Bacterial Endophytes Associated with *Eucalyptus nitens* Clones

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously, in its entirety or in part, been submitted at any other university for a degree.

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SUMMARY

Plants are colonised by a vast amount of bacteria which are found in parts such as seeds, roots, leaves and fruits while fewer are found on blossoms, stems and vascular tissue. These different parts of plants make up distinct micro ecosystems which may result in different bacterial species (endophytes) colonizing these ecosystems. Such interactions could be for life or only a short period of time and may cause no significant damage or they could be latent pathogens. Isolations of both Gram negative and Gram positive bacteria have been made from an extensive range of plant species and include bacterial genera from the following groups: Firmicutes, Actinobacteria, α Proteobacteria, β Proteobacteria, and γ Proteobacteria.

The focus of this study was the endophytic bacterial community of resistant, healthy and diseased *Eucalyptus nitens* clones, the latter of which showed symptoms of bacterial blight and die back previously described as caused by *Pantoea ananatis*. The endophytic bacteria of these sampled clones were studied using culturing dependent and independent methods. The focus was on the *Enterobacteriaceae* in order to determine whether *P.ananatis* is present as an endophyte of these clones. To obtain the isolates, standard culturing techniques were used, followed by sequence identification of the 16S rRNA as well as two housekeeping genes, *rpoB* and *gyrB*. Results obtained from the culturing study were compared to results obtained from a PCR-DGGE study of the same samples. Although no conclusion could be drawn as to which organism present caused the disease symptoms on the susceptible clones, it was seen that *Enterobacter* and *Pantoea*, were the most frequently isolated in both of the studies from all clones sampled. This implies that they are present as endophytes in the *E.nitens* clones, together with *Pseudomonas* and *Bacillus* as suggested by the DGGE study.

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CONTENTS

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS

α	-	alpha
β	-	beta
ºC	-	degrees Celsius
γ	-	gamma
µl	-	microliter
µM	-	micromolar
%	-	percentage
16S	-	16S ribosomal RNA
ACC	-	1-aminoglycopropane-1-carboxylate
bp	-	base pairs
CO_2	-	carbon di-oxide
cm	-	centimeter
CFU	-	colony forming unit
CTAB	-	hexadecyltrimethylammonium bromide
DGGE	-	denaturing gradient gel electrophoresis
dNTP	-	deoxynucleic-5'-triphosphate
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediamine-tetra-acetic acid
g/ml	-	grams per millileter
GC-rich	-	guanine/cytocine rich
gyrB	-	gyrase B gene
GyrB	-	gyrase B protein
IAA	-	indole-3-acetic acid
LB-Broth	-	Lysogeny Broth

$MgCl_2$	-	magnesium chloride
Ml	-	milliliter
mm	-	millimeter
mM	-	millimolar
O/F	-	Oxidation/Fermentation
O_2	-	oxygen
pH	-	potential of hydrogen
PCR	-	polymerase chain reaction
PGPR	-	plant-growth promoting rhizobacteria
RNA	-	ribonucleic acid
rRNA	-	ribosomal ribonucleic acid
rDNA	-	ribosomal deoxyribonucleic acid
rpoB	-	ribosomal subunit β gene
RpoB	-	ribosomal subunit β protein
rpm	-	revolutions per minute
spp.	-	species
TAE	-	Tris-acetate-EDTA
TEMED	-	tetramethylethylenediamine
TGGE	-	temperature gradient gel electrophoresis
Taq	-	*Thermus aquaticus*
t-RFLP	-	terminal-restriction fragment length polymorphism
UV	-	ultra violet
U/µl	-	Units per microliter
V	-	Volts

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 1

INTRODUCTION

CHAPTER 1

Introduction

The interactions between bacteria and plants vary dramatically. Bacteria are typically found to be present in different parts of the plant and often have an impact on the plant's health and growth (Beattie, 2007). Leaves make up the dominant part of the above ground plant tissue followed by stems, blossoms and fruits. These parts are collectively known as the phyllosphere and are subject to considerable environmental fluctuations. Nutrients leaking from the mesophyll and epidermal cells onto the surface of the phyllosphere support the growth of bacteria in this zone, also known as the epiphytic region (Leveau and Lindow 2001). A vast number of bacteria also colonize plant parts such as seeds, roots, leaves and fruits while fewer are found on blossoms, stems and vascular tissue (Beattie, 2007).

On the opposite side of the scale is the endophytic region of the plant consisting of the parts internal to the epidermis and harboring endophytic bacteria. Most research focuses on endophytic bacteria that colonize the intercellular plant tissue below and above ground. The intercellular spaces, within a living plant, consist of complex nutrient concentrations making it an attractive option for colonization by bacteria. The rhizosphere is the biggest contributor to the endophytic population of plants, with the roots yielding passage for the bacteria from the rhizosphere to the internal areas of all plant compartments. Bacterial endophytes can populate the intercellular spaces of plants for their whole life cycle or only part thereof, without any external signs of infection or without damaging their host (Ryan *et al*., 2007). It is, however, believed that some endophytes can be present in a plant host as a latent pathogen, not presenting symptoms until favourable environmental conditions arise.

Endophytes have been isolated from a wide variety of both monocotyledonous and dicotyledonous plants, including woody tree species and herbaceous crops. The endophytic bacteria range from Gram positive to Gram negative organisms belonging to the α Proteobacteria, β Proteobacteria, γ Proteobacteria, Firmicutes, and Actinobacteria (Rosenblueth

and Martínez-Romero, 2006). The most frequently isolated genera from the first three groups include *Agrobacterium, Burkholderia* and *Herbaspirillium*, and *Enterobacteriaceae* such as *Enterobacter, Klebsiella, Salmonella* and *Pantoea*, respectively. *Pantoea spp*. have been isolated as endophytes from a wide variety of plants, for example, red clover (*Trifolium pretense* L.) (Sturz *et al*., 1998), citrus plants (Araujo *et al*., 2001), grapevine (Bell *et al*., 1994) and rice (*Orzya sativa* L.) (Elbeltagy *et al*., 2000). However, some species are also plant pathogens. In 2002 Coutinho *et al*. isolated *Pantoea ananatis* as the causal agent of bacterial blight and die-back of *Eucalyptus* clones in South Africa. The disease presented itself on cuttings as initial water soaked lesions, which were later observed to often combine to form larger lesions. Brady *et al.* (2008) also isolated *Pantoea* spp. in Uganda, Uruguay and Argentina from eucalypts with similar disease symptoms to those observed in South Africa.

Typically during studies on endophytic bacteria, culturing methods will be used in enumerating the endophytic population within a plant host. One of the major problems that have occurred in endophytic studies is that varied results were obtained when the populations were described both quantitatively and qualitatively. Possible reasons for this could be due to the growth media used which might not be optimal for the growth of all endophytes and variations in growth conditions of the plant might also influence the results. Traditional culture methods alone may, therefore. Not be reliable to portray the true endophytic populations in plants. To overcome this problem, non-culture methods can be used. These methods usually make use of DNA extractions directly from the sample itself. This provides a greater likelihood that all bacterial DNA present in the sample would be extracted and considered in the analysis. It has become popular to use the 16S rRNA phylogenetic marker in describing diversity within specific natural environments, because the gene is universal and conserved throughout the domain Bacteria. The techniques applied include denaturing or temperature gradient gel electrophoresis (D/TGGE), restriction terminal fragment length polymorphism (t-RFLP) and 16S rRNA cloning methods that rely on analysis of this gene sequence (Sessitsch *et al*., 2002).

During this study the endophytic bacterial community of resistant, healthy and diseased *Eucalyptus nitens* clones, the latter of which showed symptoms of bacterial blight and die back,

were studied using culturing dependent and independent methods. The endophytic populations within the roots, stems and leaves of surface disinfected plants were determined using standard isolation methods and Denaturing Gradient Gel Electrophoresis (DGGE). This study focused on the *Enterobacteriaceae*, especially the *Pantoea* spp., in the light of their in bacterial blight and die-back. The results will be compared in order to determine the usefulness of both these techniques in examining endophytic bacterial populations.

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CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

Literature review: Bacterial Endophytes

1. INTRODUCTION

The idea of microorganisms being present inside plant tissues, as endophytes, has been in existence for more than 120 years (Hardoim *et al.*, 2008). During that time various microorganisms have been isolated including fungi, bacteria and actinomycetes (Mono *et al.*, 2007). Usually the term "endophyte" is associated with fungi, but research has now been directed towards bacteria as endophytes some of which are believed to be of benefit to the plant. Others are, however, regarded to have a neutral or even detrimental effect (Lodewyckx *et al.*, 2002). There are many definitions for the word "endophyte", but for the purpose of this review the definition as proposed by Ryan *et al.* (2007) will be used: "Those bacteria that colonize the internal tissue of the plant, showing no external signs of infection or negative effect on the plant".

Endophytes have been isolated from a vast variety of agricultural plants as well as trees, and it is highly unlikely that there are any plant species free from endophytic bacteria (Rosenblueth *et al*., 2006). Because plants fix CO2 from the atmosphere and then reduce the carbon to organic compounds, the plant interior acts as a great source of carbon, nitrogen and energy for plant associated bacteria (Hardoim *et al*., 2008). In return bacteria may play an important role in promoting plant growth. The best studied example of such a mutualistic relationship is the association between Rhizobia and plant roots (Hardoim *et al*., 2008). Numerous reports have dealt with endophytes detected in parts of plants including seed, ovules, leaves, stems, roots and tubers. Densities and diversity of bacteria present seems to vary between different plant species, different plants from the same species and different parts of the same plant (Sturz *et al*., 2000). Over all it seems that roots and below ground tissue have a higher number of bacterial endophytes than the above ground tissue. However, in 1974 De Boer and Copeman showed that potato stem tissues had a higher bacterial density than the tubers. These variations could be ascribed to differences in detection methods used and choice of growth media (Sturz *et al*., 2000).

Endophytic microorganisms hold a benefit to the plant and can be used for various other biotechnological applications. In agriculture, endophytes are used as a biological control method for pathogens, because they colonize the same ecological niche as that of the plant pathogens (Hallman *et al*., 1997). Endophytes can also enable growth stimulation of plants by the following ways: nitrogen fixation (Hurek *et al*., 2002), producing phytohormones or enhancing availability of minerals (Sturz *et al*., 2000). Endophytic bacteria can also be used to remediate contamination in the environment (Bacon and Hinton, 2007). It is a well-known fact that plants have the ability to participate in phytoremediation, but bacterial endophytes may form an integral part of this process (Walton and Anderson, 1990).

In some cases endophytes might be opportunistic or latent pathogens and infections could occur during changes in environmental conditions, for instance CO2 accumulation or O2 reduction (Lund and Wyatt, 1972). Another trigger for setting off a negative response may be the presence of other microorganisms interacting with the endophyte, and that the order in which these endophytes get introduced into the plant may affect the degree of plant-growth promotion (Sturz and Christi, 1995).

The aim of this review will be to look at endophytic bacteria and how they establish themselves within plants, especially trees. Their occurrence, role in plant health and commercial application will also be addressed. In addition, attention will be given to current methods used for the isolation and identification of endophytic bacteria.

2. ENDOPHYTIC BACTERIA

Various definitions have been applied to the word "endophyte". In most cases the term "endophyte" is associated with fungi but there is, in the literature, significant references to bacteria as endophytes (Lodewyckx *et al.,* 2002). In 1992 Kloepper *et al*. called the bacteria found within tissues internal to the epidermis, endophytes but James *et al.* (1997) reasoned that all bacteria found in the interior of the plant, including active and latent pathogens, should be considered as endophytes. One of the current definitions of an endophyte is a bacterial or fungal

organism, that spends part of or their whole life cycle colonizing the inter- and or intracellular parts of the plant causing no apparent symptoms or disease (Tan and Zou, 2001). Populations can range from a few up to few hundred and the relationship can be latent phytopathogenic to mutualistic symbiosis.

The whole idea of endophytic bacteria originated from the discovery of bacterial populations at the root cortex, which developed into the idea that bacteria can penetrate and colonize the plant (Sturz *et al*., 2000). It is generally accepted that entry occurs through wounds present due to the natural growth of the plant, through root hairs or at epidermal conjunctions (Sprent and de Faria, 1988). After colonization it has been observed that the endophytic bacteria can remain in the specific plant tissue initially colonized, or spread systemically through the plant by means of transport through the conducting elements or apoplast (Hurek *et al*., 1994, James *et al*., 1994, Quadt-Hallmann *et al*., 1997a). In 1995 McInroy and Kloeper showed that endophytes in seed developed into endophytes in seedlings, thus proving that the endophytic population of the plant could be transferred to the next generation. In general it is accepted that the soil is the main contributor of endophytic bacteria (Sturz *et al*., 2000). This idea is supported by other studies (Hollis 1951, Holt 1994, Lamb *et al*. 1996, McInroy and Kloeper 1994 and Mundt and Hinkle 1976) where it was shown that bacteria present in the roots, shoots, leaves, seeds and ovules mostly correspond to bacteria found in the soil surrounding the roots of the given plant.

Reviews by Smith (1911) and Hollis (1951) showed that research on bacteria found within plants date back as far as the 1870's with work done by Pasteur and other scientists. Initially the presence of endophytic bacteria was contributed to the ineffective methods used for surface sterilization, or the presence of latent pathogens (Hollis, 1951). Improvements in research and the introduction of molecular techniques, for studying bacterial endophytes have, however, proved otherwise.

3. ENDOPHYTIC BACTERIA ASSOCIATED WITH DIFFERENT PLANTS

Almost all vascular plant species examined to date were found to harbour endophytic bacteria and/or fungi (Sturz *et al.*, 2000). It was found that the host and the environmental conditions under which the host is growing can affect the endophyte population. The plants are seen as a complex micro-ecosystem consisting of different habitats that are exploited by a large variety of bacteria that are able to interact and to establish equilibrium. A wide variety of endophytic bacteria ranging from Gram positive to Gram negative organisms have been isolated. Members of the endophyte population that are isolated from the plant on a frequent basis and in large numbers can be considered to be dominant. The population also has members which represent the more rare species which cannot be isolated easily, because of their low numbers.

Since 1940, numerous reports have been presented on indigenous endophytic bacteria found in plant tissue such as seeds and ovules, tubers, stems and leaves, and fruits. In 1997 bacteria found in plants included 129 species representing over 54 genera, with *Pseudomonas*, *Bacillus*, *Enterobacter* and *Agrobacterium* being the most commonly isolated (Hallmann *et al.*, 1997a). Endophytes have been isolated from a variety of plant species, as shown in Table 2.1, ranging from woody tree species such as oak and pear to herbaceous crop plants such as sugar beets and maize.

Table 2.1: Examples of plants and associated endophytes

Plant	**Endophytes**	**Reference**
Pine trees	*Paenibacillus, Bacillus*	Bal *et al.*, 2000
Scots Pine	*Methylobacterium extorquens,Mycobacterium sp.*	Prittilä *et al*., 2004, Prittilä *et al*., 2005
Eucalyptus	*Bacillus, Paenibacillus, Enterococcus, Methylobacterium, Sphingomonas,*	Ferreira *et al*., 2008
Oak	*Bacillus, Pseudomonas, Erwinia, Xanthomonas*	Brooks *et al*., 1994

Citrus plants	*Bacillus spp. Enterobacter cloacae, Alcaligenes sp, Pantoea agglomerans,*	Araujo et al., 2001
Corn	*Pseudomonas, Bacillus, Corynebacterium, Enterobacter, Klebsiella, Vibrio, Burkholderia*	Lalande *et al.*, 1989, Fisher *et al.*,1992, McInroy and Kloeper, 1995
Potato	*Bacillus, Micrococcus, Pseudomonas, Flavobacterium, Xanthomonas, Agrobacterium*	Hollis *et al.*, 1951, de Boer *et al.*, 1974
Tomato	*Pseudomonadaceae, Salmonella enterica*	Samish *et al.*, 1963, Islam *et al*., 2004
Rice	*Rhizobium, Sphingomonas,Pantoea sp., Klebsiella, Serratia*	Yanni *et al*., 1997, Engelhard *et al*., 2000, Kuklinsky-Sobral *et al*., 2004,Reiter *et al*. 2003, Sandhiya *et al*., 2005
Red clover	*Serratia, Bacillus, Enterobacter, Pseudomonas, Klebsiella, Micrococcus, Pantoea, Xanthomonas*	Sturz *et al.*, 1998
Sweet Potato	*Enterobacter asburia, , Pantoea agglomerans Klebsiella, Paenibacillus*	Asis and Adachi 2003, Iniguez *et al*., 2004, Reiter *et al*., 2003
Carrot	*Rhizobium,Klebsiella terrigena, Pseudomonas putida, Pseudomonas fluorescens, Salmonella enterica*	Surette *et al*., 2003, Islam *et al*., 2004

3.1 Endophytes in trees

Bacterial endophytes associated with trees are very poorly documented and research is mainly focused on root-associated endophytes with plant growth promoting effects (Cankar *et al*., 2005).

In order to characterise the interaction between citrus plants and their endophytic bacterial species Araujo *et al*., (2001) isolated endophytes from the leaves of different citrus rootstocks. Species isolated were *Alcaligenes* sp., *Bacillus* spp. (including *B. cereus, B. lentus, B. megaterium, B. pumilus, and B. subtilis*), *Burkholderia cepacia, Curtobacterium flaccumfaciens, Enterobacter cloacae, Methylobacterium extorquens,* and *Pantoea agglomerans,* with *P. agglomerans* and *B. pumilus* being the most frequently isolated. In another study Brooks *et al*. (1994) isolated bacterial endophytes from oak trees (*Quercus fusiformes*) that survived the oak wilt pathogen *Ceratocystis fagacearum*. This was done from oak trees in Texas where oak wilt is epidemic. A total of 889 isolates were obtained, in order to evaluate them as possible biological control agents for oak wilt. Many isolates (183) showed in vitro inhibition to the pathogen. In live oaks *Pseudomonas denitrificans* decreased the number of diseased trees by 50% and crown loss by 17%. Their results indicated a potential use for the selected endophytic bacteria in the control of oak wilt (Brooks *et al*. 1994).

Seedborne endophytes of trees have also been studied (Cankar *et al*., 2005 and Ferreira *et al*., 2008). For example, by taking fresh seeds from Norway Spruce tree cones directly after cutting, Cankar *et al*. (2005) used conventional culturing methods together with molecular methods, to study the tree's seed endophyte population. Previous studies indicated that the longer seed is stored, the more its bacterial endophyte population decreased (Bacon and Hinton 1996, Mundt *et al*., 1976). Cankar *et al*. confirmed this observation as they were not able to isolate endophytes from seeds stored in a seed collection after a few years. They did, however, isolate endophytes from fresh seeds collected from three out of the four trees that were sampled during their study. Restriction Fragment Length Polymorphisms (RFLP), culturing and partial 16S rDNA sequencing were used for identification and comparison of the isolates. Most of the bacteria isolated from the seed coat were identified as *Pseudomonas* spp. and *Rahnella* spp. Cankar *et al*. (2005) concluded that the genera that they had isolated may have plant growth promoting capabilities and could be used in further studies for potential growth promotion. In 2008 Ferreira *et al*. conducted a study in order to detect and identify the endophytic community in the seeds and seedlings of *Eucalyptus* spp. Isolations were done from 10 different *Eucalyptus* spp and two hybrids and the bacteria isolated belong mainly to the following classes: Alphaproteobacteria, Firmicutes and Actinobacteria, including *Bacillus* sp., *Bacillus megaterium, Enterococcus*

mundtii, *Methylobacterium* sp., *Methylobacterium variabile*, *Methylobacterium gregens*, *Paracoccus* sp., *Paenibacillus* sp, *Paenibacillus humicus, Sphingomonas phyllosphaerae* and bacteria from the *Frankiaceae* (Actinobacteria) family. With this study they showed that endophytic bacteria, especially *Bacillus, Enterococcus, Paenibacillus and Methylobacterium*, get transferred vertically from seeds to seedlings.

3.2 Endophytes in Agricultural crops

The beneficial effects that endophytes have on agricultural crops have become a very important focal point for research. Endophytes have been isolated from a wide variety of agricultural crops such as banana (Weber *et al*., 1999), carrot (Surette *et al*., 2003), maize (McInroy and Kloepper, 1995), grapevine (Bell *et al*., 1995) and soybean (Kuklinsky-Sobral *et al*., 2004). In 2005, Kukkurainen *et al*. isolated endophytes from field-grown garden strawberry, *in vitro*-grown garden strawberry and wild strawberry. In the garden strawberry endophytes were present in all plant tissue (root, stolon, leaf stalk, leaf, flower stalk, flower, berry) and the seed, and they isolated a large diversity of bacteria with *Pantoea spp.* and *Pseudomonas spp*. most common. The endophyte populations of the wild strawberries were also well represented throughout the plant and seed, but a much lower diversity was observed. Again *Pantoea spp.* and *Pseudomonas spp.* were most commonly isolated. The *in vitro* grown garden strawberries only had bacteria in the stolon, leaf stalk and leaf and no *Pseudomonas spp.* were isolated.

As mentioned previously soil is the primary source of endophytes and, therefore, it is important to establish a strong beneficial bacterial population in the rhizosphere to ensure a healthy microfloral balance within plants (Suerette *et al*., 2003). Rotation of crops has been shown to be beneficial, not only for saving the soil and restoring the soil balance, but also for maintaining the endophytic populations of the plants (Sturz *et al*., 1998). The reason may be that crops in rotation can share the same or similar endophytic populations and, therefore, the possibility exists of utilizing beneficial relationships between plants and bacterial endophytes over successive crops to develop more sustainable crop production systems (Sturz *et al*., 1998). Surette *et al*. (2003) surveyed bacterial endophyte populations in carrot root tissue to determine their influence on plant growth *in vivo*. They compared the endophyte populations from two

different cultivars of carrot but could not detect that the bacterial endophytes showed any cultivar specificity, when populating the cultivars. On the other hand Pillay and Norwak in 1997 showed that certain plant growth promoting effects caused by endophytic bacteria could be cultivar specific. A total of 28 bacterial genera were identified but *Pseudomonas, Staphylococcus* and *Agrobacterium* were the most common. With their study they provided data supporting the view that field history, cropping strategies and management practices contribute towards improving soil microbial populations and in return have an influence on endophyte populations of agricultural crops as well.

4. ENDOPHYTES ASSOCIATED WITH DIFFERENT PARTS OF THE PLANT

After entering, the plant endophytic bacteria usually reside within the apoplast of the plant. In order to get to this location they first have to survive on the leaf plane and rhizosphere, under very hostile conditions. Bacteria on the leaf surface are subject to a vast variety of environmental stresses including UV radiation, desiccation, osmotic stress and temperature changes (Hirano and Upper, 2000). Adding to this, the leave surface is not even, making the concentration and distribution of nutrients on the surface variable and bacteria appear to confine themselves to sites which are beneficial for their survival (Leveau and Lindow, 2001). Just as is the case with the phyllosphere, the rhizosphere also makes survival of potential endophytic bacteria complex, but because of the fact that roots act as a source of organic carbon, the population density in the rhizosphere is significantly higher than anywhere else on the plant surface (Cocking, 2003). Because of the differences between the environments associated with the phyllosphere and rhizosphere respectively, and the different survival mechanisms needed to colonize these different environments, their endophytic bacteria were considered separately.

4.1 The phyllosphere and endophytes of the leaves

The above ground tissue of the plant including the leaves, stem, blossoms and fruit is collectively known as the phyllosphere and provides a good liveable environment for microorganisms (Sabaratham and Beattie, 2003). The outside or epiphytic parts include the waxy cuticle that covers the trichome and guard cells. The endophytic parts or inside parts include the substomal

cavities, intercellular spaces and the mesophyll cell surfaces. The microenvironment of these sites differs substantially and, therefore, the growth of specific organisms will be promoted. In order to colonize the endophytic parts of the plant, the bacteria will first have to survive on the external parts until a suitable mode of entry presents itself.

Microbes that live on the surface of the leaves are susceptible to environmental stress, for example, humidity, fluctuations in temperature as well as UV radiation and desiccation (Wilson *et al*., 1999). Bacteria have the ability to change the environment in order to enhance their survival (Beattie and Lindow 1999). Strategies used include amongst others increasing the nutrients on the surface of the leaf. It has been shown by Brandii *et al*. (1998) that a low nutrient value can influence the growth of the bacteria on the leaf. Another strategy is to produce extracellular polysaccharides on the surface, which Brandii *et al*. (1998) proved by observing strands of amorphous material standing out from and in between bacterial cells on a leaf using scanning electron microscopy. These extracellular polysaccharides anchor the cells on the surface of the leaf and prevent desiccation and can be seen as the analog of biofilms formed by aquatic bacteria. A study conducted by Wilson and Lindow (1994) showed that the behaviour of bacterial cells varies in a density dependent manner and that when subject to desiccation, *Pseudomonas syringae* with a high cell concentration showed a 100 fold better survival on leaves than cells associated with inocula of low cell concentrations. To avoid UV radiation the microorganisms would produce siderophores and pigment (Lindow and Brandl, 2003). Bacteria also seem to localize themselves in what is called “protected” sites on the leaves including the base of the trichomes, substomatal cavities or cracks in veins and cuticles (Leveau and Lindow, 2001).

Some fungi have the ability to use appressoria to disrupt the plant cuticle and invade epidermal cells; in contrast, bacteria have to rely mostly on natural openings or wounds for entry (Rico *et al*., 2009). Gurian-Sherman and Lindow (1993) showed that high humidity followed by rainfall causes an increase in epiphytic population densities because it provides water and cracks in the leaves that increase nutrient availability. A high inoculum density on the plant surface in return favours invasion of intercellular spaces (Melotto *et al*., 2006). This usually happens through

natural openings on the surface, biotic or abiotic wounds or through insect vectors (Beattie and Lindow, 1995). Some bacteria appear to converge at the base of open stomata because of a high sucrose concentration. High humidity will cause the stomata to open resulting in a point of entry. Stomata are not passive sites and it was seen that bacterial elicitors play a role in opening and closing of these structures (Melotto *et al*., 2006). In order for bacteria to competitively colonize infection sites they need to possess chemotactic and nutrient utilization abilities, allowing them to move toward sites where plant secretions such as glutattion and apoplastic fluid occur (Brensic and Winans 2005). It has also been shown that some symbiotic bacteria have the ability to manipulate the plant into providing entry channels, called an infection thread, promoting bacterial entrance into the plant tissue from the leave surface (Beattie, 2007).

Once the epiphytic bacteria colonize the inside of the plant they become known as endophytes and they could reside within a certain type of plant tissue or colonize the plant systemically by transport or active migration through the apoplast (Quadt- Hallmann *et al*., 1997a). The reason for having an affinity for a certain niche within the plant may be because of the different nutritional requirements bacteria have or because of their interaction with other microorganisms. Endophytes mostly colonize the apoplast or intercellular part of the plant and there are only a few incidents where intracellular colonization was reported, for instance with *Rhizobium* and *Alcaligenes faecalis* that gets enveloped by the plant within special structures (You *et al*., 1991).

4.2 The rhizosphere and endophytes of the roots

The root-soil interface or rhizosphere is a very complex environment with the presence of radial and longitudinal gradients along the different roots. These gradients are determined by chemical, physical and plant factors. Some of the factors that play a role include species and nutritional status, mineral nutrients, pH, redox potential and reducing processes, root exudates and microbial activity (Marschner, 1988). When roots grow soil compacts around it and root cells and mucilage get released into the rhizosphere. At the zone of elongation large amounts of soluble exudates are secreted and as the epidermal and cortical cells become dilapidated, more carbon-rich compounds gets released. All of these factors contribute towards making the rhizosphere the multifaceted source of organic carbon that it is (Kerry, 2000). Therefore, this environment has a

very high bacterial population density surrounding it, which is estimated to be greater than 60 times that of the bulk soil (Lynch and Whipps, 1990). In return the bacteria assist the plants in acquiring phosphorus, potassium and to some extent nitrogen (Cocking, 2003).

Before bacteria can start colonizing the roots, there are usually four steps that have to be undertaken, namely, movement of bacteria from the bulk soil towards the roots, recognition, adhesion and provision of nutrients by the host. Maintaining the bacteria before entrance is a very expensive process for the host, especially in terms of nitrogen and carbon supply (Sprent and Raven 1985). There are three accepted routes the bacteria use to enter and colonize the roots: 1) through wounds, especially where lateral and adventitious roots protrude, 2) through root hairs and 3) between undamaged epidermal cells. Successive spread of the endophytes through the roots, as through the leaves, may be via intercellular spaces or in some cases when confined by host cell walls they would break down the primary wall. They cannot, however, digest the secondary wall and, therefore, are only able to move in between cells, but not through them (Sprent and De Faria 1988).

The benefits that plants get from harbouring endophytes in the roots are well recognized although it is not clear whether plants and the associated bacteria have a preference for an endophytic rather than a rhizospheric relationship (Rosenblueth *et al*., 2006). It has been proposed that endophytic bacteria get selected by the plant and that they take on these bacteria from a large pool of rhizospheric and soil species (Rosenblueth *et al*., 2006). Biotic and abiotic stresses play a role in both these groups, but it has been shown that endophytic bacteria could be better protected from environmental stress factors than rhizospheric bacteria (Hallman *et al*., 1997a). In 1998, Germida *et al*. did a study where they showed that the bacterial population found at the root surface was more diverse than the endophytic population. Current data supports the hypothesis that the interaction between the host and the endophyte may not be limited or specific. Zinniel *et al*., (2002) showed that when analyzing the host range of a wide variety of endophytes, no strict specificity towards a host could be observed. Another important observation made was that the presence of different endophytic species in soybean depends on

plant genotype, plant age, kind of tissue sampled and the season in which the sampling was done (Kuklinsky-Sobral *et al*., 2004).

Most of the suggested infection processes are based on studies observing rhizobia. There are, however, many more bacteria associated with plant roots that have the ability to penetrate and survive as endophytes. For that reason many questions are still being asked about the infection process and whether all root associated bacteria make use of the same basic methods for entrance.

5. IMPACT OF ENDOPHYTES ON PLANT HEALTH AND RECENT DEVELOPMENTS AND APPLICATIONS

Over 300 000 plants are hosts to endophytic bacteria, showing no external signs of their presence (Ryan *et al*., 2007). Many plants have still not been fully studied with regards to their bacterial endophytic population, which leaves a lot of scope for the discovery of new and useful endophytes. Different potential applications are foreseen including the use of endophytes as plant growth enhancers and bio-control agents, as well as another application of interest, the use of endophytes for the biodegradation of pollutants in soil.

5.1 Phytobacteria contributing negatively to plant health

The fact that latent infections are prevalent for bacterial pathogens makes it difficult to distinguish between non-symptomatic infections and colonization by plant pathogens that have a delay in symptom production (Hayward 1974). Therefore, it is important to take into consideration that not all the phytobacteria present at a certain stage of the plant's life cycle will contribute beneficially toward them or the surrounding environment. Plant-associated bacteria can typically be divided into three symbiotic groups, namely, commensals, mutualists and pathogens or parasites, based on their interaction with the plant.

Commensals will usually be the bacteria that are not known to have any effect on the plant, mutualists will be the ones that have a beneficial effect on the plants and pathogens and parasites

will be the bacteria that have an adverse effect on the plant's health. A specific organism may be a commensal on one host and a pathogen on another or be a commensal for a few months before entering a pathogenic phase. It is also accepted that all pathogenic bacteria will be in a commensialistic relationship with the host during some stage of its life cycle. This relationship can be further divided into endophytism and epiphytism, depending on whether the bacteria are present within the plant tissue or on the outside of the plant. Endophytes are bacteria that are isolated from surface sterilized tissue. About 15% of endophytes isolated thus far were classified as pathogenic species, but they could have been represented by non-pathogenic strains or they were present in an environmental situation that was not suitable for development of disease (Kobayashi and Palumbo, 2000). Therefore, according to these definitions it can be concluded that the endophytic population of a plant consist of the bacteria isolated from symptomless surface sterilized plant tissue and has the potential to consist of individuals, latent pathogens, which may produce disease symptoms at some stage of their life cycle.

5.2 Recent developments and applications for endophytes

5.2.1 Promoting plant growth and development

The main focus for plant growth promoting bacteria falls on rhizobacteria because they improve nutrient cycling. They are also important for phosphate solubilization, to supply vitamins, are responsible for osmotic adjustment and oversee modification of root morphology (Ryan *et al*., 2007). Nitrogen fixation is probably the most studied aspect of plant growth promotion. Studies of nitrogen fixation by rhizobia have served as a starting point because diazotrophy is common in prokaryotes (Lodewyckx *et al*., 2002). Endophytic diazotrophs like *Azospirillum* and *Azotobacter* probably has an advantage over the root-associated diazotrophs because they position themselves inside the plants to better exploit carbon substrates provided by the plant (McInroy and Kloepper 1995). On the other hand there is no direct evidence that endophytic diazotrophs are responsible for biological nitrogen fixation even though there are many assumptions supporting this notion (Lodewyckx *et al*., 2002).

Phytohormones, with the most common one being indole-3-acetic acid (IAA), plays a role in increasing root growth and length, and has been associated with root hair proliferation (Vessey, 2003). Enzymes like 1-aminoglycopropane-1-carboxylate (ACC) deaminase is suggested to play a role in plant growth promotion by modulating the level of ethylene in developing plants (Vessey, 2003). Some plant-growth promoting rhizobacteria (PGPR) increase root respiration by making use of a compound named lumichrome, a degradation product of riboflavin. Increased root respiration has been associated with increased CO_2 in the rhizosphere which favours both bacterial growth as well as assimilation in plants (Phillips *et al*., 1999). Volatile compounds like 2,3-butaneidiol and acetoin has been found to increase the growth of *Arabidopsis thaliana* seedlings and are produced by bacteria such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* (Ryu *et al*., 2003).

5.2.2 Biological Control

The bacteria that have the ability to be bio-control agents typically colonize the same niche as the phytopathogens, creating competition between the two strains for resources and inducing plant resistance. An example is that of the patented bio-control (No. 5994117, ATCC 55732*) Bacillus subtillis* strain RRC 101, which is a non-pathogenic, biological control agent and protects maize plants against fungi. It is used against colonization by *Fusarium verticillioides* (Bacon and Hinton 1999). . This fungal endophyte of maize is universally associated with maize plants and produces a toxin which associates infected maize with animal and human oesophageal cancer. Its endophytic habitat makes it difficult to control and, therefore, using something like endophytic bacteria will make it possible to eliminate the fungi on the basis of competitive exclusion (Bacon and Hinton 2002).

Another way in which these bio-control agents can protect plants against pathogens is by colonizing the same niche as the pathogens and creating space in which the bio-control bacteria can excrete compounds to inhibit the growth of the pathogens (Berg *et al*., 2005).These are often novel compounds that prevent bacterial plant diseases as well as antifungal metabolites that have the potential to open up doors to discover new drugs in the treatment of diseases in humans, plants and animals (Strobel *et al*., 2004). The term natural products refers to naturally derived

metabolites and/or by-products produced by microorganisms, with activity against both plant and human pathogens (Strobel and Daisy 2003). Secondary metabolites produced by soil bacteria include antibiotics and volatile organic compounds that are antifungal, antibacterial and antiviral. Examples of bacteria that produce these products are *Pseudomonas*, *Bacillus* and *Burkholderia* and these groups of bacteria still represents an untapped source for such compounds (Ryan *et al*., 2007).

Due to the resistance in many microorganisms against existing anti-microbial compounds and immunocompromised individuals at risk of opportunistic pathogens, there is still a big market for new effective antibiotics, chemotherapeutic agents and agrochemicals with low toxicity and minor impact on the environment, creating opportunities for the discovery of novel natural products, although that area is still mainly unexplored (Strobel and Daisy 2003).

5.2.3 Phytoremediation

In the process of phytoremediation one can use endophytic bacteria with specific traits to degrade xenobiotic compounds present in contaminated soil (Siciliano *et al*., 2001). An example of such would be a phyto-symbiotic bacterial strain isolated from hybrid poplar trees that has the ability to degrade numerous nitro-aromatic compounds such as 2,4,6-trinitrotoluene. If natural biodegration ability, on the other hand is absent, genetically engineered strains can be created, as Lodewyckx *et al*. (2001) showed that endophytes from yellow lupin can be genetically modified to have nickel resistance, and have the ability to increase the nickel accumulation and tolerance of inoculated plants.

In 2005 Newmann and Reynolds made a summary of the advantages of using endophytic microorganisms to deal with xenobiotic pollution. They found that when it is necessary for genetic engineering of a xenobiotic degradation pathway, the best would be to use bacteria rather than plants, because they are easier to manipulate. The degrader strain will have an advantage to reach larger population sizes due to lowered competition, produced by the unique niche provided by the interior plant environment.

6. ISOLATION AND DETECTION METHODS FOR ENDOPHYTES

A problem that occurs during the study of endophytes is that it is difficult to quantitatively and qualitatively describe the populations, resulting in varied results. This problem is often due to the fact that the growth media used might not be optimal for growth of the endophytes and there may be variations between the growth conditions within the plants and the artificial growth media and growth environment created in a laboratory. Traditional culture methods may, therefore, not produce reliable results for population studies of endophytes. To overcome this problem, molecular methods can be used. These methods usually make use of direct DNA extractions, excluding the culture step. This provides a greater likelihood that most of the bacterial DNA present in the sample would be taken into consideration during analysis.

6.1 Culturing Methods

Culturing is still extremely important when investigating bacterial endophytes in order to study their morphology and physiology, but also very limiting. These limitations can be because of the small sized samples, selective growth conditions and the laborious nature of most methods (Hallmann *et al.* 1997a). The initial sample preparation procedure is of extreme importance, as it should assist in recovering the complete internal microbial population. This, however, is unlikely, as the absorbance of bacteria cells to plant cell structures or the penetration of the sterilant into plant tissues, causing it to kill some of the endophytic bacteria (Hallmann *et al.* 1997a) may result in incomplete recovery. Table 2.2 shows some preparation and isolation methods for different kinds of plants. Due to the importance of the sample preparation step, this issue will be the main focus of this section.

6.1.1 Trituration of surface sterilized material

Trituration of surface-disinfected plant tissue is the most common procedure used to isolate endophytic bacteria. Disinfectants used for this procedure include sodium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide or a combination of two or more of these chemicals followed by several washes with sterile water or buffer (Hallmann *et al.* 1997a). Tween 20/80 or

TritonX-100 can be used to reduce the tension of the solvent in order to allow the sterillant to reach the protected sites on the leave. When optimizing the sterilization, factors like plant species, age of material and plant parts used as well as concentration of the sterillant should be considered (Hallmann *et al*., 1997a). After sterilization, a sterile mortar and pestle are used to grind the plant tissue in sterile water or buffer solution (Mahaffee and Kloepper, 1997).

When monitoring the sterilization step, to ensure that the sterrilant is not penetrating into the leaf, the plant tissue can be stained with tetrazolium-phosphate buffer solution and look for colour development. Only the actively metabolizing cells will show a colour reaction and it can be assumed that the other cells have been killed by the sterilization process (Patriquin and Döbereiner, 1978). Another procedure used to decrease the chance to get contamination is to do regular sterility tests. This is done by dipping the plant tissue into nutrient broth after surface sterilization (Gagné *et al*., 1987), or transferring 0.1 ml of the final washing water to a test tube containing growth media or imprinting the tissue onto a nutrient medium (McInroy and Kloepper, 1994). Quadt-Hallmann and Kloepper (1996) demonstrated that no observed growth during these tests, does not guarantee total sterility as single cells or spores may survive in special niches. Advantages of this technique include the fact that almost any plant tissue can be used and it is suitable for describing the broad spectrum of endophytes occurring in the total plant tissue. It is, however, not a useful examination tool for bacteria from certain physical niches, for example, when comparing intercellular versus intracellular colonization (Hallmann *et al*., 1997a).

Table 2.2: Examples of methods used to isolate and characterize endophytic bacteria, using culture methods (Lodewyckx *et al*., 2002)

Plant	**Isolation method**	**Identification method**	**Reference**
Red Clover	Washing: water, detergent, 95% ethanol, 2% sodium hypochloride, rinse 3 times in sterile dH2O. Blended in Ringers,	Single colonies. Characterization: Gram stain, Oxidase test, Biolog, Mobility test.	Tanprasert *et al.* 1997

	shaken 45min in conicals. Dilution series, plated on TSA plates		
Citrus plants	Surface disinfect system	RAPD(molecular)	Araujo *et al.* 2001
Cotton	Plant tissue washed in 29% hydrogen peroxide, rinsed in 4x sterile 0.02M potassium phosphate buffer. Grounded in mortal with 9.9ml wash buffer. Serial dilution	Plated onto 3 different media: R2A (oligothrophs) TSA (heterotrophs) Sc (others) Incubated at 28C for 48-72 hrs. Identified by FAME (molecular)	McInroy and Kloepper, 1995
Corn	Sterilized by 75% ethanol 3-5% Na hypochloride, 75% ethanol, Ground in mortal with Ringers	Nutrient agar incubated at 25C for 7 days. Identified using BIOLOG	Fisher *et al.* 1992

6.1.2 Vacuum and pressure extraction

Vacuum and pressure extraction can be used to bypass the above mentioned problems concerning surface disinfection In this method a vacuum technique has been used to extract the xylem sap from roots of grapevine (Bell *et al.* 1995) and the Scholander pressure bomb to extract root sap from cotton and other agricultural crops (Hallmann *et al.* 1997b). Mahaffee *et al.* (1997) did a comparison of the bacterial community recovered from cotton roots by the trituration and pressure bomb techniques and it indicated that only a subset of the bacterial endophytes are recovered by the pressure bomb technique. *Bacillus* spp., shown to be a predominant group of the soil rhizosphere and endorhiza communities of cucumber, were only recovered by the trituration technique, whereas *Pseudomonas* spp., commonly reported as endophytes, dominated the bacterial community recovered with the pressure bomb technique as described by Hallmann *et al.* (1997b). The researchers also observed quantitative differences in the total populations recovered. The trituration technique constantly gave higher numbers of endophytic bacteria. As with the trituration technique the vacuum and pressure bomb techniques

should also include controls when being used. Tissue dipped in a bacterial suspension prior to extraction has been suggested. This is to confirm that surface contaminants will not be forced from the surface into the internal parts of the plant by the applied pressure, and ending up with external tissue contaminants appearing as endophytes in the sample. To further exclude contamination from the isolates the applied pressure should be released slowly. A drawback of this technique is that soft tissues of herbaceous plants or seedlings might not be strong enough to withstand the treatment. The practical use of this technique is, therefore, limited to certain plant species or tissue (Hallmann *et al.* 1997b).

6.1.3 Centrifugation

Another method that can be used to get the bacterial endophytes out of the plant tissue is by centrifugation, used to collect the intracellular fluid from the plant. Dong *et al.* (1994) used centrifugation to extract intracellular fluid from sugarcane. He reported that up to 10^4 CFU/ml of an acid producing bacterium were isolated. The ethanol flaming technique was used to first surface sterilize the tissue after which centrifugation at 3000 x g removed most of the apoplast. By applying cryo-scanning electron microscopy they could show that the cells were still intact and that none of the fluid from the symplast was extracted. This method is suitable for soft plant tissue, but surface sterilization is required implying that the sterilant could penetrate the inner parts of the plant and kill some of the endophytes, causing the results to not be representative of the true population.

In conclusion, when deciding on a method for the initial treatment of the sample it is important to keep in mind what type of plant tissue is used and what controls should be included when performing the technique. The physical niche to be sampled and associated plant-dependent factors should also be kept in mind. The use and comparison of different techniques offers the advantage of examining endophytic colonization and localization from different perspectives, thereby enhancing the understanding of the potential function endophytic bacteria have in their environment (Hallmann *et al.* 1997a).

6.2 Molecular techniques

Our knowledge of microbial diversity has mainly been based on culturing of these organisms but in fact it is likely that less than 1 % of microorganisms observed in nature are culturable using standard methods (Amann *et al*., 1995). Thus a large number of bacteria cannot be detected using cultivation because of their unknown growth requirements (Amann *et al*., 1995), or because they went into a viable but unculturable state (Tholozan *et al*., 1999). For this reason it has become popular to use the 16S rRNA phylogenetic marker in describing diversity within specific natural environments. 16S rRNA cloning methods that rely on analysis of this gene sequence (Sessitsch *et al*., 2002) can be used to identify these specific sequences, but setting up and sequencing of cloning libraries are, however, tedious and therefore other techniques that were developed to be applied in such cases include denaturing or temperature gradient gel electrophoresis (D/TGGE) and restriction terminal fragment length polymorphism (t-RFLP)

6.2.1 terminal-Restriction Fragment Length Polymorphism (t-RFLP)

With the terminal restriction fragment length polymorphism technique the size polymorphism of terminal restriction fragments from a PCR amplified marker is measured (Marsh *et al*., 1999). The technique consists of two different technologies namely comparative genomics and nucleic acid electrophoresis. The first technology provides the necessary insight into the design of primers which is then used in a PCR to amplify the fragment of choice. In the second step restriction enzymes are used to cut the fragments creating labeled terminal fragments appropriate for sizing on high resolution gels. Automated systems such as the ABI gel or capillary electrophoresis systems provide digital output of all the bands and may be preferred to fluorescently labeled primers, as the analysis is not limited to the terminal fragments of the digestion only (Marsh *et al*., 1999). This technique not only provides a rapid and sensitive idea of the amplification product diversity within a community, but also a comparative distribution across communities (Marsh *et al*., 1999). The major advantages of this technique is that you have a direct link to the sequencing database making it easy to perform phylogenetic inference, it is an indirect nucleic acid sequencing technique and provides a greater resolution than electrophoretic systems such as DGGE. , T-RFLP's gel analysis is immediate and provides a digital output (Marsh *et al*., 1999). The main disadvantage of t-RFLP is the fact that the

identification of organisms responsible for a particular element in a profile could be very complex if the targeted restriction site is shared by a number of bacterial groups (Ball *et al*., 2008).

6.2.2 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis is a direct analysis method developed by Muyzer *et al*. in 1993, to investigate the genetic diversity of complex microbial populations. The method is based on the electrophoresis of PCR-amplified 16S rDNA fragments through polyacrylamide gels, in a linearly increasing gradient of denaturants, and the separation of the mixed PCR products are based on the sequence-specific melting point of the specific products. With this method fragments of the same length but different base pair sequences can be separated easily, and by adding GC-rich sequence to either one of the primers the fragments' melting behaviour can be modified, to try and get as close as possible to 100% of the possible sequence variation (Muyzer *et al*., 1993).

Primers that target conserved bacterial regions are used to target the variable regions of the 16S ribosomal genes in environmental DNA extracts (Gelsomino *et al*., 1999). This is then followed by analysis using DGGE, separating amplified fragments of up to 400 bp with only a few sequence differences easily (Nubel *et al*., 1996). With this method the entire band pattern is considered to present a general "image" of the whole community (Fromin *et al*., 2002). Since its development in 1993 DGGE has been widely used in microbial ecology studies e.g. the quality control of probiotics for human consumption (Temmerman *et al*., 2003), rhizosphere bacteria studies (Stafford *et al*., 2005 and Lu *et al*., 2006), soil bacterial community studies (Brons and Elsas, 2008, Gelsomino *et al*., 1999) as well as for plant bacterial community studies (Garbeva *et al*., 2001).

There are also a number of disadvantages associated with DGGE. This may include the detection of heteroduplex molecules (Ferris and Ward, 1997) although it is not commonly experienced (Nübel *et al*., 1999), molecules produced by different operons of the same organism can

sometimes also be detected (Nübel *et al*., 1996). It is difficult to separate relatively small fragments and then co-migration of the bands, as mentioned above, is also a problem (Vallaeys *et al*., 1997). A study by Sekiguchi *et al*. (2001) suggested that a single DGGE band does not always represent a single bacterial strain and that the band which migrated to the same position in different lanes may represent different bacteria. Cloning and sequencing of the co-migrating 16S rDNA fragments, cut out from the DGGE gel, can overcome this problem (Sekiguchi *et al*., 2001). As with any other technique it is very important to cross-check results obtained, in order to ensure that the true microbial community structure was analyzed. The technique also has limited sensitivity when it comes to the detection of rare community members, but research has found that this problem can be solved by hybridization with specific probes (Straub and Buchholz, 1998).

The fact that the technique is reliable, rapid, reproducible and relatively inexpensive still makes it a popular choice for community analyses. It enables the study of the complexity and behaviour of bacterial communities, as well as the simultaneous analysis of multiple samples making it easy to follow changes in the community over time (Muyzer, 1999). It is also advantageous that one can identify community members by excising and sequencing bands from the gel or by making use of hybridization analysis, which is not possible in the case of t-RFLP (Muyzer, 1999).

7. CONCLUSION

Bacterial endophytes are a group of organisms with lots of potential applications and which have recently attracted more attention. This may be linked to the ability of some isolates to provide plants natural protection against pathogens, or the production of natural molecules that can be used in the medical field, or their potential in remediating polluted soil. It is obvious that the terminology and definitions regarding endophytes are confusing. Many researchers see the concept of symptomless, non-disease causing infection as inherent to the definition of endophytes. This infection may then lead to one of three possible relationships namely neutralism, mutualism or commensalism (Bacon and Hinton, 2007). But as James *et al.* suggested in 1997, possible or latent pathogens could also be present as endophytes in a non-

pathogenic state or under unfavourable environmental conditions. The terminology used for bacterial endophytes are mainly based on fungal interactions and terms and definitions regarding phytobacteria should be standardized to better distinguish the different types of relationships between these bacteria and their plant hosts.

Many techniques have been used in studying bacterial communities in natural ecosystems, including the more commonly used molecular techniques like DGGE/TGGE (Muyzer *et al*., 1993, Muyzer and Smalla 1998 and Muyzer 1999), and T-RFLP (Marsh 1999). As our understanding of endophytic bacteria continues to grow, the potential to capitalize on the unique characteristics and close association with plants also grow (Hallman *et al*., 1997a).

An important aspect that requires further attention is the sequencing of more genomes of plant symbiotic bacteria (Puhler *et al*., 2004). This will also require the development of bioinformatics to be able to get the most information from the genomes (Puhler *et al*., 2004).

As methods used to research bacterial endophytes get more perfected and more information is gathered about the use and ecology of these bacterial endophytes, the better understanding we will have about the specific role these bacteria have in the environment. An understanding of the mechanisms enabling these endophytic bacteria to interact with plants will be essential in order to attain the biotechnological potential of efficient plant–bacterial association for a range of applications. With the availability of complete genome sequences of key endophytic bacteria, the genes governing colonization and establishment of endophytic bacteria *in planta* can be identified (Ryan et al., 2007). This could be an important link in developing our understanding of how these bacteria operate, what gives it the ability to live inside plants and how to utilize them to our advantage.

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CHAPTER 3

DETECTING CULTURABLE BACTERIAL ENDOPHYTES FROM THE FAMILY *ENTERIOBACTERIACEAE* IN *EUCALYPTUS NITENS* CLONES

CHAPTER 3

Detecting Culturable Bacterial Endophytes from the family *Enteriobacteriaceae* in *Eucalyptus nitens* clones.

1. INTRODUCTION

Endophytic bacteria are categorized as organisms residing within plant tissue, without causing significant damage to their host (Hallmann *et al*., 1997). There are many reports describing the presence of bacterial endophytes inhabiting various plant tissues, for example, seeds, leaves and tubers. Isolations of both Gram negative and Gram positive bacteria have been made from an extensive range of plant species and include bacterial genera from the following groups: Firmicutes, Actinobacteria, α Proteobacteria, β Proteobacteria, and γ Proteobacteria, (Rosenblueth and Martínez-Romero, 2006). The most common genus found is from α Proteobacteria and is the plant symbiont *Agrobacterium* which is often involved in transferring foreign DNA into the plant genome. The most frequently isolated genera from the β Proteobacteria are *Burkholderia* and *Herbaspirillium*. *Burkholderia* includes species which are either opportunistic or are commonly found as human pathogens. The family *Enterobacteriaceae* is part of the γ Proteobacteria and most of the reported endophytes belong to this family. Genera that were reported as endophytes include *Brenneria, Pectobacterium* (Lodewyckx *et al*., 2002), *Citrobacter* (Martínez *et al*., 2003), *Enterobacter, Erwinia* (McInroy and Kloepper 1995), *Escherichia* (Ingham *et al*., 2005), *Klebsiella* (Rosenblueth *et al*., 2004), *Pantoea* (Coutinho and Venter, 2009). *Salmonella* (Cooley *et al*., 2003, Guo *et al*., 2002, Islam *et al*., 2004) and *Serratia* (Sandhiya *et al*., 2005).

Some of the bacterial endophytes isolated form *Eucalyptus* trees have been identified as possibly belonging to the genus *Pantoea* (Procópio *et al*., 2009). *Pantoea ananatis* has been implicated as the causal agent of bacterial blight and die-back of *Eucalyptus* clones in South Africa (Coutinho *et al*., 2002). Isolates belonging to this and related *Pantoea* spp. were also isolated from *Eucalyptus* trees in Uganda, Uruguay and Argentina with similar disease symptoms as those observed in South Africa (Brady *et al*., 2008; Swart, 2009). From this data the question arises whether the bacterium causing bacterial blight and dieback was introduced by insect vectors or

was it already present within the plant as an endophyte, waiting for favourable environmental conditions to trigger symptoms. As *Pantoea* species have already been recorded as endophytes, differences in the endophyte populations of susceptible and resistance clones needs to be addressed. Although the disease symptoms are only apparent on the leaves of young plants, it would be of interest to determine if isolates could be found throughout the plant as different microecosystems exist within a single plant (McInroy and Kloepper 1994).

The aim of this study was to determine which *Enterobacteriaceae* can be isolated as endophytes from different parts (namely leaves, stems and roots) from a number of *Eucalyptus nitens* clones. The clones used for isolation all differed in their susceptibility to *P. ananatis* and this ranged from highly susceptible to resistant. To obtain the isolates standard culturing techniques were used, followed by sequence identification of the 16S rRNA as well as two housekeeping genes, *rpoB* and *gyrB.*

2. MATERIALS AND METHODS

2.1 Sampling

The sampling of the plant tissue was undertaken at a nursery in White River, Mpumalanga, South Africa during November 2007. At that time, the nursery had been experiencing very high rainfall but lower than usual temperatures. Entire plants of the cold tolerant *Eucalyptus nitens* clones, resistant and susceptible to *P. ananatis*, were sampled. The ranking of clones was done by the nursery manager after careful observation of the prevalence of disease symptoms on the different clones and scored as 1 (resistant) to 5 (highly susceptible). Eight clones were sampled and Table 3.1 shows their level of susceptibility to *P. ananatis*. For the susceptible clones, three healthy and three diseased plants were sampled. Diseased plants were chosen when the leaves had distinct water-soaked lesions, whereas healthy plants had no visible symptoms. For the resistant clones three plants per clone were sampled.

2.2 Processing of samples, pure culturing and screening

The plants were divided into three main parts, namely leaves, stems and roots, and each part was labelled and a number was allocated to it. The numbers were then entered into a table, making each part of a specific plant traceable. After the documentation was completed each labeled part of the plant was processed. First each part of the plant was surface sterilized using a four step method of washing with sterile water, sterilizing for 30 seconds in a 10% sodium hypochlorite solution, followed by 30 seconds in 70% ethanol and ending off with another rinse step with sterile water. A flame sterilized forceps and scissors were used to cut the plant parts into very small pieces which were then transferred to a 50 ml Schott bottle, to which 10 ml Nutrient Broth was added. The bottles were incubated at 25 ºC for 2-5 days, or until adequate growth could be observed. With the initial surface sterilizing of the plant parts, all epiphytic organisms were removed from the plant material. This was verified by culturing of the wash water from the final step.

After incubation of the Nutrient Broth, 100 µl was plated out onto Nutrient Agar plates containing 0.01 g/ml cyclohexamide to prevent fungal growth. The plates were incubated for 1-2 days at 28 ºC. The plates were then evaluated and colonies with different morphologies and colour were chosen and re-plated onto Nutrient Agar plates to obtain pure cultures. The pure cultures were preserved on Microbank™ Beads (Pro-lab diagnostics), according to manufactures protocol, and kept at -70 ºC.

A total of 408 isolates were obtained and screened. Only cultures belonging to the family *Enterobacteriaceae* were further identified. Bacteria belonging to this family are facultative anaerobic Gram negative organisms utilizing glucose in the presence or absence of oxygen. The Hugh-Leifson test was used to determine the oxidation/fermentation properties of the isolates. Filter sterilized D-glucose was added to the O/F medium (Biolab) after autoclaving. A single colony was picked from a plate using a sterile toothpick and used to inoculate a tube of 5 ml O/F medium. Two tubes were inoculated per culture and 800 µl of sterile paraffin was added to one of the tubes to create an anaerobic environment. The tubes were incubated overnight at 28 °C. All cultures where the medium in both tubes changed from green to yellow (facultative

anaerobic), were then Gram stained. All isolates that were Gram negative and facultative anaerobic were used for further study (Table 3.2).

2.3 Partial 16S rDNA Colony PCR and Sequencing

After the first screening all the facultative anaerobic, Gram negative cultures were subjected to a colony PCR amplifying the 16S rRNA gene. One colony was picked from a pure culture plate and resuspended in 100 µl sterile distilled water and used as template. Each 50 µl PCR reaction consisted of 5 µl 10x reaction buffer, 4 µl $MgCl_2$ (2.5 µM) and 4 µl of a mixture of all four dNTP's (200 µM each), 1 µl (100 µM) each of the pA forward (5'-AGA GTT TGA TCC TGG CTC AG-3') and pH reverse primers (5'-AAG GAG GTG ATC CAG CCG CA-3') (Coenye *et al*., 1999) and 0.2 µl Super-Therm Taq DNA polymerase (5 U/µl). To this 2 µl of the resuspended colony and 32.8 µl of nuclease free water was added. The reaction was then subjected to an amplification program comprising of the following steps. Lysis of cells at 95°C for 10 minutes, followed by denaturing at 94°C for one minute, annealing at 58°C for 1 minute and elongation at 72°C for 1 minute, repeated for 30 cycles. The final annealing step was at 72°C for 7 minutes where after the reaction was kept at 4°C. Five microliter of the PCR product was loaded onto a 1% agarose gel and run for 30 minutes at 90 Volts, to confirm amplification of the target sequence.

Following amplification, the PCR product was cleaned using the DNA Clean and Concentrator Kit (Zymo Research) according to the manufacturer's recommendations. The amplified DNA was, in the final step, eluted in 30 µl sterile distilled water. The 10 µl sequencing PCR reaction contained 2 µl Big Dye Sequencing Reaction mix, 1µl 5x Sequencing Buffer, 0.3 µl (100 µM) *pD primer (5'-CAG CAG CCG CGG TAA TAC-3') (Coenye *et al*., 1999) and 4 µl of the cleaned 16S amplification product. The sequencing conditions included denaturation at 96°C for 5 seconds, followed by 25 cycles of denaturing at 96°C for 10 seconds, annealing at 55°C for 5 seconds and elongation at 60°C for 4 minutes. The reaction was kept at 4°C. The PCR products were then sequenced on an ABI Prism DNA Automated sequencer.

2.4 Partial *gyrB* Colony PCR and Sequencing

To confirm the identity of the *Pantoea* species following 16S rRNA sequencing, it was decided to amplify the *gyrB* housekeeping gene using the *gyrB*P forward (TAA RTT YGA YGA YAA CTC YTA YAA AGT) and *gyrB*02 reverse (5'-CMC CYT CCA CCA RGT AMA GTT-3') primers (Brady *et al*., 2008). Each 50 µl PCR reaction consisted of 5 µl of 10x reaction buffer, 4 µl (25 mM) $MgCl_2$, 4 µl dNTP's (200 mM each), 2 µl of each of the forward and reverse primers (100 µM), 0.3 µl (5 U/µl) Super-Therm Taq DNA polymerase and 2 µl of the resuspended bacterial colony (as described previously). The amplification conditions consisted of cell lysis at 95°C for 10 minutes, 3 cycles of denaturing at 95°C for 1 minutes, annealing at 55°C for 2 minutes 25 seconds, elongation at 72°C for 1 minute 15 seconds, 30 cycles of denaturing at 95°C for 35 seconds, annealing at 55°C for 1 minute 15 seconds and elongation at 72°C for 1 minute 15 seconds. The final elongation was at 72°C for 7 minutes. PCR products were separated on a 1% agarose gel for 30 minutes at 90V.

The amplification products showing a positive result of the correct size were purified using the DNA Clean and Concentrator kit (Zymo Research). Four microlitre of the purified product was used in a 10 µl sequencing reaction along with 2 µl Big Dye sequencing reaction mix, 1 µl 5x sequencing buffer and 0.3 µl (100 µM) sequencing primer *gyrB* 07-F (5'-GTV CGT TTC TGG CCV AG-3') (Brady *et al*., 2008). The sequencing conditions consisted of denaturation at 96°C for 5 seconds, 25 cycles of denaturation at 96°C for 10 seconds, annealing at 55°C for 5 seconds and elongation at 60°C for 4 minutes. PCR products were sequenced using the ABI Prism Automated DNA sequencer.

2.5 Partial *rpoB* Colony PCR and Sequencing

To confirm the identity of the *Enterobacter* species isolated, an *rpoB* colony PCR was done using the *rpoB* CM7 forward (5'-AAC CAG TTC CGC GTT GGC CTG-3') and *rpoB* CM31b reverse (5'-CCT GAA CAA CAC GCT CGG A -3') primers (Brady *et al*., 2008). Each reaction consisted of 5 µl of 10x reaction buffer, 4 µl (25 mM) $MgCl_2$, 4 µl dNTP's (200 µM each), 2 µl of each of the 100 µM primers, 0.3 µl (5 U/µl) Super-Therm Taq DNA polymerase and 2 µl of

the resuspended colony (as described previously). The amplification conditions consisted of cell lysis at 95°C for 10 minutes, 3 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes 15 seconds, elongation at 72°C for 1 minute 15 seconds, 30 cycles of denaturation at 95°C for 35 seconds, annealing at 55°C for 1 minute 15 seconds, annealing at 72°C for 1 minute 15 seconds, ending off with a final annealing at 72°C for 7 minutes. PCR products were separated on a 1% agarose gel for 30 minutes at 90 V.

The amplification products showing a positive result of the correct size were purified using the DNA Clean and Concentrator kit (Zymo Research). Four microliter of the purified product was used in a 10 µl sequencing reaction along with 2 µl Big Dye sequencing reaction mix, 1 µl of the 5x sequencing buffer and 0.3 µl (100 µM) sequencing primer *gyrB* 07-F (5'-GTV CGT TTC TGG CCV AG-3') (Brady *et al*., 2008). The sequencing PCR reaction consisted of denaturation at 96°C for 5 seconds, 25 cycles of denaturation at 96°C for 10 seconds, annealing at 55°C for 5 seconds and elongation at 60°C for 4 minutes. PCR products were sequenced using the ABI Prism Automated DNA sequencer.

2.6 Phylogenetic analyses

Nucleotide sequencing results were edited and blasted against the Genbank sequence database. The sequences were aligned using Clustal X (Thompson *et al.*, 1997) and the overhangs trimmed. The *rpoB* sequences were aligned with the sequences of all currently described *Enterobacter* and *Cronobacter* species. The Modeltest 3.7 programme (Posada & Crandall, 1998) was then applied to the data sets to determine the best-fit evolutionary model. Maximum likelihood and neighbour joining analyses were performed using Phyml (Guindon & Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000) respectively, by applying the models and parameters determined by Modeltest (only Maximum likelihood phylogenetic trees are shown). Bootstrap analysis with 1000 replicates was performed to assess the reliability of the clusters.

3. RESULTS

3.1 Processing of samples, pure culturing and screening

In order to be able to manage and compare the results, the physical state of the plants needed to be documented and the samples had to be divided into parts to standardise the process. Table 3.2 summarises the results obtained for the Gram reactions performed on all of the isolates. Table 3.3 provides a summary of all the isolates belonging to the family *Enterobacteriaceae.* These cultures all showed a positive result for the Hugh-Leifson test and stained Gram negative.

Most of the isolates from this study fell in the Gram negative group, but not many of them were identified as belonging to the family *Enterobacteriacae.* As indicated in Table 3.2, most of the Gram negative isolates originated from the leaf and stem material tested. Only 38 of the 408 isolates belonged to the family *Enterobacteriaceae* based on the Hugh-Leiffson and Gram staining results. From these isolates 39.5% was isolated from resistant clones and the remaining 60.5% from susceptible clones. Eighteen of 23 isolates obtained from the susceptible clones were from diseased material and the remaining 5 from healthy material, all 5 from the roots of the plants (Table 3.3). No isolates belonging to the family were obtained from healthy material of Clone 2. Clone 3, Clone 1, Clone 4 and diseased material from Clone 2 contributed 10, 7, 2 and 4 potential *Enterobacteriaceae* isolates, respectively. For the resistant group most of the isolates were acquired from the root material (8 in total) followed by 6 isolates from the leaf material and 1 stem isolate. Clone 6, Clone 7, Clone 5 and Clone 8 contributed 6, 5, 3 and 1 isolates, respectively.

The Gram stain results that were obtained from the remaining isolates were either Gram positive or Gram negative rods with the Hugh-Leiffson results being a colour change only in the one tube, indicating an aerobic organism. Because of the fact that only Gram negative, facultative anaerobic cultures underwent 16S rRNA sequencing, the presence and identity of other possible genera present in the sampled symptomatic, asymptomatic and resistant plant material was not determined.

3.2 Partial 16S rDNA Colony PCR and Sequencing

The 16S rRNA gene was amplified for the selected strains. These strains all yielded a PCR amplification product of about 1500 bp. All these products were sequenced and the Blast results are summarized in Table 3.3. Figure 3.1 shows the phylogenetic analysis of the 16S amplification.

In order to determine the diversity of the species from the *Enterobacteriaceae* isolated from the plants, the 16S rRNA was amplified and sequenced. Type strain sequences from *Pantoea, Enterobacter*, *Cronobacter*, *Erwinia, Tatumella* and *Kluyvera* were obtained from GENBANK and included in the analysis. The *Enterobacteriaceae* isolates sequenced formed 12 distinct clusters within the phylogram (A to I) (Figure 3.1). The isolates were mostly from Clones 1 and 3, and these where scattered throughout the 12 clusters. From this data it can also be said that the *Enterobacteriaceae* from both asymptomatic and symptomatic plants had higher species diversity than those occurring in the other clones. Most of the other species belonging to the *Enterobacteriacae* were isolated from the roots of the different plants and they were identified as *Enterobacter* and *Erwinia* species. The remaining isolations present in the phylogram were isolated from either leaves or stem material. Possible genera that these isolates belonged to where either *Pantoea* or *Enterobacter*. None of the type strains, however, clustered within groups A and B although the blast results indicated that these isolates could belong to either *Enterobacter* or *Pantoea*. Groups C, D, E, F, G, H and I all clustered in the vicinity of some of the *Enterobacter* type strains whereas groups J and K clustered with *Pantoea agglomerans* and *Pantoea eucalypti,* respectively.

3.3 Partial *gyrB* Colony PCR and Sequencing

The *gyrB* gene was amplified and sequenced in order to confirm the identity of the possible *Pantoea* species provisionally identified from the partial 16S rRNA sequencing results. Correct amplification resulted in a band size of approximately 650 bp. Table 3.4 summarizes the blast results for the *gyrB* sequences, and Figure 3.2 the phylogenetic tree drawn with the partial *gyrB* sequences.

The Maximum Likelyhood Phylogram constructed for the *gyrB* sequencing results grouped the isolates into four distinct groups as indicated in Figure 3.2. Isolates from diseased Clone 3 (61d) and resistant Clone 5 (238c and 239b) clustered with *Pantoea eucallypti* and isolates from a different diseased plant of Clone 3 (3d, 4c, 6c, 7b and 7d) and a diseased plant of Clone 1 (12c) clustered with *Pantoea vagans*. All of these groupings were supported with good bootstrap values. The isolates in groups C and D on the other hand did not have good bootstrap support and blast results against GenBank indicated that they were possible *Enterobacter* spp.

3.4 Partial *rpoB* Colony PCR and Sequencing

In order to identify the possible *Enterobacter* isolates, amplification and sequencing of the *rpoB* gene was performed. PCR amplification products for the *rpoB* gene migrated to about 650 bp on an agarose gel when compared to a 100 bp marker run simultaneously. Table 3.5 indicates the blast results for the *rpoB* amplification of possible *Enterobacter* isolates. All of the *rpoB* sequences blasted as possible *Enterobacter* spp. Figure 3.3 shows that the sequences grouped well within the phylogenetic tree constructed. The phylogenetic analysis of these *Enterobacter* isolates indicated five distinct groups (Figure 3.3 groups A-E). The type strains of *Enterobacter mori* and *Enterobacter asburiae* clustered in groups A and C, respectively. Groups B, D and E and isolates 30b and 116c did not cluster with a known *Enterobacter* sp.

4. DISCUSSION

During the first recorded outbreak of bacterial blight and die-back caused by *Pantoea ananatis*, it was noted that the optimal environmental conditions for the development of the disease was at lower day temperatures (between 20-25°C) accompanied by high humidity (Coutinho *et al*., 2002). In the nursery sampled in this study, outbreaks of the disease, however, appeared during the summer months under high temperatures and after the first heavy rains (Mr Sean de Haas, nursery manager, personal communication). At the time the plants were sampled, temperatures had been lower than usual mostly between 25°C and 28°C and more than the average rainfall had occurred, meaning that the environmental conditions were closer to the conditions described

in the original disease description. During this time the outbreak in the White River area was mostly restricted to cuttings from Clone 1. This clone was also ranked as the most susceptible to the disease. The other susceptible clones showed some disease symptoms but were much less severely affected and symptoms were limited to leaf spots. These kinds of less severe symptoms were also noted in the description of the disease (Coutinho *et al*., in 2002). Clone 4 being the least susceptible had no infected plants, even at the time when the nursery was revisited 2 months after the initial sampling.

Culturing is currently not used as the preferred method for environmental studies due to the fact that only a small part of the total known bacterial species can actually be cultured on the available media (Amann *et al.,* 1995). We did manage to isolate *Enterobacteriaceae* from the plant material, but only 9.3% of the isolates belonged to this family and only two genera were positively identified. It could be that possible *Enterobacteriaceae* were competitively outnumbered by the other species present or that they were in a viable but non culturable state. This statement implies that the bacteria is present in the sample and metabolically active, but does not develop into colonies on the culture media routinely used (Oliver 2005). In literature when isolations of endophytes from woody species were studied using the culture dependent method, at the most 1, 2 and in many cases no genera from the *Enterobacteriaceae* were found. However, isolates obtained have included *Pantoea agglomerans* and *Enterobacter cloacae* isolated from Citrus plants (Arau'jo *et al*., 2002) and *Erwinia* from Oak (Brooks *et al.,* 1994).

As expected for the *Enterobacteriaceae* (Dauga, 2002) a number of isolates could not be identified to species level when using the partial 16S rRNA sequences. Sequencing of the *gyrB* gene provided a reliable means of identifying the possible *Pantoea* isolates whereas the *ropB* gene was chosen in an attempt to better identify the possible *Enterobacter* isolates (Brady et al., 2008; Mollet, 1997). *Pantoea eucalypti* was identified from a diseased as well as a resistant plant during this study and *Pantoea vagans* from only diseased plants. *Pantoea eucalypti* and *Pantoea vagans* were described in 2009 by Brady *et al*., and were isolated from plants presenting typical bacterial blight and die back symptoms. *Pantoea eucalypti* isolated from the rhizosphere of *Lotus tenuis* on the other hand was said to show in vitro plant growth promoting capabilities

(Castagno *et al.*, 2011), showing that it could be a useful bacterial endophyte. Although no *Pantoea ananatis* isolates were identified we did isolate *Pantoea vagans* from disease material. This species is a common plant epiphyte, of which Strain C9-1, isolated from apple (Ishimaru *et.al.*, 1988) is registered as a biocontrol agent against fire blight caused by *Erwinia amylovora*. In the case of this study it maybe a common endophytes of eucalyptus.

With regards to the *Enterobacter* species obtained, the following isolates clustered with *Enterobacter mori*: 181a, 121c, 103a, 109b, 109c, 168a, 151b, 211b, 252 and 109a while 128c, 118b, 128a, 128b and 118a clustered with *Enterobacter asburiae*. *E. mori was* recently described by Zhu *et al*. (2011) and found to be associated with bacterial wilt in *Morus alba,* and were isolated from the roots of symptomatic *M. alba* plants. In this study *E. mori* was isolated only from the roots of Clone 6 (a resistant clone), Clone 7 (a resistant clone) and Clone 3 (a susceptible but symptomless clone) at the time of sampling *Enterobacter asburiae* has been isolated as an endophyte previously from, for example, sweet potato (Asis and Adachi 2003) and cotton and sweet corn (Mcinroy and Kloepper, 1995). Groups B, D and E (Figure 3.3) could possibly be novel *Enterobacter* species because they did not have high similarity values to the *Enterobacter* type strain sequences closest to them. It is, however, not possible to assign them to a species with the support of only one housekeeping gene, therefore, more genes should be sequenced and DNA-DNA Hybridizations should be performed on the unknown strains in order to classify them.

5. CONCLUSIONS

In this study the culture dependent method and sequencing of the 16S rDNA, *gyrB* and *rpoB* genes indicated that the most frequently isolated genera from the *Enterobacteriaceae* were *Enterobacter* and *Pantoea*. Three possible *Enterobacter* spp. isolated seem to be undescribed species as none of them grouped with a high similarity with any of the known *Enterobacter* species used in the phylogenetic analysis at the time. In the case of the *Pantoea* isolates, *Pantoea vagans* and *Pantoea eucalyptii* were identified. *Pantoea ananatis* was not isolated either from the disease, healthy or resistant clones. This could be because *Pantoea ananatis* was

present but out competed by the other species present. From this study it appears as if *Pantoea eucalypti*, *P. vagans*, *Enterobacter asburiae and Enterobacter mori* were isolated as endophytes from resistant and susceptible plant respectively.

6. TABLES

Table 3.1: Susceptibility of *Eucalyptus nitens* clones to *Pantoea ananatis*

Clone Name	**Susceptibility/Resistance**
Clone 1	5
Clone 2	4
Clone 3	4
Clone 4	3
Clone 5	1
Clone 6	1
Clone 7	1
Clone 8	1

Table 3.2: Results for the Gram reactions done on all of the isolated cultures.

Clone			Leaves		Stem		Roots	
			Gram +	Gram -	Gram +	Gram -	Gram +	Gram -
	H*	A	3	1	2	3	2	1
		B	1	1	2	1	0	2
		C	2	3	1	1	1	2
		A 07/07	3	2	1	3	0	4

Clone 1	D**	B 07/07	1	2	1	3	1	0
		A	5	7	7	5	3	0
		B	0	3	3	3	1	2
		C	4	4	1	1	0	1
Clone 3	H	A	2	3	5	2	0	2
		B	3	0	2	2	1	1
		C	5	4	0	1	0	1
	D	07/07	4	11	2	7	1	2
		A	8	3	3	5	1	2
		B	6	4	3	3	2	0
Clone 4	H	A	5	5	2	3	0	1
		B	5	1	3	0	0	2
		C	4	3	3	1	0	1
	D	No diseased material collected						
Clone 2	H	A	6	3	0	1	0	3
		B	2	2	0	1	1	1
		C	1	2	0	2	1	1
	D	A	4	5	0	4	0	3
		B	3	3	0	1	0	2
		C	3	4	1	6	1	1

Clone 5	R***	A	1	5	3	1	0	2
		B	3	4	1	0	0	3
		C	0	8	0	3	0	3
Clone 6	R	A	0	4	0	1	1	2
		B	0	3	0	1	0	2
		C	3	1	1	0	0	1
Clone 7	R	A	0	2	0	3	0	6
		B	0	6	2	1	0	3
		C	5	9	3	3	0	2
Clone 8	R	A	0	1	2	2	0	3
		B	1	0	1	1	1	1
		C	3	1	3	0	2	0

* H = healthy

** D = diseased

*** R = resistant

Table 3.3: Cultures belonging to the family *Enterobacteriaceae* chosen for inclusion in this study. D-represents diseased plant, H-represents healthy plants, R-represents resistant plants.

Sample number	Sample Name	Hugh-Leifson	Gram Stain
3d	Clone 3(D) Leaves 07/07	+	-
4c	Clone 3(D) Leaves 07/07	+	-
6c	Clone 3(D) Stem 07/07	+	-
7b	Clone 3(D) Stem 07/07	+	-
7d	Clone 3(D) Stem 07/07	+	-
12b	Clone 1(D)A Leaves 07/07	+	-
14c	Clone 1(D)A Stem 07/07	+	-
15a	Clone 1 (D) A Roots 07/07	+	-
20b	Clone 1(D)B Stem 07/07	+	-
30b	Clone 1(D)A Leaves	+	-
31b	Clone 1(D)A Leaves	+	-
61d	Clone 3(D)A Stem	+	-
62a	Clone 3(D)A Stem	+	-
62d	Clone 3(D)A Stem	+	-
81a	Clone 2(D)A Roots	+	-
81a2	Clone 2(D)A Roots	+	-
88a2	Clone 2(D)B Roots	+	-
88b	Clone 2(D)B Roots	+	-
103a	Clone 7(R)A Roots	+	-
109a	Clone 7(R)B Roots	+	-
109b	Clone 7(R)B Roots	+	-

109c	Clone 7(R)B Roots	+	-
116c	Clone 7(R)C Roots	+	-
118a	Clone 6(R)A Leaves	+	-
118b	Clone 6(R)A Leaves	+	-
121c	Clone 6(R)A Roots	+	-
128a	Clone 6(R)C Leaves	+	-
128b	Clone 6(R)C Leaves	+	-
128c	Clone 6(R)C Leaves	+	-
151b	Clone 1(H)A Roots	+	-
168a	Clone 3(H)A Roots	+	-
181a	Clone 3(H)C Roots	+	-
205a	Clone 4(H)A Roots	+	-
211b	Clone 4(H)B Roots	+	-
223b	Clone 8 (R) B Roots	+	-
238c	Clone 5 (R) A Leaves	+	-
239b	Clone 5 (R) A Stem	+	-
252c	Clone 5(R)C Roots	+	-

Table 3.4: Results from a BLAST analysis in Genbank for the partial *gyrB* sequences of strains used in this study.

Sample number	Result	% Similarity
3d	*Pantoea sp. (LMG24201/24196)*	99
4c	*P.vagans (LMG 24201)*	98
6c	*Pantoea sp. (LMG24199)*	99
7b	*Pantoea vagans*	99
7d	*Pantoea vagans*	98
12b	*Pantoea vagans*	99
14c	*Enterobacter sp.*	96
15a	*Enterobacter/Brenneria*	93
20b	*Enterobacter sp.*	96
30b	*E.cloacae*	95
31b	*E.cloacae*	91
61d	*Pantoea eucalypti*	98
62a	*E.cloacae*	95
62d	*E.cloacae*	95
81a	*Enterobacter cloacae*	90
81a2	*Enterobacter cloacae*	92
88a2	*E.cloacae*	94
88b	*E.cloacae*	96
103a	*E.cloacae*	97
109a	*E.cloacae*	95
109b	*E.cloacae*	97

109c	*E.cloacae*	90
116c	*E.cloacae*	93
118a	*E.cloacae*	97
118b	*E.cloacae*	97
121c	*E.cloacae*	96
128a	*E.cloacae*	97
128b	*E.cloacae*	97
128c	*E.cloacae*	97
151b	*E.cloacae*	96
168a	*E.cloacae*	96
181a	*E.cloacae*	96
205a	*E.cloacae*	94
223b	*E.cloacae*	92
238c	*Pantoea eucalypti* strain LMG 24198	98
239b	*Pantoea sp. (LMG24198/24197/24199)*	96
252c	*E.cloacae*	97

Table 3.5: Results from a BLAST analysis in Genbank for the partial *rpoB* sequences of strains used in this study

Sample number	Result	% Similarity
15a	*Enterobacter cloacae/Enterobacter asburiae*	95
30b	*Enterobacter cloacae*	98
31b	*Enterobacter cloacae*	97
62a	*Enterobacter cloacae*	97
62d	*Enterobacter cloacae*	97
81a	*Enterobacter cloacae/ Enterobacter asburiae*	98
81a2	*Enterobacter cloacae*	96
88b	*Enterobacter cloacae*	98
103a	*Enterobacter cloacae*	98
109a	*Enterobacter cloacae*	97
109b	*Enterobacter cloacae*	98
109c	*Enterobacter cloacae*	98
116c	*Enterobacter cloacae*	99
118a	*Enterobacter cloacae*	99
118b	*Enterobacter cloacae*	97
121c	*Enterobacter cloacae*	99
128a	*Enterobacter asburiae*	97
128b	*Enterobacter cloacae*	99
128c	*Enterobacter cloacae/ Enterobacter asburiae*	98
151b	*Enterobacter cloacae*	97
168a	*Enterobacter cloacae*	97

181a	*Enterobacter cloacae*	98
205a	*Enterobacter/Klebsiella*	97
211b	*Enterobacter cloacae*	96
223b	*Enterobacter cloacae*	96
252c	*Enterobacter cloacae*	99

7. FIGURES

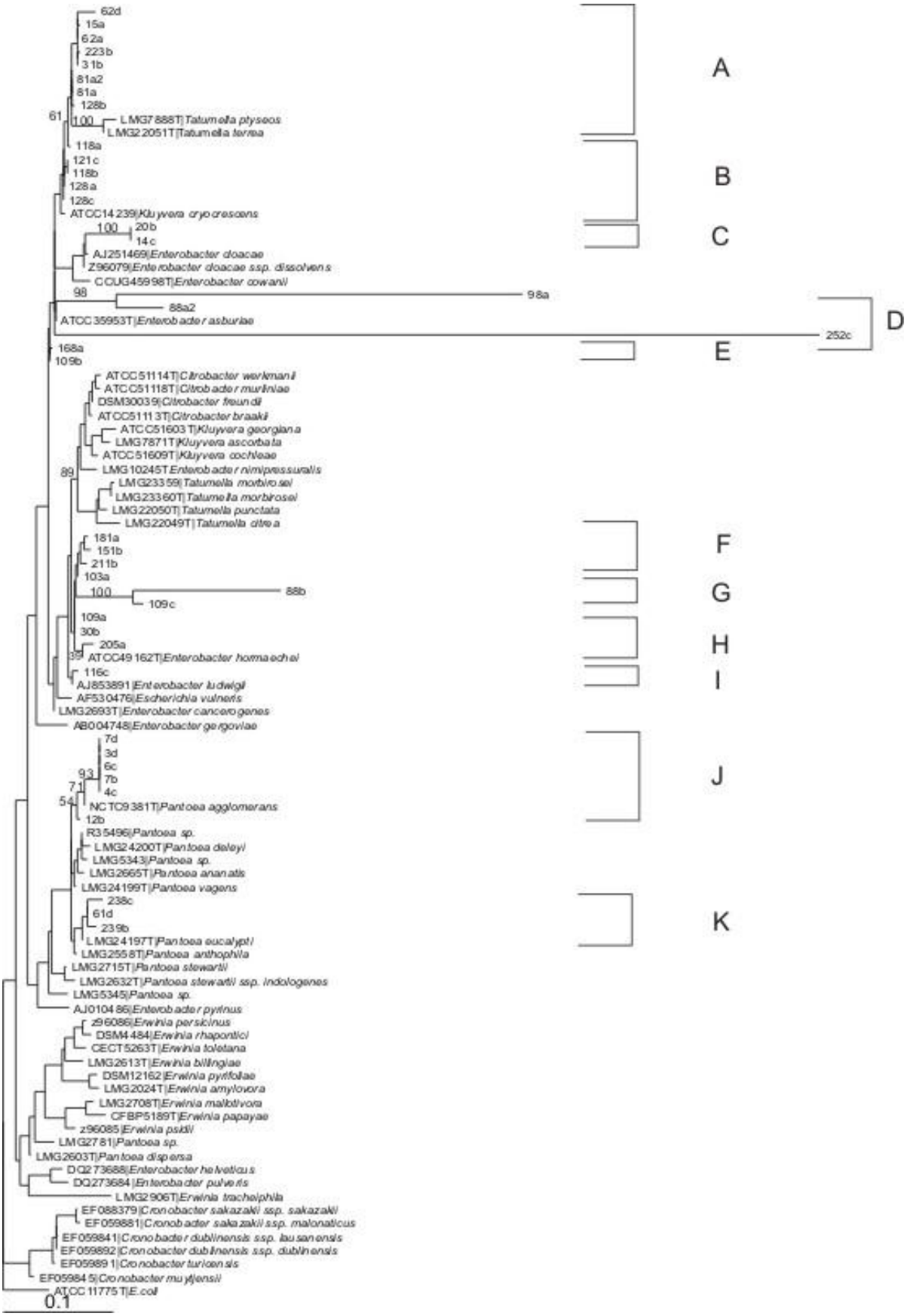
62d
15a
62a
223b
31b
81a2
81a
128b
61
100
LMG7888T|Tatumella ptyseos
LMG22051T|Tatumella terrea
118a
121c
118b
128a
128c
ATCC14239|Kluyvera cryocrescens
100
20b
14c
AJ251469|Enterobacter cloacae
Z96079|Enterobacter cloacae ssp. dissolvens
CCUG45998T|Enterobacter cowanii
98
98a
88a2
ATCC35953T|Enterobacter asburiae
252c
168a
109b
ATCC51114T|Citrobacter werkmanii
ATCC51118T|Citrobacter murliniae
DSM30039|Citrobacter freundii
ATCC51113T|Citrobacter braakii
ATCC51603T|Kluyvera georgiana
LMG7871T|Kluyvera ascorbata
ATCC51609T|Kluyvera cochleae
89
LMG10245T|Enterobacter nimipressuralis
LMG23359|Tatumella morbirosei
LMG23360T|Tatumella morbirosei
LMG22050T|Tatumella punctata
LMG22049T|Tatumella citrea
181a
151b
211b
103a
100
88b
109c
109a
30b
205a
99
ATCC49162T|Enterobacter hormaechei
116c
AJ853891|Enterobacter ludwigii
AF530476|Escherichia vulneris
LMG2693T|Enterobacter cancerogenes
AB004748|Enterobacter gergoviae
7d
3d
6c
93
7b
71
4c
54
NCTC9381T|Pantoea agglomerans
12b
R35496|Pantoea sp.
LMG24200T|Pantoea deleyi
LMG5343|Pantoea sp.
LMG2665T|Pantoea ananatis
LMG24199T|Pantoea vagens
238c
61d
239b
LMG24197T|Pantoea eucalypti
LMG2558T|Pantoea anthophila
LMG2715T|Pantoea stewartii
LMG2632T|Pantoea stewartii ssp. indologenes
LMG5345|Pantoea sp.
AJ010486|Enterobacter pyrinus
z96086|Erwinia persicinus
DSM4484|Erwinia rhapontici
CECT5263T|Erwinia toletana
LMG2613T|Erwinia billingiae
DSM12162|Erwinia pyrifoliae
LMG2024T|Erwinia amylovora
LMG2708T|Erwinia mallotivora
CFBP5189T|Erwinia papayae
z96085|Erwinia psidii
LMG2781|Pantoea sp.
LMG2603T|Pantoea dispersa
DQ273688|Enterobacter helveticus
DQ273684|Enterobacter pulveris
LMG2906T|Erwinia tracheiphila
EF088379|Cronobacter sakazakii ssp. sakazakii
EF059881|Cronobacter sakazakii ssp. malonaticus
EF059841|Cronobacter dublinensis ssp. lausannensis
EF059892|Cronobacter dublinensis ssp. dublinensis
EF059891|Cronobacter turicensis
EF059845|Cronobacter muytjensii
ATCC11775T|E.coli
0.1
A
B
C
D
E
F
G
H
I
J
K

Figure 3.1: A Phylogenetic tree constructed with partial 16S rDNA sequences of the presumptive *Enterobacteriaceae* cultures isolated from plant parts of different *Eucalyptus* n*itens* clones, and type strains from selected genera in the family *Enterobacteriacae*. The tree was constructed based on sequences of approximately 600 bp using the Maximum Likelyhood method. The results of a 1000 bootstrap trials are shown at the nodes. All isolate are indicated by their original sample number.

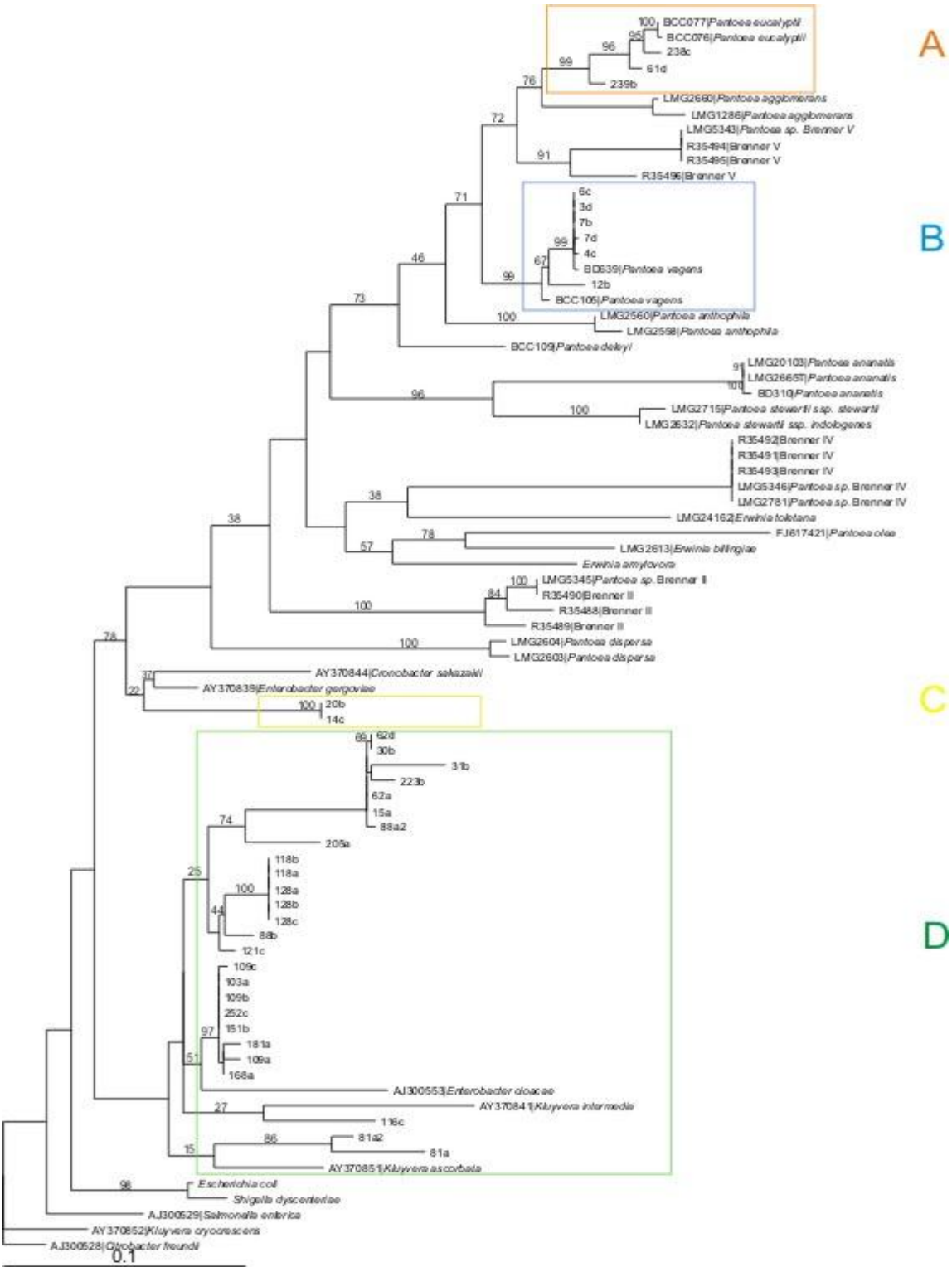

Figure 3.2: A Phylogenetic tree constructed with the partial *gyrB* sequences of cultures obtained from different *Eucalyptus nitens* clones, The *gyrB* sequence for the type strains of the *Pantoea, Erwinia, Enterobacter* and *Kluyvera* were included. The tree was constructed on sequences of approximately 550 bp using the Maximum Likelyhood method. The results of a 1000 bootstrap trials are shown at the nodes. All isolate are indicated by their original sample number. The blocks indicate the groups formed by the isolated strains

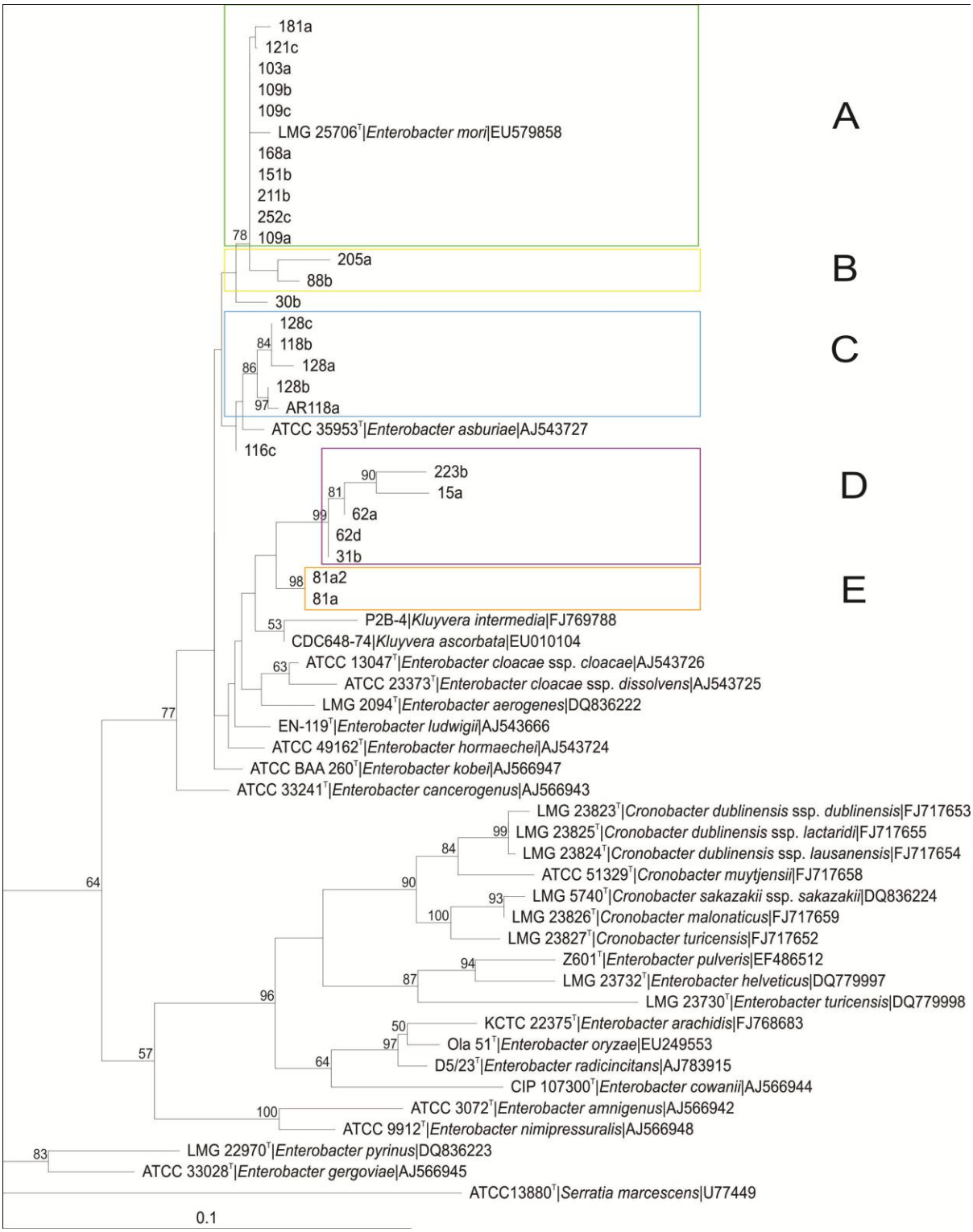

Figure 3.3: A Phylogenetic tree constructed with partial *rpoB* sequences of the cultures isolated form different parts of *Eucalyptus nitens* clones. Sequences for the type strains of *Enterobacter* and *Cronobacter* were included. The tree was constructed based on a sequences of approximately 600 bp using the Maximum Likelyhood method. The results of a 1000 bootstrap trials are shown at the nodes. *Citrobacter marcescens* was used as the outgroup. All isolate are indicated by their original sample number.

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CHAPTER 4

ANALYSING BACTERIAL ENDOPHYTES IN *EUCALYPTUS NITENS* CLONES, USING PCR-DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

CHAPTER 4

Analysing bacterial endophytes in *Eucalyptus nitens* clones, using PCR - Denaturing Gradient Gel Electrophoresis (DGGE).

1. INTRODUCTION

The leaves, stems and roots of plants make up distinct microecosystems which may result in different bacterial species (endophytes) colonizing these ecosystems (McInroy and Kloepper 1994). Such interactions could be for life or only a short period of time and may cause no significant damage or they could be latent pathogens (Hallmann *et al*., 1997). These microecosystems are complex and cultivation of these bacteria can be very difficult. It is widely recognised that only a fraction of known bacteria can be cultured. This may be due to the fact that the bacteria within a certain community might be interdependent upon each other in order to survive (Muyzer *et al*., 1998). A lack of knowledge of the preferred growth requirements and conditions for these organisms (Muyzer *et al*., 1998) could be another reason. Subsequently, in order to get a better representation of the organisms present in an environmental sample, it is important to use molecular techniques to compliment the traditional microbiological procedures (Muyzer *et al*., 1998).

Denaturing Gradient Gel Electrophoresis (DGGE) is one such technique, providing a pattern of the genetic diversity present within a microbial community, also known as genetic fingerprinting. The technique depends on the electrophoresis of amplified 16S rDNA fragments trough a gradient polyacrylamide gel, separating fragments of the same length but different nucleotide sequences (Muyzer *et al*., 1993). By making use of this technique 50% of the sequence variants within a sample can be detected and by adding a GC-clamp to one of the amplification primers, the detection rate can be increased to 100%. This can be achieved because separation is based on a partially melted double stranded DNA molecule showing a decreased electrophoretic mobility in polyacrylamide gels containing a linear gradient of DNA denaturants (Muyzer *et al*., 1998).

The aim of this study was to determine the diversity of bacterial genera present in different parts of *Eucalyptus nitens* clones from a nursery using a 16S rDNA based molecular technique. It the

light of the role played by various *Pantoea* species in bacterial blight and die-back of some of these clones, attention was specifically given to the occurrence of the *Enterobacteriaceae* and especially the *Pantoea* spp. in this tissue. The clones tested ranged from highly susceptible to resistant to *P. ananatis*, the cause of bacterial blight and dieback of *Eucalyptus.*

2. MATERIALS AND METHODS

2.1 Sampling

The same samples used for the culturing of endophytes were used for this part of the study. But only some of the seedlings were included, those were the ones identified in the previous chapter as definitely harbouring *Enterobacteriaceae* as endophytes. For detail on sampling, please refer to Chapter 3 Section 2.1.

2.2 Processing of samples and DNA extraction

The samples were processed as described in Chapter 3 Section 2.2. At the onset of the project some difficulties were experienced with finding suitable methods for the extraction of bacterial DNA from the processed samples. Initially the plant tissue was grounded using a sterile mortar and pestle in 1 ml sterile water. Both the ZR Soil Microbe DNA Kit™ (Zymo Research from Inqaba Biotech) as well as the conventional CTAB method (Adapted from Current Protocols, unit 2.4) was assessed for extracting DNA. Alternatively the tissue was cut into small pieces, added to a Phosphate buffer and then sonicated for 5 minutes. The phosphate buffer was thereafter collected and spun down at 7500 rpm for 10 minutes followed by DNA extraction using the Qiagen® DNA extraction kit. After a period of trial and error the phosphate buffer was replaced with nutrient broth in order to enrich for the bacterial cells.

After incubation in Nutrient Broth, 1 ml was transferred to a sterile 1.5 ml eppendrof tube and centrifuged at 7500 rpm for 10 minutes and the supernatant was removed. Thereafter the pelleted cells were used in a DNA extraction using the Qiagen® DNeasy Blood and Tissue Kit according to the protocol for Gram positive organisms. Visualization of the DNA was done on a 1% agarose gel ran at 90V for 30 minutes.

2.3 16S PCR amplification using GC-Clamp

The isolated DNA for the chosen samples were subject to a PCR targeting the V3 region of the 16S gene using primers pA8f (5'- AG AGT TTG ATC CTG GCT CAG-3') with a GC-clamp added to the 5' end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') (Fjellbirkeland *et al*., 2001) and PRUN518r (5'-ATT ACC GCG GCT GCT GG-3') (Øvreas *et al*., 1997). Each 50 µl reaction consisted of 5 µl 10x PCR buffer, 4 µl of a mixture of all four dNTP's (200 µM each), 1 µl each of the forward and reverse primers (10 µM), 0.3 µl Supertherm Taq DNA Polymerase (5 U/µl) and 1 µl of the genomic DNA (100 ng/µl). The amplification conditions included an initial denaturing step at 95°C for 10 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 1 minute followed by a final elongation at 72°C for 10 minutes. Five microliters of each of the PCR products was separated on a 1% agarose gel at 90 V for 30 minutes. A 100 base pair marker was used to compare the size of the amplification products to determine their approximate size.

The final forward and reverse primers used in this study were determined from multiple experiments testing different primer pairs for amplification of the V3 region of the 16S gene (Table 4.2) to ensure that the amplification gave a single product i.e. be as specific as possible as well as yield enough product for further analysis. The aim was to get the best band resolution during denaturing gradient gel electrophoresis. As different primer pairs give different product lengths, it was not only important to find the correct pair for the study, but also a product length suitable for further identification studies (Chen *et al*., 2008).

2.4 Denaturing Gradient Gel Electrophoresis

2.4.1 Preparation of the DGGE gradient polyacrylamide gel

To prepare an 8% polyacrylamide gel with a ureum gradient, a 100% ureum acrylamide solution (54 ml 30% Acrylamide/bisacrylamide (Sigma), 84 g ureum, 80 ml 100% formamide, 4 ml 50x TAE, up to a volume of 200 ml) and 0% ureum acrylamide solution (54 ml 30% Acrylamide/bis-Acrylamide (Sigma), 4 ml 50x TAE, up to a volume of 200 ml) were used with Ammonium

Persulphate (100 µl) and TEMED (8 µl) added for polymerisation. The Gradient of the denaturing gel contained between 30% and 55% denaturing solution, and the gel was prepared using the Model 475 Gradient Delivery System (Bio-Rad) and casted onto 16.0 x 16.0 cm glass plates separated by 1.0 mm spacers. The prepared gradient gels were left to polymerase for 1 hour after which the 20 well combs were inserted between the two glass plates and a stacking gel, containing no denaturant, was poured and allowed to set for 20 minutes.

In order to get optimum separation of the amplified gene products from the different species represented in the mixed genomic DNA extractions, runs on gels with larger and smaller gradients were compared. The gradients evaluated included 35% - 70%, 30% - 80%, 40% -90% and 30% - 55%.

2.4.2 Sample preparation and running of the DGGE gel.

The tank of the DCode™ Universal Mutation Detection System (Bio-Rad) was filled with 7 litres of 1x TAE buffer (Tris-Base, Glacial acetic acid, 0.5 M EDTA) and heated to a temperature of 60 °C while stirring. The wells of the prepared gels were washed and filled with pre-warmed buffer from the tank of the system. Forty five microlitres of the PCR amplification product were added to 5 µl loading dye (Bromophenol blue, Xylene cyanol and 1x TAE buffer) mixed and the 50 µl sample/loading dye mixture was loaded into the wells. The system run for 10 minutes at 50 V until the buffer temperature reached 60°C again. This was followed by a second session of 3.5 hours at 200 V. The tank was intermittently re-filled with 1x TAE buffer up to a volume of 7 litres during the run, to compensate for the buffer lost due to condensation. A need to test different run times and electrical current conditions also became necessary and therefore gels ran at a voltage of 70 V for 16 hours were compared to gels ran for 3.5 hours at 200 V, to determine the best conditions for the study.

After completion of the run the gel plates were removed and separated to release the acrylamide gels for transfer to a staining buffer consisting of 0.01% SyBR Gold staining solution. Staining was carried out at 22 °C for 30 minutes with shaking. The separated bands were captured using

a UV gel documentation system. Bands of interest were determined by visualizing captured images and excised using a Dark Reader™ (Clare Chemical Research, Inc.). Excised bands were transferred to a clean Eppendorf tube and 30 µl of sterile distilled water was added. The bands were kept at 4 °C overnight or were stored at -20 °C for future analysis.

2.5 Sequencing and identification of bands

The stored bands were subject to another round of 16S rDNA amplification using the same primers and conditions as stated in 2.3, but excluding the GC-clamp. PCR amplification product clean-up was performed using the Zymo Research DNA Clean and Concentrator Kit and a Sequencing PCR set up containing 2 µl Big Dye Sequencing Reaction mix, 1 µl 5x Sequencing Buffer, 0.3 µl (100µM) PRUN 518r primer (Øvreas *et al*., 1997), 4 µl cleaned amplification product and nuclease free water to a total volume of 10 µl . The sequencing conditions included denaturation at 96 °C for 5 seconds, followed by 25 cycles of denaturing at 96 °C for 10 seconds, annealing at 55 °C for 5 seconds and elongation at 60 °C for 4 minutes. The PCR products were sequenced on an ABI Prism DNA Automated sequencer. Nucleotide sequence results were edited and blasted against GenBank,

2.5.1 Cloning

Cloning of selected DGGE bands were performed using the CloneJET™ PCR Cloning Kit (Fermentas), according to the manufacturer's protocol with the following modifications. The ligation reaction was incubated at 22 °C for 2 hours. For the transformation into competent *Escherichia coli* JM109 cells ($1x10^6$), 25 µl thawed cells were added to 2 µl ligation reaction and incubated on ice for 20 minutes followed by "heatshock" at 42 °C for 50 seconds and another ice incubation for 2 minutes. After addition of 800 µl LB-Broth to the transformation reaction and incubation at 37 °C for 1.5 hours with shaking (150 rpm), 100 µl of the transformation reaction was plated onto LB/Ampicillin agar (100 µg/ml). Five colonies were picked from the plate after incubation at 37°C for 24 hours and screened by PCR using the pJET1.2 forward and reverse sequencing primers (10 µM) as indicated in the instruction manual. Amplification products were visualized on a 1% agarose gel with running conditions of 90 V for 30 minutes.

Bands with the correct size, as compared to a 100 bp ladder, were excised and purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Sequencing reactions were set up as previously described in 2.5 and the resulting nucleotide sequences were edited and blasted against GenBank.

3. RESULTS

3.1 Sampling

Seedlings identified to harbour members of the *Enterobacteriaceae* based on the culturing experiment (Chapter 3), were used in this study. This was to ensure that the results obtained from this culture independent method could be compared with the culture dependent method. It was however also possible to determine the presence of bacteria from other groups because of the use of a non-culturing, PCR-DGGE approach followed by16S sequencing, these results will also be mentioned in this chapter. Clones were ranked in order of susceptibility by the nursery manager after careful observation of the prevalence of disease symptoms on the different clones. For further information see Chapter 3 point 3.2.

3.2 Processing of samples and genomic DNA extractions

Different options for isolating the bacterial DNA from the plants were considered. With the initial grinding of the plant tissue problems were experienced in obtaining bacterial DNA from the samples. Two different kits, Qiagen and ZymoResearch were tested as well as an adaptation of the CTAB method. The adapted CTAB method of extraction was used first, but no distinct bands were seen when visualized on agarose gels and based on the observed smears there might have been ethanol left in the sample. With the second approach it was seen that when the ground material was added to the ZR Bashing Bead™ Lysis tube and the extraction carried out according to the instruction manual, bands were visible on the agarose gel, but the concentration was very low, indicating that it would be necessary to separate the bacterial cells from the processed plant material first, before performing a DNA extraction. As an alternative to the second method, the ground material were transferred to phosphate buffer and placed on a rotary shaker at 22 °C in order to dislodge the bacterial cells and trap them in the buffer before

concentration and extraction. The Qiagen kit was used for extraction, but no bands were visualized.

After various efforts it was concluded that by grinding the plant material, isolation of plant DNA (chloroplast) were favoured above bacterial DNA. The plant material was therefore cut rather than ground in order to minimise the disruption of the plant cells. Due to the low levels of bacterial cells it was decided to first do an enrichment step in nutrient broth which was then followed by a DNA extraction of the pelleted cells using the Qiagen DNeasy Kit (Figure 4.1). Adequate amounts of bacterial DNA were extracted when this approach was followed.

3.3 16S rDNA PCR amplification

Genomic DNA samples used for 16S rDNA amplification and DGGE analysis were selected based on the plants from which *Pantoea* and/or *Enterobacter spp*. were isolated (Results Chapter 3 point 3.3). Of the 261 genomic DNA extractions that were performed a total of 118 were selected (Table 4.1). Due to the high number of samples that required DGGE analysis, DNA obtained from the same plant parts of a specific plant were combined in equal amounts for the PCR reaction. These pooled DNA sets were given an alphabetical letter as sample name whereas the single DNA isolations were still represented by the original numerical code. After pooling a total of 85 DNA samples were analysed.

An amplification product of 510 bp was obtained. The primer pair **518r** and **pA8f** gave a longer product than those obtained from the other tested primer pairs (Table 4.2) and this PCR protocol also gave more reproducible results. It was decided to use the above mentioned primer pair in the study, seeing that the longer product will increase the possibility of detecting differences between the different species present in the samples. A longer initial amplification product also ensures a longer template for further identification, ensuring better identification results.

3.4 Denaturing Gradient Gel Electrophoresis

Optimization of the DGGE protocol was required in order to obtain the best results for this study. After a series of experiments it was found that the 30% - 55% gradient in the denaturing gel solution yielded the best separation of the amplified gene products as can be seen in Figure 4.2. It was also found that running the gel for 3.5 hours at 200 V produced the same results as a gel ran at 70 V for 16 hours. The longer runs had the added disadvantage that the tank could not always be refilled with buffer to compensate for the evaporated buffer which often resulted in an error and aborting of the run. The PCR consisted of a total volume of 50 ul of which 45 ul was added to the loading dye and loaded onto the gel to make sure as much as possible of the amplification product was used in the run.

After determining that the 30 - 55% gradient resulted in the best separation of bands, the technique and results became more reproducible. It was seen that the samples contained a rather large diversity of species and that they were present in varying numbers, which can be seen by the number of bands for each sample as well as the brightness of the bands (Figure 4.2). It could clearly be seen that there were similarities as well as differences in the banding pattern of the different samples (Figure 4.2). The average number of bands that were observed were between 6 and 10, with the lowest being only 2. Some had similar bands present whereas specific bands were absent from certain samples.

3.5 Sequencing and identification of bands

Sequencing results from the bands indicated that cloning of some of the bands would be necessary. This was due to the fact that the bands from different bacterial genera co-migrated on the gel and was observed as one single band. When these bands were subjected to sequencing without cloning nonsense sequences that cannot be blasted against GenBank were obtained. It was found that the larger the population present in a specific isolation the more likely it was that cloning would be necessary for the bands obtained. Thus the bands that did not gave useful sequencing results for this round of sequencing, were subject to cloning to separate the bands of the different 16S copies present. It was also observed that multiple copies of the 16S rRNA were

present in some bacteria and that these copies often resulted in multiple bands during separation with DGGE (Figure 4.3).

3.5.1 Cloning

Separation of the co-migrating bands was accomplished by making use of the CloneJET™ PCR Cloning Kit. Five colonies were picked per sample in order to include all possibilities of bacteria present in the combined gel band. On the agarose gel, bands with a size of +/- 560 bp were representative of the insert amplified from the plasmid. Table 4.3 shows the results for the excised bands that were sequenced as well as the bands that were cloned.

The genera most commonly identified were *Pseudomonas, Bacillus, Enterobacter*, *Xanthomonas/Stenotrophomonas* and *Pantoea*. *Pantoea* was isolated from the leaves of healthy and diseased Clone 3, healthy Clone 4 and resistant Clone 5 cuttings as well as from root and stem material from the rest of the clones. *Pseudomonas* and *Bacillus* were wide spread and found in leaves, stem and root material of all the clones used in the study. The root material from all the clones showed the highest diversity followed by the leaf and then the stem material. *Clostridium* was identified in the roots of the resistant Clone 7 clones as well as the diseased root material from the Clone 1 and Clone 3 cuttings. Chloroplast DNA was also identified in one sample. *Xanthomonas /Stenotrophomonas* were identified in all of the clones except for Clone 5.

4. DISCUSSION

The initial processing and direct DNA extraction methods used in this study resulted in the detection of chloroplast DNA and not bacterial DNA. Similar observations were made by Garbeva *et al*. (2001), in a study done on the diversity of bacterial endophytes in potato plants. Following these results a decision was made to cut the plant parts into small pieces and not to grind the material. After sectioning the material, samples were either incubated in phosphate buffer or nutrient broth with added cyclohexamide. Bacterial DNA was detected for both methods but when DGGE patterns of the phosphate buffer incubation and broth incubation were compared, greater diversity could be seen for the broth incubation. Although it is not a truly

culture free approach the broth incubation followed by DNA extraction with the Qiagen® DNeasy Blood and Tissue Kit was used for the rest of the project as it yielded results which illustrated greater diversity of the bacteria present in the tissue.

A longer 16S rDNA sequence allows for more accurate identification when it is compared to verified nucleotide sequences. Some difficulty was experienced in finding the optimal primer pair. The addition of the GC-clamp to the forward primer increases the melting temperature of the DNA piece to detect close to 100% of all possible sequence variations without letting the amplification product separate completely (Sheffield *et al*., 1989). The problem arising with the addition of the clamp is, however, that it increases the annealing temperature of the forward primer substantially, causing the annealing temperatures of the forward and reverse primers to differ quite significantly. A large difference makes it difficult to optimize the PCR and improve the primer binding specificity. After comparing the use of reverse primer 518 with different forward clamp primers, yielding amplification products ranging from 161 bp to 510 bp, it was decided to use the PRUN518 reverse and pA8 forward primers (510 bp).

For the 85 bacterial samples that were used in this study (Table 4.1) a total of 340 bands were excised and sequenced. The excised bands were re-amplified to exclude the GC-clamp and the DNA purified to be sure that the product for sequencing was of a good quality. After analysing the sequencing results using BioEdit, it was seen that some of the sequences had background, meaning that there were more than one sequence that were overlapping and these needed to be separated using cloning, before it could be blasted against the Genbank Database. Cloning was done for 182 of the 340 bands that were excised. It was seen that almost half (51%) of the bands that had to be cloned were sampled from susceptible plants showing diseased symptoms, 26.7% from resistant symptomless plants and 22.2% from healthy symptomless susceptible plants. This shows that diseased plants had a higher amount of bacteria present, increasing the chances of bands co-migrating. Another observation that was made was that healthy plants showed less endophytic bacterial diversity than was present in diseased plants. The species richness was also much higher in the diseased plants compared to the healthy plants (Table 4.3).

If one looked overall at the different parts of the plants of both susceptible and resistant clones it can be seen that both the diversity and richness in the stem of the plants were the lowest when compared to that of the leaves and roots. But when the diversity of bacteria within the leaves and roots were compared, the difference was not that noticeable. When comparing results from all the susceptible clones, bacterial populations of healthy plants to that of diseased plants, it was seen that the healthy plants had a lower diversity and, for some species, a lower richness than that of the diseased plants. When Table 4.3 is considered it can be seen that a total of 29 different genera were identified from all the plants studied. The most represented genera were *Bacillus*, *Pseudomonas,* a possible *Xanthomonas/Stenotrophomonas* sp., *Enterobacter* and *Pantoea.* The genus isolated most frequently from all three parts (roots, stem, leaves) of the same plant was *Pseudomonas*. *Bacillus* on the other hand was also isolated frequently from plants of all the clones tested, but it was rarely isolated simultaneously from all three parts of the same plant. Other species detected during this study have also previously been isolated from *Eucalyptus*. These include endophytes such as *Arthrobacter, Delftia, Acinetobacter, Herbaspirillium, Brevundimonas, Variovorax, Sphingomonas, Agrobacterium, Comamonas, Rhizobium and Acidiovorax* (Lodewyckx *et.al*., 2002) and *Paenibacillus, Clostridium* and *Microbacterium* (Rosenblueth and Martinez-Romero, 2006).

Of the *Enterobacteriaceae* detected, only *Enterobacter* and *Pantoea* were frequently detected from all the plants studied. *Enterobacter* was present in high numbers in all of the diseased plants screened. *Enterobacter* spp. were only found in the stems of one Clone 4 and one Clone 2 cuttings of the healthy clones but was present in all 6 diseased plants processed and screened. The two other genera belonging to the family *Enterobacteriaceae* isolated were *Erwinia* and *Klebsiella*, They originated from the stem of Clone 6 cuttings and the stem of Clone 1 diseased cuttings, respectively. *Klebsiella* has previously been isolated from agricultural plants such as wheat, sweet potato, rice, soybean, banana, maize, sugarcane and carrot (Rosenblueth and Martinez-Romero, 2006). *Erwinia* has been reported by Kuklinsky-Sobral *et.al.* (2004) in soybean. *Pantoea* was found in 4 out of 6 healthy plants included in this study, and from 2 out of 6 diseased plants. Possible explanations for this can be that no diseased material for Clone 4, which was also ranked susceptible to bacterial blight and die-back, could be sampled. This means that the overall representation of diseased material was lower, thus reducing the chances

of detecting possible *Pantoea* present. The enrichment step in the processing part of the study could have favoured the faster growers or the bacteria that were initially more abundant in the sample, for example, the *Bacillus* and *Pseudomonas* spp. Another possibility is that a *Pantoea* sp. was not responsible for the diseased symptoms observed on the plants. When the bacterial diversity of the clones were compared in order of decreasing susceptibility, it was seen that a higher diversity of bacteria were isolated from the most susceptible clones, i.e. Clone 1 and Clone 3. This could be because the defence mechanisms of the diseased plants had already been overcome and this would then allow opportunistic bacteria to colonize the tissue.

Other species such as *Bacillus*, *Pseudomonas* and a possible *Xanthomonas*/*Stenotrophomonas* were found in almost evenly balanced numbers throughout the whole plant of both susceptible and resistant clones and can, therefore, be considered to be common endophytes of this host. They are commonly found as endophytes in other plant species (Lodewyckx *et al*., 2002). Most of the beneficial effects that bacteria have on their host plant are accomplished by using similar mechanisms to those which Plant-Growth-Promoting Rhizobacteria (PGPR) use on their hosts (Höflich et al., 1994.) This is because most of the endophytes come from the rhizosphere and are usually facultative endophytes i.e. can spend their lifecycle outside of plant tissue in the rhizosphere (Di Fiori and Del Gallo, 1995). Rosenblueth and Martinez-Romero (2006) and Hallmann and Berg (2006) have shown that root colonizing rhizosphere bacteria such as Pseudomonas and Bacillus are often also found as inhabitants of the internal tissues of the plant. *Bacillus polymyxa*, a rhizosphere bacterium, has been shown to have plant growth promoting effects on lodge pole pine (Bent and Chanway, 1998). In 1991 Frommel *et al*. showed that the presence of Pseudomonas in the roots of potato plants stimulated root branching and root hair formation in those plants.

Enterobacter spp. has been isolated as endophytes from maize, soybean, citrus plants and sweet potato (McInroy and Kloepper 1995, Kuklinsky-Sobral *et al*., 2004, Araujo *et al*., 2002; Hinton *et al*., 1995, Asis and Adachi 2003). The *Enterobacter* spp. detected in this study could also be possible endophytes, but as they were resent in high numbers within the diseased plant material, it is possible that they only entered after the defence system of the plants were compromised.

Zhu *et. al.,* (2011) described the novel species *Enterobacter mori* which is associated with bacterial wilt in *Morus alba* and was isolated from the roots of mulberry trees. In the culturing part of this study *Enterobacter* was isolated from the roots of diseased plants of Clones 1 and 2. Because this study only focused on sequencing the 16S gene, identification could only be made up to genus level. In 2009 Brady *et al*. isolated *Enterobacter cowanii* from *Eucalyptus* in Uruguay that showed symptoms of bacterial blight and dieback. Although they did not conclude that *Enterobacter cowanii* was the causal agent of the symptoms, they did suggest that these bacteria are present as endophytes in *Eucalyptus* trees showing blight and dieback symptoms. To further identify those specific *Enterobacter* spp. detected in this DGGE study, one should target another gene such as the *rpoB* gene (Mollet *et al*., 1997).

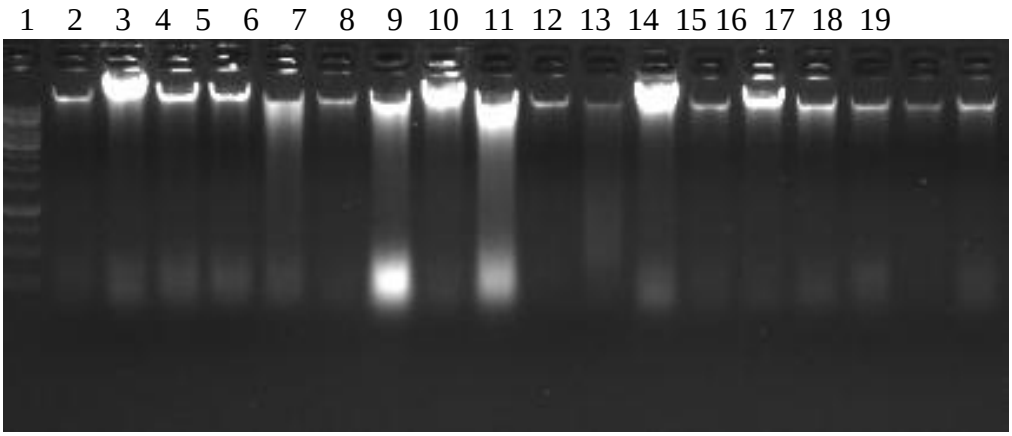
In previous studies *Pantoea* species have been isolated from many different plants including rice and soybean (Kuklinsky-Sobral *et al*., 2004; Verma *et al*., 2004), with *Pantoea agglomerans* isolated from citrus plants and sweet potato (Araujo *et al*., 2001, 2002; Asis and Adachi 2003). Therefore as *Pantoea* spp were isolated in this study from healthy and diseased, as well as from resistant clones, it can be stated that *Pantoea* spp. are endophytic in these *E. nitens* clones sampled from the nursery. We can, however, not state exactly what *Pantoea* species were isolated as another housekeeping gene needs to be sequenced in order to confirm their identity (Brady *et al*., 2008). High numbers of *Pantoea* were expected especially from the diseased material. The reason for the lower levels might be that the disease that presented itself on the plants sampled were not caused by *Pantoea* as suspected, but rather by a different bacterial pathogen. However, to support these results, further studies should be carried out, not only to confirm this statement, but also to confirm the status (as endophytes or latent pathogens) of the genera isolated as possible endophytes.

5. CONCLUSION

In this study a total of 85 bacterial DNA samples were used and a total number of 340 DGGE bands were excised and sequence resulting in the detection of 29 different genera. Although we could not succeed in using a total culture free 16S DGGE procedure, it was seen that the healthy plants showed less diversity in the bacterial genera present with the species richness being much

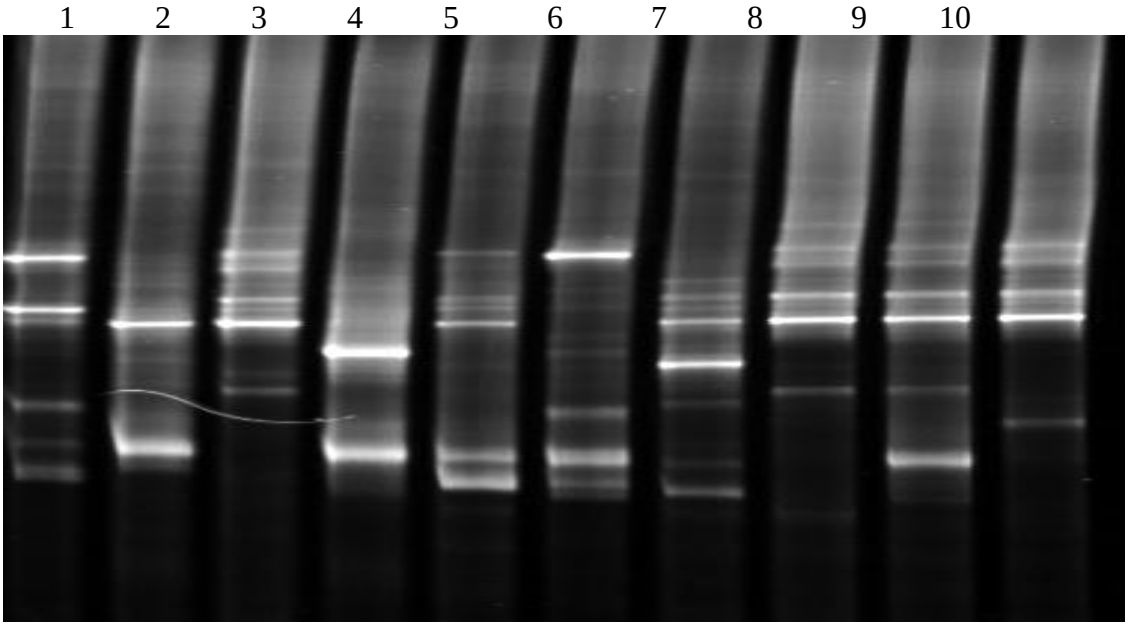
higher in diseased plants than in those of the healthy plants. A higher diversity of genera was also isolated from the two most susceptible clones, i.e. Clone 1 and Clone 3. This could have been because of the fact that the resistance of the plants was already compromised making it susceptible to infection by secondary colonizers. The genera mostly isolated included *Pseudomonas, Bacillus, Enterobacter, Xanthomonas/Stenotrophomonas* and *Pantoea,* with *Enterobacter, Pantoea, Erwinia* and *Klebsiella* representing the family *Enterobacteriaceae.* The focus was only on sequencing the 16S gene, and identification of the isolates could therefore only be made up to genus level. There is a possibility that the *Enterobacter* isolated from this study were present as endophytes in the *Eucalyptus* plants tested. It could be said that some of the *Pantoea* isolated were present as endophytes because they were isolated from healthy, diseased and symptomless plants. Further studies are, however, necessary to validate the findings seeing as the species of Pantoea and *Enterobacter* spp were not identified.

6. FIGURES



Lane 1: 1kb Marker Lanes 2-19: Samples

Figure 4.1: An agarose gel showing a DNA extraction done using the Qiagen DNeasy Extraction Kit after enrichment of the cut plant material.



Lanes 1-10: Examples of samples run on DGGE polyacrylamide gel

Figure 4.2: An 8% DGGE gel run for ten DNA samples after amplification of the V3 region of the 16S rDNA with primers pA8f-GC and PRUN518r.

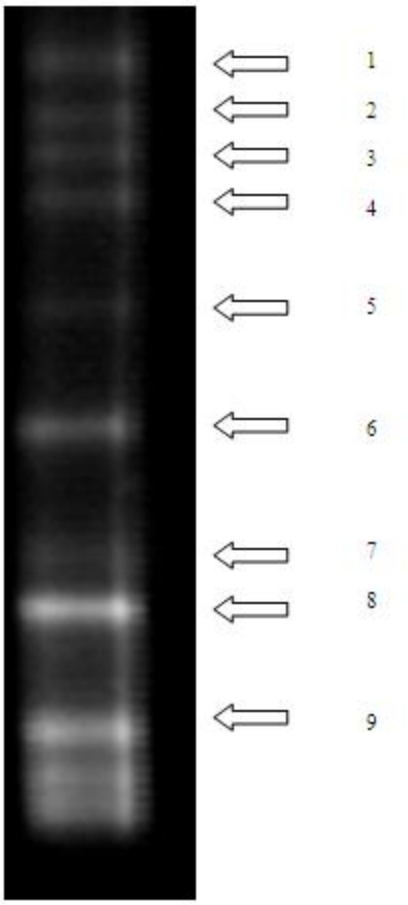


Figure 4.3: An image of a lane cut from a DGGE gel showing the numbers of copies of the 16S rRNA gene associated with the type strain of Pantoea ananatis (LMG 2665).

7. TABLES

Table 4.1: The list of plant samples included in the DGGE study

Condition	Plant Name	Plant Part	Sample Name
Healthy	Clone 1 A (11/07)	Leaves	D
		Stem	E
		Roots	151
	Clone 3 A (11/07)	Leaves	V
		Stem	W
		Roots	168
	Clone 3 C (11/07)	Leaves	F
		Stem	G
		Roots	181
	Clone 2 A	Leaves	H
		Stem	136
		Roots	137

	Clone 4 A (11/07)	Leaves	Z
		Stem	204
		Roots	205
	Clone 4 B (11/07)	Leaves	AA
		Stem	BB
		Roots	211
Diseased	Clone 3 (07/07)	Leaves	A,R
		Stem	8,S
		Roots	9
	Clone 3 A (07/07)	Leaves	53,54,55,56,57,58
		Stem	59,60,61,62
		Roots	63
	Clone 1 A (07/07)	Leaves	B
		Stem	C
		Roots	15
	Clone 1 B (07/07)	Leaves	T
		Stem	U
		Roots	21
	Clone 1 A (11/07)	Leaves	28,29,30,31
		Stem	32,33,34,35
		Roots	36
	Clone 2 A (11/07)	Leaves	75,76,77
		Stem	78,79
		Roots	81
	Clone 2 B (11/07)	Leaves	X
		Stem	Y
		Roots	88
Resistant	Clone 7 A	Leaves	CC
		Stem	102
		Roots	103
	Clone 7 B	Leaves	DD
		Stem	108
		Roots	109
	Clone 7 C	Leaves	K
		Stem	115
		Roots	116
	Clone 6 A	Leaves	EE
		Stem	120
		Roots	121
	Clone 6 B	Leaves	FF
		Stem	125
		Roots	126
	Clone 6 C	Leaves	L
		Stem	130
		Roots	131

	Clone 5 A	Leaves	M
		Stem	239
		Roots	240
	Clone 5 C	Leaves	GG
		Stem	251
		Roots	251
	Clone 8	Leaves	HH
		Stem	222
		Roots	223

Table 4.2: A list of the different primer pairs tested in this study

Reverse Primer	Forward Primer	Product Length
518r (5'-ATT ACC GCG GCT GCT GG-3') (Øvreas *et al*., 1997)	**F357** (5'-TAC GGG AGG CAG CAG-3') (Chen *et al*., 2008)	161bp
	F338 (5'- ACT CCT ACG GGA GGC AGC AG-3') (Cocolin *et al*.,2001)	180bp
	pA8f (5'- AGA GTT TGA TCC TGG CTC AG – 3') (Fjellbirkeland *et al*., 2001)	510bp

Table 4.3: Results for the 16S sequencing done on the bands excised from the DGGE Gels

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CHAPTER 5

CONCLUSION

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Conclusion

Endophytic bacteria are typically present in parts of the plant internal to the epidermis, and may have an impact on the plant's health and growth (Beattie, 2007). Endophytic bacteria can reside inside the plants as natural flora and increase the plant's fitness or as latent pathogens, waiting for ideal environmental conditions to present symptoms. During most studies on detecting endophytic bacteria, the popular approach is to use culturing techniques. Many researchers, however, also commonly use molecular techniques such as cloning libraries, terminal-Restriction Fragment Length Polymorphism t-RFLP (Sessitsch *et al*., 2002), and denaturing gradient gel electrophoresis (DGGE). DGGE was found to be reliable, rapid, reproducible and relatively inexpensive and it makes a popular choice for community analyses (Muyzer, 1999). In this study a culturing approach was compared to a molecular based DGGE approach to determine the endophytic population in *Eucalyptus nitens* clones, focusing mostly on the *Enterobactericeae*.

The genera identified throughout this study included *Bacillus, Pseudomonas, Xanthomonas/Stenotrophomonas, Arthrobacter, Enterobacter, Paenibacillus, Clostridium, Delftia, Lynsinibacillus, Acinetobacter, Achromobacter, Tisserella, Exiguibacterium, Herbaspirillium, Pantoea, Brvundimonas, Variovorax, Sporosarcina, Microbacterium, Sphingomonas, Enterococcus, Brevibacillus, Agrobacterium, Labedella, Erwinia, Comamonas, Rhizobium, Klebsiella and Acidiovorax*. Most of these were identified from the DGGE study because it was the only method that allowed for the 16S rDNA screening of the dominant population present. The genera most commonly isolated during the DGGE study were *Bacillus, Pseudomonas, Xanthomonas/Stenotrophomonas, Enterobacter and Pantoea.* The *Bacillus, Pseudomonas* and *Enterobacter* were evenly spread throughout the leaves, stem and roots of all the clones sampled, whereas *Pantoea* was not isolated from Clones 1, 6 and 8 in the DGGE study. In comparison to this the culturing study showed that *Pantoea eucalypti* was isolated from the leaves and stem material of clones 5 and 3, respectively, and *Pantoea vagans* from the stem and leaves of clone 3 and the leave material of clone 1. *Enterobacter* on the other hand in this study was isolated from every clone from either the leaves, stem or root material. Most

isolates were made from the roots of healthy and resistant clones (clones 1, 3 and 4 and clones 7, 6 and 5, respectably), followed by the roots and leaves of diseased clones (clones1 and 2 and clone1) and lastly the stem of a diseased clone (clone 3).

When looking at the total bacterial population isolated from susceptible and resistant clones in the DGGE study, it was seen that the susceptible clones showed a much higher richness and diversity, especially in the diseased samples. This is an indication of how weakened the diseased plant's resistance is, making it a target for colonization. The symptomless samples of the susceptible clones harboured a total of 13 different genera., The symptomless plants and plants showing symptoms had a total of 11 genera in common, namely *Pseudomonas, Bacillus, Xanthomonas/Stenotrophomonas, Arthrobacter, Brevundimonas, Agrobacterium, Pantoea, Exiguobacterium, Sporosarcina, Paenibacillus and Enterobacter*. The susceptible clones compared to the resistant clones had 9 genera in common including *Pseudomonas, Bacillus, Xanthomonas/Stenotrophomonas, Brevundimonas, Agrobacterium, Pantoea, Sporosarcina, Paenibacillus Sphingomonas, Acinetobacter, Clostridium, and Herbaspirillium*. Each of the healthy, diseased and resistant clones had genera that were specific to their respective groups. For the healthy samples of the susceptible clones the genera Rhizobuim and *Variovorax* were unique, for the susceptible clones showing symptoms it was *Lynsinibacillus, Delftia, Klebsiella, Tissierella, Microbacterium, Enterococcus* and *Brevibacillus*. The genera specific to the resistant clones were *Acidiovorax, Erwinia* and *Comamonas*. *Arthrobacter* and *Exiguobacterium* were common genera isolated from the susceptible clones but they were not isolated from the resistant clones. The diseased and resistant clones shared the genera *Herbaspirillium, Clostridium, Achromobacter, Acinetobacter* and *Sphingomonas* which were not isolated from the susceptible symptomless plants.

During the culturing study the following observations were made. In the course of sampling Clone 1 was the most susceptible. The *Enterobacteriaceae* isolated made up a total of 9.3% and two genera, *Enterobacter* and *Pantoea,* were identified. This corresponded to some other studies found in literature where culturing was used. Usually in these studies only one to two and in many cases none of the genera isolated belonged to the *Enterobacteriaceae*. Reasons for this

could be that they have been competitively outnumbered by other species present or that they were in a state that made them unculturable during that time. No *P. ananatis* isolates were identified, but *Pantoea vagans* (from diseased material) and *P. eucalypti* (from diseased as well as resistant plants) were found. *Enterobacter mori* and *E. asburiae* were also identified. There were, however, groups of *Enterobacter* isolates that could not be identified because they did not cluster close enough to any of the known *Enterobacter* strains that were available at that stage. It was concluded that *P. eucalypti, P. vagans, E. asburiae* and *E. mori* occur as endophytes in susceptible and resistant *E. nitens* clones.

When looking at the PCR-DGGE Study, it was difficult to totally exclude culturing from the study because of the fact that the plant's choloroplast DNA totally outnumbered the bacterial DNA present. Therefore an enrichment of the plant material in Nutrient Broth followed by a total DNA extraction was followed. During the DGGE band sequencing it was found that the diseased plant had a higher diversity of genera present than the resistant or symptomless plants. Because, for this part of the study, the means of identifying genera other than those belonging to the family *Enterobacteriaceae* were performed, those results were also included, but the main focus remained the *Enterobacteriaceae*. Twenty nine different genera were identified from the study at the end with the most representative ones being *Bacillus, Pseudomonas*, a possible *Xanthomonas/Stenotrophomonas* sp., *Enterobacter* and *Pantoea. Erwinia* and *Klebsiella* were also identified, but only in two cases. Only 16S sequencing was performed on the bands and therefore identification could only be done up to genus level. *Enterobacter* was identified in high numbers in all the diseased material but was only found in the stems of 2 healthy clones. *Pantoea* was identified from healthy and diseased plants but their representation in the diseased material was lower, therefore it was concluded that *Pantoea* spp. are present as endophytes in these *E.nitens* clones. Higher numbers of *Pantoea* were expected though, especially from the diseased material. Reasons for these unanticipated results could have been that disease that presented itself was not caused by *Pantoea*, but rather by a different pathogen, but further studies needs to be done to confirm this statement.

When the culturing and PCR-DGGE techniques are compared it is difficult to say whether there was a big difference found in the total bacterial population, mainly because only *Enterobacteriaceae* were targeted in the culturing study. When the *Pantoea* and *Enterobacter spp* isolated from both of these studies are compared it was seen that the results were similar in the case of the *Enterobacter spp. Enterobacter* was identified in all of the clones tested (1-7) in both the culturing and DGGE studies. In the case of *Pantoea* it was isolated from clones 1, 3 and 5 in the culturing study and from clones 2, 3, 4, 5 and 7 in the DGGE study, only clone 1 had no *Pantoea* identified in the DGGE study.

It can be said that from both studies it was concluded that *Pantoea* and *Enterobacter* are definitely endophytic to these *E.nitens* clones. It is still however unsure as to which bacterium was responsible for the disease symptoms because no *P.ananatis* was identified in the culturing study. It is possible though that there was a *P.ananatis* found in the DGGE study, but this was not confirmed because identification of the bands could not be done up to species level. Because of the fact that only *Enterobacteriaceae* were targeted and there is no knowledge of the other species isolated in the culturing study, no comparison can be drawn for them, but according to the DGGE results, it looks like *Bacillus* and *Pseudomonas* are definitely also common endophytes of these *E.nitens* clones. The data suggests that different *E. nitens* clones harbour a wide diversity of bacteria and endophytes and that these bacteria are not necessarily involved in causing disease.

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