UNIVERSITEIT VAN PRETORIA
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Epiphytic and endophytic members of the *Enterobacteriaceae* associated with healthy *Eucalyptus trees*

Submitted by

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A dissertation submitted in partial fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

In the Faculty of Natural and Agricultural Sciences, Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

September 2006

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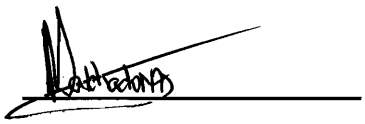
Declaration

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



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November 2006

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Acknowledgements

- I would like to thank my parents, Musiiwa Makhado, Mafanedza, and Thavhadziawa Ramutumbu, for unconditionally loving me and supporting me even in hopeless situations. Whenever I felt like giving up, you were there for me.
- My uncle Khathu Netshisaulu and the family for believing in me and encouraging me at all times.
- Pieter de Maayer, a friend, mentor, a chaperone, thank you so much for helping me. You are a one truly amazing "fella".
- Lorinda Swart, thank you for your assistance with the AFLPs. It was much appreciated.
- My Supervisor Prof. Teresa Coutinho, what can I say, you've been a supervisor and a great support. Thank you for your guidance and your love. Even though I went through difficult times, you never stopped believing in me.
- My Co-Supervisor, Prof. Fanus Venter, thank you so much for guiding me through this process.
- Profs. Mike and Brenda Wingfield, thank you for your inspiration.
- NRF and TPCP, for financial support.
- My colleagues in the Denison Lab, as well as the FABI team and TPCP, thank you for the laughter and the help.
- My late uncle, Alfred M Ramutumbu, thank you for your encouragement and inspiration despite poor health, this is not the last one.
- Finally and above all, I would like to thank God for giving me life and strength for this achievement.

------ooOoo------

Preface

Eucalyptus species, clones and hybrids are planted extensively along the eastern coastal belt of South Africa. They occupy an area of approximately 600000 ha. A number of fungal pathogens attack this host causing substantial economic losses each year. Only two bacterial diseases have been reported to infect *Eucalyptus* locally, namely, *Ralstonia solanacearum* and *Pantoea ananatis*. A third pathogen, *Xanthomonas eucalypti*, has been reported to infect *E. citriodora* in Australia.

Pantoea ananatis was first reported in South Africa in 2002 as a pathogen of *Eucalyptus*. It has subsequently been found to occur on onion seed and maize in this country. The disease caused by this bacterial pathogen, blight and die-back, was initially widespread occurring in all areas were *Eucalyptus* was grown commercially. Today, it is rarely found outside of the nursery environment where it readily infects seedlings, cuttings and hedge plants. A number of clones of the hybrid, *E. grandis* x *E. nitens* (GN), has been found to be very susceptible to *P. ananatis*.

Chapter One of this thesis presents a review on the *Enterobacteriaceae* found associated with plants as epi- and endophytes. The aim of the review is to consider the prospect of members of this bacterial family surviving on and in various plant hosts without causing disease symptoms. Colonization of bacterial epi- and endophytes is considered as well as the potential role they may play as phytopathogens. This sets the stage for the rest of the thesis and highlights the importance of having an understanding of the etiology of pathogens which allows management strategies to be put into place.

To date, to our knowledge, no studies have been undertaken to identify epi- or endophytes on *Eucalyptus*. Little is known about the etiology and epidemiology of bacterial blight and die-back caused by *P. ananatis*. Thus in Chapter Two, analyses are performed on healthy *Eucalyptus* tissue in order to determine whether this species and/or any other member of the *Enterobacteriaceae* are present as epiphytes or endophytes on this host.

Chapter 1

Enterobacteriaceae found associated with plants as epi-and endophytes: a literature review

INTRODUCTION

Eucalyptus spp. are versatile trees that grow in a variety of climatic conditions and over a diverse range of habitats (Turnbull, 2000). Eucalypts were initially introduced into the tropical and subtropical regions of the Southern Hemisphere for fuel wood, windbreaks and land reclamation purposes since early times (Poynton, 1979; Potts and Pederick, 2000). Over the years, eucalypts have become a major source of diverse commercial forest products such as pulpwood, fibreboard, sawn timber, poles, mine timber props, charcoal, honey and essential oils (Sedjo, 1999; Turnbull, 1999). South Africa has a long history of planting eucalypts, since it does not have its own natural timber resources (Poynton, 1979). It is currently the third largest *Eucalyptus* growing country in the world with nearly 600 000 ha of eucalypt plantations (Schönau *et al*., 1994; Owen and Van der Zel, 2000). These plantations are mainly distributed along the eastern coastal belt of South Africa, and comprise an assortment of species and hybrids that are planted in various habitats with diverse climatic conditions (Herbert, 1994).

A number of fungal diseases cause severe damage to eucalypts in South Africa, notably Mycosphaerella leaf blotch (Crous and Wingfield, 1996), Botryosphaeria (Smith *et al*., 1994) and Colletogloeopsis canker (Wingfield *et al.,* 1997). Thus far, three bacterial diseases have been reported on this host, namely, bacterial wilt caused by *Ralstonia solanacaerum* (Coutinho *et al*., 2000), bacterial dieback associated with *Xanthomonas eucalypti* (Truman, 1974) and blight and dieback caused by *Pantoea ananatis* (Coutinho *et al*., 2002). With the exception of *X. eucalypti*, both *R. solanacearum* and *P. ananatis* have been reported on this host in South Africa. In 1999, Van Zyl *et al.* (1999) also found two *Pantoea* spp. in a synergistic relationship with the fungal pathogen *Colletogloeopsis zuluence*.

In 1998, a severe disease was noted in a nursery in Kwa-Zulu Natal on ramets of an *E. grandis* x *nitens* (GN) hybrid clone (Coutinho *et al*., 2002). The disease subsequently spread to other nurseries in the Northern Province, Kwa-Zulu Natal and Mpumalanga Provinces of South Africa. It has been observed on a number of different *Eucalyptus* species, including *E. grandis*, hybrids and clones. This disease is characterized by typical bacterial blight symptoms, including tip dieback and leaf

spots. The leaf spots are watersoaked and subsequently coalesce to form large lesions. The spread of the pathogen appears to radiate from the leaf petiole into the main vein and consequently into the surrounding tissues. The leaves are prematurely abscised as the leaf petioles become necrotic and the tree appears scorched as the disease advances. With repeated infections the plants become stunted. New shoots are formed and the trees take on a bushy appearance. Little is known about the etiology or the epidemiology of this disease and this thus prompted an investigation into whether this pathogen has the ability to survive on or in its host as an epi- or endophyte.

In this chapter, literature on the *Enterobacteriaceae* associated with plants either an endophytes and/or epiphytes is reviewed.

ENTEROBACTERIACEAE

According to Brenner and Farmer III (2005), the family *Enterobacteriaceae* incorporates a group of Gram negative, facultatively anaerobic rod-shaped bacteria, approximately 0.3-1.0 x 1.0-6.0 µm in size. They are generally motile by peritrichous flagella. Most bacteria in this family grow well and are most metabolically active at 25-35°C. They are chemoorganotrophic, having both a respiratory and fermentative metabolism. With the exception of *Shigella dysenteriae* O group I and *Xenorhabdus* they are catalase positive and the majority are oxidase negative.

At present, the family *Enterobacteriaceae* contain over 44 genera and 176 species. Members have been isolated from a wide variety of sources, including soil, fresh and salt water, fruits and vegetables, grains, meats and eggs, a wide variety of plants, insects, animals and humans (Brenner and Farmer III, 2005). As its name suggests, this family includes numerous organisms specific to the gastrointestinal tract of humans and animals and a number of them are important pathogens of these hosts. *Salmonella enterica*, *Shigella flexnerii* and *Yersinia pestis* are some of the most devastating human and animal pathogens and have been associated with nosocomial, zoonotic and foodborne disease outbreaks (Beane *et al.*, 1990). This family also includes a number of important plant pathogens, mainly belonging to the genera *Pantoea*, *Pectobacterium, Erwinia, Brenneria* (Brenner and Farmer III, 2005) and *Dickeya* (Samson *et al.*, 2005). The genera *Erwinia* and *Pectobacterium*

contains some of the most well-studied plant pathogens and these are responsible for plant diseases ranging from soft rots, blights and wilts to localized necrosis on a broad range of host plants, including apples, potatoes, cucumbers, sugarcane and cacti (Brenner and Farmer III, 2005). *Erwinia amylovora* causes fire blight of pomaceous plants, a typical necrotic disease that affects apples, pears, raspberries and several ornamental trees (Johnson and Stockwell, 1998). *Pectobacterium carotovorum* subsp. *carotovorum* and subsp. *atrosepticum* and *Dickeya chrysanthemi* cause soft rot of potatoes (Scheissendoppler and Cate, 1996) and this devastating disease costs the agricultural sector worldwide millions of dollars annually.

A number of *Enterobacteriaceae,* other than *P. ananatis*, have been isolated from trees. In particular, the genus *Brennaria* contains several very important tree pathogens. *Brennaria alni* causes bark canker disease of alder, while *Brenneria salicis* causes watermark disease in willows (Hauben *et al.*, 1998). Bark necrosis of Persian walnut has been ascribed to *Brennaria nigrifluens* (Wilson *et al.*, 1957) and *Brenneria quercina* causes shoot blight in oaks (Hauben *et al.*, 1999). *Enterobacter cancerogenus* and *Enterobacter nimmipressuralis* cause cankers on poplar and wetwood disease of elm, respectively (Dickey and Zumoff, 1988; Brenner *et al.*, 1986), while *Erwinia billingae* has been isolated from stem cankers and diseased blossoms of pear, apple, cherry and elm (Mergaert *et al.*, 1999).

PLANT – MICROBE INTERACTIONS

The intimate relationship that exists between microbes and plants ranges from commensualistic and mutualistic to those that are detrimental to the plant's health (Lugtenberg *et al.*, 2002; Hirano and Upper, 2000). Microbes, in their interactions with plants, often use the same mechanisms, whether the microbe is beneficial or pathogenic, although in different combinations and for different purposes (Abramovitch and Martin, 2004). Plant recognition by microbes and concomitant recognition of the plant by the microorganism is considered to be the key initial event in the response of plants to microbes. This recognition can occur through adhesins, fimbriae, flagella, and type III & IV secretion systems (Lugtenberg *et al*, 2002). Subsequent colonization occurs by different means. Some microorganisms colonise the surface of plants and survive as part of the epiphytic microflora while others

occupy spaces within plants tissues and this is referred to as endophytic colonization (Brencic and Winans, 2005).

1. Bacterial epiphytes

Bacterial epiphytes are bacteria that are reside on the surface of leaves, roots and stems of plants (Hirano and Upper, 2000; Smith and Hayasaka, 1982). They are primarily associated with the leaves and are found on the epidermis, surfaces below the leaves, and in the apoplast of the mesophyll cells, but not internal to plant cells or tissues (Hirano and Upper, 2000., Beattie and Lindow 1999). The epiphytic microflora consists mainly of members of the genera *Pseudomonas*, *Flavobacterium*, *Xanthomonas* and *Erwinia* (Weyman-Kaczmarkowa and Pędziwilk, 2001a). These organisms flourish as a result of the sugars and organic acids exuded by the plants (Weyman-Kaczmarkowa and Pędziwilk, 2001b). Most of the bacterial plant pathogens colonize the surfaces of healthy plants as epiphytes before they colonize internally to initiate disease (Beattie and Lindow, 1994). The dynamics of individual populations within the epiphytic community are determined by rates of immigration, emigration, growth, and death (Hirano *et al.*, 1992).

The relationship between plant and epiphyte is not superficial. Monier and Lindow (2004) suggested that, as is the case in endophytic colonization, density-dependent expression of traits is involved in epiphytic interactions with plants. They also speculated that cells in aggregates in which compounds would accumulate such signals might have a different epiphytic fitness than more solitary cells given that some bacteria produce cell signalling compounds. Bacterial genes conferring pathogenicity and inducing disease symptoms in plants also contribute to the fitness of epiphytic bacteria (Beattie and Lindow., 1994). Pathovars of the same species of phytopathogenic bacteria have been found in various degrees as epiphytes on the leaves of their susceptible host and non-host plants (Hirano and Upper. 1983). The epiphytic phase of the pathogens represents an important aspect of their epidemiology and a stage at which chemical and biocontrol is aimed. However little is known about the genes and phenotypes that contribute to the ability of

bacteria to grow on leaves and survive the variable physical environment in this habitant (Beattie and Lindow, 1994).

Limited research has been carried out on bacterial epiphytes of trees. One study was conducted to analyse the epiphytic microflora on poplar, with the intention of identifying possible native antagonists to the poplar necrosis fungus *Dothichiza populea*. However, the bacteria were simply identified on a morphological basis and not to the generic or species level (Weyman-Kaczmarkowa and Pędziwilk, 2001).

1.1 Colonization by bacterial epiphytes

Bacterial colonization of plants occurs preferentially at certain sites on the leaf. These include the stomata, guard cells, trichomes and the epidermal cell wall junction, especially in the groove along the veins (Monier and Lindow, 2004; 2005). Steps in colonization strategies include modification of leaf habitat, aggregation, ingression, and egression. (Beattie and Lindow, 1999).

Because leaf surfaces are exposed to environmental factors such as fluctuating high and low temperatures, leaf age, UV radiation from sunlight, relative humidity, excessive drought or moisture and nutrient depletion, they present a hostile environment for bacterial colonization for epiphytes (Dulla *et al.*, 2005). However, a diverse collection of bacterial species are able to grow and maintain large population sizes on leaves (Upper *et al.*, 2003; Monier and Lindow, 2005). Bacteria can modify their environment to enhance and cope with the conditions of the leaf environment, either by increasing local nutrient concentration, by production of extracellular polysaccharides (EPS) to prevent desiccation when water becomes scarce, and by production of pigments to protect against the effects of UV radiation (Beattie and Lindow, 1999).

1.2 Potential phytopathogens as epiphytes

Given the economic importance of plant pathogenic bacteria, the processes that mediate their epiphytic existence on plants have received much attention. A much addressed question is whether traits that confer virulence are also

required for epiphytic fitness (Lindow and Leveau, 2002). Pathogenicity does not appear to be required for growth of bacteria in the phyllosphere under conditions of high relative humidity; however, it is involved in the ability to access and multiply in certain protected sites in the phyllosphere and in growth on dry leaves (Wilson *et al.* 1999).

1.3 *Enterobacteriaceae* as epiphytes

The most studied Gram negative epiphyte is *Pseudomonas syringae*, which is not a member of the *Enterobacteriaceae* (Monier and Lindow, 2005., Hirano and Upper, 2000., Mercier and Lindow, 2000., Knoll and Schreiber, 2000., Kinkel *et al*, 2000). A number of members of the family *Enterobacteriaceae* have been identified as epiphytes on a number of commercial crops (Azad *et al*., 2000; Coplin and Kado, 2001; Gitaitis et al., 2002b; Khetmalas et al., 1996; Paccola-Merelles et al., 2001; Pujol and Kado, 2000). *Klebsiella planticola,* for example, has been isolated as an epiphyte from the roots of red clover and cress (Korhonen *et al*., 1983).

An epiphytic phase in the disease cycle has been recorded for *P. agglomerans* which intensifies the symptoms in basal bacteriosis of wheat caused by *Pseudomonas syringae* (Pasichnyk *et al*., 2005). *Erwinia amylovora* has also been found as an epiphyte on weeds growing in orchards (Gvozdiak and Lukach, 2001). Miller and Schroth (1972) reported the presence of an epiphytic population of *E. amylovora* on pear.

Watanabe *et al*. (1996) reported *P. ananatis* as an epiphyte and pathogen of rice in Japan. Cother *et al.* (2004) also suggested that *P. ananatis* may be present as an epiphyte on rice in Australia. *P. ananatis* was recovered as an epiphyte from 25 asymptomatic weed species in onion fields in the United States, where it has been described as causative agent of central rot. The weeds included Florida beggarweed and pusley, crabgrass, sicklepod, Texas millet, tall verbena and yellow nutsedge, as well as several crops including Bermuda grass, cowpea and soybean (Gitaitis *et al.,* 2002b). *P. ananatis* has been identified as an epiphyte on apples, and one strain *P. ananatis* CPA-3

has proven to be effective in the post-harvest control of *Penicillium expansum* (Torres *et al.*, 2005).

2. Bacterial endophytes

Endophytic bacteria are defined as those that inhabit and form a close relationship with, living plant tissues within the interior of the plant, particularly in the leaves, branches, roots, and stems, without causing disease symptoms or forming symbiotic structures (Zinniel *et al.*, 2002; Iniguez *et al.*, 2005). Of the four types of interactions between bacteria and plants, namely as a pathogen, symbiont, endophyte or epiphyte, the endophytic interactions are the least studied and least understood (Iniguez *et al.*, 2005). The presence of endophytic bacteria has been reported for many different plant species and tissues of plants at a variety of growth stages. Endophytic bacteria are found in virtually every plant on earth, including a wide range of agricultural and horticultural crops (Sturz *et al.*, 2000). In contrast, almost nothing is known about the bacterial endophytes of trees. There have been occasional reports of endophytic bacteria in asymptomatic trees, but little is known about their diversity, colonization sites and influence on plant growth (Chanway, 1997; 1998). Most plants have not been studied for their bacterial endophytes and these could be rich and reliable sources of genetic diversity and novel, undescribed bacterial species (Strobel *et al.*, 2004).

Until recently, the term endophyte was applied almost exclusively to fungi, including the mycorrhizal fungi (Chanway, 1996). However a large array of Gram positive and Gram negative bacterial endophytes have been isolated, including members of the genera *Bacillus*, *Curtobacter Clavibacter, Microbacterium, Micrococcus, Erwinia, Agrobacterium, Rhizobium, Pantoea, Enterobacter, Acetobacter, Xanthomonas, Burkolderia, Pseudomonas, Klebsiella, Escherichia, Agrobacterium* and *Serratia* (Vega *et al.*, 2005; Jimẽnez-Salgado *et al.*, 1997; Araujo *et al.*, 2001; Elvira-Recuenco and van Vuurde, 2000; Zinniel *et al.*, 2002; Quadt-Hallmann *et al.*, 1997; Marcell and Beattie, 2002; McInroy and Kloepper, 1995; Sturz and Christie, 1995; Hallmann *et al.*, 1997). As endophytic bacteria are being better analysed, it has become clear that they can confer several important benefits to plants,

such as greater resistance to stress conditions, production of phytohormones that promote plant growth and nitrogen fixation from the atmosphere (Loiret *et al.*, 2004; Strobel *et al.*, 2004; Quadt-Hallmann *et al.*, 1997). Thus endophytic bacteria are receiving increasing scientific and commercial attention for their potential improvement in the quality of crops (Elviro-Recuenco and van Vuurde, 2002). The close relationship between plant and bacterium is advantageous for the endophyte. As bacteria proliferate inside the plant tissues, they are likely to interact more closely with the host, face less competition for nutrients, and are more protected from adverse changes in the environment, than bacteria on the surface (Reinhold-Hurek and Hurek, 1998).

A few reported endophytic bacteria are known plant pathogens that inhabit symptomless susceptible hosts or nonhost plants or they may reside in tissues on which symptom development has not been noted (Misaghi and Donndelinger, 1990; Hirano and Upper, 2000). Phytopathogenic bacteria are generally better colonists of plants than bacteria that do not cause disease (Hirano and Upper, 2000). The plant may remain symptomless until external factors trigger disease development; and latent infections may be a function of low pathogen number (Shekhwat *et al.*, 1984., Schuld *et al.*, 1992., Sturz *et al.*, 2000). Endophytes may also be aggressive saprophytes or opportunistic pathogens (Strobel *et al.*, 2004).

2.1 Entry and spread of endophytes in a plant

The mechanisms by which endophytic bacteria enter the plants are poorly understood (Mahaffee *et al.*, 1997). Bacterial endophytes may move to the host plant by a number of mechanisms, including wind action, attachment to soil particles, via water, on agricultural equipment and by vectors, including humans, birds, insects, bacteriophagous nematodes and weeds (Azad *et al.*, 2000; Gitaitis *et al.*, 2002b, 2003., Cother *et al.*, 2004., Walcott *et al.*, 2002., Paccola-Meirelles, 2001., Wells *et al.*, 2002., Watanabe *et al.*, 1996., Khetmalas *et al.*, 1996., Misaghi and Donnderlinger, 1990., Schwartz *et al.*, 2003., Baird and Gitaitis, 1997., Watanabe and Sato, 1999). Soil and seeds are believed to be the major sources from which endophytic bacterial populations originate (Sturz *et al.*, 2000; McInroy and Kloepper, 1995). Mundt

and Hinkle (1976) identified 395 endophytic bacteria in ovules and seeds of 27 plant species, comprising 19 genera and 46 species, suggesting many seedlings are already colonized by endophytes prior to germination and seedling development. They concluded that bacteria present within seeds provides the plant with a protective mechanism that would normally function to prevent bacterial parasitism (Mundt and Hinkle, 1976).

The lack of penetration structure renders bacteria unable to exert mechanical or physical forces to penetrate intact epidermal cells. However, bacteria may enter intact plant tissues by invagination of the root hair cell, by penetration of the junction between root hair and adjacent epidermal cells, or enzymatic process involving degradation of cell wall-bound polysaccharides (Quadt-Hallmann *et al.*, 1997). Bacteria may enter through undifferentiated meristematic root tissue (Mahaffee *et al.*, 1997). They might also enter the epidermis through passive plant uptake by transpiration (Quadt-Hallmann *et al.*, 1997). Alternatively, bacterial endophytes enter plant tissue through germinating radicles, abrasion or wounds including broken trichomes, stomata, lenticles, hydatodes, foliar damage due to windblown soil particles, rain or hail (Zinniel *et al.*, 2002., Hallmann *et al.*, 1997., Gitaitis *et al.*, 2002a, 2003., Azad *et al.*, 2000; Bell *et al.*, 1995; Cother *et al.*, 2004., Vega *et al.*, 2005). Watanabe *et al*, (1996) found high levels of *Erwinia* on and within rice leaves as epiphyte and endophyte. They speculated that the *P. ananatis* and *P. agglomerans*, gained entry to phloem through leafhopper (*Nilaparvata lugens*) wounds and then multiplied rapidly in rice.

Once endophytic bacteria are inside the plant, they may either become localized at the point of entry or spread throughout the plant (Hallmann *et al.*, 1997, Mahaffee *et al.*, 1997). Systemic spread of endophytic bacteria has been demonstrated for an *Erwinia* spp. in cotton, which was recovered from the roots, stems, and unopened flowers, as well as from the mesocarp and endocarp of bolls (Misaghi and Donndelinger, 1990).

2.2 Colonization and establishment of endophytes

Internal colonization of plant tissues by bacteria is considered to be primarily intercellular, with most reports stress the importance of xylem vessels as reservoirs of large populations of bacteria. Dong *et al.* (1994) reported the presence of endophytic bacteria from the intercellular spaces of sugarcane stem parenchyma. Intracellular endophytic bacteria have also been described within the cytoplasm, cell walls, epidermal cells root hairs and parenchyma cells (Quadt-Hallmann et al., 1997). Many factors affect the efficiency of endophyte colonization and these include factors such as gnotobiotic conditions, temperature, humidity, nutrition, plant age and species, host genotype and inoculum density (Pillay and Nowak, 1997; Igniguez *et al.*, 2005).

2.3 Potential uses of bacterial endophytes

Endophytic bacteria colonize the niche similar to the phytopathogenic, which might favor them as candidates for biocontrol agents (Hallmann *et al.*, 1997). They may operate through the production of antifungal or antibacterial agents, siderphore production, nutrient competition, niche exclusion or indirectly through the induction of systematic acquired host resistance or immunity (Chen *et al.*, 1995., Kloepper *et al.*, 1980., Liu *et al.*, 1995). *P. carotovorum* subsp. *carotovorum* is inhibited by numerous endophytic bacteria including *P. agglomerans*, *Curtobacterium luteum* and *Pseudomonas* spp. (Sturz *et al.*, 1999). *Enterobacter cloacae*, an endophyte isolated from maize exhibited *in vitro* antibiosis against *Fusarium verticillioides* (Hinton and Bacon, 1995).

Endophytic bacteria are of agricultural interest because they can enhance plant growth; reduce disease symptoms caused by plant pathogens; and improve the nutrition of plants through nitrogen fixation from the atmosphere (Sturz *et al*, 2000; Igniguez *et al*, 2005; Boddey *et al*, 2003). Loiret *et al.* (2004) isolated a *Pantoea* strain as an endophytic nitrogen fixing bacterium from sugarcane. They can increase disease resistance and decrease frost damage as well as contribute to the control of plant parasitic nematodes, and

insects (Sturz and Matheson, 1996; Chen *et al.*, 1995; Sturz *et al.*, 1998; Hallmann *et al.*, 1999; Azevedo *et al.*, 2000).

In some cases bacterial endophytes can increase seedling emergence and promote plant establishment under adverse or stressful conditions (Chanway, 1997). Bacterial endophytes have been associated with growth promotion of several crops including potato, tomato, lettuce, maize, cucumber, cotton and can thus preserve or enhance crop yield (Sturz and Christie, 1995; Reiter *et al.*, 2002; Asis and Adachi, 2003; Hinton and Bacon, 1995; Nowak *et al.*, 1995; Zinniel *et al.*, 2002; Bensalim *et al.*, 1998). Plant growth effects include increase in plant height, root and shoot biomass, potato and tuber production, root leaf-hair formation, and lignification of xylem vessels (Sturz and Christie, 1995; Pillay and Nowak, 1997; Sturz *et al.*, 1997; Nowak, 1995).

2.4 Potential phytopathogens as endophytes

Sabratnam and Beattie (2003) hypothesized that endophytic colonization is primarily a property of pathogens. This hypothesis was supported by the results of Wilson *et al*, (1999), who found that in laboratory studies, endophytic population in bean leaves increased for several pathogens but remained very low or undetectable for several non-pathogens. A number of phytopathogenic bacteria have been isolated as endophytes from a diverse range of host plants. These include members of the genera *Enterbacter, Erwinia, Pantoea, Pseudomonas, Xanthomonas* and *Agrobacterium* (Asis and Adachi, 2003; Bell *et al.*, 1995; Kuklinsky-Sobral *et al.*, 2004; Manulis and Barash, 2003; Hallmann *et al.*, 1997; Sabreatnam and Beattie, 2003).

2.5 *Enterobacteriaceae* as endophytes

A number of members of the family have been isolated as endophytes, including *Enterobacter cloacae* and *Klebsiella pneumonia* on maize (Hinton and Bacon, 1995; Chelius and Triplett, 2000), *Enterobacter asburiae* on cotton (Quadt-Hallmann *et al.*, 1997) and *Klebsiella* spp. and *Enterobacter cloacae* on banana (Martínez *et al.,* 2003). *Pantoea agglomerans* has been isolated as an endophyte from a number of plants, such as sweet potato (Sessitch *et*

al., 2004; Asis and Adachi, 2004), and rice (Mukhopadhay *et al*., 1996). *P. agglomerans* colonizes intercellular spaces in the root cortex of maize and stem mesophyll cells in wheat (Ruppel *et al.,* 1992). Cambours *et al.* (2005) found that the frost dieback of willows was associated with bacterial endophytes which included *P. agglomerans. Pectobacterium. carotovorum* subsp. *carotovorum* and subsp. *atrosepticum*, as well as *Dickeya chrysanthemi* have been isolated from asymptomatic potatoes, as endophytes. Symptoms were only noted when environmental conditions were favourable for disease development (Scheissendoppler and Cate, 1996). *Pantoea ananatis* was isolated as a nitrogen-fixing symbiont from the dune grasses *Ammophila arenaria* and *Elymus mollis* (Dalton *et al*., 2004). This bacterium was isolated from the rhizome and stem tissues. Sessitsch *et al.* (2004) isolated *P. ananatis* from the stems of potato plants and suggested their use as biological control agent against fungi pathogenic to potato.

Of particular concern is the isolation of several enteric pathogens of humans and animals from plants as endophytes and this includes *Salmonella*, *Escherichia coli* and *Klebsiella pneumonia* (Dong *et al*., 2003; Iniguez *et al*., 2005; Lindow and Leveau, 2002). These bacteria colonise food plants and with a growing trend towards eating fresh fruit and vegetables, there is an increased risk of exposure towards these foodborne pathogens for the consumer (Lindow and Leveau, 2002; Iniguez *et al*., 2005).

CONCLUSIONS

The majority of research on plant-associated microorganisms has focused on phytopathogens. Given that these organisms threaten a sustainable source of nutrition world-wide, this is not surprising. The epiphytic phase associated with many phytopathogens has been shown to be an important part of their life cycle. Endophytic bacteria are receiving increasing interest as they can contribute to the health, growth and development of plants (Kuklinsky-Sobral *et al.,* 2004). They have also been reported to be potential biological control agents against both bacterial and fungal pathogens (Kinkel *et al.,* 2000). The *Enterobacteriaceae* in particular have received a lot of attention as endophytes and epiphytes. This can be explained by their relative abundance on plants, and also by the fact that many plant diseases

have been ascribed to members of this family. As many enterobacterial species are associated with human and animal diseases, it is necessary that their biology is understood as fully as possible, including their plant-association habits.

Some information is known about the epi- and endophytes associated with economically important agricultural crops. This information is largely lacking for tree species, especially those grown for commercial forestry purposes. To date, to our knowledge, no studies have been undertaken to identify epi- or endophytes on *Eucalyptus*. *Pantoea ananatis* has been identified as the causative agent of blight and dieback on *Eucalyptus* in South Africa, but little is known about the etiology and epidemiology of this disease. There is thus an inherent need to analyse whether this species or any other member of the *Enterobacteriaceae* are present as epiphytes or endophytes on this host.

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Chapter 2

Epi-and endophytic *Enterobacteriaceae* associated with *Eucalyptus* trees in South Africa

ABSTRACT

In order to manage an outbreak of a disease, an understanding to the etiology and epidemiology of the pathogen is needed. Bacterial blight and die-back, caused by *Pantoea ananatis* in South Africa, is problematic in nurseries as well as in newly established plantations of *Eucalyptus.* Little is known about the etiology and epidemiology of this specific disease although it is better understood in other hosts infected by this pathogen. In this study, one aspect of the etiology of the disease was investigated and that was whether this pathogen was able to reside epi- and endophytically on/in *Eucalyptus* leaves of different ages. The second question was whether other members of the *Enterobacteriaceae* are present in or on leaves of this host. Isolations were performed and strains were characterized both phenotypically and genotypically. Only 11% and 13% of stains isolated as epi- and endophytes from different *Eucalyptus* clones, respectively, belonged to the *Enterobacteriaceae*. Two percent and 15% of strains isolated as epi- and endophytes from different weed species, respectively, belonged to this family. The majority of epiphytes were isolated from young leaves while most endophytes were isolated from mature leaves. Based on partial 16S rDNA sequencing results the strains belonging to the *Enterobacteriaceae* belonged to the Genera *Pantoea, Enterobacter, Citrobacter, Cedecea* and *Raultella*. All suspected *Pantoea* strains were subjected to Amplified Fragment Length Polymorphisms (AFLPs) and only six could be confirmed as belonging to this genus and were *P. ananatis*.

INTRODUCTION

Forestry in South Africa contributes significantly to the economy. Forestry, wood and paper contributed 14.4 billion rand to annual gross domestic product in the past two years (Forestry SA, 2004; Forestry SA, 2003). *Eucalyptus* species, hybrids and clones account for more than 50% of newly afforested areas in South Africa (Anonymous, 1996; Forestry SA, 2003). South Africa is currently

the third largest *Eucalyptus* growing country in the world with nearly 600 000 ha of *Eucalyptus* plantations (Owen and Van der Zel, 2000; Schönau *et al.*, 1994;).

The most significant bacterial pathogen infecting *Eucalyptus* and causing bacterial blight and dieback in South Africa is *Pantoea ananatis* (Coutinho *et al.*, 2002). This pathogen infects this host in nurseries and in newly established plantations where it causes leaf-spots and following repeated infection leads to trees becoming stunted and eventually dying. The disease is widespread in South Africa occurring in all areas where *Eucalyptus* is grown commercially.

Pantoea ananatis, formerly classified as *Erwinia ananas* (syn. *Erwinia uredovora*) (Mergaert *et al.,* 1993), belongs to the family *Enterobacteriaceae*. It is a plant-associated pathogen that is a Gram negative organism, rod-shaped, yellow pigmented and is motile by means of a peritrichous flagellum. It is also non-spore forming and facultatively anaerobic. The type strain of *P. ananatis* is LMG2665, which was isolated from a pineapple in Brazil. The name of this species has its origin from the generic name of the pineapple, *Ananas comosus,* the source from which it was first isolated.

Pantoea ananatis can infect a wide range of plant hosts. It has been found to cause leaf spot of maize (Paccola-Meirelles *et al.*, 2001); necrotic leaf blotch disease of sudangrass (Azad *et al.* 2000); leaf blight, seed stalk rot and bulb decay of onion (Gitaitis and Gay, 1997); a postharvest disease of cantaloupe fruit (Bruton *et al.,* 1991) and brown spot of honey melons (Wells *et al.,* 1987). It has recently been found to occur on onion seed (Goszczynska *et al.*, 2006b) and causing brown rot of maize (Goszczynska *et al.*, 2006a) in South Africa.

The epidemiology of plant diseases caused by *P. ananatis* on different hosts is relatively unknown. What has been established is that the pathogen enters its host through flowers (Serrano, 1928) and/or wounds created by feeding insects (Gitaitis *et al.*, 2003; Watanabe *et al.*, 1996; Wells *et al.*, 1987), mechanical

injury (Serrano, 1928) and plant to plant contact during high winds (Azad *et al.*, 2000). *P. ananatis* is a common inhabitant of the gut microflora of brown plant hoppers (*Nilaparvata lugens*) (Watanabe *et al.,* 1996), mulberry pyralid (*Glyphodes pyloalis*) (Takahashi *et al.,* 1995) and tobacco thrips (*Frankliniella fusca*) (Gitaitis *et al*., 2003; Wells *et al.,* 1987). The development of hopper burn symptoms on rice was found to be accelerated when *P. ananatis* was present on the leaf surfaces. Gitaitis *et al*. (2003) were also able to show that tobacco thrips vector *P. ananatis* in onion fields.

In this study, we investigated whether or not *P. ananatis* was present as an epi- and/or endophyte in healthy *Eucalyptus* leaves and whether other *Enterobacteriaceae* were associated with this host in a similar manner.

MATERIALS AND METHODS

Plant material

Healthy leaf material of different ages was collected from *E. grandis* x *E. nitens* hybrid clones, GN015, GN055, GN065 GN108, GN121 and GN188. The material was harvested in summer from White River in Mpumalanga province, South Africa. The trees were located in nurseries where young growth shoots were routinely harvested for use as propagation material. The leaves collected were classified as "young", first two, fully expanded leaves from the tip of the branch, and "mature", the fifth leaf pair from the tip of the branch. Efforts were made to try and selected branches of the same age. Based on field observations, GN 108 is resistant and GN015, GN055, GN065 GN121 and GN188 are susceptible to *P. ananatis*.

Weeds growing in the vicinity of an outbreak of bacterial blight and die-back in *E. grandis* x *E. nitens* hedges in White River were collected. Weeds were identified to genus level as *Senecio* sp., *Oxalis* sp., *Portulaca* sp. and *Bidens* sp. Entire

plants were collected but only the leaves were examined for the presence of epi- and endophytic *Enterobacteriaceae*.

Epiphytic isolations

Leaves were washed in sterile distilled water to remove dust and other debris. Three leaves from each clone and of different ages were placed in 50 ml Zwittergent reagent (0.04 g EGTA, 0.12g Tris-base, 0.1g peptone, 0.04g 3-dodecyuldimethyl-ammonia, dH_20 to make up 90ml) (LeChevallier *et al*., 1984) and sonicated for 10 minutes. The top 1 ml was removed and placed in a sterile Eppendorf tube. A loopful of the suspension was streaked on to Nutrient Agar (NA) (Biolab Pty Ltd) and the Petri dishes incubated at 30 ºC for 48 hours. The age of all leaves used in the experiment was recorded. Thirty leaves of each clone and age were subjected to this procedure.

Endophytic isolations

Leaves were washed in sterile distilled water to remove dust and other debris. They were then subjected to surface sterilization using the following procedure: 1:9 dilution of a commercial bleach solution containing sodium hypochlorite for 5 minutes, 70% ethanol for 3 minutes and then the leaves were washed twice with sterile distilled water. Leaves were placed into a mortar which contained 1 ml sterile distilled water and crushed to a fine paste with a pestle. The resulting suspension was streaked on to Nutrient agar (NA) and the Petri dishes incubated at 30 ºC for 48 hours. Thirty leaves of each clone and age were subjected to this procedure.

Bacterial strains collected

All bacterial colonies appearing on Nutrient Agar were purified and stored at -70 ºC in beaded cryovials (Microbank™, Pro-Lab, Richmond Hill, Canada). Strains

identified as belonging to the *Enterobacteriaceae* were placed in the Bacterial Culture Collection (BCC) in the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Characterization and identification of strains isolated

Phenotypic characterization

All strains were characterized based on Gram reaction, cell and colony morphology on Nutrient Agar (NA), and the utilization of glucose in an oxidative and/or fermentative manner (Hugh Leifson oxidation/fermentation reaction). Strains that were Gram negative, rod shaped and Hugh Leifson positive, indicating that they were members of the *Enterobacteriaceae*, were selected for further characterization.

Indole production

The method as described by Miller & Wright (1982) was used to determine indole production. All strains were plated on to media containing 10 g tryptone, 1g L-tryptophan and 15 g agar per litre. Petri dishes were incubated for 48 h at 28 °C. p-dimethylamino-cinnamaldehyde (1g in 100 ml of 10% hydrochloric acid) was spotted in a grid on filter paper in a Petri dish. A loopful of the bacterium to be tested was placed on the treated filter paper. A blue-green colouration of the treated area within 10 sec indicated a positive reaction. A negative reaction was denoted by a colourless or light pink colouration of the treated area.

Oxidase test

The method described by Keane *et al.* (1970) was used to determine oxidase production. As inocula, 24 h strains grown on nutrient agar supplemented with 1% glucose were used. A loopful of growth was placed on filter paper

impregnated with 1% (w/v) aqueous tetra methyl-p-phenylenediamine dihydrochloride solution. Strains were considered to be oxidase positive when a purple colour developed within 10 sec. and oxidase negative if no colour developed after 60 sec.

Catalase test

The catalase test involved adding hydrogen peroxide to each strain growing on NA. If the strain produced catalase, they converted the hydrogen peroxide and oxygen gas evolved. The evolution of gas causes bubbles to form and is indicative of a positive test.

Growth on PA 20 medium

PA 20 medium was developed by Goszczynska *et al.* (2006c) to isolate *P. ananatis* from onion seed. It was found to be useful method for detecting *P. ananatis* from *Eucalyptus* leaves showing typical symptoms of blight (data not shown). The media contains NaCl 20g, K_2HPO_4 1g, $NH_4H_2PO_4$ 1g, $MgSO_4.7H_2O$ 0.2g, 2ml of 0.075% aq. Crystal violet, 1ml of 1.6% aq. solution of Bromothymol Blue and Agar 15g. The pH was adjusted to 8.0 with 1.0 N NaOH. After autoclaving and cooling to 50 °C, filter sterilized solutions of 3g of D (+) Arabitol dissolved in 5ml water and 2ml of 1% aq solution of thallium nitrate were added. All strains identified as belonging to the *Enterobacteriaceae* were streaked onto this media and incubated at 28 °C for between 4-7 days.

Molecular characterization

DNA Extraction

Two methods of extracting DNA were employed. The first method was by using the ™ tissue kit (Qiagen) and the isolated DNA was stored at -20 ºC. The second

method involved placing the bacterial strains in 1ml sterile distilled water in a 1.5 ml Eppendorf tube and incubating the tube in boiling water for 10 minutes. Thereafter the Eppendorf tubes were centrifuged for 1 minute at 13000 rpm, the supernatant removed and the DNA placed into clean, sterile 1.5/1.0 ml Eppendorf tubes.

Sequencing of the 16S rRNA gene

The 16S rRNA gene of all strains isolated belonging to the Enterobacteriaceae were amplified using the universal primers 16F27pA (5'-AGAGTTTGATCCTGGCTCAG'3') and 16R1522pH (5'-AAGGAGGTGATCCAGCCGCA-3'). Each PCR mixture contained 10 µl reaction buffer (with $MgCl_2$), 10 µl dNTPs (250 mM), 1 µ1 U Taq polymerase, 5 µl DNA (50 – 100 ng), 1 µl each of the two primers and72 µl nuclease free water. The DNA was amplified in a Perkin-Elmer PCR machine programmed as follows: a denaturing step consisting of 94 °C for 5 minutes, 30 amplification cycles, with each cycle consisting of 94 °C for 1 minute, 58 °C for 1 min, and 72 °C for 3 minutes; and the final extension step consisting of 72 °C for 10 min. After amplification, the PCR product was visualized on a 1% agarose gel stained with ethidium bromide. Successful PCR reactions were purified using the Roche PCR purification kit.

The purified PCR products were sequenced with the primers 16F27pA and 16R 1622pH as well as with the internal primers FpD (5' CAG CAG CCG CGG TAA TAC 3'), FO (5' AAC TCA AAG GAA TTG ACG G 3'), F3 (5' AGT CCC GCA ACG ACG GCA AC 3'), R Gamma (5' ACT GCT GCC TCC CGT AGG AG 3'), RpD (5' GTA TTA CCG CGG CTG CTG 3'), 16R685 (5' TCT ACG CAT TTC ACC GCT AC 3'), and 3R (5' GTT GCG CTC GTT GCG GGA CT 3') using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were analysed using an ABI Prism 377 DNA sequencer and edited with programme BIOEDIT. The GenBank/EMBL databases were used for

homology searches using the BLAST programme (National Center for Biotechnology Information, US National Institutes of Health, Bethesda, MD). A selection of 16S rDNA sequences obtained from a BLAST search were aligned with the partial sequences of the strains isolated in this study using CLUSTALX (). The alignment was trimmed of the overhangs before parsimony analysis. Heuristic searches with maximum parsimony used stepwise (simple) addition and tree-bisection-reconnection to produce a phylogenetic tree (PAUP 4.0b3, D.L. Swofford, Illinois Natural History Survey, Campaign). Bootstrap values were obtained from the same data matrix.

Fluorescent Amplified Fragment Length Polymorphism (F-AFLP) analysis

Strains identified as belonging to the Genus *Pantoea* after partially sequences were BLASTed in Genbank were subjected to F-AFLP analysis. Genomic DNA was extracted using a DNeasy™ tissue kit (Qiagen) and stored at -20 ºC. Between 100-150 ng of DNA from each of the strains was digested with 12 U *Eco*RI (Roche) and 8 u *Mse*I (Roche) in 5 x Restriction/Ligation buffer (50 mM Tris HAc, 50 mM MgAc, 250 mM KAc, 25 mM DTT). The digestion reaction was incubated at 37 ºC for 2 hours, then heated at 70 ºC for 15 minutes. Double-stranded adaptors, 5 pmol *Eco*RI and 50 pmol *Mse*I, were added to the 15 µL digestion mixture, together with 5 x Restriction/Ligation buffer, 0.3 mM ATP and 1 U T4 DNA Ligase (Roche). The ligation reaction was incubated at 20 ºC for 2 hours and then diluted 1:10 with nuclease-free water.

A pre-amplification reaction was performed and contained 1 x Reaction buffer, 1.5 mM $MgCl_2$, 250 µM dNTP's, 100 pmol each of Eco-00 (5'-GACTGCGTACCAATTC-3') and Mse-00 primer (5'-GATGAGTCCTGACTAA-3'), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 2 µL diluted ligation reaction. Amplification was carried out in a GeneAmp Thermal Cycler (Applied Biosystems). The amplification conditions included denaturation at 94 ºC for 3 minutes, 20 cycles of denaturation at 94 ºC for 30 seconds, annealing of

primers at 56 ºC for 1 minute and elongation at 72 ºC for 1 minute, and extension at 72 ºC for a further 5 minutes. Following pre-amplification, each reaction was diluted 1:50 with nuclease-free water .

The selective amplification reaction, in a total volume of 20 µL, contained 1 x Reaction buffer, 1.5 mM $MgCl_2$, 250 µM dNTP's, 0.5 pmol fluorescently-labelled Eco-C primer (5'-GACTGCGTACCAATTCC-3'), 2.4 pmol Mse-GC primer (5'-GATGAGTCCTGAGTAAGC-3'), 1 U Taq DNA polymerase (Southern Cross Biotechnologies) and 5 µL diluted pre-amplification reaction. Amplification was carried out in a GeneAmp Thermal Cycler (Applied Biosystems). The selective PCR conditions included denaturation at 94 ºC for 5 minutes, 9 cycles of denaturation at 94 ºC for 30 seconds, annealing of primers at 65 ºC for 30 seconds and elongation at 72 ºC for 1 minute, where the annealing temperature decreases by 1 ºC/cycle until 56 ºC is reached. This was followed by 23 cycles of denaturation at 94 ºC for 30 seconds, annealing of primers at 56 ºC for 30 seconds, and elongation at 72 ºC for 1 minute, and a further 5 minutes of extension at 72 ºC.

Fragment separation

The separation of F-AFLP fragments was performed using a LI-COR IR2 automated sequencer (LI-COR Biosciences). Polyacrylamide gels were prepared using 20 µL Long Ranger gel stock solution (8 % Long Ranger gel solution (LI-COR Biosciences), 7 M urea, 10 x TBE buffer), 150 µL 10 % ammonium persulphate and 15 µL TEMED for polymerisation. Gels were poured using the LI-COR gel casting apparatus and left to polymerise for 60 minutes. A 30 minute pre-run was performed at 1 500 V and 35 W to equilibrate the ions in the gel and running buffer. The selective amplification reactions were mixed with an equal volume of formamide loading buffer (95 % formamide, 20mM EDTA, bromophenol blue). The mixture was heated at 90 ºC for 3 minutes and then cooled on ice for 10 minutes. 0.8 µL of each sample was loaded onto the

sequencing gels, along with an IRD-700 labelled sizing standard at each end of the gel. The gels were run for 4 hours at 1 500 V and 42 W with 0.8 x TBE running buffer.

Gel analysis

The band patterns from the gels were analysed using GelCompar (Applied Maths). Gels were normalised by aligning the 700 bp sizing standards included in each gel, and the area between 50-700 bp was analysed. Following analysis, a UPGMA dendrogram was constructed using the Dice similarity coefficient. An optimisation setting of 0.1 % and a tolerance setting of 0.2 % was applied to the analysis. Type strains of all *Pantoea* spp. were included in the analysis.

RESULTS

Epiphytic isolations

Of the 43 strains isolated as epiphytes from *Eucalyptus* leaves, 10 (23%) belonged to the *Enterobacteriaceae* (Table 1). The majority of the other strains isolated belonged to the *Pseudomonaceae*. One strain belonging to this group was isolated from weeds (*Senecio* sp.). All strains were Gram negative, short rods, Hugh Leifson test was positive, catalase positive and oxidase negative. Seven and three strains were isolated as epiphytes from young and mature *Eucalyptus* leaves, respectively.

Endophytic isolations

Of the 47 strains isolated as endophytes from *Eucalyptus* leaves, 12 (26%) belonged to the *Enterobacteriaceae* (Table 1). The majority of the other strains isolated belonged to the *Pseudomonaceae*. Six strains belonging to this group were isolated from weeds (two from *Senecio* sp., three from the *Bidens* sp. and

one from the *Oxalis* sp.). Strains belonging to this group were all Gram negative, short rods, Hugh Leifson test was positive, catalase positive and oxidase negative. Four and eight strains were isolated as endophytes from young and mature *Eucalyptus* leaves, respectively.

Characterization and identification of strains isolated

Phenotypic characterization

Strains obtained from both epiphytic and endophytic isolations from both *Eucalyptus* and weeds were shown to belong to the *Enterobacteriaceae* based on their Gram stain, cell morphology, their ability to ferment glucose and all were catalase positive and oxidase negative. Colony colour, indole test results and the ability to grow on PA20 differed between strains (Table 2).

Molecular characterization

Sequencing of the 16S rRNA gene

A BLAST search of partial sequences of the 16S rRNA gene of all epiphytes and endophytes belonging to the *Enterobacteriaceae* revealed that the majority of strains belonged to the Genus *Pantoea* (Table 3). A selection of 13 strains not belonging to this family were also sequenced and BLASTed. The majority of these strains, 62%, belonged to the Pseudomonad group.

A neighbour-joining tree (Saitou & Nei, 1987) was constructed using Bionumerics 4.0 (Applied Maths, Inc., Belgium). A 550 base pair fragment of the 16S rDNA gene were obtained for all strains. These strains were compared to a number of type strains in the *Enterobacteriaceae* (Fig. 1). The tree showed clearly that the sequences of the strains isolated in this group belong to a number of different species in the *Enterobacteriaceae* including *Enterobacter*, *Citrobacter, Cedecea*,

Raultella and *Pantoea*. There were only two strains, BCC 656 and BCC 671, were similar to *P. ananatis*.

Fluorescent Amplified Fragment Length Polymorphism (F-AFLP) analysis

Seventeen strains had tentatively been identified as belonging to the Genus *Pantoea* based on their partial sequence similarity to strains in Genbank (Table 3). When the fingerprint profiles were compared to the seven type strains of *Pantoea*, BCC540, BCC542, BCC656, BCC661, BCC737 and BCC739 were found to belong to this genus and their identity could be confirmed as *P. ananatis* (Fig. 2). BCC542 and BCC656 were isolated as epiphytes whereas BCC540 and BCC739 were isolated as endophytes from young *Eucalyptus* leaves. BCC661 was isolated as an epiphyte from *Senecio* leaves and BCC739 as an endophyte from *Oxalis* leaves.

DISCUSSION

In this study we were able to show that *P. ananatis* exists as both an epi- and endophyte in healthy, young *Eucalyptus* leaves of the susceptible GN clones 055 and 188. This pathogen was also isolated as an epiphyte on a *Senecio* sp. and as an endophyte from an *Oxalis* sp. The identity of *P. ananatis* was confirmed using F-AFLPs. This method was described by Brady *et al.* (2006) as being reliable in distinguishing the seven species in the Genus *Pantoea* from each other. The fact that the two strains, BCC 656 and 671, found to be similar to *P. ananatis* based on partial 16S rDNA sequences, were not found to group with this species in the F-AFLP tree, clearly indicates that this techniques has limited application in identification of *Pantoea* strains to species level

Pantoea ananatis is a common epiphyte on many plants. Gitaitis *et al.* (2002) detected and cultured this bacterium as an epiphyte from 25 asymptomatic weed species including crabgrass, sicklepod, yellow nutsedge, and from crop plants

such as Bermuda grass, cowpea and soybean. It has also been reported as an epiphyte on rice plants (Watanabe *et al.*, 1996), maize (Paccola-Meirelles *et al.*, 2001), barley, buckwheat, uredospores of *Ustilago* smut of corn (Coplin and Kado, 2001), cotton lint (Chun and Perkins, 1997); mulberry (Takahashi *et al.*, 1995) and poplar trees (Zeng *et al.*, 1999).

Pantoea ananatis has been reported less frequently as an endophyte than an epiphyte. Sessitsh *et al.* (2004) reported the occurrence of *P. ananatis* as an endophyte in field-grown potatoes. This bacterium was shown to possess some antifungal activity against diseases of potato notably *Verticillium dahliae, Rhizoctonia solani, Sclerotinia sclerotiorum* and *Phytophthora cactorum*. *P. ananatis* was also found endophytically in stem and rhizome tissue of the dune grasses, *Ammophila arenaria* and *Elymus mollis*, from Oregon (Dalton *et al.,* 2004). These isolates were found to fix nitrogen in these hosts.

In addition to *P. ananatis*, a number of other members of the *Enterobacteriaceae* were isolated as epiphytes and endophytes from healthy *Eucalyptus* leaves. These included *Enterobacter, Citrobacter, Cedecea* and *Raultella* spp. *Enterobacter* spp. have been found on human skin and plants as well as in soil, water, sewage, intestinal tracts of humans and animals, and some dairy products. *E. cloacae* has been found to suppress damping off caused by *Pythium ultimum* in carrot, cotton, cucumber, lettuce, radish, tomato and wheat (Kageyama and Nelson, 2003). *E. asburiae* has been isolated as an endophyte in sweet potato stems in Japan but its exact role in this host has still to be elucidated (Asis and Adachi, 2003). *Citrobacter spp.* are enteric organisms that inhabit the intestinal tract of humans and animals. They have also been found in soil, water, sewage and food (Troth *et al*, 2006). .Originally, all *Cedecea* isolates had been recovered from humans (Brenner, 1991). However, *C. lapagei* and a *Cedecea* sp. were isolated from laboratory-reared oriental fruit flies, *Dacus dorsalis* (Jang and Nishijima, 1990). One strain of *C. davisae* has also been isolated from vegetables (Österblad *et al.*, 1999).

This study has provided evidence that an important pathogen of *Eucalyptus, P. ananatis*, is able to reside in its host and in weeds in close proximity to the host as both an epi- and endophyte. Preventing optimal environmental conditions which would allow the bacterium to enter the host through, for example stomata, should be considered in the nursery environment. Weed control is another important management strategy that must be taken into consideration, particularly in areas where hedges are planted.

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Table 1 Epi- and endophytic bacteria isolated from *Eucalyptus* and weed leaves

	Bacterial strains isolated from:		Strains belonging to the *Enterobacteriaceae*	Identity of *Enterobacteriaceae* based on BLAST results of partial 16s DNA sequences	BCC No.
	Young leaves [YL]	Mature leaves [ML]			
GN108					
Epiphytes	4	0	2 [YL]	*Enterobacter cloacae* *Erwinia* sp.	653 652
Endophytes	3	0	2 [YL]	*Pantoea agglomerans* *Pantoea agglomerans*	663 736
GN121					
Epiphytes	4	3	1 [YL] 2 [ML]	*Pantoea ananatis* *Pantoea ananatis* *Enterobacter* sp.	319 309 311
Endophytes	4	3	0		
GN188					
Epiphytes	7	8	3 [YL] 1 [ML]	*Pantoea ananatis* *Enterobacter* sp. *Enterobacter ludwigii* *Pantoea agglomerans*	656 646 674 649
Endophytes	8	14	1 [YL] 8 [ML]	*Enterobacter cloacae* *Pantoea agglomerans* *Enterobacter cloacae* *Enterobacter cloacae* *Enterobacter cloacae* *Pantoea agglomerans* *Pantoea ananatis* *Enterobacter cloacae* *Pantoea dispersa*	660 655 673 665 654 659 662 668 737
GN055					
Epiphytes	3	4	1 [YL]	*Pantoea ananatis*	542
Endophytes	4	4	1 [YL]	*Pantoea ananatis*	540
GN015					
Epiphytes	4	6	0		
Endophytes	3	2	0		
GN156					
Epiphytes	0	0	0		
Endophytes	1	1	0		
***Senecio* sp.**					
Epiphytes	4	1	1 [YL]	*Pantoea ananatis*	661
Endophytes	3	5	1 [YL] 1 [ML]	*Pantoea ananatis* *Enterobacter* sp.	666 667
***Bidens* sp.**					
Epiphytes	4	5	0		
Endophytes	8	3	2 [YL] 1 [ML]	*Pantoea agglomerans* *Pantoea ananatis* *Enterobacter endosymbiont*	669 671 672
***Oxalis* sp.**					
Epiphytes	4	1	0		
Endophytes	1	2	1 [YL]	*Pantoea ananatis*	739

Table 2 Phenotypic characteristics of epiphytic and endophytic strains belonging to the *Enterobacteriaceae* isolated in this study

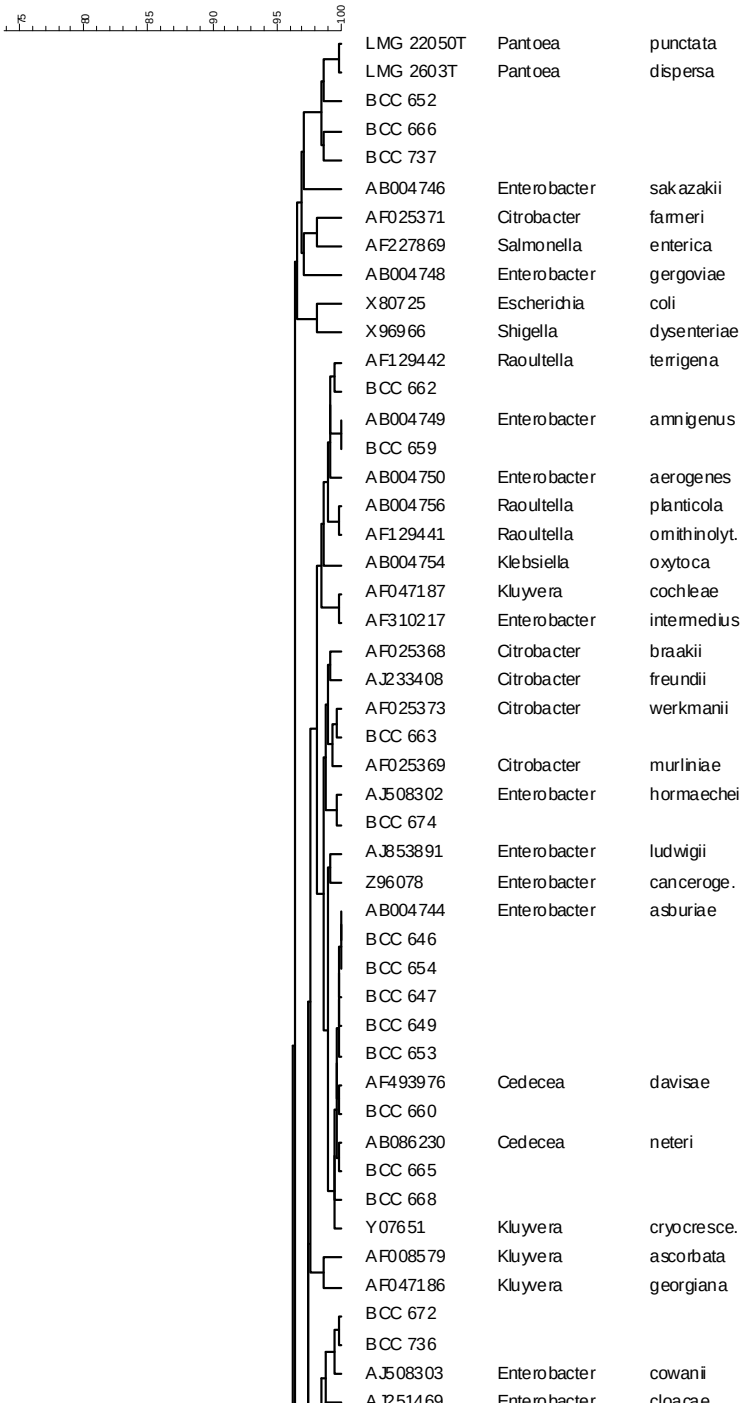
Strain No.	Host	Plant material	Colony colour	Growth on PA 20	Indole reaction
Epiphytic strains:					
BCC 309	GN121	Mature leaves	Yellow	+	-
BCC 311	GN121	Mature leaves	Yellow	+	-
BCC 319	GN121	Young leaves	Yellow	+	-
BCC 542	GN055	Young leaves	Yellow	+	-
BCC 646	GN188	Mature leaves	White	-	-
BCC 649	GN 188	Mature leaves	White	-	-
BCC 652	GN 108	Young leaves	White	+	-
BCC 653	GN 108	Young leaves	White	-	-
BCC 656	GN188	Young leaves	Yellow	+	+
BCC 661	*Senecio* sp.	Leaves	Yellow	+	-
BCC 674	GN 188	Young leaves	White	-	-
Endophytic strains:					
BCC 540	GN055	Young leaves	Yellow	+	-
BCC 654	GN188	Young leaves	Yellow	-	-
BCC 655	GN 188	Mature leaves	White	+	-
BCC 659	GN188	Young leaves	Yellow	+	-
BCC 660	GN188	Young leaves	Yellow	+	-
BCC 662	GN188	Mature leaves	Yellow	+	-
BCC 663	GN108	Young leaves	Yellow	+	-
BCC 665	GN188	Mature leaves	Yellow	-	-
BCC 666	*Senecio* sp.	Leaves	Yellow	+	-
BCC 667	*Senecio* sp.	Leaves	White	+	-
BCC 668	GN188	Young leaves	White	-	-
BCC 669	*Bidens* sp.	leaves	Yellow	-	-
BCC 671	*Bidens* sp.	Leaves	Yellow	-	-
BCC 672	*Bidens* sp.	Leaves	Yellow	+	-
BCC 673	GN188	Young leaves	White	+	-
BCC 736	GN108	Young leaves	Yellow	+	-
BCC 737	GN188	Young leaves	Yellow	+	-
BCC 739	*Oxalis* sp.	Leaves	Yellow	+	+

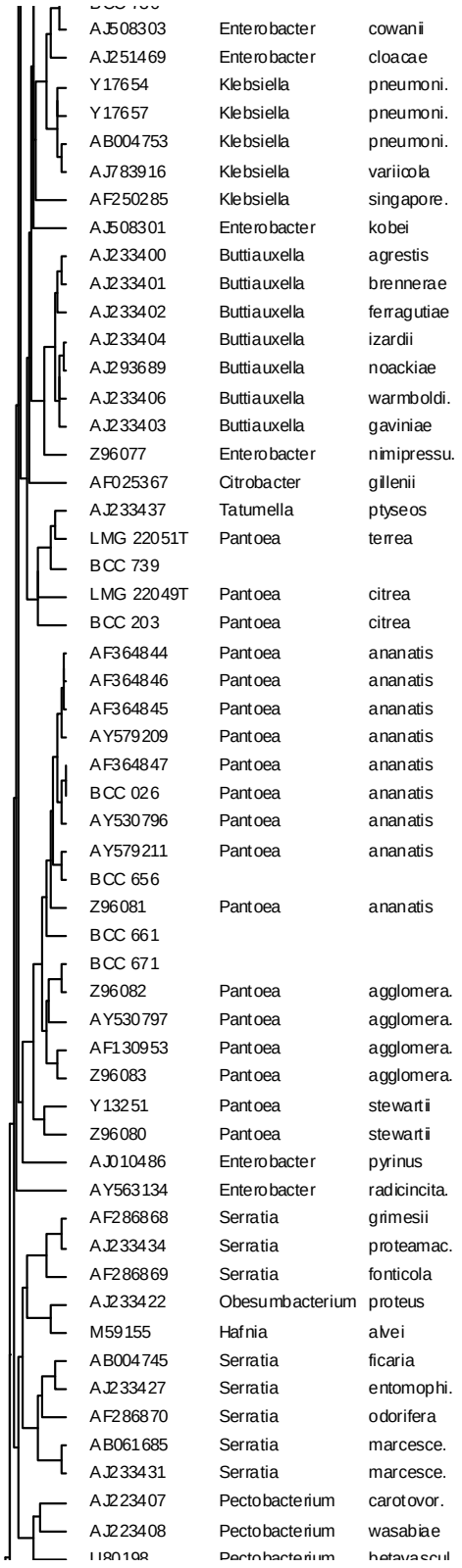
Table 3 Blast results for partial 16S rDNA sequences obtained for the *Enterobacteriaceae* isolated as epi- and endophytes in this study

Strain number	Identified as	Percentage homology
Epiphytic strains:		
BCC 309	*Pantoea ananatis*	95%
BCC 311	*Enterobacter* sp.	94%
BCC 319	*Pantoea ananatis*	100%
BCC 542	*Pantoea ananatis*	100%
BCC 646	*Enterobacter* sp.	98%
BCC 649	*Pantoea agglomerans*	100%
BCC 652	*Erwinia sp.*	99%
BCC 653	*Enterobacter cloacae*	100%
BCC 656	*Pantoea ananatis*	97%
BCC 661	*Pantoea ananatis*	98%
BCC 674	*Enterobacter* sp.	97%
Endophytic strains:		
BCC 540	*Pantoea ananatis*	100%
BCC 654	*Enterobacter cloacae*	97%
BCC 655	*Pantoea agglomerans*	100%
BCC 659	*Pantoea agglomerans*	100%
BCC 660	*Enterobacter cloacae*	100%
BCC 662	*Pantoea ananatis*	98%
BCC 663	*Pantoea agglomerans*	98%
BCC 665	*Enterobacter cloacae*	100%
BCC 666	*Pantoea ananatis*	98%
BCC 667	*Enterobacter* sp.	100%
BCC 668	*Enterobacter cloacae*	100%
BCC 669	*Pantoea agglomerans*	99%
BCC 671	*Pantoea ananatis*	98%
BCC 672	*Enterobacter endosymbiant*	100%
BCC 673	*Enterobacter cloacae*	100%
BCC 736	*Pantoea agglomerans*	99%
BCC 737	*Pantoea dispersa*	98%
BCC 739	*Pantoea ananatis*	97%

Pairwise (OG:100%,UG:0%) (FAST:2,10) Gapcost:0%

16S rRNA




AJ508303 Enterobacter cowanii
AJ251469 Enterobacter cloacae
Y17654 Klebsiella pneumoni.
Y17657 Klebsiella pneumoni.
AB004753 Klebsiella pneumoni.
AJ783916 Klebsiella variicola
AF250285 Klebsiella singapore.
AJ508301 Enterobacter kobei
AJ233400 Buttiauxella agrestis
AJ233401 Buttiauxella brennerae
AJ233402 Buttiauxella ferragutiae
AJ233404 Buttiauxella izardii
AJ293689 Buttiauxella noackiae
AJ233406 Buttiauxella warmboldi.
AJ233403 Buttiauxella gaviniae
Z96077 Enterobacter nimipressu.
AF025367 Citrobacter gillenii
AJ233437 Tatumella ptyseos
LMG 22051T Pantoea terrea
BCC 739
LMG 22049T Pantoea citrea
BCC 203 Pantoea citrea
AF364844 Pantoea ananatis
AF364846 Pantoea ananatis
AF364845 Pantoea ananatis
AY579209 Pantoea ananatis
AF364847 Pantoea ananatis
BCC 026 Pantoea ananatis
AY530796 Pantoea ananatis
AY579211 Pantoea ananatis
BCC 656
Z96081 Pantoea ananatis
BCC 661
BCC 671
Z96082 Pantoea agglomera.
AY530797 Pantoea agglomera.
AF130953 Pantoea agglomera.
Z96083 Pantoea agglomera.
Y13251 Pantoea stewartii
Z96080 Pantoea stewartii
AJ010486 Enterobacter pyrinus
AY563134 Enterobacter radicincita.
AF286868 Serratia grimesii
AJ233434 Serratia proteamac.
AF286869 Serratia fonticola
AJ233422 Obesumbacterium proteus
M59155 Hafnia alvei
AB004745 Serratia ficaria
AJ233427 Serratia entomophi.
AF286870 Serratia odorifera
AB061685 Serratia marcesce.
AJ233431 Serratia marcesce.
AJ223407 Pectobacterium carotovor.
AJ223408 Pectobacterium wasabiae

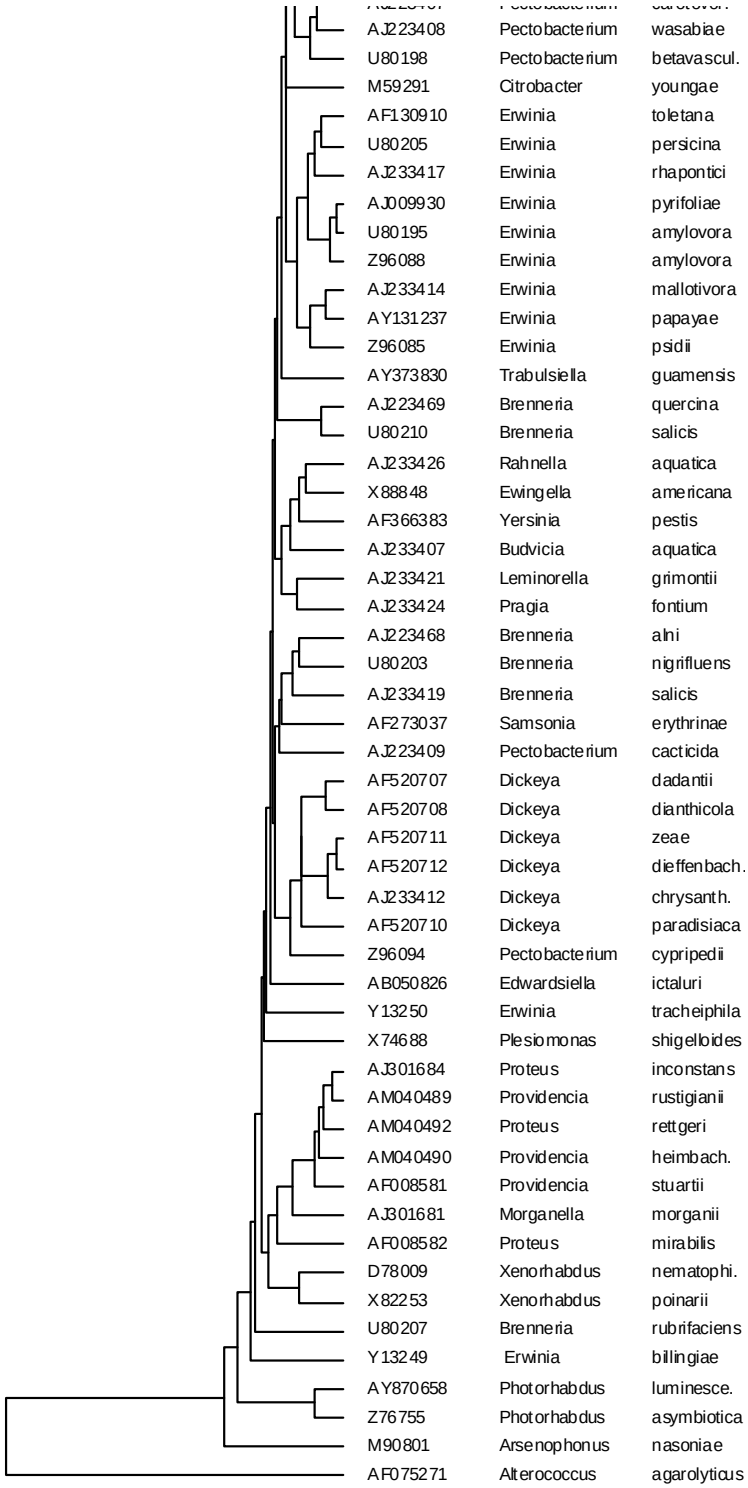
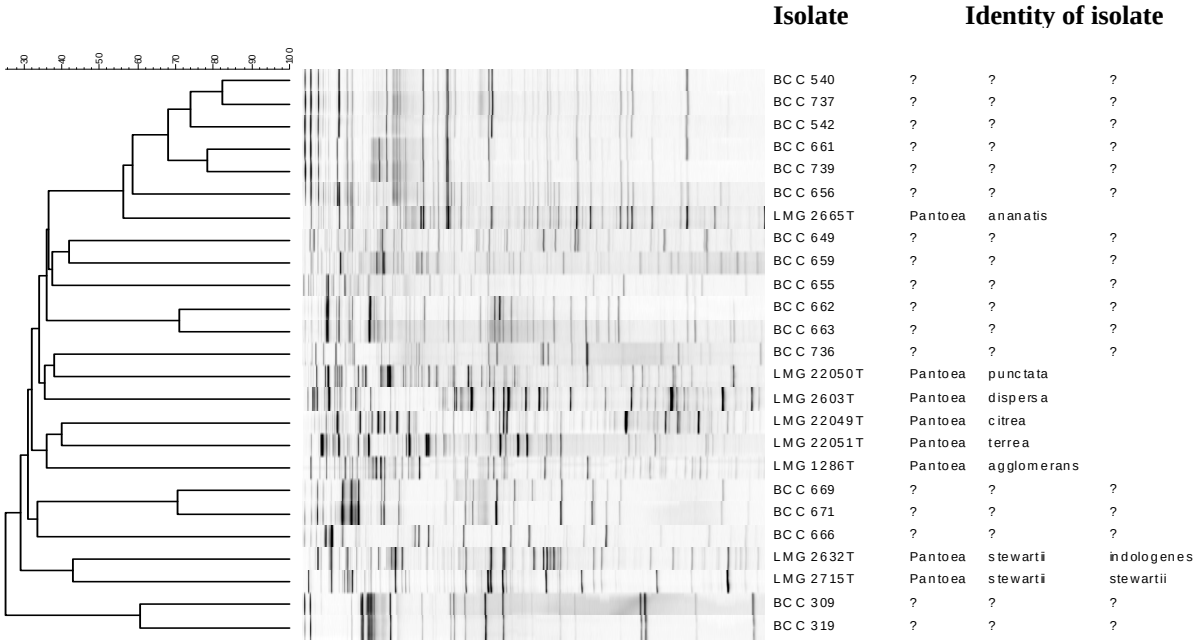

AJ223408 Pectobacterium wasabiae
U80198 Pectobacterium betavascul.
M59291 Citrobacter youngae
AF130910 Erwinia toletana
U80205 Erwinia persicina
AJ233417 Erwinia rhapontici
AJ009930 Erwinia pyrifoliae
U80195 Erwinia amylovora
Z96088 Erwinia amylovora
AJ233414 Erwinia mallotivora
AY131237 Erwinia papayae
Z96085 Erwinia psidii
AY373830 Trabulsiella guamensis
AJ223469 Brenneria quercina
U80210 Brenneria salicis
AJ233426 Rahnella aquatica
X88848 Ewingella americana
AF366383 Yersinia pestis
AJ233407 Budvicia aquatica
AJ233421 Leminorella grimontii
AJ233424 Pragia fontium
AJ223468 Brenneria alni
U80203 Brenneria nigrifluens
AJ233419 Brenneria salicis
AF273037 Samsonia erythrinae
AJ223409 Pectobacterium cacticida
AF520707 Dickeya dadantii
AF520708 Dickeya dianthicola
AF520711 Dickeya zeae
AF520712 Dickeya dieffenbach.
AJ233412 Dickeya chrysanth.
AF520710 Dickeya paradisiaca
Z96094 Pectobacterium cypripedii
AB050826 Edwardsiella ictaluri
Y13250 Erwinia tracheiphila
X74688 Plesiomonas shigelloides
AJ301684 Proteus inconstans
AM040489 Providencia rustigianii
AM040492 Proteus rettgeri
AM040490 Providencia heimbach.
AF008581 Providencia stuartii
AJ301681 Morganella morganii
AF008582 Proteus mirabilis
D78009 Xenorhabdus nematophi.
X82253 Xenorhabdus poinarii
U80207 Brenneria rubrifaciens
Y13249 Erwinia billingiae
AY870658 Photorhabdus luminesce.
Z76755 Photorhabdus asymbiotica
M90801 Arsenophonus nasoniae
AF075271 Alterococcus agarolyticus

Fig. 2 F-AFLP dendrogram of suspected *Pantoea* spp. isolated from *Eucalyptus* and weed leaves as epi- and endophytes



Summary

Studies presented in this thesis, highlights the importance of determining whether members of the Enterobacteriaceae can be associated with plants as epi- and endophytes. In particular, whether the causal agent of blight and die-back of Eucalyptus can survive both epi- and endophytically on/in its host as well as in weeds grown in close proximity to these hosts. This knowledge allows one a better understanding of the etiology and epidemiology of this disease. Appropriate management strategies can now be provided and the impact of the disease lessened in the nursery environment.

Chapter One presents an evaluation of the potential importance of *Enterobacteriaceae* as epi- and endophytes on/in plants. Some information is known about the epi- and endophytes associated with economically important agricultural crops. This information is largely lacking for tree species, especially those grown for commercial forestry purposes. Many *Enterobacteriaceae* occur both epi- and endophytically on/in plants including *Pantoea ananatis*. This pathogen is known to occur epiphytically on weeds as well as on its hosts where under ideal environmental conditions it is capable of causing disease symptoms. As an endophyte, *P. ananatis* occurs in dune grass where it fixes nitrogen and in sweet potato where it is believed to protect the plant against fungal pathogens.

Chapter Two analyses healthy leaves, both young and mature, removed from various clones of the hydrid, *E. grandis* x *E. nitens*, for the presence of bacterial epi- and endophytes. *Enterobacteriaceae* were also isolated and these included *Pantoea* spp. and *Enterobacter* spp. *P. ananatis* was isolated both epi- and endophytically on/in healthy *Eucalyptus* tissue as well as from leaves removed from weeds growing in close proximity to the diseased plants.

This thesis clearly indicates that *P. ananatis* can occur both epi- and endophytically in healthy *Eucalyptus* tissue. The movement of planting material into new environments where bacterial blight and die-back does not occur should be restricted. Irrigaton practices in nurseries should be reviewed to prevent the accumulation of water on the

plant surface which will allow for entry of the pathogen into the host through natural openings. Another management strategy that must also be recommended is that stringent weed control be implemented in the nursery environment.