea</i> sp. 2	64.5	<i>P. dispersa</i>		0.481	<i>Enterobacter cloacae</i>	97
BCC 380	“	“	64.5	“	99	0.613	<i>P. dispersa</i>	99
BCC 381	Uruguay	<i>Pantoea</i> sp. 2		<i>P. agglomerans</i>		0.305	<i>P. ananatis</i>	99
BCC 382	“	“		“		0.480	“	99
BCC 383	“	<i>Aeromonas hydrophila</i>	95.4	“		0.390	<i>P. agglomerans</i>	99
BCC 574	“	<i>Pantoea</i> sp. 2		“		0.395	<i>P. ananatis</i>	99



Table 2: Results obtained with API 20E and Biolog Identification Systems as well BLAST homology searches using the Genbank/EMBL database with percentage identity significance, similarity indices and percentage homology, respectively (continued)

Isolate	Country of Origin	API 20E		Biolog			BLAST Homology Results	
		Identity	% Significance	Identity	% Probability ^a	Similarity Index	Result	% Homology
BCC 583	“	“		“		0.405	“	99
BCC 611	“	“		“		0.417	“	99
BCC 754	“	“		“		0.343	“	99
BCC 756	“	<i>Pantoea</i> sp.3		“	95	0.554	“	99
BCC 757	“	“		“		0.448	“	99
BCC 758	“	“		“	87	0.534	<i>P. agglomerans</i>	99
BCC 759	“	“	95.5	“	96	0.541	“	99
BCC 760	“	“		“	99	0.603	<i>P. ananatis</i>	99
BCC 761	“	“		“		0.341	“	99
BCC 761	“	“		“		0.495	<i>P. agglomerans</i>	99
BCC 763	“	“		“	95	0.601	<i>P. ananatis</i>	99
BCC 764	“	“		“	96	0.609	<i>P. ananatis</i>	99
BCC 916	“	<i>Pantoea</i> sp. 2		“		0.371	<i>P. ananatis</i>	99

^a Percentages probability are obtained for profiles where the Similarity index is higher than 0.5



Table 3: Summary of results obtained by API and Biolog systems as well as 16S rDNA gene sequence and AFLP band pattern analyses with provisional isolate identifications made in this study

Isolate	Origin	API:	BIOLOG	16S rDNA sequence analysis	AFLP analysis	Identity
BCC 105	Uganda	<i>Pantoea</i> sp. 3	<i>P. agglomerans</i>	<i>P. agglomerans</i> group	<i>P. vagens</i> group	<i>Pantoea vagens</i>
BCC 107	“	“	“	“	“	“
BCC 109	“	“	“	“	Cluster 1 ^a	<i>Pantoea</i> sp.
BCC 208	“	<i>Pantoea</i> sp. 3	<i>P. agglomerans</i>	“	<i>P. vagens</i> group	<i>Pantoea vagens</i>
BCC 691	“	“	“	“	“	“
BCC 210	“	<i>Pantoea</i> sp. 2	<i>P. dispersa</i>	<i>P. dispersa</i> group	<i>P. dispersa</i> group	<i>Pantoea dispersa</i>
BCC 211	“	Unacceptable Profile	<i>Enterobacter</i>	“	Cluster 1 ^a	<i>Pantoea</i> sp.
BCC 212	“	<i>Pantoea</i> sp. 2	<i>P. dispersa</i>	“	<i>P. dispersa</i> group	<i>Pantoea dispersa</i>
BCC 213	“	Unacceptable Profile	“	<i>Erwinia</i> group	Cluster 12 ^b	<i>Pantoea</i> sp.
BCC 378	“	Unacceptable Profile	<i>P. agglomerans</i>	<i>P. dispersa</i> group	<i>P. dispersa</i> group	<i>Pantoea dispersa</i>
BCC 380	“	“	“	“	“	“
BCC 381	Uruguay	<i>Pantoea</i> sp. 2	<i>P. agglomerans</i>	<i>P. ananatis</i> group	<i>P. ananatis</i> group	<i>Pantoea ananatis</i>
BCC 382	“	“	“	“	“	“
BCC 383	“	<i>Aeromonas hydrophila</i>	“	<i>P. agglomerans</i> group	<i>P. agglomerans</i> group	<i>Pantoea agglomerans</i>
BCC 574	“	<i>Pantoea</i> sp. 2	“	<i>P. ananatis</i> group	<i>P. ananatis</i> group	<i>Pantoea ananatis</i>
BCC 583	“	“	“	“	“	“
BCC 611	“	“	“	<i>P. ananatis</i> group	<i>P. ananatis</i> group	<i>Pantoea ananatis</i>
BCC 754	“	“	“	“	“	“
BCC 756	“	<i>Pantoea</i> sp.3	“	<i>P. agglomerans</i> group	<i>P. eucalyptii</i> group	<i>Pantoea eucalyptii</i>
BCC 757	“	“	“	“	“	“



Table 3: Summary of results obtained by API and Biolog systems as well as 16S rDNA gene sequence and AFLP band pattern analyses with provisional isolate identifications made in this study (continued)

Isolate	Origin	API:	BIOLOG	16S rDNA sequence analysis	AFLP analysis	Identity
BCC 758	Uruguay	<i>Pantoea</i> sp. 3	<i>P. agglomerans</i>	<i>P. agglomerans</i> group	<i>P. vagens</i> group	<i>Pantoea vagens</i>
BCC 759	"	"	"	"	"	"
BCC 760	"	"	"	"	<i>P. eucalyptii</i> group	<i>Pantoea eucalyptii</i>
BCC 761	"	"	"	"	<i>P. vagens</i> group	<i>Pantoea vagens</i>
BCC 762	"	"	"	"	<i>P. vagens</i> group	"
BCC 763	"	"	"	"	<i>P. vagens</i> group	"
BCC 764	"	"	"	"	<i>P. vagens</i> group	"
BCC 916	"	<i>Pantoea</i> sp. 2	"	<i>P. ananatis</i> group	<i>P. ananatis</i> group	<i>Pantoea ananatis</i>

^a Isolates BCC 109, 207 and 211 grouped apart from other *Pantoea* sp. after AFLP band pattern analysis (Fig 2)

^b Isolate BCC 213 from Thailand formed a single group, grouping closest to isolate BD390, a reference strain from union in South Africa

Figure 1: Phylogenetic tree constructed from unknown Uganda, Thailand and Uruguay isolates with selected members of the *Enterobacteriaceae*. Seven type strains of genus *Pantoea* are indicated in red. Uganda isolates are indicated in green, Thailand isolates indicated in blue and Uruguay isolates are indicated in cyan. Bootstrap values are indicated above the branches.

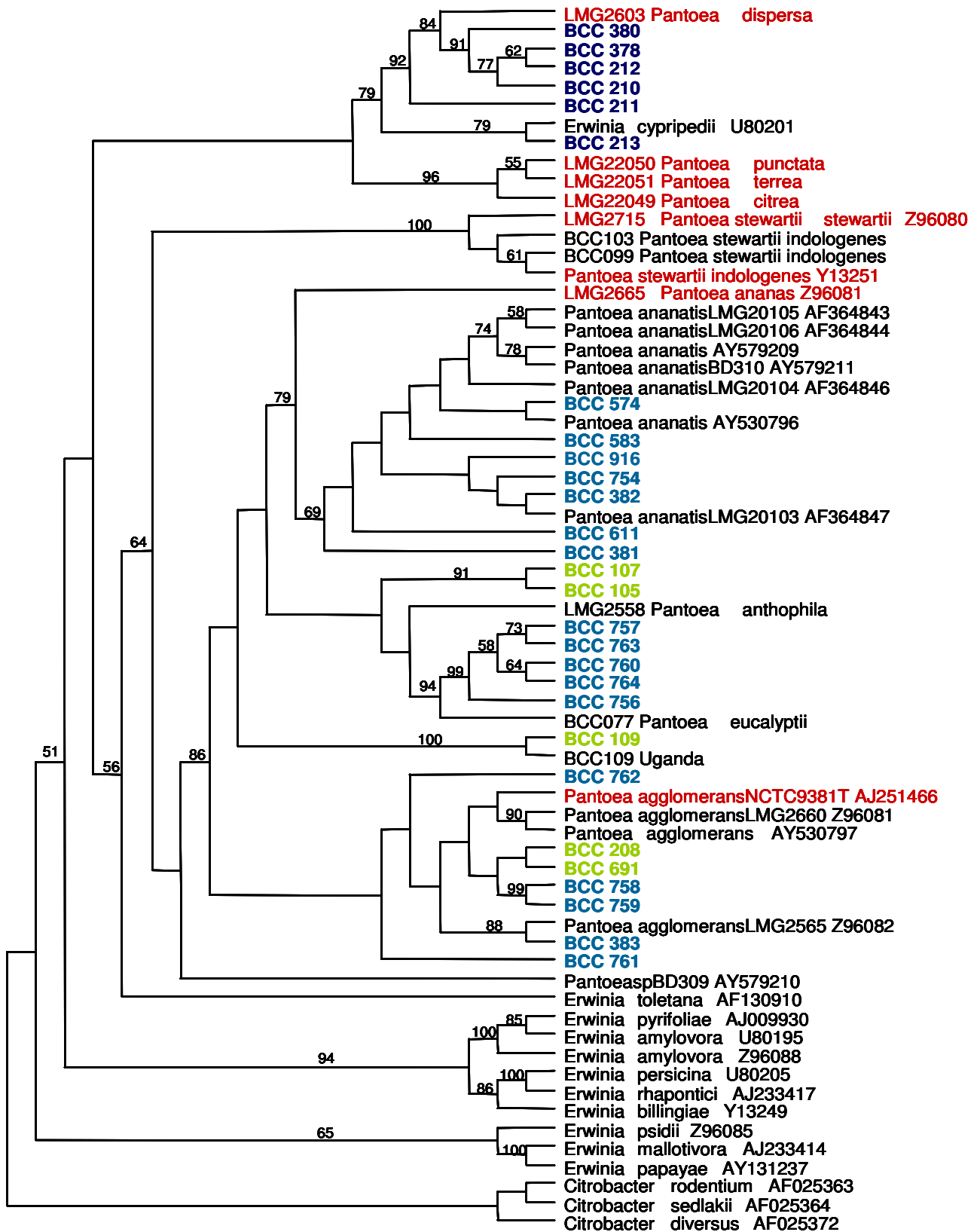


Figure 2: UPGMA dendrogram constructed using the Pearson correlation coefficient after analysis of AFLP band patterns obtained from unknown Uganda, Thailand and Uruguay isolates, with selected references strains including type strains of the seven existing *Pantoea* species. Uganda isolates are indicated in green, Thailand isolates in blue and Uruguay isolates in cyan. The seven existing type strains are indicated in red.



Pearson correlation [0.0% -100.0%]

10 20 30 40 50 60 70 80 90 100

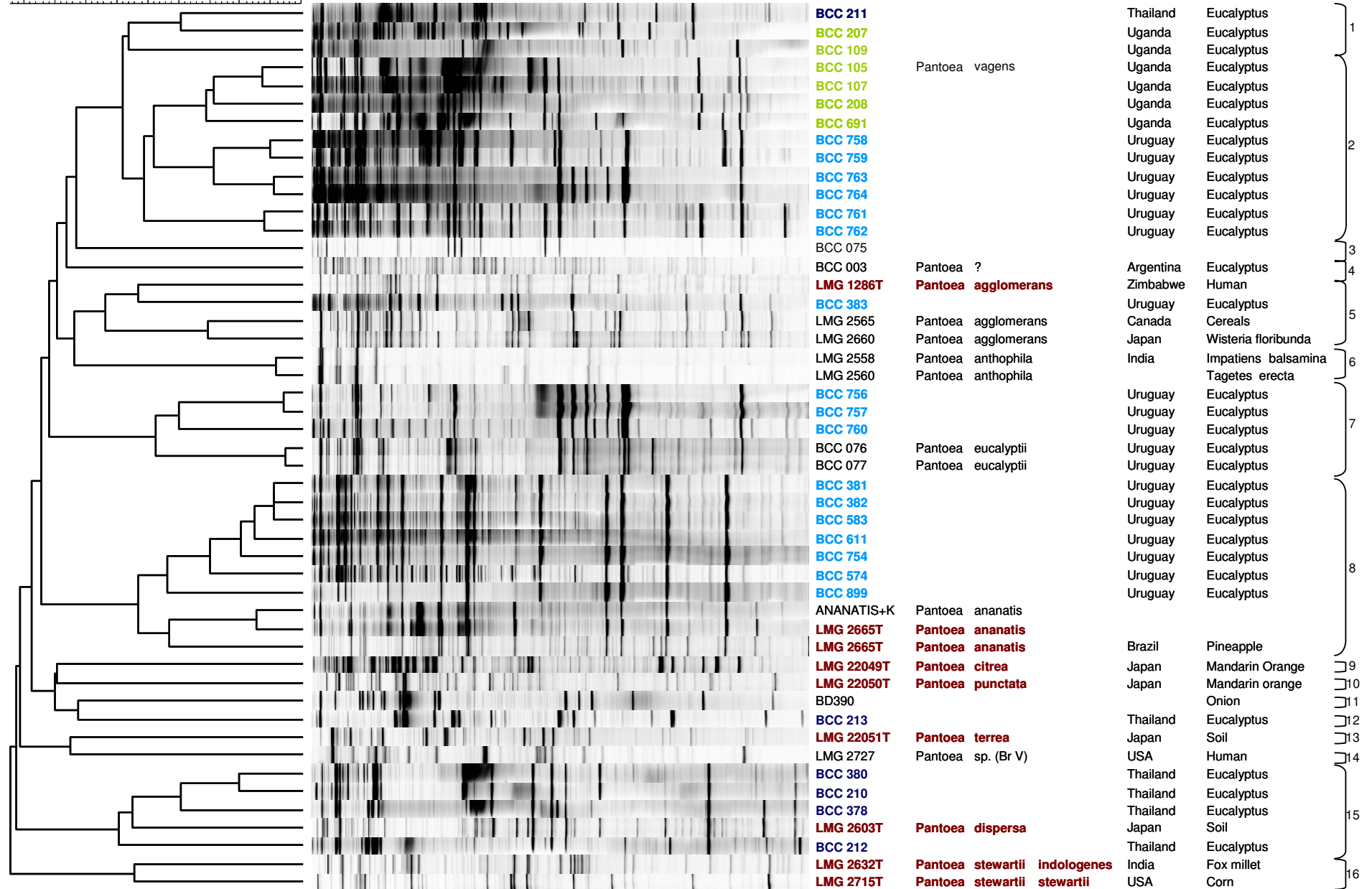


Figure 3: Hypersensitive response (HR) results obtained with Uganda, Thailand and Uruguay isolates. **A:** Negative Control (sterile saline solution), **B:** Positive Control *P. ananatis* LMG 20103, **C & D:** BCC 105 and BCC 208 from Uganda, **E & F:** BCC 212 and BCC 380 from Thailand, **G & H:** BCC 574 and BCC 382 representing *P. ananatis* isolates from Uruguay, **I & J:** BCC 761 and BCC 763 representing *P. vagens* isolates from Uruguay, **K & L:** BCC 756 and BCC 760 representing *P. eucalyptii* isolates from Uruguay

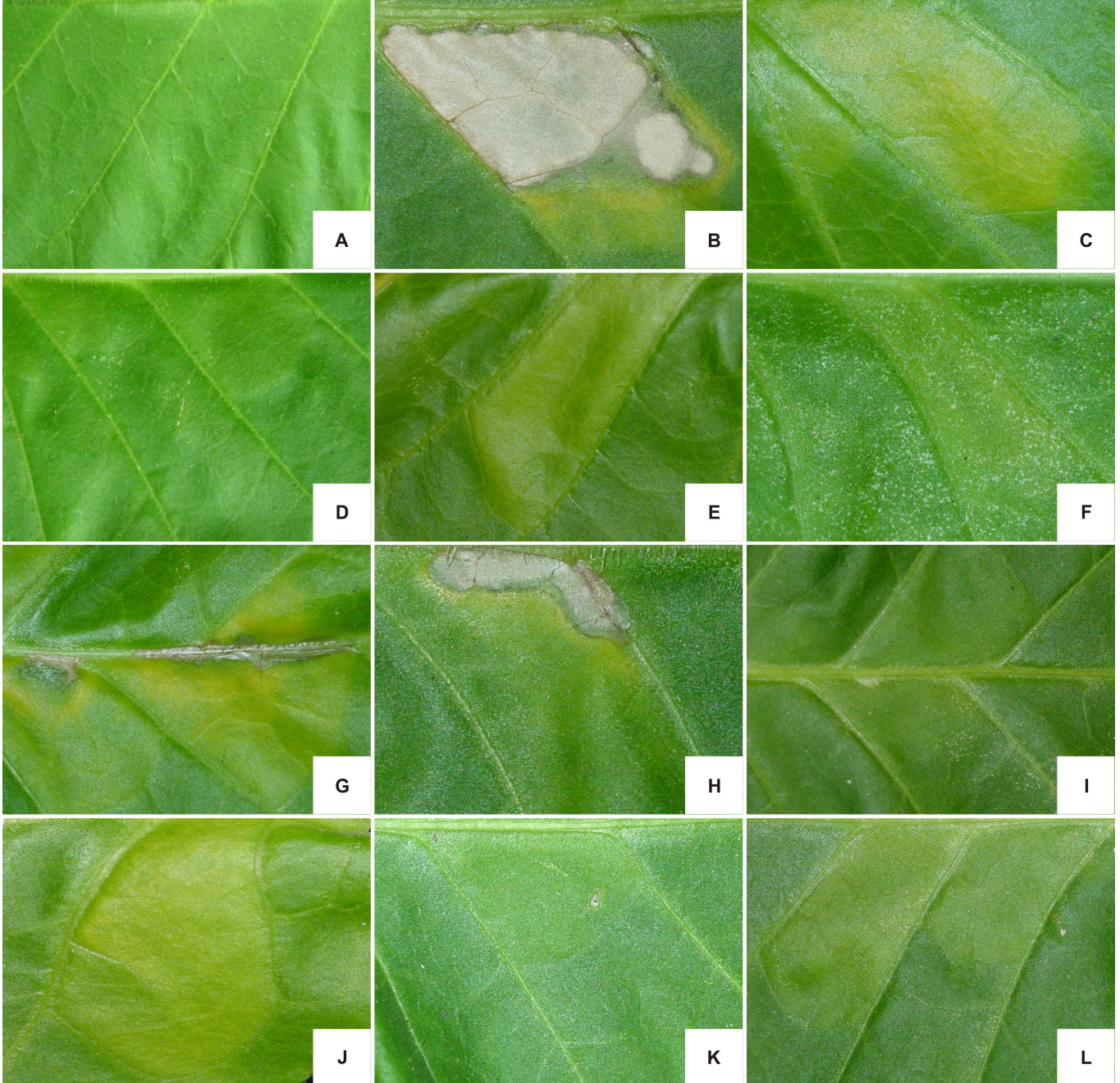
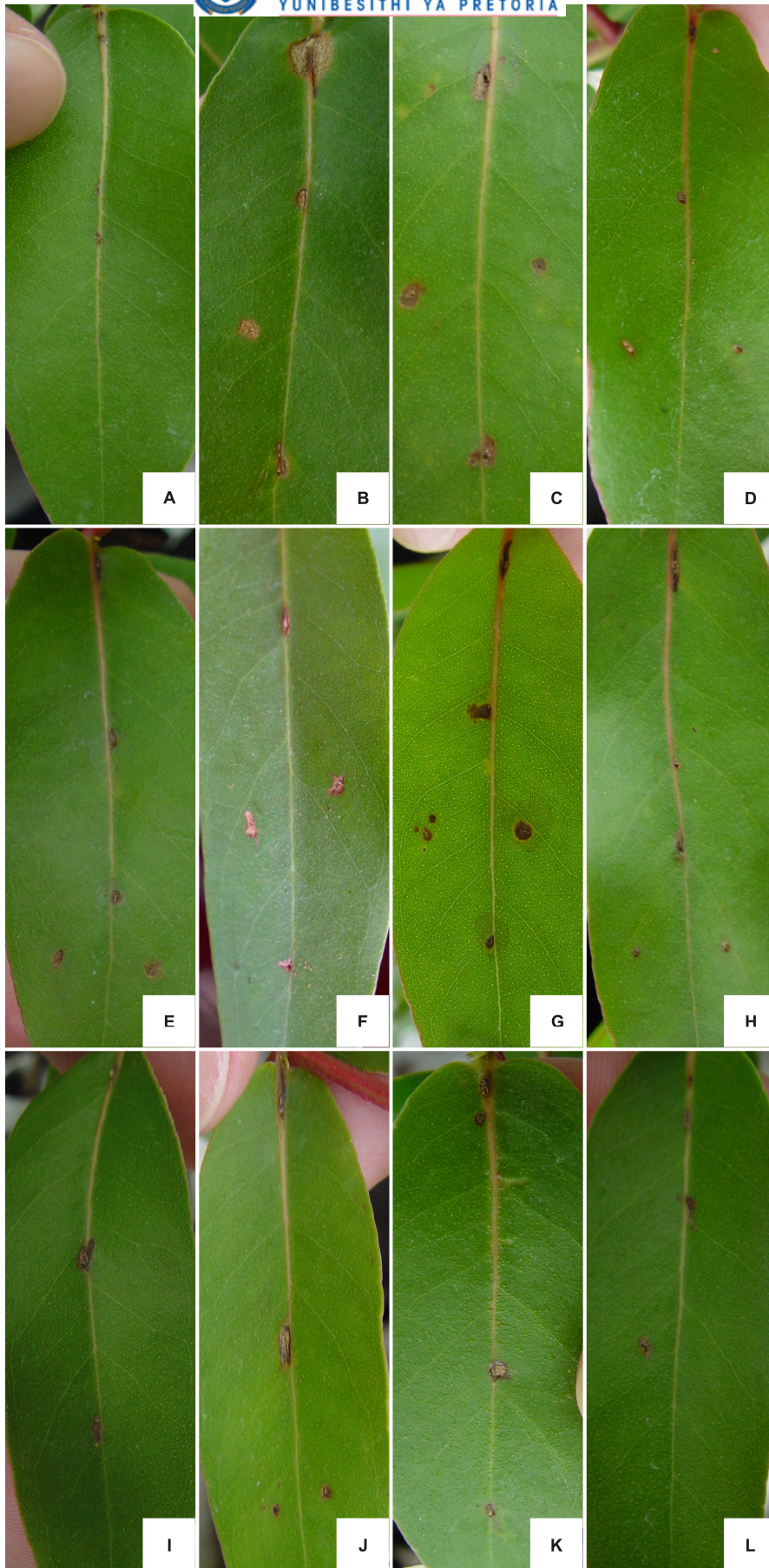


Figure 4: Pathogenicity screening results obtained with isolates from Uganda, Thailand and Uruguay. **A:** Negative Control (sterile saline solution), **B:** Positive Control *P. ananatis* LMG 20103, **C & D:** BCC 105 and BCC 208 from Uganda, **E & F:** BCC 212 and BCC 380 from Thailand, **G & H:** BCC 574 and BCC 382 representing *P. ananatis* isolates from Uruguay, **I & J:** BCC 761 and BCC 763 representing *P. vagens* isolates from Uruguay, **K & L:** BCC 756 and BCC 760 representing *P. eucalyptii* isolates from Uruguay



Summary

Plantations of *Eucalyptus* spp. are expanding world-wide to serve growing global requirements for timber and pulp products. Together with this expansion, there has been a concomitant increase in diseases affecting these trees. Most of these are caused by fungi but there are a growing number of diseases caused by bacterial pathogens. Very little is known about them and the focus of this study was to consider species in the genus *Pantoea* and their association with diseases on *Eucalyptus*. *Pantoea* spp. are known pathogens of agricultural crops in South Africa and elsewhere in the world. They are also ubiquitous occurring in diverse ecological niches. Despite their prevalence, little is known about their association with plants, particularly where they occur as pathogens.

The first chapter of this thesis presents an overview of the important aspects concerning the identification and classification of bacterial pathogens. Different techniques used for bacterial identification and classification were considered. These techniques are classified into different levels, based on their complexity and level of data resolution. As techniques have developed and been refined, our understanding of how organisms are related to each other has increased. An overview of the taxonomic history of the genus *Pantoea* was used to illustrate this point.

An interaction between two *Pantoea* spp. and *Colletogloeopsis zuluense*, a serious fungal pathogen of *Eucalyptus* has been reported in the past. In the second chapter of this thesis, I considered the view that pathogenicity of *C. zuluense* is enhanced when infection occurs in conjunction with the two *Pantoea* spp. The identities of the two *Pantoea* spp. were confirmed as *Pantoea ananatis* and *Pantoea stewartii* subsp. *indologenes*. Both greenhouse and field inoculation trials with the two *Pantoea* spp. and *C. zuluense* failed to confirm that there is an increase in pathogenicity of *C. zuluense* when these bacteria are present.

Studies in chapter three of this thesis, considered the identity of bacteria associated with diseased *Eucalyptus* leaf material from Uganda, Thailand and

Uruguay. Symptoms observed in these countries were very similar and they were also similar to those of bacterial blight observed in South Africa. The majority of isolates obtained from Thailand were identified as *Pantoea dispersa*, based on both phenotypic and DNA-based data. This is the first report of *Pantoea dispersa* associated with disease on *Eucalyptus*. Uganda isolates were identified as *Pantoea vagens* prov.nom., a new species in the genus *Pantoea*. The majority of Uruguay isolates were identified as either *Pantoea ananatis* or *Pantoea vagens* prov.nom. The remaining isolates from Uruguay were found to belong to *Pantoea eucalyptii* prov.nom., a proposed new *Pantoea* sp., as well as *Pantoea agglomerans*. Pathogenicity results showed that the majority of isolates from all three countries were moderately pathogenic, eliciting moderate reactions in both tobacco and susceptible *Eucalyptus grandis* x *nitens* hybrid clones.

Overall, results of studies presented in this thesis showed that *Pantoea* spp. can exist in complex interactions with both plants and fungi. These interactions are, however, not necessary for bacterial survival. We believe that the majority of *Pantoea* spp. are opportunistic pathogens based on their ability to selectively enter into interactions as well as occur epiphytically on plants. Variability in pathogenicity, both observed in this study and previously reported, further supports this view. Additional studies are needed to determine the conditions conducive to disease development in order to fully understand the threat these pathogens pose to global forestry.