Characterisation

of Rhizobacterial Communities

of *Eucalyptus* Species

and Hybrids

By

Melanie Patrick

Dissertation submitted in fulfillment of the requirements for the degree

Magister Technologiae: Agriculture,

Department of Life Sciences,

Faculty of Health and Environmental Sciences,

Central University of Technology, Free State

BLOEMFONTEIN

NOVEMBER 2013

Declaration

Declaration with regard to independent work:

I, Melanie Patrick (student number 212086219), hereby declare that this research project submitted to the Central University of Technology, Free State, for the degree Magister Technologiae: Agriculture is my own independent work. This research project was conducted at the Central University of Technology, Free State, under the supervision of Prof Annabel Fossey and co-supervised by Dr Olga de Smidt.

Signature of student **Date**

Melanie Patrick

I certify that this statement is correct.

Signature of supervisor **Date**

Prof Annabel Fossey

Acknowledgements

First and foremost I would like to thank my supervisor, Prof Annabel Fossey and co-supervisor Dr Olga de Smidt for their time, guidance, patience, and expertise. They helped broaden my spectrum of knowledge, something no one can take away from me. Secondly, a big thank you to my parents, fiancé and brother for their love, motivation, and support throughout my studies. Finally to our Heavenly Father, without Him and these inspirational people this project could not have been possible.

In addition I would also like to thank the following people who were of great assistance:

- Bryn Pollard and Tammy Swain for their help with the sample collection at Sunshine Seedling Services and the ICFR respectively,

- Chricilia Jordaan at SGS for the FAME analysis, and

- Nel-Marié at the Biotechnology Department at the University of the Free State for her assistance with the sequencing.

Abstract

Introduction: Good quality *Eucalyptus* is of importance to South Africa's pulp and paper industry. Limited land is available for forestry, therefore *Eucalyptus* with genotypes for good pulp and paper qualities, particularly hybrids, are bred and cloned via cuttings. Although these *Eucalyptus* clones keep the favourable genotypes in the population, many have difficulty with rooting. Research has shown that rhizobacteria can improve rooting. Thus, one strategy to enhance the rooting of cuttings is to use rhizobacterial preparations. The aim of this study was to characterise rhizobacterial communities of *Eucalyptus* hybrid and species and identify possible plant-growth promoting rhizobacteria (PGPR).

Materials and methods: Rhizospheric samples were collected from *Eucalyptus* hybrids and species. The rhizobacterial communities were characterised using fatty acid methyl esters (FAME) analysis and denaturing gradient gel electrophoresis (DGGE). DGGE fragments were further sequenced to identify rhizobacteria.

Results and discussion: FAME analysis successfully achieved a broad characterisation of the *Eucalyptus* hybrid and species rhizobacterial communities based on their fatty acid composition. Myristic acid (C14:0) was the most abundant fatty acid. DGGE profiles gave a molecular profile of the *Eucalyptus* hybrid and species rhizobacterial communities based on their DNA composition. *Nitrosomona eutropha* was present in all samples which illustrates a nitrogen-rich environment. *Adhaenbacter aquaticus* was unique to the better rooting *Eucalyptus* hybrid GU111.

Conclusion: This study provided some insight into the diversity of rhizobacterial communities of *Eucalyptus* hybrids and species. Possible PGPR were identified and the observation made that the nature of the soil environment changes with the aging of the associated host. These findings allow further investigation into the formulation of potential rhizobacterial preparations for rooting enhancement of *Eucalyptus* cuttings.

Table of Contents

Chapter 5

Rhizobacterial community profiles using denaturing gradient gel electrophoresis

Chapter 6

Discussion and conclusion

Acronyms

AOCS	American Oil Chemists' Society
AM	Arbuscular mycorrhizal
ARC	Adventitious rhizogenesis in cuttings
ARDRA	Amplified rDNA restriction analysis
AVP1	Arabidopsis vacuolar pyrophosphatase
BSA	Bovine serum albumin
gDNA	Genomic deoxyribonucleic acid
DGGE	Denaturing gradient gel electrophoresis
dNTP	Deoxyribonucleotide triphosphates
DNA	Deoxyribonucleic acid
FAME	Fatty acid methyl ester
GA2O	Gallium-induced oxidation
GC	Gas chromatography
ICFR	Institution for Commercial Forestry Research
KZN	KwaZulu-Natal
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
PLFA	Phospholipid fatty acids
RAPD	Random amplified polymorphic DNA
RISA	Ribosomal intergenic spacer analysis
rRNA	ribosomal ribronucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SSCP	Single strand conformation polymorphism
TPSI	Trehalose-6-plwsphate synthase
TGGE	Temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism

UPGMA	Unweighted pair group method with an arithmetic mean algorithm
U.S.A	United States of America
UV	Ultra-violet
WRI	Wattle Research Institute

List of Tables

List of Figures

Chapter 1
Introduction

1.1 Background

Plantation forests cover 1.3 million hectares of South Africa's land (Pogue, 2008). Commercial plantation forests are major contributors to the South African economy. South Africa plays an important role in the saw timber and pulp and paper industry and is one of only two countries in Africa that significantly contribute to this industry (Shackleton *et al.,* 2007; Pogue, 2008). The South African plantation forests are made up of eucalypts, pine and black wattle (Komakech *et al.,* 2009). Pine is the major forestry contributor comprising 52% of plantation forests (Pogue, 2008). *Eucalyptus* hybrids and species are the main resource for the high demanding saw timber and pulp and paper industry, occupying 39% of plantation forests (Swain & Jones, 2004; Pogue, 2008). The remaining plantation forests consist of black wattle, also a source for high quality material for the pulp and paper industry (Chetty, 2001; Beck & Fossey, 2007).

Eucalyptus plantations in South Africa are made up of hybrids and species. There are over 700 *Eucalyptus* species (Grattapaglia & Sederoff, 1994; Ishii, 2009; Cupertino *et al.,* 2011). *Eucalyptus* species with superior genotypes are identified and bred. The superior genotypes are bred to produce phenotypes with characteristics important mostly for the pulp and paper industry. The ability of *Eucalyptus* species to hybridise also allows tree breeders to rapidly combine years of evolutionary diversity into one genotype. A major advantage associated with hybridisation includes better adapted *Eucalyptus* hybrids to less favourable environmental conditions (Komakech *et al.,* 2009).

Eucalyptus hybrids are multiplied through cuttings, producing clones. In this way uniformity is achieved and favourable genotypes are kept in the population. In contrast, a major disadvantage of cloning is the range of rooting ability. Some *Eucalyptus* cuttings demonstrate poor rooting resulting in substantial production and financial losses. Increased production costs and the limited availability of land for forestry are important factors affecting this industry's sustainability. To meet the increasing demand, plantation forest companies need to increase their outputs. The forestry industry is focusing on improving rooting percentages of clonal cuttings.

There is a variety of rooting enhancing strategies being investigated. One such strategy involves the addition of hormones to stimulate adventitious rooting (Whiting *et al.,* 2011). More recently, a novel strategy is to use rhizobacteria to stimulate rooting (Díaz *et al.,* 2009). Rhizobacteria are found in the environment known as the rhizosphere which is in direct contact with the roots. The microbial diversity in the rhizosphere can be beneficial to the host plant (Asghar *et al.,* 2004). Díaz *et al.* (2009) has shown that rhizobacteria can increase the rooting of cuttings. The rhizobacteria that increase the overall well-being and production of plants are referred to as plant growth promoting rhizobacteria (PGPR). The mechanisms by which PGPRs enhance rooting ability are not completely known. There are suggestions that the rhizobacteria produce plant hormones and fix nitrogen asymbiotically (Gutierrez Mañero *et al.,* 1996; Kennedy *et al.,* 1997). Another suggestion is that the PGPR produce siderophores against harmful microorganisms (Flaishman *et al.,* 1996).

Recent studies confirm that treatments of cuttings with non-pathogen bacteria such as *Bacillus* and *Pseudomonas* induced root formation in some plants (Patena *et al.,* 1988; Esitken *et al.,* 2003). A similar effect has been seen in forestry species, including *Pinus taeda*, *Pinus elliotii*, *Pinus contorta*, *Picea glauca* and *Pseudotsuga menziessii* (Chanway *et al.,* 1991; O'Neill *et al.,* 1992; Enebak *et al.,* 1998). Research regarding the use of rhizobacterial treatments on *Eucalyptus* hybrids and species is limited, however in Brazil the rooting ability of *Eucalyptus grandis* cuttings has also been increased through rhizobacterial stimulation (Teixeira *et al.,* 2007). The rhizobacterial preparations seem to be somewhat species specific (Long *et al.,* 2008) therefore, to make rhizobacterial preparations for the rooting enhancement of *Eucalyptus* hybrid and species cuttings, their rhizobacterial communities first need to be studied and characterised.

1.2 Aim and objectives

The aim of this study was to characterise the rhizobacterial communities of *Eucalyptus* hybrids and species. This forms part of a large project in which rhizospheric preparations will be tested on poor rooters to establish whether they will enhance rooting frequencies as shown in previous studies.

The following objectives were devised in order to meet this aim:

- To identify and sample rhizospheres of *Eucalyptus* hybrids and species;
- To broadly characterise rhizospheric communities on a biochemical level using fatty acid methyl esters (FAME).
- To further characterise rhizobacterial communities by producing rhizobacterial community profiles. This is achieved using a molecular approach of polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE);
- To identify possible plant-growth promoting rhizobacteria (PGPR).

1.3 Layout of chapters

Chapter 1: Introduction

The introduction provided a general description of the study, including the problem statement as well as the main aim and objectives.

Chapter 2: Literature review

The literature review covered the literature available on all the aspects of this study.

Chapter 3: Sample collection

Chapter 3 covered the different aspects of sample collection. These were all included in one chapter as the same samples were used for both FAME and DGGE analysis.

Chapter 4: Broad characterisation of *Eucalyptus* rhizospheric communities using fatty acid methyl ester analysis

This chapter described the use of a biochemical method (FAME analysis) to achieve a broad characterisation of *Eucalyptus* hybrid and species rhizospheric communities. In this chapter differences among and between *Eucalyptus* hybrid and species rhizospheric communities were also determined statistically. The content of this chapter has been submitted for publication in Applied Soil Ecology as follows: Patrick, M.A., Fossey, A., de Smidt, O. Broad characterisation of *Eucalyptus* rhizospheric communities using fatty acid methyl ester analysis (Appendix A).

Chapter 5: Rhizobacterial community profiles using denaturing gradient gel electrophoresis

Chapter 5 discussed the molecular process of DGGE and how it successfully determined the rhizobacterial community profiles of *Eucalyptus* hybrids and species. The content of this chapter has been submitted for publication in Southern Forests as follows: Patrick, M.A., de Smidt, O., Fossey, A. Characterising *Eucalyptus* hybrid and species rhizobacterial communities in KwaZulu-Natal, South Africa (Appendix B).

Chapter 6: Discussion and conclusion

In the final chapter the overall observations of both analyses were discussed with considerations to keep in mind as well as suggestions to further the development a rhizobacterial preparation to enhance the rooting ability of *Eucalyptus* hybrid and species cuttings.

Chapter 2
Literature review

2.1 Introduction

Forestry has been described as the Earth's lungs (WWF, 2012). Similar to a human body that is unable to function without lungs, planet Earth can not function without forests (WWF, 2012). Forests are not just about the living environment such as plants, animals and microorganisms but rather the relationship between these living organisms and the non-living environment including soil, climate and water (WWF, 2012).

Forests play an important role worldwide (DAFF, 2012). The benefits of forestry were noted as early as 400 BC by Plato, the Greek philosopher and mathematician (Bauhus *et al.*, 1998). Plato realised that the loss of forests leads to soil erosion and the disappearance of water springs (Bauhus *et al.*, 1998). Plato's observations demonstrate the importance of forests for soil protection and conservation. Forests display a wide range of functions; they provide wood from trees, nutrition for animals, grazing and have medicinal purposes (WWF, 2012). Furthermore forests also maintain a favourable climate, act as shelted belts (Figure 2.1a) and increase biodiversity (Finnigan & Poulton, 2005; DAFF, 2012). In addition to environmental benefits the careful and well planned establishment of trees provide economic benefits (Finnigan & Poulton, 2005; DAFF, 2012). Economic benefits are mainly attributed to the production and sale of non-timber and timber products such as sawlogs (Figure 2.1b), veneer and pulpwood (DAFF, 2012).

a) 

b) 

Figure 2.1: Benefits of forestry: a) shelted belts and b) harvested logs (Finnigan & Poulton, 2005).

2.2 Forestry in South Africa

In South Africa, the forestry industry is made up of three resources: woodlands, plantation forests and natural forests (Pogue, 2008). Woodlands are the largest forest resource, while natural forests are on a decline (Bauhus *et al*., 1998; Pogue, 2008). Plantation forests occupy the designated land available for plantations in South Africa and are becoming a focus for research (Pogue, 2008; DAFF, 2012). Figure 2.2 illustrates that some products and services are associated with all forestry resources while others are specific to a particular source (Pogue, 2008). The main purpose and motivation for the development of plantation forests is the production of wood, paper and pulp (Pogue, 2008). The paper and pulp industry accounted for 56% of the forestry resources in 2004 and acts as an important source of income and employment in South Africa (Pogue, 2008).

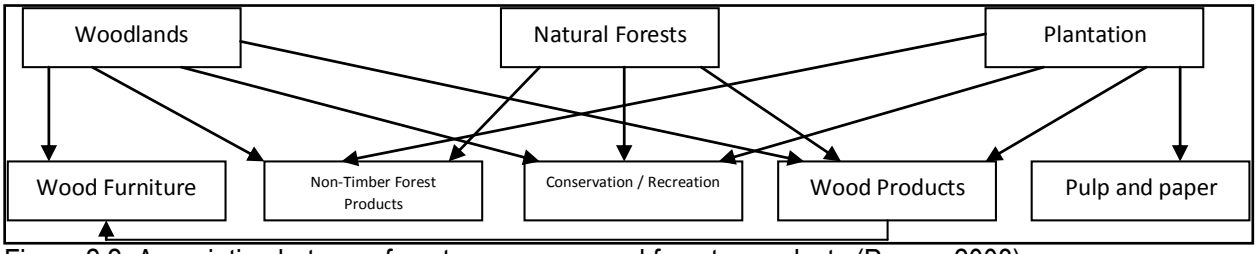


Figure 2.2: Association between forestry resources and forestry products (Pogue, 2008).

The history of South African plantation forestery dates back to the late 17th century during Dutch Colonial rule in the Cape (Pogue, 2008). In 1896 a *Eucalyptus* plantation was established in the Western Cape (Pogue, 2008). The sole purpose of *Eucalyptus* plantations was to supply fuel for those travelling to the Kimberley diamond fields (Pogue, 2008; Forestry South Africa, 2012). After World War I the Department of Forestry was established with the intention to improve the management of South African natural and plantation forests (Pogue, 2008).

South Africa has become a world leader in plantation forestry and its products (Beck & Dunlop, 2001; Pogue, 2008; Jacobs & Punt, 2010). The province of Mpumalanga is home to most of the plantation forests (40.8%) in South Africa, followed by KwaZulu-Natal (39.6%) (Pogue, 2008). Democratisation in 1994 allowed the well established South African pulp and paper industry entry into the competitive international market (Pogue, 2008). Sappi, formed in 1936 and Mondi, formed in 1967 are two main companies in South Africa that are responsible for the operation of pulp mills (Pogue, 2008; The Wood Foundation, 2012). Sappi and Mondi are both in the top twenty international pulp and paper companies (Pogue, 2008; The Wood Foundation, 2012). In addition, these companies provide three main types of occupation; professional, skilled trades and machine operators (Pogue, 2008). The plantation forestry sector not only creates direct employment in the mentioned three areas but also provides employment to other individuals in the primary and secondary transport of timber (Pogue, 2008). The employment opportunities provided by the forestry industry increases the employment rate, especially in rural areas where employment opportunities are scarce (Pogue, 2008).

The plantation forestry resource in South Africa is comprised largely of three species; black wattle, pine and eucalypt (Pogue, 2008). None of these species are indigenous to South Africa (Pogue, 2008). These species are used for their fast growth and characteristics required by the industry (Fossey, 2009). Black wattle belongs to the species *Acacia mearnsii* and is a hardwood that accounts for the least of the three species' to plantation forests (Beck & Dunlop, 2001; Pogue, 2008). Black wattle is the basis of the wattle industry in South Africa (Chetty, 2001). Black wattle is used to produce high quality vegetable tannin in their bark, which is used for tanning, especially in the treatment of leather (Chetty, 2001). Tannin is the reason for wattles profitable market (Chetty, 2001). Pine is a softwood that comprises most of the plantations (51%) in South Africa (Forestry South Africa, 2012). Pine trees are a cheap and local source of saw logs (Chetty, 2001; Pogue, 2008). *Pinus patula, Pinus elliotti, Pinus taeda* and *Pinus gregii* are the most

important commercial pine species (Chetty, 2001). The *Eucalyptus* genus consists of over 700 species and are the most widely planted trees globally (Grattapaglia & Sederoff, 1994; Ishii, 2009; Cupertino *et al*., 2011). *Eucalyptus* is a source of hardwood fibre (Chetty, 2001). The construction of Mondi Richards Bay Pulp and Liner Board Mill in 1983 resulted in a shift from pine saw logs and pulpwood to hardwood fibre (Chetty, 2001).

2.3 *Eucalyptus*

There has been a global expansion of *Eucalyptus* trees (Cortinas *et al.,* 2010). In tropical and subtropical regions *Eucalyptus* is the most widely cultivated hardwood genus (dos Santos *et al.,* 2004). *Eucalyptus* trees have a wide range of adaptability which puts it in high demand (dos Santos *et al.,* 2004; Pogue, 2008). The southern hemisphere has the added advantage of a shorter timber growth cycle of *Eucalyptus* (Grattapaglia & Sederoff, 1994; Pogue, 2008). In the southern hemisphere *Eucalyptus* takes approximately nine years to achieve reasonable size for pulping, which is a considerably shorter time frame compared to the northern hemisphere where it could be anything around 50 years (Pogue, 2008). In South Africa, *Eucalyptus* trees make up 39% of plantations (Forestry South Africa, 2012). The economic importance of *Eucalyptus* stems mostly from its use as saw timber and pulp for paper products (Swain & Jones, 2004; Pogue, 2008). Other products include poles, mining timber, fuel wood and 'non-wood' products such as tannin, honey and essential oils, (Chetty, 2001; Rahim *et al.,* 2003; Pogue, 2008; Andreote *et al.,* 2009a).

The major components of wood, which is in high demand from commercial plantations for pulp and paper production, include cellulose, lignin and hemicelluloses (Sharma & Ramamurthy, 2000; Kien *et al.,* 2009). Together the wood components make up the extracellular matrix of the cells, as shown in Figure 2.3 (Baucher *et al.,* 1998). Cellulose is the main component in the secondary cell wall and contributes about 40 to 50% of the dry weight of wood (Kien *et al.,* 2009). *Eucalyptus* trees have a high cellulose content (Kien *et al.,* 2009). Lignin is mainly found in the secondary thickenings of tissues that play a part in conduction and support (Baucher *et al.,* 1998). Other functions of lignin include; rigidity, strength and ensures the walls are hydrophobic and water impermeable (Baucher *et al.,* 1998). The lignin is a key component to good quality paper production (Baucher *et al.,* 1998).

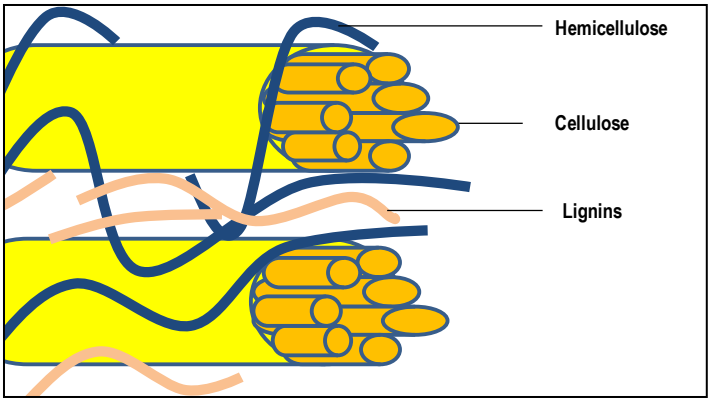


Figure 2.3: Major components of wood include hemicelluloses, cellulose and lignin.

2.3.1 *Eucalyptus* hybrids and species

The genus *Eucalyptus* from the family Myrtaceae is indigenous to Australia, Papua New Guinea and Philippines (Grattapaglia & Sederoff, 1994; Ishii, 2009; Cupertino *et al.,* 2011). *Eucalyptus grandis*, *Eucalyptus urophylla*, *Eucalyptus camaldulensis* and *Eucalyptus globulus* are the most important economic *Eucalyptus* species (Eldridge *et al.,* 1993; Cupertino *et al.,* 2011). *E. grandis* (Flooded Gum / Rose Gum) is considered easy to cultivate, have rapid growth and a variety of uses (Chetty, 2001; MacMahon *et al.,* 2010). *E. urophylla* (Timor Mountain Gum) is also able to grow rapidly (Bhumibhamon & Maid, 2009). *E. camaldulensis* (Murray Red Gum / Red Gum / River Gum) has the widest natural distribution because of its drought resistance (MacMahon *et al.,* 2010; Girijashanker, 2012). The ability to adapt to changing rainfall and temperature regimes is a promising characteristic of *E. camaldulensis* (MacMahon *et al*., 2010; Girijasjanker, 2012). Lastly, *E. globulus* (Blue Gum / Tasmanian Blue Gum) is able to grow on a range of soils and does require good drainage (California Invasive Plant Council, 2012). *E. globulus* is important for the cellulose and paper industry because of its low lignin content and frost resistance (de Almeida *et al.,* 2010).

South African *Eucalyptus* plantations are located in different climatic areas (Komakech *et al.,* 2009). However, humid, warm temperate and subtroptical regions are preferred (Komakech *et al.,* 2009). *E. grandis* which makes up 80% of the total *Eucalyptus* plantations in South Africa has been the favourite species in these climatic conditions because of the species' ability to grow quickly and provide adequate wood properties (Malan & Arbuthnot, 1995; Chetty, 2001; Komakech *et al.,* 2009; MacMahon *et al.,* 2010). Other pure eucalypt species found in South Africa include *Eucalyptus elata*, *Eucalyptus fastigata*,

Eucalyptus macarthurii, and *Eucalyptus nitens* (Gardner, 2007). Dating back to the 1980s and 1990s the major South African forestry companies expanded their forestry land-bases into areas that do not display the desired climatic conditions (Gardner, 2007; Komakech *et al.,* 2009). Within the first few years the forest companies realised that the *Eucalyptus* species they were growing were not as environmentally adapted as they had expected and other options had to be investigated (Gardner, 2007; Komakech *et al.,* 2009).

The high demand for pulp and paper has necessitated the forestry industry to identify eucalypt genotypes that are better adapted to the drier and warmer, or drier and colder environments (Gardner, 2007; Komakech *et al.,* 2009). Tests carried out by the Institute of Commercial Forestry Research (ICFR) showed that *Eucalyptus longirostrata* (Grey Gum) displayed growth potential in Zululand, South Africa, because of its resistance to poor, shallow soils (Henderson, 1976; Gardner, 2007). The ever increasing demand for pulpwood demands that timber outputs be increased (Bailléres *et al.,* 2002; Little *et al.,* 2003). This increased demand can be achieved by either expanding into additional land areas or by increasing the amount of timber attainable from the existing land (Bailléres *et al.,* 2002; Little *et al.,* 2003).

2.4 Breeding strategies

Successful breeding programmes are a top priority of the forestry industry (Little *et al.,* 2003). Breeding programmes have both short and long term effects (Lee *et al.,* 2005). Short term effects can be noticed almost immediately (approximately two years) and focus on rapid tree production for biomass production and biofuel (Lee *et al.,* 2005; Harfouche *et al.,* 2012). The long term effects can only be seen over time (more than 10 years) and include improvement of entire tree populations, important for carbon sequestration (Lee *et al.,* 2005; Harfouche *et al.,* 2012).

Eucalyptus breeding strategies can be a prolonged, intricate process as represented in Figure 2.4 (Harfouche *et al.,* 2012). The first step in the breeding process is the collection of seeds or cuttings from natural populations (Harfouche *et al.,* 2012). The usage of seeds is a means of sexual reproduction while the usage of cuttings is a means of asexual reproduction (Chetty, 2001). These seedlings or cuttings are established in seed or stool beds and selected on their survival and rooting ability (Harfouche *et al.,* 2012). From the seed or stool beds the seedlings or cuttings are transferred for early field testing (this is the start of the breeding cycle) where growth, crown form, pest resistance and wood quality is monitored (Lee *et al.,*

2005; Harfouche *et al.,* 2012). Superior genotypes are identified from early field tests and established in a breeding orchard (Lee *et al.,* 2005; Harfouche *et al.,* 2012). The superior genotypes undergo a series of crossing and testing to develop advanced generation breeding populations, including specific hybrids. The best performing genotypes are further propagated by cuttings and used directly in clonal plantations (Harfouche *et al.,* 2012).

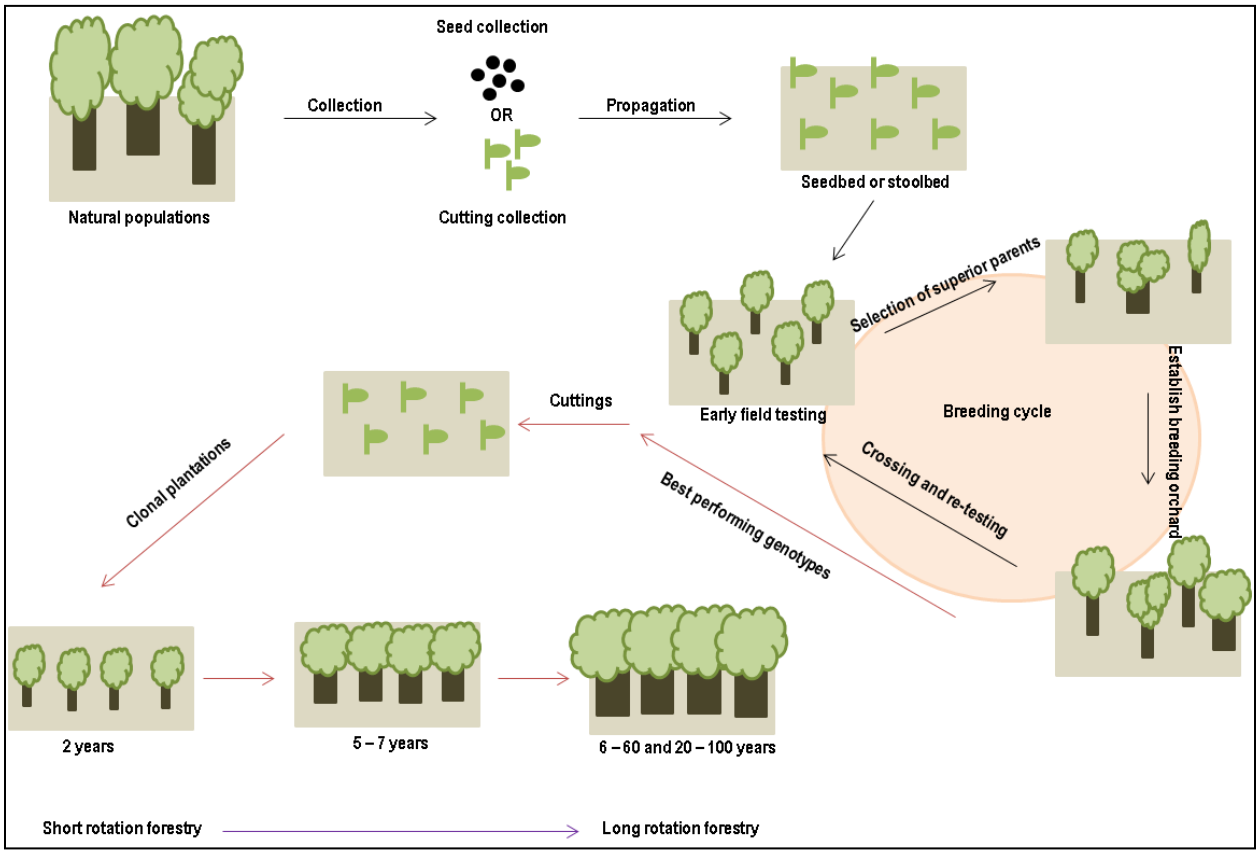


Figure 2.4: Breeding strategies of *Eucalyptus* (adapted from Harfouche *et al.,* 2012).

2.4.1 Controlled pollination

Pollination of *Eucalyptus* is mostly achieved by insects (House, 1997). However, birds play a role in pollination of *Eucalyptus* with larger flowers (House, 1997). The flowers of *Eucalyptus* do not have the characteristics needed to attract specialised pollinators (House, 1997). Therefore although honeybees and ants are common visitors to the flowers, they are not efficient pollinators (House, 1997). Understanding

pollination and what makes it successful is important in many *Eucalyptus* breeding programmes (House, 1997).

Controlled pollination allows researchers to produce and improve *Eucalyptus* using seeds (House, 1997). A widely used method for controlled pollination in *Eucalyptus* involves the bud being emasculated below the operculum and removing the anthers, leaving the style and stigma undamaged (Figure 2.5) (House, 1997). The emasculated buds on the branch are then isolated and pollen collection occurs during the operculum lift stage, the pollen is then sieved and placed in a vial (House, 1997). The pollen is ultimately used to pollinate the female stigma (House, 1997).

Figure 2.5: *Eucalyptus* controlled pollination (Gene Technics, 2012).

Controlled pollination has the potential to improve seed yield, control the level of outcrossing in seeds, increase the knowledge of both parents when it comes to breeding and achieving interspecific hybridisation in *Eucalyptus* species (House, 1997). On the one hand trees grown from seeds maintain a wide genetic base for the selection and development of superior trees (Chetty, 2001). On the other hand, trees that arise from seeds vary greatly (Chetty, 2001).

2.4.2 Hybridisation

Eucalyptus species have the ability to hybridise because of their low reproductive barriers (Fossey, 2009). Hybridisation is the crossing of diverse species and allows years of evolutionary diversity to be combined into one genotype (Ellstrand *et al.,* 1996). The process of hybridisation combines desirable traits of two selected species or parents and captures its benefits in the offspring (Figure 2.6) (Bisht *et al.,* 1999). The

combination of desirable genes makes it possible for the hybrids to gain certain characteristics (Eldrige *et al.,* 1994). These characteristics include resistance to diseases and the ability to adjust to different climates where pure species would not be able to survive (Eldrige *et al.,* 1994; Potts & Dungey, 2004; Cupertino *et al.,* 2011).

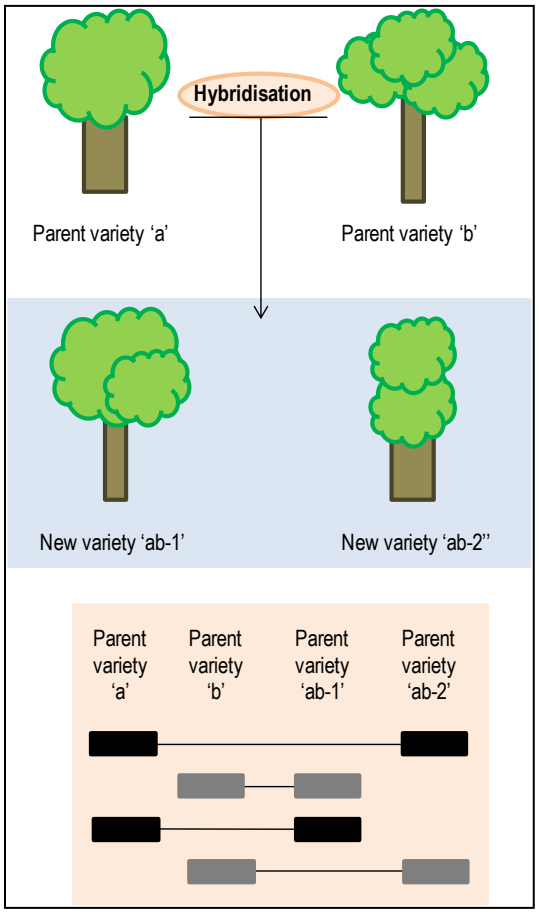


Figure 2.6: Principle of hybridisation.

The advantages displayed by hybridisation allow better-performing and better-adapted *Eucalyptus* hybrids to be bred and grown in harsh environmental conditions (Chetty, 2001; Komakech *et al.,* 2009). Hybridisation allows the *Eucalyptus* plantations to extend to drier and hotter, as well as cooler and more frost susceptible areas (Chetty, 2001). The most common *Eucalyptus* hybrids include *E. grandis* × *E. camaldulensis*, *E. grandis* × *E. urophylla* and *E. grandis* × *E. tereticornis* (Chetty, 2001). *E. grandis* and its

hybrids with *E. camaldulensis* and *E. urophylla* are predominant along the north coast of South Africa (Gardner, 2007).

2.4.3 Clonal propagation

The alternative reproductive method used in *Eucalyptus* breeding programmes is clonal propagation (Figure 2.7) (Beck & Dunlop, 2001). Through tissue culture, clonal propagation has the potential to provide high multiplication rates of uniform genotypes (Beck & Dunlop, 2001). Clonal propagation has the capability of eliminating seed production (Furze & Cheswell, 1985; Chetty, 2001). In addition, clonal propagation has the potential of obtaining certain genotypes one to two years after selection (Furze & Cheswell, 1985; Chetty, 2001). This is a significant difference compared to the four to 10 years using sexual reproductive methods (Furze & Cheswell, 1985; Chetty, 2001).

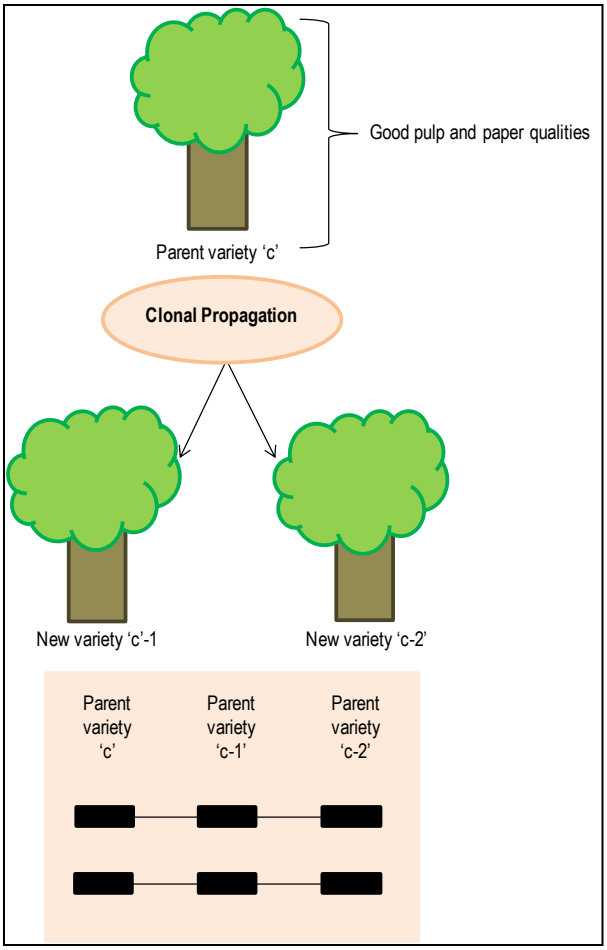


Figure 2.7: Process of clonal propagation.

The forestry industry recognised the benefits of clonal propagation as far back as the 1970s (Winton, 1970; Sunshine Seedlings Services, 2012). Small trees (*Populus tremula* L.) were produced from shoots initiated on roots growing from callus tissue (Winton, 1970). The advantages of clonal propagation are becoming more and more visible in South Africa as the field trials reach their rotation age (Sunshine Seedling Services, 2012). The industry's intention is to use clonal propagation in the attempt to increase the timber yields for pulp and paper on existing timber land (Sunshine Seedlings Services, 2012).

In the last two decades cloning of *Eucalyptus* hybrids and species have undergone continuous trial and testing resulting in substantial progress for forest companies' breeding programmes (de Assis *et al.,* 2004). *Eucalyptus* clones have been beneficial by solving problems associated with diseases such as canker (*Cryphonectria cubensis*) and improving the productivity of *Eucalyptus* hybrids and species (de Assis *et al.,* 2004). The focus of *Eucalyptus* cloning has included the importance of industrial requirements such as manufacturing of pulp and paper (de Assis *et al.,* 2004). Therefore, the establishment of vegetative propagation method is important in the development of tree breeding programmes in which cloning systems result in adaptive genetic gains (de Assis *et al.,* 2004).

Micropropagation and macropropagation, illustrated in Figure 2.8a and Figure 2.8b are forms of clonal propagation (Ezekiel, 2010). Micropropagation is expensive but captures adaptive genetic gains quicker than macropropagation (Ezekiel, 2010). The micropropagation process can occur along two routes (Chetty, 2001). One route, called somatic embryogenesis is a means of asexual induction of embryos from somatic cells (Chetty, 2001). The embryos then go through the normal developmental processes (Hussey, 1978; Chetty, 2001). The second route is referred to as organogenesis (Chetty, 2001). Organogenesis is a process whereby plant organs are induced by plant growth hormones to stimulate the developmental processes (Ammirato, 1986; Chetty, 2001). Macropropagation involves removing a section (cutting) of stem, leaf, or root tissue from the parent or donor plant (Ezekiel, 2010). The cuttings are treated with plant growth regulators and grown under controlled environmental conditions (Ezekiel, 2010).

Stem cuttings are a successful technique in *Eucalyptus* propagation (Elster & Perdama, 1999; Chetty, 2001; Titon *et al.,* 2006). The success of stem cuttings is due to its ease in handling compared to the development of micropropagated plants (Elster & Perdama, 1999; Chetty, 2001; Titon *et al.,* 2006). There

are different types of propagation systems for stem cuttings (Ezekiel, 2010). All the systems focus on the basis that the cuttings are well supplied with water at the cutting base and grown in the ideal environment that is air tight, humid and includes intermittent mist (Ezekiel, 2010). The clones created by stem cuttings are genetically identical to their parent (Beck & Dunlop, 2001; Chetty, 2001). Stem cuttings ensure that desirable traits are kept in the population with limited variation (Beck & Dunlop, 2001; Chetty, 2001). Mini-cuttings, illustrated in Figure 2.8c are a similar form of propagation successful in *Eucalyptus* (de Assis *et al.,* 2004). Mini-cuttings are very much like the discussed cuttings in concept and methodology but differ in their origin of structures used for cloning (Titon *et al.,* 2006). The mini-cuttings are produced from auxiliary sprouts of plants (de Assis *et al*., 2004).

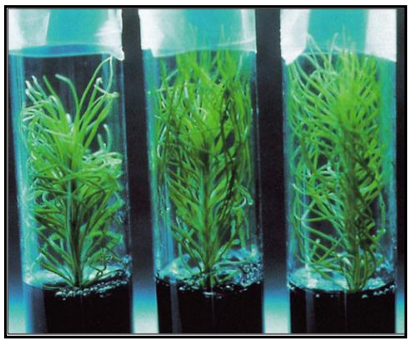
a)  b)  c) 

Figure 2.8: a) micropropagation (courtesy of A. Fossey), b) macropropagation (courtesy of A. Fossey) and c) minipropagation (GAP Gardens, 2012).

Cloning techniques have gained popularity as a research and management tool for plant production (Díaz *et al*., 2009). As described, this technique is capable of maintaining genetic gains obtained from improvement programmes (Grattapaglia *et al.,* 1995; Díaz *et al*., 2009). The enhanced plants are transferred to clonal plantations resulting in higher productivity and uniformity (Grattapaglia *et al.,* 1995; Díaz *et al*., 2009). An increase in wood production and quality together with reduced production costs are advantageous effects of clonal propagation (Merkle & Dean, 2000; Díaz *et al*., 2009). These positive clonal propagation results increase profitability and simplify production for the *Eucalyptus* breeder. Clonal propagation of *Eucalyptus* hybrids have been relatively successful in Australia, Brazil, Chile, New Zealand, Portugal, Spain, and South Africa (Campbell *et al.,* 2003; Gasper *et al.,* 2005; Titon *et al.,* 2006).

2.4.4 Transgenic plants

Transgenic plants, those that have altered genomes, are used in breeding programmes to improve phenotypic characteristics (dos Santos *et al.,* 2004; Andreote *et al.,* 2008; Andreote *et al.,* 2009b). A study conducted by Wu *et al.* (1995) showed that transgenic potatoe plants exhibited strong resistance to a bacterial soft rot disease. A different study by Romero *et al*. (1997) integrated the *TPSI* gene into the genome of tobacco plants which caused morphological alterations and increased drought tolerance. Transgenic plants are used in forestry plant-breeding programmes with the aim of improving yield and wood quality (Andreote *et al.,* 2008; Andreote *et al.,* 2009b). With the knowledge that wood quality is affected by lignin, Hu *et al.* (1999) showed that repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees.

2.5 Rooting ability of *Eucalyptus* clones

The rooting ability of *Eucalyptus* hybrids and species is affected by cloning because rooting is a genetic characteristic (de Assis *et al*., 2004). The *Eucalyptus* hybrids and species that are developed through clonal propagation display a variety of rooting percentages (Barralho & Wilson, 1994; Díaz *et al*., 2009; Peralta *et al.,* 2012). Some *Eucalyptus* hybrids and species tend to root fairly easy, such as juvenile tissue of *E. grandis* and hybrids between *E. grandis* × *E. urophylla* (Hartmann *et al.,* 1990). Many other *Eucalyptus* species (*Eucalyptus citriodora*, *Eucalyptus maculate*, *Eucalyptus paniculata* and *Eucalyptus cloeziana*) and hybrids display low rooting percentages, often to low to be viable for the high demanding industry (Sasse & Sands, 1997; Luckman & Menary, 2002; de Assis *et al*., 2004). Thus, variable rooting capacity is the most common drawback of propagation through cuttings (Barralho & Wilson, 1994; Díaz *et al.,* 2009; Peralta *et al.,* 2012).

Stem-cuttings do not develop a main tap root as is the case in seedlings but rather display adventitious rooting (Díaz *et al.,* 2009). Adventitious rhizogenesis in cuttings (ARC) is a unique and intricate process, vital in plant propagation (Fogaca & Fett-Neto, 2005). There are three phases in the ARC process (Fogaca & Fett-Neto, 2005). The first is induction, during this period molecular and biochemical steps occur but no morphological events are clearly observed (Fogaca & Fett-Neto, 2005). The second phase is known as initiation, this is when cell division takes place (Fogaca & Fett-Neto, 2005). Root meristems and root

primordial are formed during the initiation phase (Fogaca & Fett-Neto, 2005). Expression is the third and final phase in which root growth and emergence out of cuttings is visible (Fogaca & Fett-Neto, 2005). Collectively, the latter two stages are known as the root formation phase (Fogace & Fett-Neto, 2005).

A cuttings ability to form adventitious roots tends to decline as the age of donor plants increase (Ezekiel, 2010). Morphological, anatomical and biochemical changes occur as a plant matures (Ezekiel, 2010). Poor rooting can be as a result of any one or a combination of changes in these characteristics (de Assis *et al.,* 2004; Peralta *et al.,* 2012). Although cloning older tree material with a higher lignin content improves pulping properties there is a decline in its propagation success because higher doses of rooting hormone are required for root formation (Beck & Dunlop, 2001; de Assis *et al*., 2004; Ezekiel, 2010).

2.6 Rooting enhancement strategies

A plant benefits significantly from an enhanced root system (Werner *et al.,* 2010). The roots essential functions are to ensure water and nutrient uptake, store reserves, synthesis specific compounds and anchor the plant (Werner *et al.,* 2010). An enhanced root system can improve drought tolerance and also facilitate better leaf contents (Werner *et al.,* 2010). The enhancement of the root system has proven difficult as access to roots and their environments are a challenge compared to aerial plant organs (Bais *et al.,* 2006; Werner *et al*., 2010). According to Werner *et al*. (2010) research is now focusing on manipulation of root architecture for rooting enhancement.

2.6.1 Phytohormones

Rooting capability can be enhanced by treating cuttings with specific plant hormones (phytohormones) (Whiting *et al.,* 2011). The phytohormones together with environmental factors such as nutrients and light regulate plant growth (Davies, 2004; Werner *et al.,* 2010; Whiting *et al*., 2011). Researchers are able to keep potted flowering plants short with hormone treatment (Whiting *et al.,* 2011). Seedless grapes are treated with phytohormones in a similar way to increase the size of the fruit (Whiting *et al.,* 2011).

Phytohormones are synthesised endogenously and allow plants to react sufficiently to external conditions (García de Salamone *et al*., 2006). Although phytohormone's primary function is to convey information between different parts of the plant, not all of them affect the same plant process (Davies, 2004). There are five groups of phytohormones; auxins, gibberellins, cytokinins, abscisic acid and ethylene (García de Salamone *et al*., 2006).

Auxin was the first phytohormone to be discovered (Davies, 2004). Tropism is controlled by auxin and determines the direction in which a plant grows (Whiting *et al.,* 2011). Two types of tropism include geotropism and phototropism (Whiting *et al.,* 2011). Geotropism is under the influence of gravity while phototropism is influenced by light (Whiting *et al*., 2011). Gravity causes auxin to accumulate in the lower side of a horizontal stem, causing the cells to enlarge faster and turning the stem upright (Whiting *et al.,* 2011). Similarly, under the influence of sunlight the auxin concentration on the shaded side stimulates cell elongation resulting in the stem turning towards the sun (Whiting *et al.,* 2011). Produced in the terminal buds, auxin is transported downwards to stimulate root growth (Davies, 2004; Blakeslee *et al*., 2005; Fogaca & Fett-Neto, 2005; Whiting *et al.,* 2005). During the induction phase of the ARC process, higher auxin concentrations are needed and then become inhibitory during the formation phase (Fogaca & Fett-Neto, 2005).

Gibberellin synthesis and its response point is the opposite to that of auxin (Whiting *et al.,* 2011). Gibberellin is synthesised in the roots and its response point is the terminal buds, affecting flowering rather than rooting (Whiting *et al*., 2011). Figure 2.9 shows the transportation routes of auxin and gibberellins respectively.

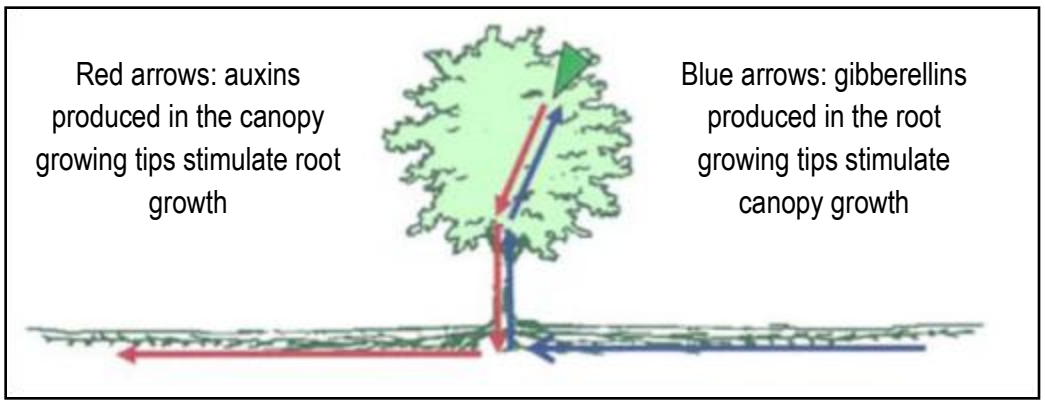


Figure 2.9: Auxin and gibberellin transport routes (Whiting *et al*., 2011).

Cytokinin, together with gibberellins, plays a role in cell division (Whiting *et al*., 2011; Band *et al.,* 2012). Although, according to Pullman and Timmis (1992) exposure to cytokinin over a long period of time has shown to be associated with increased rooting, it generally acts as a negative growth regulator, inhibiting root growth (Werner *et al.,* 2010). However, its production should not be altered because it is required to stimulate shoot growth and its deficiency can inhibit the young shoot to grow (Werner *et al*., 2010).

The remaining two hormones are often regarded as less important but both have vital roles. Abscisic acid is regarded as the stress hormone (Whiting *et al.,* 2010). During times of stress abscisic acid is able to reduce plant growth by inhibiting the effects of the other hormones (Whiting *et al.,* 2010). Ethylene is a gaseous hormone produced in all higher plants (Plant hormones, 2012). Rooting can be stimulated by ethylene because it increases auxin concentration and catabolises cytokinin (Fogaca & Fett-Neto, 2005).

Genetic factors controlling phytohormones have the potential to influence the root system (Werner *et al.,* 2010). Mutations in, or the manipulation of genes controlling phytohormones can have an effect on plant organ size and its growth (Werner *et al*., 2010). The AVP1 gene controls auxin transport and if over expressed can cause shoot and root enhancement (Gonzalez *et al*., 2009). The GA2O-oxidase1 enzyme plays a role in gibberellins production and can result in taller plants with larger leaves (Gonzalez *et al*., 2009). Down regulation of HOG1 gene which is a cytokinin binding protein results in an increase leaf size and seed yield (Gonzalez *et al*., 2009). The over expression of other plant growth regulators also cause larger leaves and flowers (Gonzalez *et al.,* 2009).

2.6.2 Soil and rhizosphere

Soil plays a vital, if not the most important role in root growth and formation (Hinsinger *et al*., 2009). The entire rooting system of plants is found in the soil (Hinsinger *et al*., 2009). Plants acquire most necessities directly from the soil (Morgan *et al*., 2005). The soil inhabitants, including prokaryotes, eukaryotes and invertebrates can influence root formation, function and enhancement (Erturk *et al*., 2010; Ranjard *et al*., 2000; Hinsinger *et al*., 2009).

The majority of Earth's biodiversity is found in soil (Lynch *et al*., 2004; Lambers *et al*., 2009). Biodiversity describes the variety of life in a particular ecosystem (Armstrong *et al*., 1998). It includes the set of species, their genetic material and their environment (Armstrong *et al*., 1998; Lynch *et al.,* 2004). Biodiversity is a function of time (evolution) and space (geographic distribution) (Lynch *et al.,* 2004). The basis of soil research has been on the relationship between biodiversity and function (O' Donnell *et al.*, 2001). To understand this relationship it is important to understand the link between genetic diversity and community structure as well as the relationships between community structure and function (Figure 2.10) (O' Donnell *et al.,* 2001).

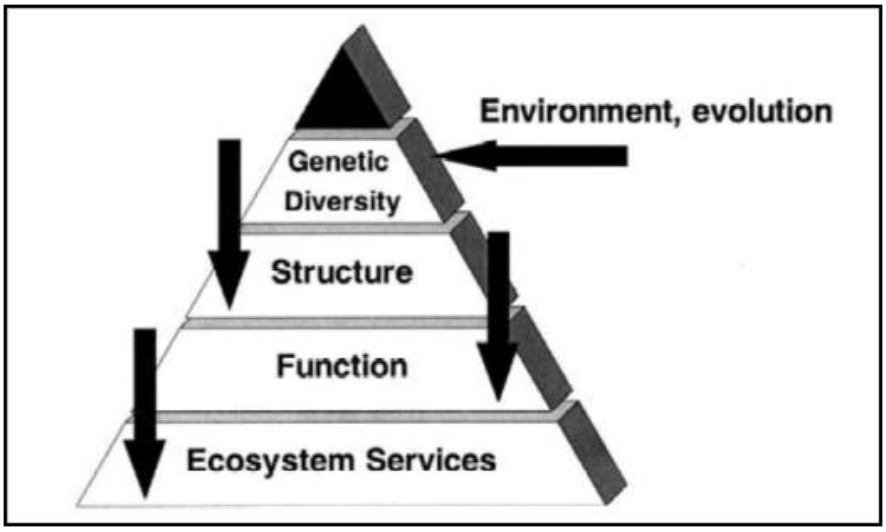


Figure 2.10: Relationship between diversity, function and ecosystem services in soils (O' Donnell *et al.,* 2001).

Bacteria, a domain of prokaryotes play an important part of the soil microflora (Erturk *et al*., 2010). The bacteria are found in abundance and it is estimated that one gram of soil contains more than 5 000 different bacterial strains (Ranjard *et al.,* 2000; Kirk *et al*., 2004). Root formation can be induced through treatment with non-pathogenic bacteria such as some *Bacillus* and *Pseudomonas* species (Pantena *et al.,* 1988; Esitken *et al.,* 2003; Erturk *et al*., 2010). *Bacillus* and *Pseudomonas* have improved rooting in 'Golden Delicious' apples (Patena *et al.,* 1988) and wild cherry softwood and semi-hardwood cuttings (Esitken *et al.,* 2003). Most of these bacteria, including other enhancing bacteria, such as *Agrobacterium rhizogenes,* are believed to be in the rhizospheric environment of the root (Díaz *et al.,* 2009).

The environment in direct contact with the roots is the rhizosphere (Hartmann *et al.,* 2008). The rhizosphere forms a layer between the bulk soil and the plant root (Figure 2.11a) (Buée *et al.,* 2009). Termed by Hiltner (Figure 2.11b) in 1904 the word 'rhizosphere' is derived from the Greek word 'rhiza' meaning root, and 'sphere' meaning field or influence (Morgan *et al*., 2005; Berg & Smalla, 2009: Hartmann *et al.,* 2008). Hiltner's definition focused on the idea that plant nutrition, health and quality is mainly influenced by the microbial composition of the rhizosphere (Berg & Smalla, 2009; Hartmann *et al*., 2008). It is considered that the rhizosphere represents the most dynamic habitat on Earth (Hinsinger *et al*., 2009).

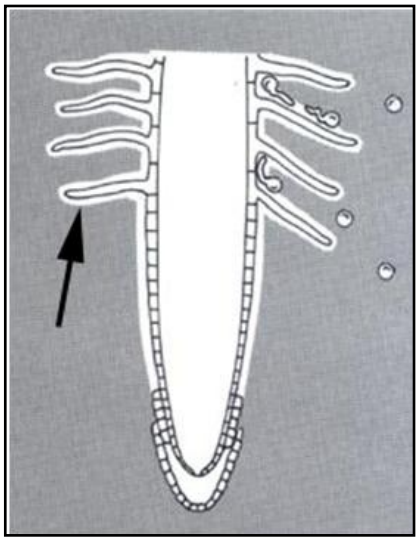
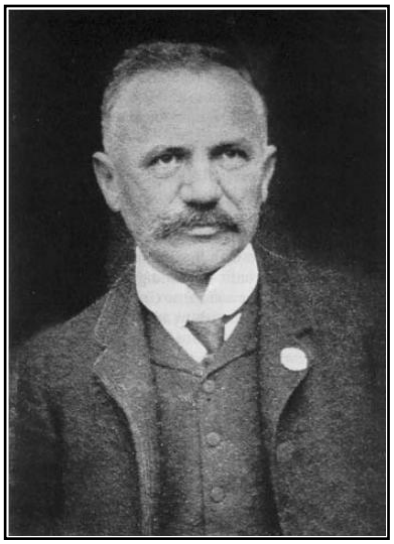
a)  b) 

Figure 2.11: a) Rhizospheric environment in direct contact with the root and surrounding soil (Apsnet, 2012) b) Dr Lorenz Hiltner (Hartmann *et al.,* 2008).

The rhizospheric microbial diversity is made up of prokaryotes, eukaryotes, viruses and fungi (Buée *et al*., 2009). Microbial populations in the rhizosphere are thought to be double those found in the surrounding soil (Buée *et al*., 2009). The plant and associated microorganisms use the rhizosphere as a channel of communication (Bais *et al.,* 2006). The communication channel is maintained through the rhizosphere's own ecosystem (Bais *et al.,* 2006). The rhizosphere's ecosystem comprises of root secretions and associated microorganisms (Bais *et al.,* 2006). The channel is a two way process between the host plant (macrosymbiont) and the microorganism (microsymbiont) (Lambers *et al.,* 2009). The microorganisms release phytohormones beneficial to roots and the carbon compounds given off by roots enhance microbial activity (Morgan *et al.,* 1995).

The rhizosphere has three interacting components; the rhizospheric soil, the rhizoplane, which is the root surface and the root itself (Barea *et al.,* 2005). Rhizodeposition is produced by the root (Buée *et al*., 2009). Carbon compounds that are continuously released from living plant roots make up the rhizodeposition (Buée *et al.,* 2009). The amount and type of carbon released determines different characteristics of the rhizosphere (Lambers *et al.,* 2009). These characteristics involve chemical, physical and biological interactions and make up the rhizosphere ecology (Hinsinger *et al.,* 2009).

Many different interactions occur in the rhizosphere (Barea *et al*., 2005). The interactions can involve dead or living plant material (Barea *et al.,* 2005). Interactions include root-root, root-insect and root-microbe interactions (Bais *et al*., 2006; Buée *et al*., 2009). Many of these interactions are regarded as neutral, but some can be regarded as either negative or positive (Bais *et al.,* 2006; Buée *et al.,* 2009). Negative interactions cause plant diseases and death (Bais *et al.,* 2006; Buée *et al.,* 2009). Positive interactions promote growth and well-being of plants (Bais *et al.,* 2006; Buée *et al.,* 2009). Plant growth-promoting bacteria (PGPB) are responsible for positive interactions (Bais *et al.,* 2006; Buée *et al.,* 2009). PGPB include nitrogen fixers, phosphate solubilisers, phytohormone producers, siderophore synthesisers, mineral uptake enhancers and plant development enhancers (Bashan & Holguin, 2002; Erturk *et al.,* 2010). The mechanisms of PGPB are not fully understood but it is thought that they alter the phytohormone pathways (Erturk *et al*., 2010). One hypothesis is that the PGPB cause production of auxins and inhibit ethylene synthesis (Erturk *et al.,* 2010). The hormonal alteration results in morphological alterations such as root hair development and root elongation (Erturk *et al.,* 2010).

A novel idea is to use rhizospheric microbes to stimulate rooting of forestry species (Díaz *et al*., 2009). Research showed that rhizobacteria (bacteria situated in the rhizosphere) have the ability to increase rooting of *Eucalyptus* stem-cuttings (Barazani & Friedman, 1999; Díaz *et al.,* 2009; Erturk *et al*., 2010). Effects of rhizobacteria stimulation have also been seen in conola (Asghar *et al.,* 2004) and wheat (Khalid *et al.,* 2004) where growth and yield were increased. The rhizobacteria that increase the overall well being and production of plants are referred to as plant growth promoting rhizobacteria (PGPR). The mechanisms by which PGPRs enhance rooting ability are not completely understood. PGPR are defined by three intrinsic characteristics (Barea *et al.,* 2005). The first is the rhizobacteria must be able to colonise the root (Barea *et al.,* 2005). Secondly, rhizobacteria must be able to survive and multiply in microhabitats (Barea *et al.,* 2005). Lastly, rhizobacteria must be able to promote plant growth (Barea *et al,* 2005).

2.7 Microbial diversity of the rhizosphere

Microorganisms form part of the community structure and function in natural ecosystems, such as the rhizosphere (Dubey *et al*., 2005; Malave-Orengo *et al*., 2010; Chowdhury & Dick, 2012). Community structure and diversity are defined by species richness and evenness (Liu *et al.,* 1997). Species richness looks at the number of species in a community (Liu *et al*., 1997). Species evenness refers to the size of species population within a community (Liu *et al*., 1997).

Understanding the functional activity of microorganisms in an ecosystem requires an estimate of the microbial diversity (Malave-Orengo *et al*., 2010). Characterising soil microbial communities is a challenge (Malave-Orengo *et al.,* 2010). Limited methodology makes characterising the vast phenotypic and genotypic diversity of soil microbial communities difficult (Kozdrój & van Elsas, 2000; Malave-Orengo *et al.,* 2010). Many microorganisms are unculturable because the suitable media is unknown (Giraffa & Neviani, 2001). Culture-dependent methods are only able to identify a small fraction (about 5%) of mostly unimportant microorganisms (Cavigelli *et al.,* 1995; Kozdrój & van Elsas, 2000). The development of culture-independent methods has allowed microbial diversity in soil to be determined at different levels (Kozdrój & van Elsas, 2000; Dahllöf, 2002; Andreote *et al.,* 2008).

Biochemical analysis allows a broad-scale characterisation of communities (Cavigelli *et al.,* 1995). Phospholipid fatty acids (PLFA) and fatty acid methyl esters (FAMEs) are two biochemical methods that have the ability to characterise whole micro communities relatively quickly (Cavigelli *et al.,* 1995). The results of both these techniques are analysed using gas chromatography (Cavigelli *et al.,* 1995). Molecular methods allow the microorganisms of natural ecosystems to be analysed on a genetic level (Fantroussi *et al.,* 1999; Giraffa & Neviani, 2001). These molecular methods can detect, identify and characterise microorganisms successfully because all organisms contain genetic material (Fantroussi *et al*., 1999; Giraffa & Neviani, 2001). The genetic make-up, base sequence of deoxyribonucleic acid (DNA) differs between related and non-related species (Dubey *et al.,* 2005).

Polymerase chain reaction (PCR) is a process in which a target sequence of DNA is amplified millions of times (Marschner, 2007), Efficiency, reliability, reproducibility and the ability to give qualitative and quantitative information of different microbial populations are advantages of PCR (Marschner, 2007). The PCR success has led to modifications of the process to further enhance its capabilities (Giraffa & Neviani, 2001; Dubey *et al.,* 2005). These modified processes include real time PCR, Reverse Transcription (RT)-PCR, Touchdown PCR and Nested-PCR (Dubey *et al.,* 2005). Other PCR-based fingerprinting techniques involve terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA), random amplified polymorphic DNA (RAPD), denatured gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP) and ribosomal intergenic spacer analysis (RISA) (Dubey *et al.,* 2005). These methods, which are illustrated in a flow diagram in Figure 2.12, provide relevant information about whole microbial community structures, especially in soil (Dubey *et al.,* 2005).

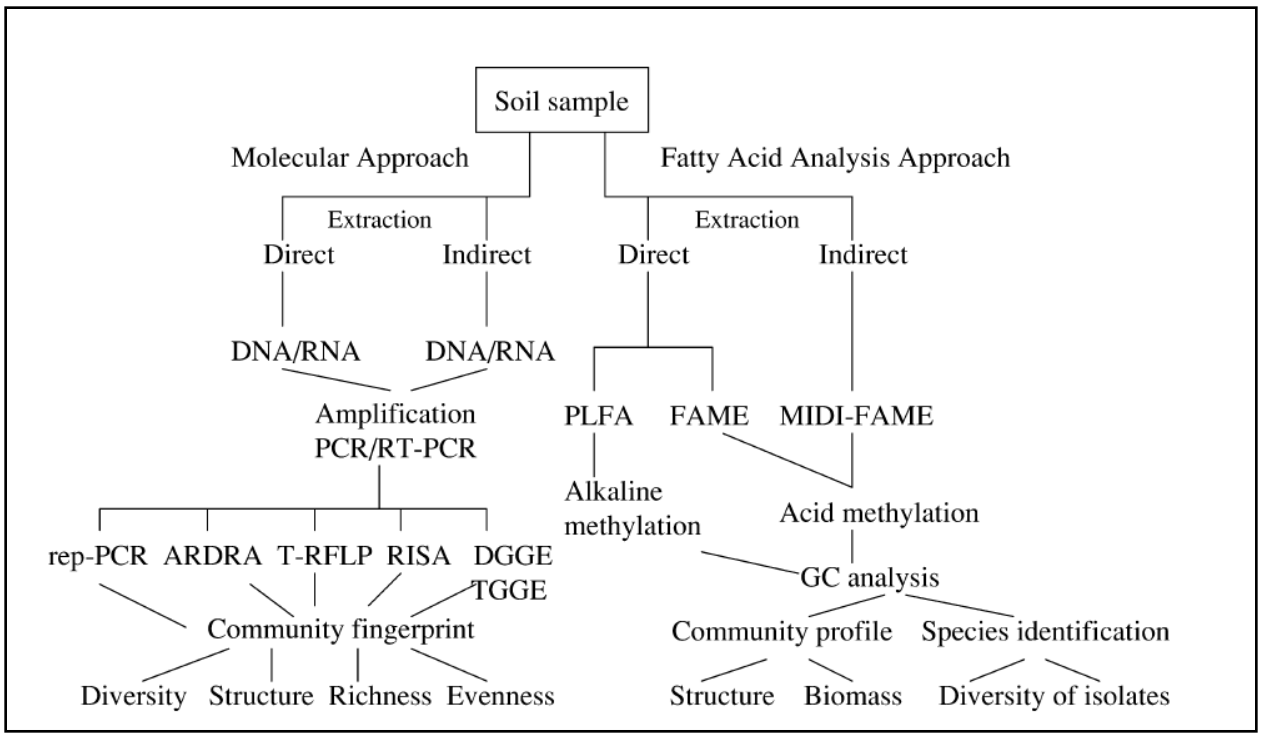


Figure 2.12: Fingerprinting techniques (Kozdrój & van Elsas, 2001).

Chapter 3
Sample collection

3.1 Introduction

The aim of this study was achieved by sampling rhizospheric soil from different *Eucalyptus* hybrids and species. The sample collection process was a once-off process. The samples were obtained in close proximity and time of each other to eliminate as many environmental differences as possible, particularly temperature.

3.2 Site description

The province of KwaZulu-Natal (KZN) in South Africa was the source of sample collection. Pietermaritzburg and its surrounding areas is where many of South Africa's plantation forestry occurs (Pogue, 2008). Rhizospheric samples were obtained from two separate nurseries in the Pietermaritzburg area (illustrated in Figure 3.1). One was a private commercial nursery, Sunshine Seedlings Services where *Eucalyptus* hybrid rhizospheric samples were collected. The other nursery was a research nursery located at the Institute for Commercial Forestry Research (ICFR), where *Eucalyptus* species rhizospheric samples were collected.

a) 

b) 

Figure 3.1: a) Map of South Africa showing the location of sample collection (Malinda Morisson, 2012). b) Close-up satellite image of the nurseries in relation to Pietermaritzburg (Google Maps, 2013).

Sunshine Seedlings Services started in 1982, boasts a production of over 50 million seedlings annually (Sunshine Seedlings Services, 2012). Sunshine Seedlings has expanded to become South Africa's leader in the containerised seedling industry (Sunshine Seedlings Services, 2012). Over the past decade Sunshine Seedlings have been actively involved in *Eucalyptus* clonal forestry (Sunshine Seedlings Services, 2012). They focus on clones of hybrids between *Eucalyptus grandis* × *Eucalyptus nitens* (GN), *Eucalyptus grandis* × *Eucalyptus urophylla* (GU) and *Eucalyptus grandis* × *Eucalyptus camaldulensis* (GC) (Sunshine Seedlings Services, 2012).

The rooting percentage of macro cuttings varies but can be low in some *Eucalyptus* genotypes. Sunshine Seedlings have taken a step away from macro cuttings and moved towards a much younger tip cutting (Sunshine Seedlings Services, 2012). The tip cuttings are taken from younger mother plants, situated inside the tunnels (Sunshine Seedlings Services, 2012). Usage of more juvenile tissue has improved the rooting of these cuttings (Sunshine Seedlings Services, 2012). The plantlets that originate from these micro cuttings show a rooting system very similar to that of seedlings (Sunshine Seedlings Services, 2012). The tunnels found at Sunshine Seedling Services are shown in Figure 3.2.

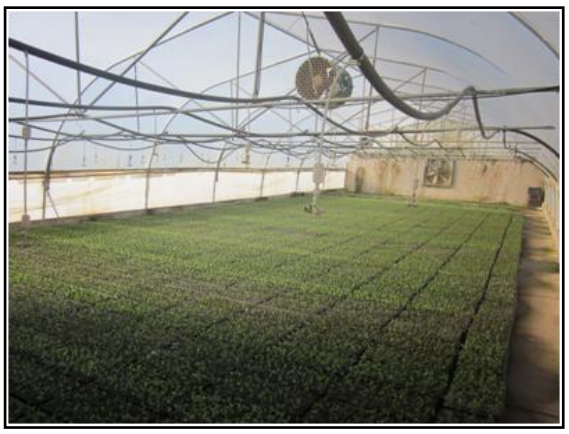
a)  b) 

Figure 3.2: Tunnels situated at Sunshine Seedling Services a) the tunnels from the outside and b) the inside. (Photographs courtesy of M.A Patrick.)

The ICFR, formed in 1947 as the Wattle Research Institute (WRI) is recognised internationally for its forestry research (ICFR, 2012). The ICFR focuses on developing technology and expertise in growing and managing trees in a sustainable, profitable and responsible way (ICFR, 2012). This is done to benefit the forestry industry in South Africa (ICFR, 2012). Research by the ICFR has extended to *Eucalyptus* species with the intention to develop improved material through comprehensive breeding strategies (ICFR, 2012). The *Eucalyptus* species focused on by the ICFR are mostly commercially grown cold tolerant species (ICFR, 2012). These species include *Eucalyptus nitens*, *Eucalyptus macarthurii*, *Eucalyptus dunnii* and *Eucalyptus smithii* (ICFR, 2012). The ICFR *Eucalyptus* species are all grown from seedlings.

3.3 Soil sampling

A total of 62 soil samples were collected in June 2012 from *Eucalyptus* hybrid and species rhizospheres as well as potting soil from each nursery which served as controls. Thirty rhizospheric samples were obtained from Sunshine Seedlings Services *Eucalyptus* cuttings (Figure 3.3). The Sunshine Seedling rhizospheric samples originated from two approximately two year old hybrid genotypes, *E. grandis* × *E. nitens* (GN) and *E. grandis* × *E. urophylla* (GU). Ten rhizospheric samples were taken from the *Eucalyptus* hybrid GU111, and a further 20 rhizospheric samples from two different GN hybrids namely, GN018B and GNPP2107. An additional four Sunshine Seedling control samples were collected. These control samples contained the final potting soil product that had been sprayed to get rid of pathogens before planting cuttings.

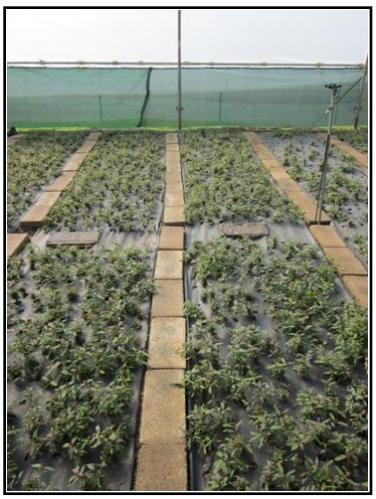
a)  b) 

Figure 3.3: Sunshine Seedlings Services *Eucalyptus* hybrid cuttings from which rhizospheric samples were collected a) *E. grandis* × *E. urophylla* and b) *E. grandis* × *E. nitens.* (Photographs courtesy of M.A Patrick.)

At the ICFR nursery 28 samples were collected. The ICFR rhizospheric samples came from seedlings from three different species; *E. macarthurii* (five rhizospheric samples), *E. smithii* (five rhizospheric samples) and *E. nitens* (four rhizospheric samples). These rhizospheric samples were taken from seedlings planted in bags and were approximately six months old. An additional 10 rhizospheric samples were taken from younger *E. nitens* seedlings (*E. nitens*229), still in trays. The plants from which these rhizospheric samples were collected are illustrated in Figure 3.4. The ICFR control samples contained the final soil before planting seedlings; namely the potting mix from GROMOR (previously known as National Plant Food) from Cato Ridge, KZN.

a)  b)  c)  d) 

Figure 3.4: ICFR *Eucalyptus* species from which rhizospheric samples were collected a) *E. macarthurii* b) *E. smithii* c) *E. nitens* and d) the younger *E. nitens*229. (Photographs courtesy of M.A Patrick.)

Each rhizospheric sample contained approximately 30 g of soil from the area around the rooting system. The *Eucalyptus* hybrids and species were identified for sampling and the rhizospheric soil of these plants was collected and placed in glass bottles to prevent any fatty acid contamination. A unique number was assigned to each sample. Each sample number started with its nursery of origin, the Sunshine Seedling samples with 'SS' and the ICFR samples with 'ICFR'. Samples were further numbered numerically in order of sampling. A 'C' was also included in the sample number of the controls. Table 3.1 illustrates the different aspects of the sample collection including the sample number, collection date, nursery and tree origin as well as whether the sample was obtained from a seedling or cutting. After the samples were collected they were placed on ice to ensure the microbial population did not change during transportation. A portion of each sample was transferred to a sterile falcon tube and stored at -20°C for preservation and DNA analysis.

Table 3.1: Specifications of sample collection.

Sample number	Nursery origin	Tree origin	Seedlings / cuttings
SS1	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS2	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS3	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS4	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS5	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS6	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS7	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS8	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS9	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS10	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GN018B	Cutting (clone)
SS11	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS12	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS13	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS14	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS15	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS16	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS17	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS18	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS19	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS20	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GNPP2107	Cutting (clone)
SS21	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS22	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)

Sample number	Nursery origin	Tree origin	Seedlings / cuttings
SS23	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS24	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS25	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS26	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS27	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS28	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS29	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS30	Sunshine Seedlings Nursery	*Eucalyptus* hybrid GU111	Cutting (clone)
SS C1	Sunshine Seedlings Nursery	Potting soil (control)	N/A
SS C2	Sunshine Seedlings Nursery	Potting soil (control)	N/A
SS C3	Sunshine Seedlings Nursery	Potting soil (control)	N/A
SS C4	Sunshine Seedlings Nursery	Potting soil (control)	N/A
ICFR32	ICFR	*E. macarthurii*	Seedling
ICFR33	ICFR	*E. macarthurii*	Seedling
ICFR34	ICFR	*E. macarthurii*	Seedling
ICFR35	ICFR	*E. macarthurii*	Seedling
ICFR36	ICFR	*E. macarthurii*	Seedling
ICFR37	ICFR	*E. smithii*	Seedling
ICFR38	ICFR	*E. smithii*	Seedling
ICFR39	ICFR	*E. smithii*	Seedling
ICFR40	ICFR	*E. smithii*	Seedling
ICFR41	ICFR	*E. smithii*	Seedling
ICFR42	ICFR	*E. nitens*	Seedling

Sample number	Nursery origin	Tree origin	Seedlings / cuttings
ICFR43	ICFR	*E. nitens*	Seedling
ICFR44	ICFR	*E. nitens*	Seedling
ICFR45	ICFR	*E. nitens*	Seedling
ICFR46	ICFR	*E. nitens229*	Seedling
ICFR47	ICFR	*E. nitens229*	Seedling
ICFR48	ICFR	*E. nitens229*	Seedling
ICFR49	ICFR	*E. nitens229*	Seedling
ICFR50	ICFR	*E. nitens229*	Seedling
ICFR51	ICFR	*E. nitens229*	Seedling
ICFR52	ICFR	*E. nitens229*	Seedling
ICFR53	ICFR	*E. nitens229*	Seedling
ICFR54	ICFR	*E. nitens229*	Seedling
ICFR55	ICFR	*E. nitens229*	Seedling
ICFR C1	ICFR	Potting soil (control)	N/A
ICFR C2	ICFR	Potting soil (control)	N/A
ICFR C3	ICFR	Potting soil (control)	N/A
ICFR C4	ICFR	Potting soil (control)	N/A

Chapter 4

Broad characterisation of *Eucalyptus* rhizospheric communities using fatty acid methyl ester analysis

4.1 Introduction

Analysis of whole-soil fatty acid methyl esters (FAMEs) was used as a tool for broad taxonomic characterisation of the rhizospheric microbial communities based on the average fatty acid composition of microbial membranes. Fatty acids are important building blocks of cellular material (Kaneda, 1999; Ichihara & Fukubayashi, 2010). The cell membrane is made up of lipids whose physical, chemical and physiological properties are determined by fatty acids (Kaneda, 1999; Ichihara & Fukubayashi, 2010). Fatty acids influence fluidity, integrity and permeability of cell membranes as well as the activities of membrane-bound enzymes (Eder, 1995; Kaneda, 1999; Islam *et al.,* 2009). Classification of fatty acids is based on their biosynthetic relationships together with the mechanisms by which fluidity is controlled (Kaneda, 1999).

A fatty acid is made up of carboxylic acids with long hydrocarbon chains (Mallory & Sayler, 1984; Virtual Chembook, 2012), illustrated in Figure 4.1a. Although fatty acids are similar in chemical nature, they are a diverse group of compounds (Mallory & Sayler, 1984; Virtual Chembook, 2012). The number of carbons that form part of the hydrocarbon chain vary between fatty acids (Eder, 1995; Virtual Chembook, 2012). Naturally occurring fatty acids have chain lengths containing anything from four to 24 carbons, but are normally between 11 and 19 (Eder, 1995; Virtual Chembook, 2012). Fatty acids form part of either the straight-chain fatty acid family or the branched-chain fatty acid family (Kaneda, 1999). The straight-chain fatty acids, also known as saturated fatty acids, contain no double bonds in their structure (Virtual Chembook, 2012). Branched-chain fatty acids (unsaturated fatty acids) on the other hand, contain double bonds in their structure (Virtual Chembook, 2012) (Figure 4.1b). Saturated fatty acids have a lower melting point than unsaturated fatty acids because their straighter structure allows them to stack closer together (Virtual Chembook, 2012). In contrast, the double bonds in unsaturated fatty acids cause bends which prevent close interactions (Virtual Chembook, 2012).

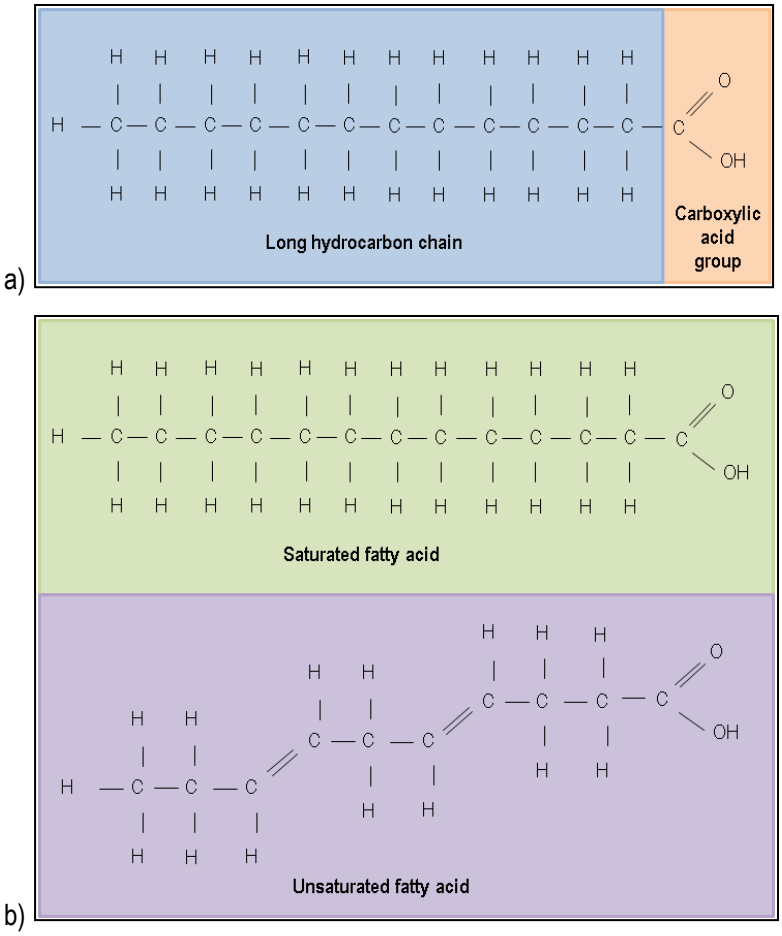


Figure 4.1: a) Essential features of fatty acids and b) saturated and unsaturated fatty acid structures.

Unsaturated fatty acids can either be in *cis* or *trans* configuration (Kaneda, 1999; Virtual Chembook, 2012). The *cis* configuration of unsaturated fatty acids is more common in nature (Kaneda, 1999; Virtual Chembook, 2012). When adjacent hydrogen atoms are on the same side as the double bond, the fatty acid is said to be in the *cis* configuration (Kaneda, 1999; Virtual Chembook, 2012). The *cis* configuration causes the chain to bend thus; the more double bonds present in *cis* configuration results in a reduction in flexibility (Kaneda, 1999; Virtual Chembook, 2012). The *trans* configuration differs from the *cis* configuration in that the two hydrogen atoms in the double bond are bound at the opposite sides of the double bond (Kaneda, 1999; Virtual Chembook, 2012). Therefore, chains in a *trans* configuration contain no bends (Kaneda, 1999; Virtual Chembook, 2012). It is unlikely that fatty acids with a *trans* configuration are formed naturally (Kaneda, 1999; Virtual Chembook, 2012).

Fatty acids are named in a specific manner (Cavigelli *et al.,* 1995). Examples of naming fatty acids include C12:0, C14:1, C18:2*cis*9*cis*12 and C18:1at9. A "C" at the beginning of a fatty acid name represents carbon atoms (Cavigelli *et al.,* 1995). The number of carbon atoms in the fatty acid is indicated by the number after the "C" and before the colon (Cavigelli *et al.,* 1995). The colon is an indicator of a double bond, which is followed by a number that indicates the number of double bonds (Cavigelli *et al.,* 1995). In the case of unsaturated fatty acids, *cis* or *trans* follows the number of double bonds indicating its configuration (Cavigelli *et al.,* 1995). Numbers that follow the configuration represent the position of double bonds relative to the carboxyl end of the molecule (Cavigelli *et al.,* 1995). If the configuration is unknown "at" is used instead of *cis* or *trans* (Cavigelli *et al.,* 1995).

Fatty acids extracted from soils are mainly from microorganisms, but can also include those from plant residues and other soil insects (Marschner, 2007). FAME analysis is able to detect fatty acids from living and non-living microorganisms (Marschner, 2007). However most FAMEs are derived from living microorganisms as the fatty acids of non-living biomass are rapidly decomposed in soil (Marschner, 2007).

Each microorganism has a unique fatty acid profile (Cavigelli *et al.,* 1995; O' Donnell *et al*., 2001). These fatty acid profiles are made up of different fatty acid combinations (Cavigelli *et al.,* 1995; O' Donnell *et al.,* 2001). Therefore, microbial communities are a mixture of different fatty acid combinations (Cavigelli *et al.,* 1995; O' Donnell, *et al.,* 2001). A change in a community's fatty acid profile indicates a change in the microbial composition of the community (Glucksman *et al*., 2000; Kirk *et al*., 2004).

Studying fatty acid profiles is becoming a popular method of characterising soil microbial communities (Marscher, 2007). Cavigelli *et al.* (1995) undertook *in situ* assessments of the spatial distributions of soil microbial communities using FAME analyses to better understand the patterns of microbial diversity. Results indicated that microbial communities of soil samples obtained from *Zea mays L.* located in SW Michigan, in the northern part of the U.S.A. Corn Belt were similar (Cavigelli *et al.,* 1995). In another study, FAME analyses was carried out to determine the differences in soil microbial communities of grass/legume pastures that were induced by an increase in salinity and alkalinity in South Australia (Pankhurst *et al.,* 2001). These results showed a shift towards a bacterial dominated microbial community (Pankhurst *et al.,* 2001). More recently, in 2009, Islam *et al.* attempted to observe the long term effects of the application of certain fertilisers on soil microbial communities of a rice-based cropping system in the Republic of Korea.

These results showed that the compost amendments promoted the abundance of Gram-positive bacterial FAMEs (Islam *et al.,* 2009). Although the significance of this is not yet fully understood FAME analyses successfully determined that there had been a shift in the microbial community (Islam *et al.,* 2009).

4.2 Principle of FAME analysis

The successful analysis of fatty acids requires, firstly, their removal from lipids in the microbial cellular membranes (Keneda, 1999; Carrapiso & Garcia, 2000). There are four steps to successfully remove fatty acids from these lipids (Sasser, 2009). These steps include saponification, methylation, extraction and sample cleanup (Sasser, 2009). During the saponification process the saponifiable lipids, which are normally triacylglycerols and phospholipids are derivatised producing esters (Chowdhury & Dick, 2012). In the methylation step, esters are heated together with alkali producing methyl ester derivatives, which are the FAMEs (Chowdhury & Dick, 2012). The methylation process ensures the FAMEs are able to be readily volatised (Chowdhury & Dick, 2012). A base can also be used as a catalyst during methylation (Carrapiso & Garcia, 2000). Usage of a base speeds up the process and only requires mild heating temperatures (Carrapiso & Garcia, 2000). Equation 1 and 2 show the derviatisation using an acid and base respectively (Carrapiso & Garcia, 2000):

Acid: $R'\text{-}CO\text{-}CH + CH_3\text{-}OH \overset{H^+}{\leftrightarrow} R'\text{-}CO\text{-}OCH_3 + HOH$ (1)

Base: $R'\text{-}CO\text{-}OH + CH_2N_2 \rightarrow R'\text{-}CO\text{-}OCH_3 + N_2$ (2)

In the third step, FAMEs are then extracted into an organic phase and quantified by means of gas chromatography (GC) (O' Donell *et al.,* 2001; Chowdhury & Dick, 2012). In the final sample clean-up step, sodium hydroxide dissolved in distilled water is added to the extract to prevent contamination during the GC process (O' Donell *et al.,* 2001; Chowdhury & Dick, 2012). The GC produces chromatograms (series of peaks) which reveal the FAME characteristics of the particular microbial community (O' Donell *et al.,* 2001; Chowdhury & Dick, 2012). A typical chromatogram is made up of a number of peaks, as shown in Figure 4.2 (Grob & Barry, 2004). For qualitative analysis, the retention time is used, which is the time taken from the point of injection until the peak maximum (Kaneda, 1999; Grob & Barry, 2004). FAMEs can be identified by comparing their retention times to those of known commercial standards (Eder, 1995). The peak area

determines peak concentration and therefore, shows the amount of fatty acid present in the sample (Eder, 1995; Grob & Barry, 2004).

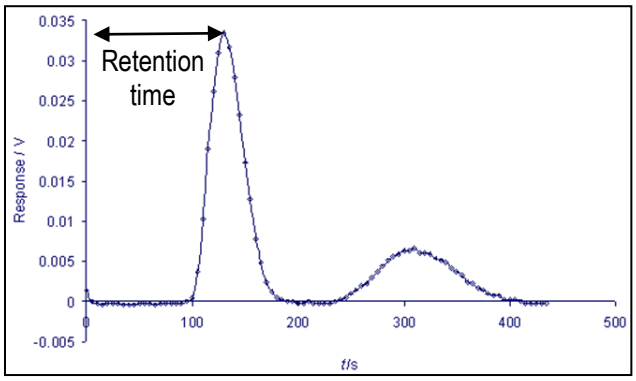


Figure 4.2: Example of a chromatograph (AnalChemVoc, 2012).

4.3 Materials and methods

4.3.1 Pooling of samples

Collected rhizospheric soil samples from the same *Eucalyptus* species and hybrids as well as the respective potting soils used as controls, were pooled together in such a way that a whole-soil FAME profile from each *Eucalyptus* species, hybrid and control could be obtained (Table 4.1). This resulted in a total of nine pooled samples ranging from 20 to 25 g of soil. The nine pooled samples represented nine specific microbial communities originating from four *Eucalyptus* hybrid and three *Eucalyptus* species rhizospheric samples, as well as two control soil samples.

Table 4.1: Origin, soil sample and collective name for pooled samples representing specific communities subjected to FAME analysis.

Nursery origin	Source soil samples	Collective name for pooled sample
Sunshine Seedlings	SS2; SS4; SS6; SS8; SS10	*Eucalyptus* hybrid GN018B
Sunshine Seedlings	SS12; SS14; SS16; SS18; SS20	*Eucalyptus* hybrid GNPP2107
Sunshine Seedlings	SS22; SS24; SS26; SS28; SS30	*Eucalyptus* hybrid GU111
Sunshine Seedlings	SS C1; SS C2; SS C3; SS C4	Control: Sunshine Seedlings potting soil
ICFR	ICFR32; ICFR33; ICFR34; ICFR35; ICFR36	*E. macarthurii*
ICFR	ICFR37; ICFR38; ICFR39; ICFR40; ICFR41	*E. smithii*
ICFR	ICFR42; ICFR43; ICFR44; ICFR45	*E. nitens*
ICFR	ICFR46; ICFR48; ICFR50; ICFR52; ICFR54	*E. nitens229*
ICFR	ICFR C1; ICFR C2; ICFR C3; ICFR C4	Control: ICFR potting soil

4.3.2 Generation of FAME profiles

FAME analyses were performed on the nine pooled samples by SGS laboratories, Cape Town, South Africa. The AOCS Official Method Ce2-66 was used to prepare fatty acids for gas chromatography. The AOCS Official Method Ce2-66 protocol is confidential but follows the general four-step procedure of FAME preparation which includes saponification, methylation, extraction and sample clean-up. FAMEs were then analysed by gas chromatography using the Agilent GC, model 7890A (Agilent Technologies). FAME profiles were produced for each sample representing a specific community. The peak areas of the FAME profiles were recorded by ChemStation (Hewlett Packard).

4.3.3 Determination of significant difference among and between FAME profiles

Pearson Chi Square Tests were performed to determine whether the difference among and between *Eucalyptus* hybrids and *Eucalyptus* species rhizospheric communities were of any significance. Three

Pearson Chi Square statistics were calculated using the peak areas of the saturated fatty acids at a significance level of α = 0.05 using the formula shown in Figure 4.3.

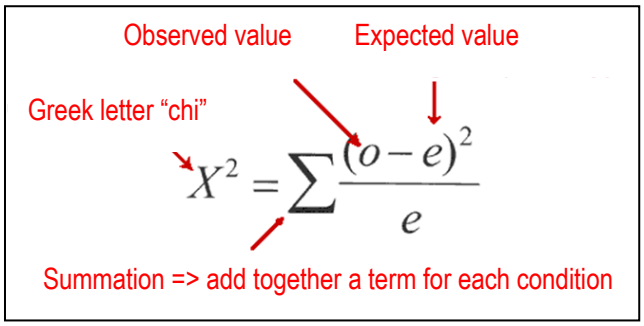


Figure 4.3: Chi Square Test equation (Chi Square Test, 2013)

The Pearson Chi Square results were tested under the following hypotheses:

1. For comparison among *Eucalyptus* hybrid rhizospheric communities:
   H_o: there is no difference in fatty acid composition among *Eucalyptus* hybrid rhizospheric communities
   H_a: there is a difference in fatty acid composition among *Eucalyptus* hybrid rhizospheric communities
   with 10 values being able to vary independently (df = 10).

2. For comparison among *Eucalyptus* species rhizospheric communities:
   H_o: there is no difference in fatty acid composition among *Eucalyptus* species rhizospheric communities
   H_a: there is a difference in fatty acid composition among *Eucalyptus* species rhizospheric communities
   with 15 values being able to vary independently (df = 15).

3. For comparison between *Eucalyptus* hybrid and species rhizospheric communities:
   H_o: there is no difference in fatty acid composition between *Eucalyptus* hybrid and species rhizospheric communities
   H_a: there is a difference in fatty acid composition between *Eucalyptus* hybrid and species rhizospheric communities
   with 30 values being able to vary independently (df = 30).
   .

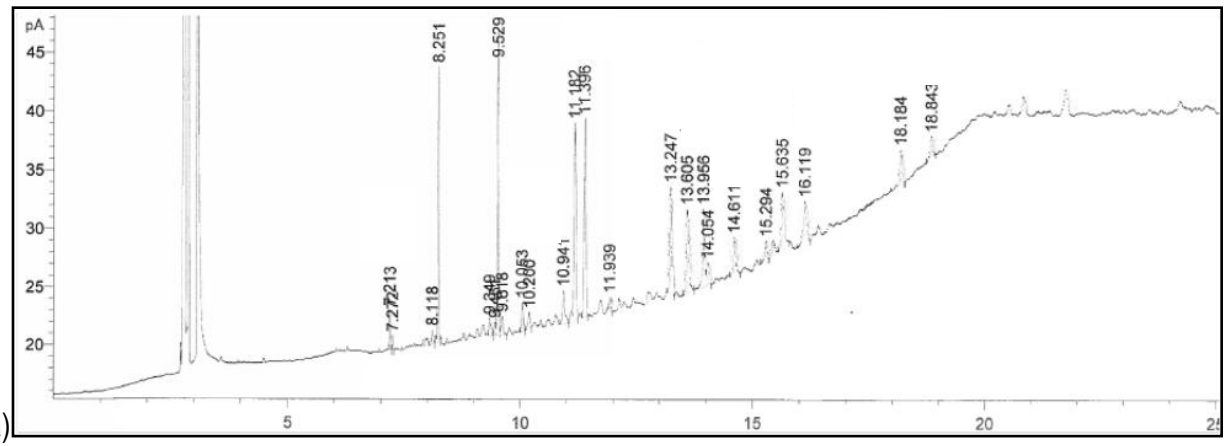
4.4 Results

4.4.1 FAME profiles

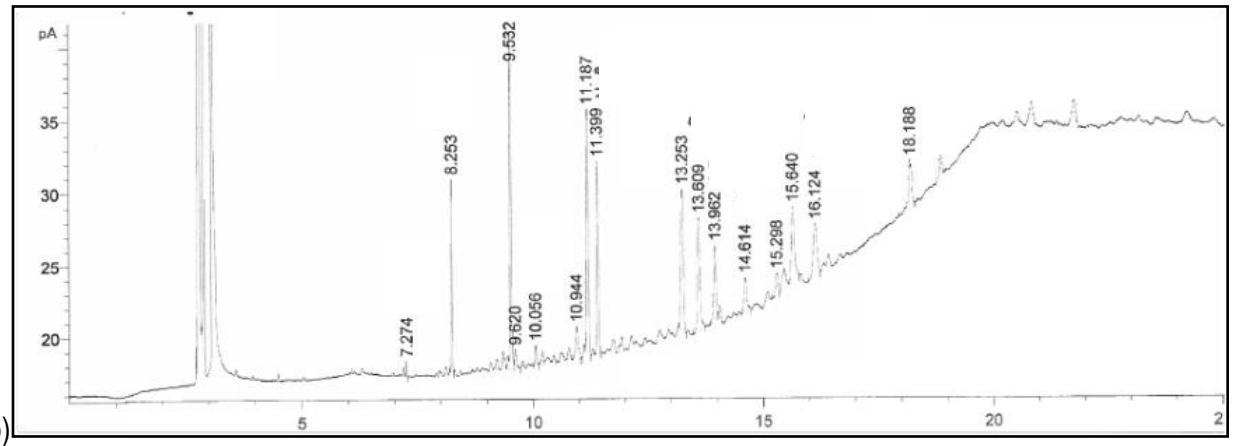
Gas chromatograms (FAME profiles) showed the overall fatty acid composition of each sample. The rhizospheric samples from the *Eucalyptus* hybrids GN018B, GNPP2107 and GU111 demonstrated similar FAME profiles (Figure 4.4). In contrast, the FAME profile of the *Eucalyptus* hybrid control had fewer peaks and therefore less fatty acids than the *Eucalyptus* hybrid samples. All FAME profiles had retention times within 22 minutes.

The rhizospheric soils from the *Eucalyptus* species, *E. macarthurii*, *E. smithii*, and *E. nitens229*, as well as the *Eucalyptus* species control showed similar FAME profiles; with the exception of *E. nitens* whose FAME profile had fewer peaks and therefore fewer fatty acids (Figure 4.5). When compared to the *Eucalyptus* hybrid rhizospheric communities, the *Eucalyptus* species rhizospheric communities FAME profiles were more variable with notably more FAMEs.

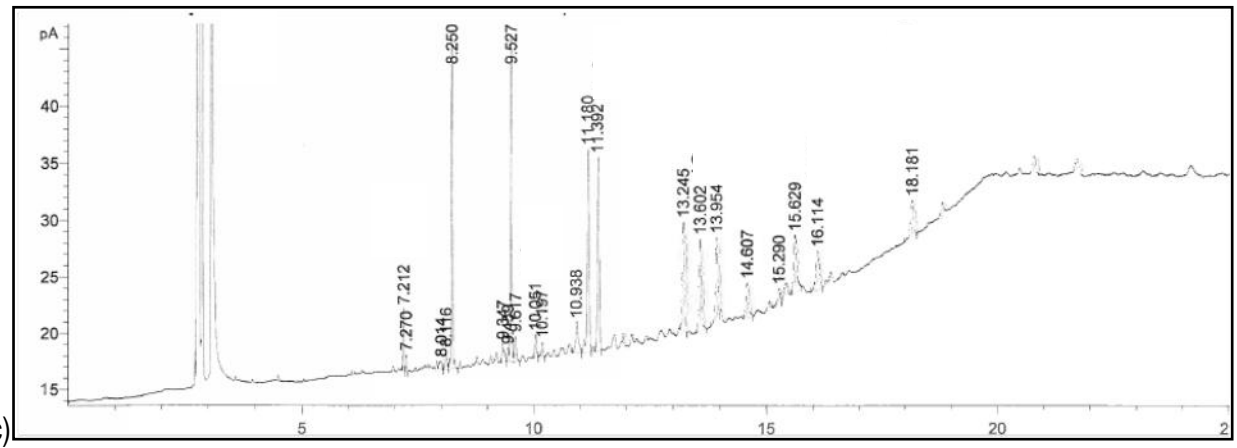
a)


pA
45
40
35
30
25
20
8.251
9.529
11.182
11.396
13.247
13.605
13.956
14.611
15.294
15.635
16.119
18.184
18.843
14.054
11.939
10.941
8.118
7.213
5
10
15
20

b)


pA
35
30
25
20
9.532
11.187
11.399
8.253
13.253
13.609
13.962
14.614
15.298
15.640
16.124
18.188
7.274
9.620
10.056
10.944
5
10
15
20

c)


pA
40
35
30
25
20
15
8.250
9.527
11.180
11.392
13.245
13.602
13.954
14.607
15.290
15.629
16.114
18.181
7.270
7.212
10.938
5
10
15
20

d)

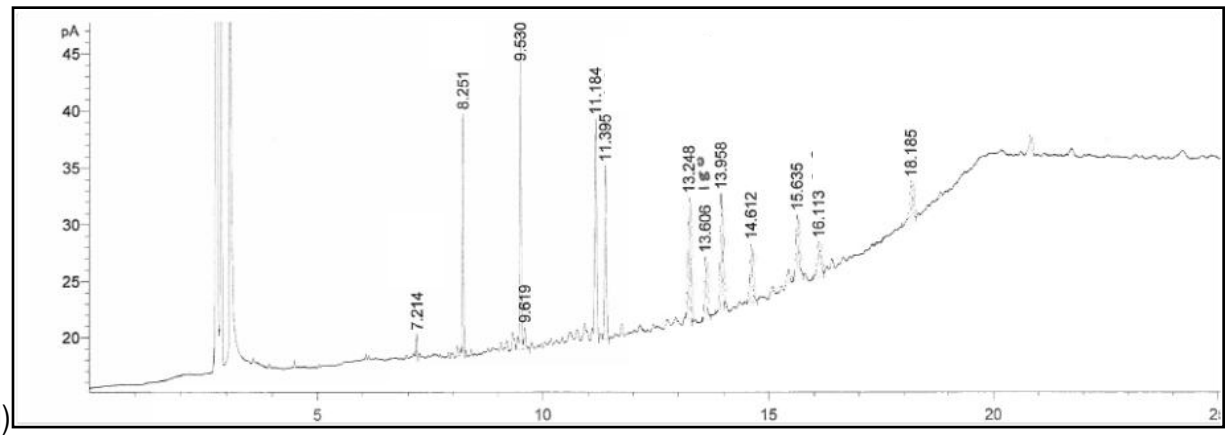
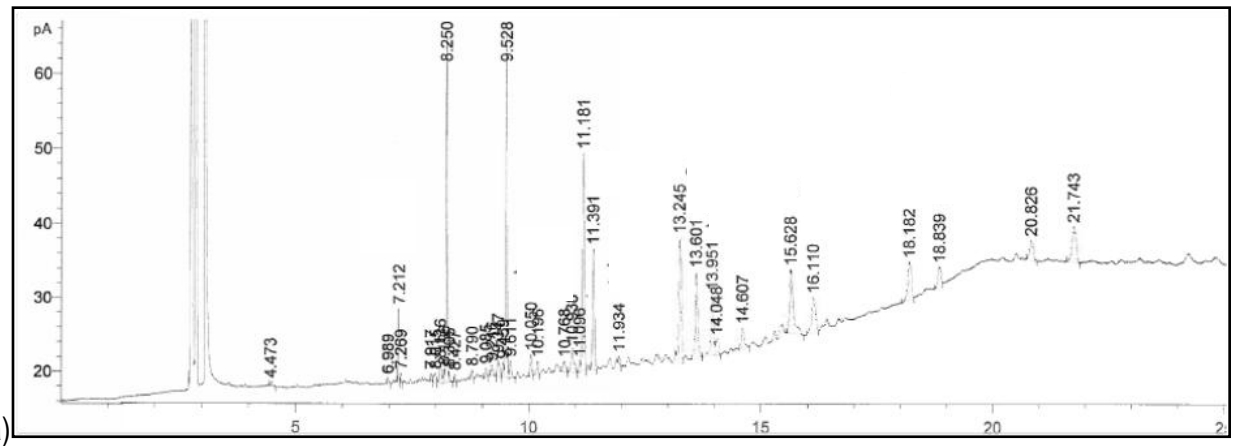
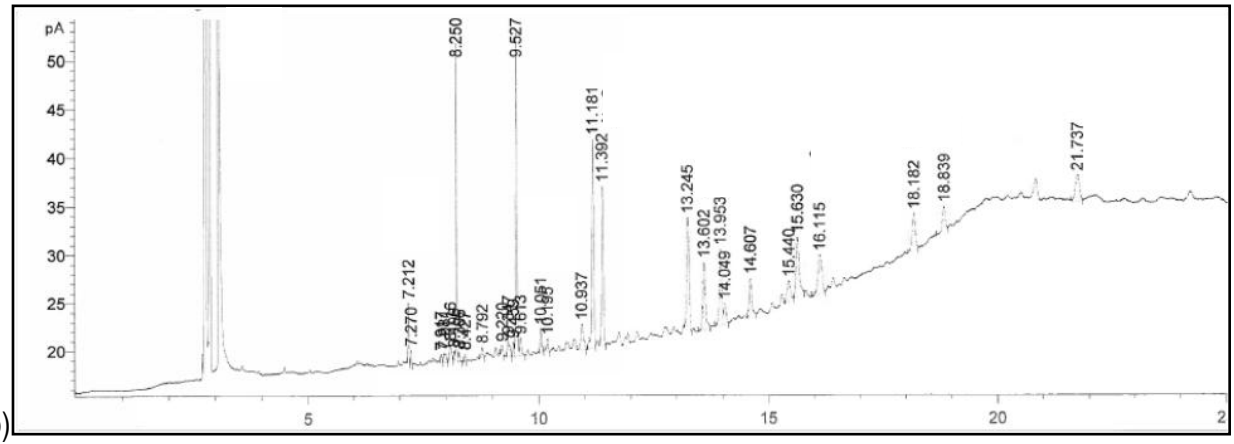


Figure 4.4: FAME profiles of the Eucalyptus hybrids a) GN018B, b) GNPP2107, c) GU111 and d) control

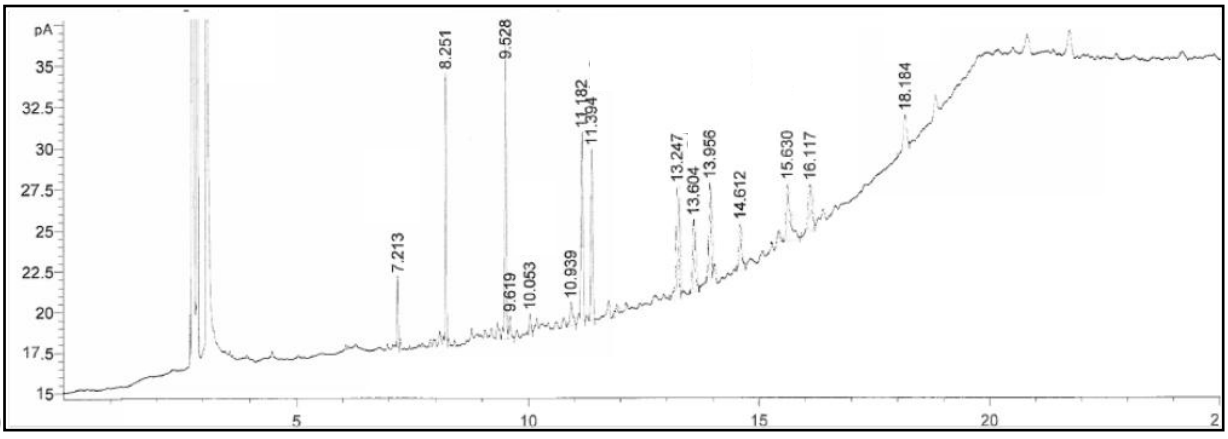
a)


pA
8.250
9.528
11.181
11.391
13.245
13.601
13.951
14.048
14.607
15.628
16.110
18.182
18.839
20.826
21.743
7.212
4.473
6.989
7.269
8.790
10.050
10.768
11.098
11.934

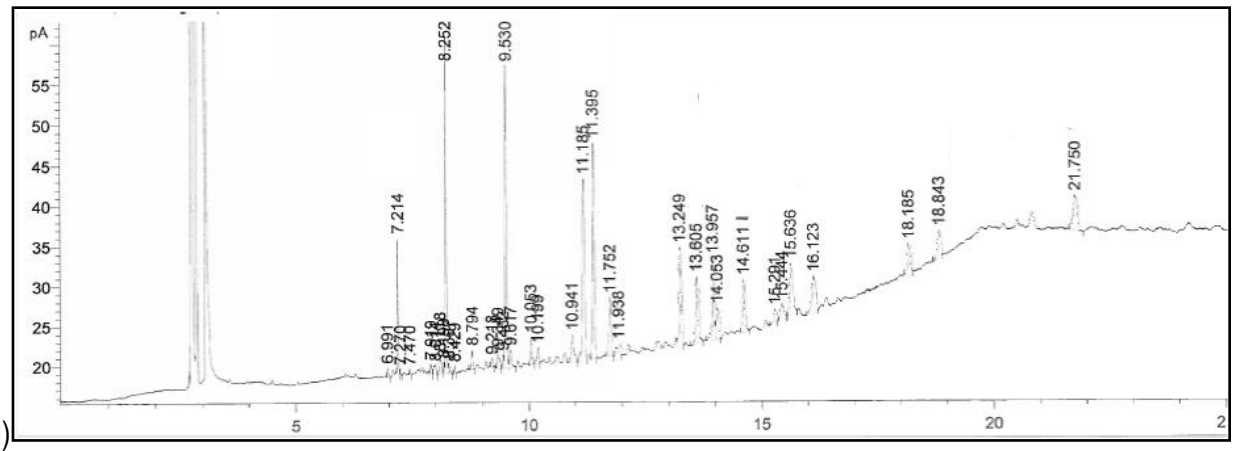
b)


pA
8.250
9.527
11.181
11.392
13.245
13.602
13.953
14.049
14.607
15.440
15.630
16.115
18.182
18.839
21.737
7.212
7.270
8.792
10.937

c)


pA
8.251
9.528
11.182
11.394
13.247
13.604
13.956
14.612
15.630
16.117
18.184
7.213
9.619
10.053
10.939

d)



e)

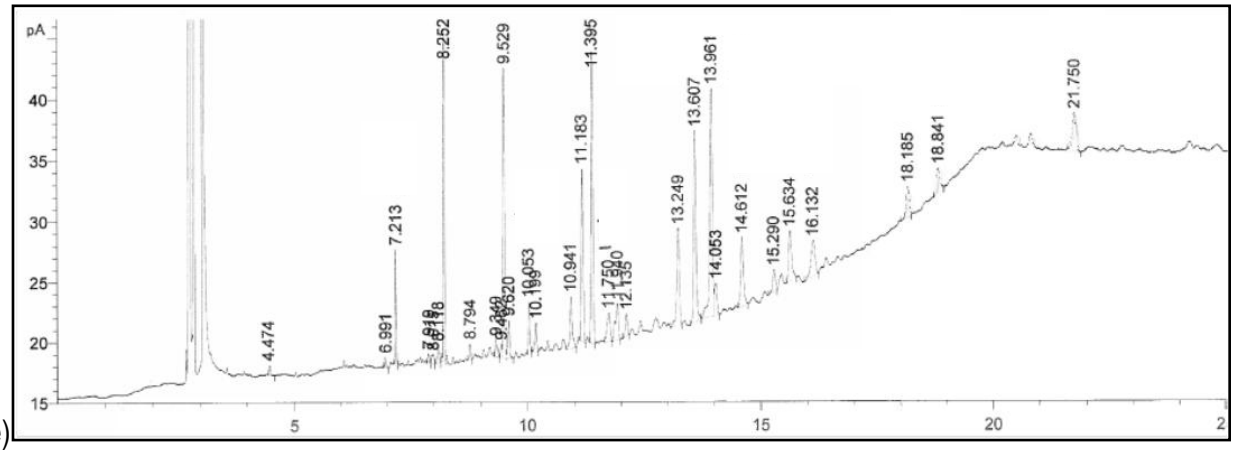


Figure 4.5: FAME profiles of the *Eucalyptus* species a) *E. macarthurii*, b) *E. smithii*, c) *E. nitens*, d) *E. nitens229* and e) control.

A total of 18 different fatty acids were detected from the *Eucalyptus* hybrid and species rhizospheric communities, of which 13 could be identified. The concentrations of the different fatty acids were calculated from the peak areas of the FAME profiles and expressed as a percentage. All 13 identified fatty acids had an even number of carbon atoms (indicated by the number before the colon in the abbreviation) ranging from 10 to 24 carbons (Table 4.2). Eight of these 13 fatty acids did not contain double bonds (indicated by the number after the colon in the abbreviation) in their structure and were therefore saturated. Four fatty acids had a single double bond, whereas one fatty acid, eicosatrienoic acid (C20:3*cis*11*cis*14*cis*17), had three double bonds in its structure. Sixteen of the detected fatty acids were found in soil communities from both *Eucalyptus* hybrids and species. The remaining two fatty acids, palmitoleic acid (C16:1) and lignoceric acid (C24:0) were only detected in some of the *Eucalyptus* species rhizospheric communities and in none of the *Eucalyptus* hybrid rhizospheric communities. Although all the communities contained most of the detected fatty acids, only the *Eucalyptus* species control contained all 18 detected fatty acids. The *Eucalyptus* hybrid control contained 13 of the detected fatty acids; this was also the community with the least number of detected fatty acids.

Table 4.2: Fatty acid percentage in each sample representing a specific community.

Fatty acid abbreviation	Fatty acid name	*Eucalyptus* hybrid communities				*Eucalyptus* species communities				
		GN 018B	GN PP2107	GU 111	C	*E. macarthurii*	*E. smithii*	*E. nitens*	*E. nitens 229*	C
C10:0	Capric acid	1.1	0.4	1.8	0.7	1.9	1.6	2.3	3.3	2.2
C12:0	Lauric acid	9.6	6.7	12.2	9.3	10.9	10.4	10.2	10.3	7.9
C14:0	Myristic acid	13.7	15.7	14.6	16.9	14.0	14.0	15.3	11.6	8.9
C14:1	Myristoleic acid	1.4	1.2	1.1	0.0	0.92	1.1	1.0	1.1	1.6
U1	Unknown 1	2.5	2.8	2.5	1.5	0.0	2.1	2.3	1.9	2.4
U2	Unknown 2	12.2	14.0	12.6	14.2	11.5	12.0	11.9	9.6	6.9
C16:0	Palmitic acid	11.3	11.0	11.1	11.4	6.3	8.6	10.9	10.5	11.2
C16:1	Palmitoleic acid	0.0	0.0	0.0	0.0	0.34	0.0	0.0	3.4	1.9
U3	Unknown 3	7.7	10.2	7.9	10.0	7.7	7.7	9.5	6.0	5.2
C18:0	Steric acid	6.5	8.8	7.0	5.4	5.9	5.0	6.1	4.4	9.8
C18:1 *cis*9	Oleic acid	8.0	5.3	5.7	9.2	5.8	6.8	7.2	7.5	14.6
C18:2 *cis*6	Linoleic acid	3.1	3.0	3.0	5.4	1.7	2.9	4.1	3.5	3.9
U4	Unknown 4	1.6	1.7	1.2	6.7	0.0	0.0	0.0	1.4	1.2
U5	Unknown 5	5.1	7.0	5.2	0.0	6.6	5.2	7.9	4.0	3.1
C20:0	Arachidic acid	5.1	6.2	4.4	4.5	3.9	4.5	6.1	3.7	3.2
C20:3 *cis*11*cis*14*cis*17	Eicosatrienoic acid	3.5	5.0	3.5	4.8	3.6	3.5	3.8	2.4	1.9
C22:0	Behenic acid	1.9	0.0	0.0	0.0	1.6	2.1	0.0	2.1	1.8
C24:0	Lignoceric acid	0.0	0.0	0.0	0.0	4.0	3.0	0.0	3.7	3.3

C = control

The fatty acid complements of the *Eucalyptus* hybrid and species rhizospheric samples (including their respective controls) were for the most very similar. Of the 18 detected fatty acids, the saturated even group was the most abundant. The *Eucalyptus* species rhizospheric communities revealed an additional saturated even fatty acid, which was not present in the *Eucalyptus* hybrid rhizospheric samples (marked in bold in Table 4.3). Similarly, the *Eucalyptus* species rhizospheric communities also had an additional monosaturated fatty acid, when compared to the *Eucalyptus* hybrid rhizospheric communities (marked in bold in Table 4.3).

Table 4.3: Fatty acids found in *Eucalyptus* hybrid and species communities respectively.

***Eucalyptus* hybrid communities and control**		***Eucalyptus* species communities and control**	
Saturated even	**Monosaturated**	**Saturated even**	**Monosaturated**
C10:0	C14:1	C10:0	C14:1
C12:0	C18:1*cis*9	C12:0	**C16:1**
C14:0	C18:2*cis*6	C14:0	C18:1*cis*9
C16:0	C20:3*cis*11*cis*14*cis*17	C16:0	C18:2*cis*6
C18:0		C18:0	C20:3*cis*11*cis*14*cis*17
C20:0		C20:0	
C22:0		C22:0	
		C24:0	

The overall total mean percentage of each detected fatty acid was calculated. These means varied from less than 1% to approximately 14%. Myristic acid (C14:0) was the most abundant fatty acid with the highest percentage (Figure 4.6). Other fatty acids with high percentages included lauric acid (C12:0), palmitic acid (C16:0) and an unknown fatty acid (U2). The fatty acid with the lowest overall mean percentage was palmitoleic acid (C16:1).

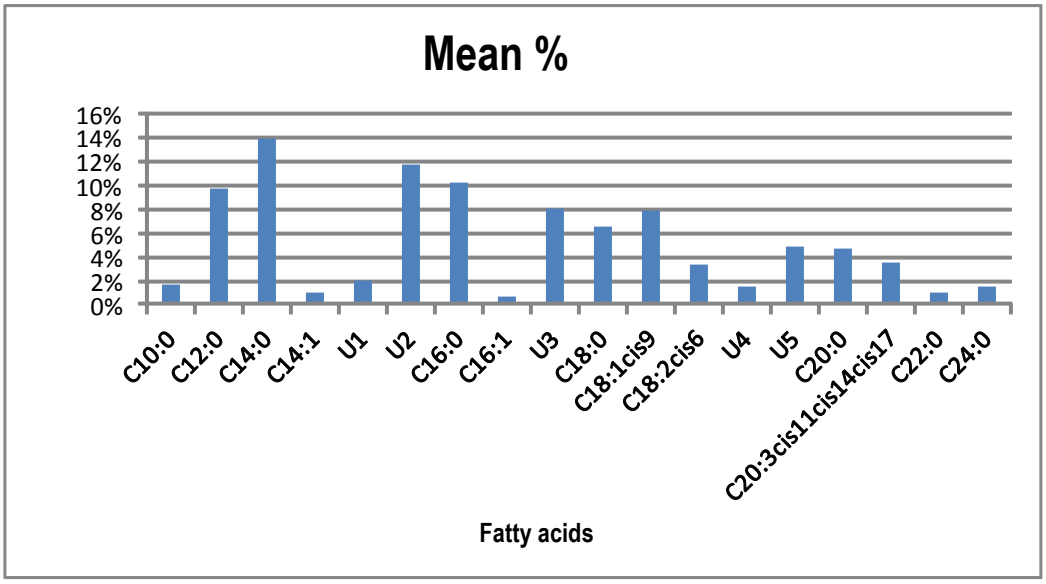


Figure 4.6: Bar graph indicating mean fatty acid percentage.

4.4.2 Comparisons of FAME profiles

The Pearson Chi Square Test was used to ascertain if significant differences occurred among *Eucalyptus* hybrid rhizospheric communities, among *Eucalyptus* species rhizospheric communities and between *Eucalyptus* hybrid and species rhizospheric communities. The test revealed no significant differences among the different *Eucalyptus* hybrid rhizospheric communities (Table 4.4) and among the different *Eucalyptus* species rhizospheric communities (Table 4.5) as well as between *Eucalyptus* hybrid and species rhizospheric communities (Table 4.6).

Table 4.4: Observed and expected peak areas for *Eucalyptus* hybrid rhizospheric communities.

Fatty acid	***Eucalyptus* hybrid rhizospheric communities**			Total
	GN018B	**GNPP2107**	**GU111**	
	o (e)	**o (e)**	**o (e)**	
C10:0	5.0 (4.3)	-	7.8 (4.8)	12.8
C12:0	40.6 (39.8)	22.7 (32.5)	53.1 (44.3)	116.4
C14:0	58.1 (59.7)	53.0 (48.7)	63.5 (66.2)	174.6
C16:0	47.7 (45.5)	37.3 (37.2)	48.2 (50.5)	133.2
C18:0	27.6 (30.0)	29.6 (24.5)	30.6 (33.3)	87.8
C20:0	21.6 (21.2)	21.1 (17.3)	19.3 (23.5)	62.0
Total	200.6	163.7	222.5	586.8

H_a was rejected at a significant level of $\alpha = 0.05$ ($X^2 = 10.51$; df = 10)

o = observed, e = expected

Table 4.5: Observed and expected peak areas for the *Eucalyptus* species rhizospheric communities.

Fatty acid	***Eucalyptus* species rhizospheric communities**				Total
	E. macarthurii	***E. smithii***	***E. nitens***	***E. nitens229***	
	o (e)	**o (e)**	**o (e)**	**o (e)**	
C10:0	14.0 (16.6)	8.8 (12.5)	6.2 (7.3)	24.0 (16.6)	53.0
C12:0	79.7 (74.2)	55.8 (56.0)	27.6 (32.4)	73.5 (74.1)	236.6
C14:0	102.6 (94.8)	75.4 (71.6)	41.4 (41.4)	83.0 (94.7)	302.4
C16:0	45.7 (61.5)	46.3 (46.5)	29.3 (26.9)	75.0 (61.4)	196.3
C18:0	43.5 (37.2)	27.0 (28.1)	16.4 (16.2)	31.8 (37.2)	118.7
C20:0	28.8 (30.0)	24.1 (22.7)	16.3 (13.1)	26.6 (30.0)	95.8
Total	314.3	237.4	137.2	313.9	1002.8

H_a was rejected at a significant level of $\alpha = 0.05$ ($X^2 = 18.08$; df = 15)

o = observed, e = expected

Table 4.6: Observed and expected peak areas for *Eucalyptus* hybrid and the *Eucalyptus* species rhizospheric communities.

Fatty acid	***Eucalyptus* hybrid rhizobacterial communities**			***Eucalyptus* species rhizospheric communities**				Total
	GN 018B o (e)	**GN PP2107 o (e)**	**GU 111 o (e)**	***E. macar-thurii* o (e)**	***E. smithii* o (e)**	***E. nitens* o (e)**	***E. nitens229* o (e)**	
C10:0	5.0 (8.3)	-	7.8 (9.1)	14.0 (13.0)	8.8 (9.8)	6.2 (5.7)	24.0 (13.0)	65.6
C12:0	40.6 (44.6)	22.7 (36.4)	53.1 (49.4)	79.7 (69.8)	55.8 (52.7)	27.6 (30.5)	73.5 (69.7)	353.0
C14:0	58.1 (60.2)	53.0 (49.1)	63.5 (66.8)	102.6 (94.3)	75.4 (71.2)	41.4 (41.2)	83.0 (94.2)	477.0
C16:0	47.7 (41.6)	37.3 (33.9)	48.2 (46.1)	45.7 (65.2)	46.3 (49.2)	29.3 (28.4)	75.0 (65.1)	329.5
C18:0	27.6 (26.0)	29.6 (21.3)	30.6 (28.9)	43.5 (40.8)	27.0 (30.8)	16.4 (17.8)	31.8 (40.8)	206.5
C20:0	21.6 (19.9)	21.1 (16.3)	19.3 (22.1)	28.8 (31.2)	24.1 (23.6)	16.3 (13.6)	26.6 (31.2)	157.8
Total	200.6	163.7	222.5	314.3	237.4	137.2	313.9	1589.4
H_a was rejected at a significant level of $\alpha = 0.05$ ($X^2 = 40.80$; df = 30)								

o = observed, e = expected

4.5 Discussion and conclusion

The fatty acid contents of the respective *Eucalyptus* hybrids, species and control soil samples varied in type and quantity of fatty acid present. Myristic acid (C14:0) was found to be the most abundant fatty acid in all the *Eucalyptus* rhizospheric communities. In contrast, palmitoleic acid (C16:1) and lignoceric acid (24:0) were unique to the *Eucalyptus* species rhizospheric communities.

Palmitic acid (C16:0) and palmitoleic acid (C16:1) differ only by a single bond, yet their quantities in the *Eucalyptus* rhizospheric communities varied greatly, where palmitic acid occurred in vastly greater concentrations than palmitoleic acid. It has been shown that palmitic acid is associated with bacteria (Zhang & Rock, 2008), while palmitoleic acid is associated with arbuscular mycorrhizal (AM) fungi (Lynch *et al.,* 2004). Singh *et al.* (2008) stated that AM fungi were a major factor in determining the bacterial assemblage, but that the bacteria did not influence the AM fungi in return. Therefore, it can be argued that the presence of AM fungi in the rhizospheres of the *Eucalyptus* species influenced the particular bacterial assemblage in *Eucalyptus* species rhizospheres, while their absence in the *Eucalyptus* hybrid rhizospheres resulted in a substantially different bacterial assemblage in *Eucalyptus* hybrid rhizospheres.

Bradley (1970) reported in his studies that lauric acid (C12:0) has an inhibitory effect on bacteria that contain palmitic acid (C16:0) as a major component in their cell membrane. However, this does not appear to be the case for *Eucalyptus* rhizospheric communities where lauric acid (C12:0) as well as palmitic acid (C16:0) occur together in abundance.

The fatty acid diversity of *Eucalyptus* species and hybrid rhizospheres supported the findings of Peiffer *et al.* (2013), who showed that field environments influenced rhizospheric diversity. Even though the *Eucalyptus* samples were collected from around the same town, Pietermaritzburg, there are differences that could influence the fatty acid compositions. These differences include among others, the potting soils used, the treatment of soils as well as the trees, the different genotypes, the way the trees are handled in the nurseries and the availability of water. The potting soil used for the placement of *Eucalyptus* hybrid cuttings is usually treated to get rid of pathogens and is regarded as 'clean' soil, therefore the low concentrations and diversity of bacteria in this soil sample is not surprising (University of California Agriculture and Natural Resources, 2013). On the other hand, the untreated control soil sample used for the planting of the *Eucalyptus* species demonstrated similar fatty acid diversity and concentrations as their *Eucalyptus* species counterparts.

In conclusion, FAME analysis provided a means to rapidly identify and quantify the presence of fatty acids of rhizospheric communities of *Eucalyptus* hybrids and species.

Chapter 5
Rhizobacterial community profiles using denaturing gradient gel electrophoresis

5.1 Introduction

Denaturing gradient gel electrophoresis (DGGE) was originally described by Myers and colleagues in 1987. The purpose of DGGE was to identify gene mutations (Myers *et al*., 1987; Muyzer *et al*., 1993; Marschner, 2007; Nakatsu, 2007). Muyzer and colleagues later adapted DGGE for microbial community analyses (Muyzer *et al.,* 1993; Nakatsu, 2007; Marschner, 2007). A microbial community's genetic diversity can successfully be described using DGGE profiling (Muyzer & Smalla, 1998). DGGE is reliable, reproducible, quick and relatively inexpensive (Giraffa & Neviani, 2001; Nakatsu, 2007).

The application of DGGE as a method to analyse the diversity of microbial communities has recently gained importance as is evident in its increased application since 1997 (Giraffa & Neviani, 2001; Nakatsu, 2007). DGGE was successfully used to study bacterial communities responsible for the composting process of rice straw (Cahyani *et al.,* 2003), to monitor two aerated lagoons of a wastewater treatment plant (Moura *et al.,* 2006) and reveal that petroleum-hydrocarbon plume caused variation in microbial communities at a petroleum-hydrocarbon contamination site (Kao *et al.,* 2009). DGGE has also been applied to the study of rhizospheric microbial communities such as in cordgrass (Lovell *et al.,* 2000) and chrysanthemum (Lovell *et al.,* 2000; Duineveld *et al.,* 2001).

5.2 Principle of DGGE

DGGE is a culture-independent and PCR-dependent technique (Muyzer & Smalla, 1998). Generally, amplified DNA fragments migrate through an agarose gel and separated according to their fragment length (Giraffa & Neviani, 2001; Marschner, 2007). DGGE is able to further separate the DNA fragments according to their different nucleotide sequences using a denaturing gradient (Dubey *et al*., 2005;

Marschner, 2007). The DNA fragments are separated on a vertical polyacrylamide gel containing denaturing chemicals, formamide and urea (Muyzer & Smalla, 1998; Marschner, 2007; Justé *et al.,* 2008). The different nucleotide sequences alter the melting properties of DNA fragments which affect their mobility (Dubey *et al.,* 2005; Justé *et al*., 2008). The mobility of DNA fragments is determined by the guanine and cytosine (GC) content (Dubey *et al*., 2005; Marschner, 2007). The unique melting property of DNA fragments allows separation and detection of fragments with a resolution power of a single nucleotide (Giraffa & Neviani, 2001; Marschner, 2007).

DNA fragments migrate through a denaturing polyacrylamide gel at a rate determined by their molecular weight (Muyzer & Smalla, 1998; Dubey *et al.,* 2005). As the fragments reach higher denaturing conditions, depending on their unique sequence compositions they begin to melt (Muyzer & Smalla, 1998; Dubey *et al*., 2005). The DNA strands start to separate and cause a change from a helical molecule to a partially melted molecule (Figure 5.1) (Muyzer & Smalla, 1998; Dubey *et al*., 2005). DNA fragments with high GC content migrate further through the gel than those with a lower GC content (Dubey *et al*., 2005; Marschner, 2007).

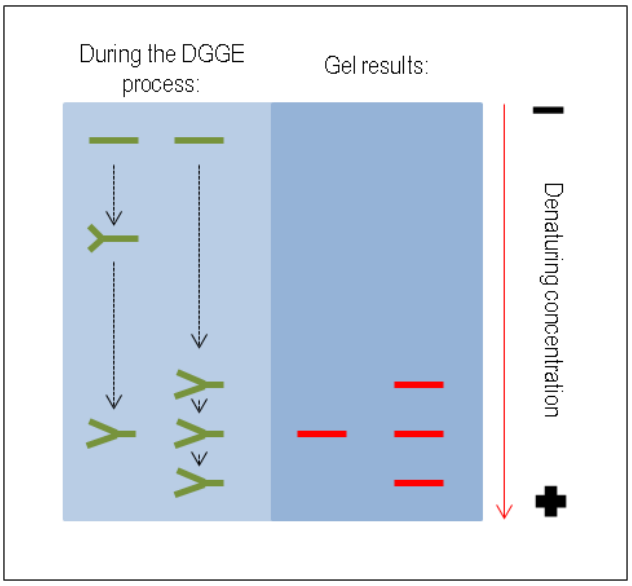


Figure 5.1: DNA fragments migrating through a denaturing gradient gel.

One of the two primers used in DGGE analyses contains a GC tail at the 5'-end known as a clamp (Muyzer & Smalla, 1998; Marschner, 2007). The clamp acts as a high melting domain which avoids complete

separation of the amplifying DNA strands (Muyzer & Smalla, 1998; Marschner, 2007) (Figure 5.2). In this way the clamp prevents the smearing of banding patterns which occurs when the DNA strands are completely separated (Muyzer & Smalla, 1998; Marschner, 2007).

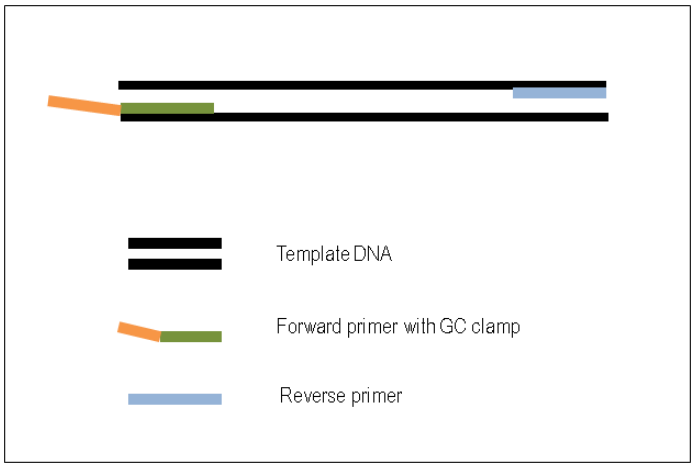


Figure 5.2: Role of different primers used in the amplification of DNA using DGGE.

5.3 Materials and methods

Successful characterisation of bacterial communities using a molecular approach follows a step-wise procedure. Firstly, DNA is extracted from the soil (Dubey *et al.,* 2006), secondly, the extracted DNA is pre-screened for usability by targeted amplification of the 16S rRNA gene (this gene was the focus because of its presense in almost all bacteria) using PCR, and lastly amplification of a shorter fragment within the same gene with a modified primer set is obtained and these fragments are separated in a urea gradient (DGGE). The resulting bands are then excised from the DGGE gel, re-amplified and compared to the NCBI database for identification.

5.3.1 DNA Extraction

The DNA extraction process requires 500 mg of soil. Soil was weighed out while taking care that no environmental contamination occurred. A 2 ml eppendorf tube was placed in a 15 ml tube and a pipette tip used to transfer the soil from falcon tubes to the eppendorf tubes. Genomic DNA (gDNA) was extracted

from a total of 42 soil samples (Table 5.1) using the FastDNA® SPIN Kit for soil (MP Biomedicals). The detailed protocol for DNA extraction using the FastDNA® SPIN Kit for soil can be found in appendix C.

DNA quality and quantity were determined in two ways. Firstly, by separation in a 0.8% agarose gel stained with 0.05% Goldview (Guangzhou Geneshun Biotech) and visualisation under UV light with the Molecular Imager® Gel Doc™ XR system (Bio-Rad). Secondly, DNA quality and quantity was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

5.3.2 16S rDNA amplification

A 1 300 bp fragment of the 16S rRNA gene was amplified using the primer set 63-F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387-R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.,* 1998). Each 25 µl PCR mixture contained 0.5 µl of extracted gDNA (the same amount of each sample was used to prevent bias), 0.5 µM of each primer, 0.2 mM dNTPs, 2.5 µl of a 10 × PCR buffer, 1 U of SuperTherm Taq DNA polymerase (JMR Holdings) and 1 mg/ml BSA (Fermentas). Amplification was performed using the C1000™ Thermal Cycler (Bio-Rad) under the following conditions; initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for a minute and 30 seconds. A final elongation step was incorporated at 72°C for 5 minutes followed by a cooling step at 4°C for 5 minutes. PCR product amplification was confirmed by agarose gel electrophoresis (1%).

5.3.3 DGGE-PCR product amplification

Once the suitability of extracted gDNA for PCR application was verified, a modified primer set was used to amplify a shorter 625 bp segment of the 16S rRNA gene for DGGE analysis. The primer set 341-F^{GC} with a 40 bp 5' GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3') and 907RM (5'-CCG TACG ATT CMT TTG AGT TT-3') were used (Muyzer *et al*., 2005; Martínez-Alonso *et al.,* 2010). Each PCR mixture (50 µl) contained 1.5 µl of the extracted gDNA (the same amount of each sample was used to prevent bias), 0.5 µM of each primer, 0.2 mM dNTPs, 5 µl of a 10 × PCR buffer and 1 U of SuperTherm Taq DNA polymerase (JMR Holdings). Touchdown PCR amplification was performed using the C1000™ Thermal Cycler (Bio-Rad) under the following conditions;

initial denaturation at 95°C for 3 minutes followed by 10 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for 1 minute. An additional 20 cycles of denaturation at 95°C for 3 minutes, annealing at 55°C for 45 seconds and elongation at 72°C for 1 minute was performed. A final elongation step was incorporated at 72°C for 6 minutes followed by a cooling step at 4°C for 3 minutes. PCR product amplification was confirmed by agarose gel electrophoresis (1.5%). In order to reduce possible inter-sample PCR variation, two sets of PCRs were performed as independent duplicates and pooled before loading on the DGGE gel.

Initially, all 42 samples were analysed with DGGE to establish diversity, consistency or deviation among representative samples of the same *Eucalyptus* hybrid or species. Eventually, 5 µl of gDNA extracted from all soil samples representing each *Eucalyptus* hybrid, species or control were pooled and used as template for further analyses (Table 5.1). This resulted in a total of nine pooled samples ranging from 20 to 25 µl of gDNA. The nine pooled samples represented nine specific microbial communities originating from three *Eucalyptus* species and four *Eucalyptus* hybrid rhizospheric samples, as well as two control soil samples.

Table 5.1: Origin, soil sample and collective name for pooled samples representing specific communities analysed with DGGE.

Nursery origin	Source soil samples	Collective name for pooled sample
Sunshine Seedlings	SS2; SS4; SS6; SS8; SS10	*Eucalyptus* hybrid GN018B
Sunshine Seedlings	SS12; SS14; SS16; SS18; SS20	*Eucalyptus* hybrid GNPP2107
Sunshine Seedlings	SS22; SS24; SS26; SS28; SS30	*Eucalyptus* hybrid GU111
Sunshine Seedlings	SS C1; SS C2; SS C3; SS C4	*Eucalyptus* hybrid potting soil (control)
ICFR	ICFR32; ICFR33; ICFR34; ICFR35; ICFR36	*E. macarthurii*
ICFR	ICFR37; ICFR38; ICFR39; ICFR40; ICFR41	*E. smithii*
ICFR	ICFR42; ICFR43; ICFR44; ICFR45	*E. nitens*
ICFR	ICFR46; ICFR48; ICFR50; ICFR52; ICFR54	*E. nitens229*
ICFR	ICFR C1; ICFR C2; ICFR C3; ICFR C4	*Eucalyptus* species potting soil (control)

5.3.4 DGGE analysis

The bacterial diversity of all the soil samples was analysed using the DCode Universal Mutation Detection System (Bio-Rad) essentially according to the protocol described by Muyzer *et al.* (1993). Gels of 6% polyacrylamide with a denaturing gradient range of 40 to 60% (100% denaturant is defined as a mixture of 7 M urea and 40% deionised formamide) were used. Twenty five µl of the amplified PCR product were loaded *per* well. Separation was performed at 60°C at 100 V for 17 hours for single sample analysis and 130 V for 5 hours for pooled samples. All the gels were stained with 0.05% GelStar® (Lonza) for 10 minutes and de-stained with distilled water for 5 minutes. The images were visualised and captured using the Molecular Image® Gel Doc™ XR system and DGGE band patterns analysed with Quantity One® 1-D Analysis imaging software (Bio-Rad). A 5% band intensity threshold was set for the band selection process. A band-matching process, based on a 1.5% position tolerance was used to obtain presence-absence matrixes, allowing the classification of individual bands according to their positions in the gel and calculation of their frequency among soil samples. The Quantity One® 1-D Analysis imaging software (Bio-Rad) also described cluster analysis pattern similarities among different soil samples using unweighted pair-group method with an arithmetic mean algorithm (UPGMA) to generate a densitometric profile (Martinez-Alsono *et al.,* 2010). Dominant bands for further discussion were selected based on band intensity (≥ 1.892).

5.3.5 Sequencing

Selected bands were excised from the DGGE gel on a DarkReader (Clare Chemicals Research), suspended in 50 µl nanopure water and incubated at 50ºC for 6.5 hours. The excised bands were re-amplified using primers 341-F (5'-CCT ACG GGA GGC AGC AG-3') and 907RM (5'-CCG TACG ATT CMT TTG AGT TT-3') (Muyzer *et al.,* 2005). Each PCR mixture (25 µl) contained 5 µl of the excised band mixture, 0.5 µM of each primer, 0.2 mM dNTPs, 2.5 µl of a 10 × PCR buffer, 1 U of SuperTherm Taq DNA polymerase (JMR Holdings) and 1 mg/ml BSA (Fermentas). Touchdown PCR amplification was performed using the C1000™ Thermal Cycler (Bio-Rad) and PCR product amplification confirmed as described in section 5.3.3.

Band positions on the DGGE gel were each assigned a unique number using Quantity One Software. At least three different representatives of each DGGE band position were sequenced. The sequences were obtained directly from 1 µl of the re-amplified product using the Big Dye® Terminator v3.1 Cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems®). Primer 341-F was used for sequencing except in cases where single (unique) bands were detected on the DGGE gel. These bands were sequenced in both directions using primer 907RM as well. The reactions were carried out using the C1000™ Thermal Cycler (Bio-Rad). Reaction conditions consisted of initial denaturation at 96ºC for 1 minute, followed by 25 cycles of denaturation at 96ºC for 10 seconds, annealing at 50ºC for 5 seconds and 60ºC for 4 minutes. An additional cooling step was incorporated at 4ºC for 5 minutes. An EDTA/Ethanol precipitation protocol, attached as appendix D, was followed for sequencing clean-up. Sequences representing the same band position were aligned using Clustal Omega. Further analyses of the sequences were performed with the basic local alignment search tool BLAST program which was compared to the 16S ribosomal RNA sequences (Bacteria and Archea) database. Public databases presently comprise mostly 16S rRNA that originates from environmental PCR and cloning or from metagenomes. Even when doing biodiversity analyses, it is generally best not only to find out which are the most similar sequences present in the public databases [nucleotide collection (nr/nt)], but also which well-known cultured species is closest. All sequences were deposited in the GenBank database under accession numbers indicated in Table 5.4.

5.4 Results

5.4.1 Extracted DNA

The extracted DNA's nanodrop readings illustrated a nucleic acid concentration average of 27.8 ng/µl. *Eucalyptus* hybrids control (SS C1) had the lowest nucleic acid concentration (1.8 ng/µl), while the highest nucleic acid concentration (72 ng/µl) was obtained from the *Eucalyptus* species control (ICFR C1). The mean A_{260}/A_{280} ratio was 1.9 which was in range of the expected ratio of approximately 1.8 indicating 'pure' DNA samples.

5.4.2 Amplified 16S PCR

Pre-screening amplification of the bacterial 16S rRNA gene (1 300 bp) was successful in all 42 samples. The presence of a band just below the 1 500 bp mark of the GeneRuler™ 1-kb Plus DNA ladder in all lanes of a 1% agarose gel proved successful amplification (Figure 5.3).

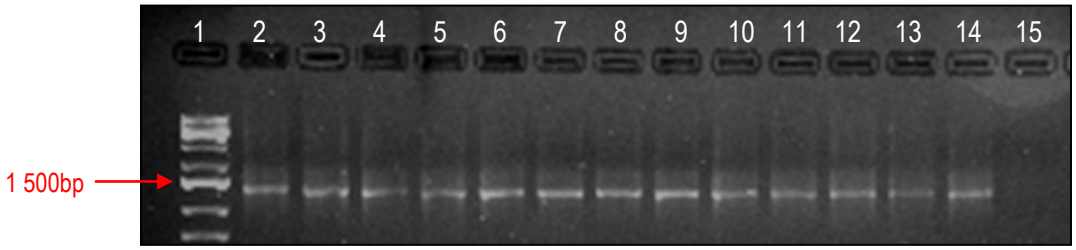


Figure 5.3: Representative agarose gel electrophoresis (1%) of 16S PCR products from 13 samples. Lanes: 1 – 1kb+ ladder, 2 – sample ICFR C1, 3 – sample ICFR C2, 4 – sample ICFR C3, 5 – sample ICFR C4, 6 – sample SS C1, 7 – sample SS C2, 8 – sample SS C3, 9 – sample SS C4, 10 – sample SS10, 11 – sample SS18, 12 – sample SS26, 13 – sample ICFR43, 14 – sample ICFR48, 15 – non-template control.

Amplification of a shorter segment of the 16S rRNA gene (625 bp) from the pooled gDNA, specific for DGGE analysis was also successful. The presence of a band just above the 500 bp mark of the GeneRuler™ 1-kb Plus DNA ladder in all lanes of a 1.5% agarose gel is illustrated in Figure 5.4.

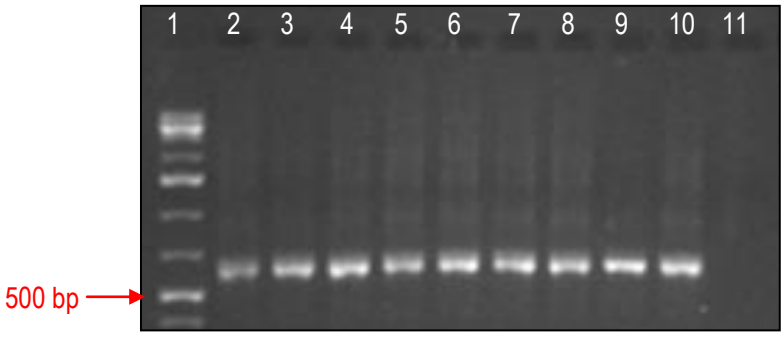


Figure 5.4: Agarose gel electrophoresis (1.5%) of PCR products amplified from pooled gDNA using DGGE primers for 9 samples. Lanes: 1 – 1kb+ ladder, 2 – pooled GN018B sample, 3 – pooled GNPP22107 sample, 4 – pooled GU111 sample, 5 – pooled *E. macarthurii* sample, 6 – pooled *E. smithii* sample, 7 – pooled *E. nitens* samples, 8 – pooled *E. nitens229* sample, 9 – pooled SS control sample, 10 – pooled ICFR control sample, 11 – non-template control.

5.4.3 Analysed DGGE

The UPGMA results, based on the obtained DGGE profiles showed that rhizospheric gDNA extracted from the same *Eucalyptus* hybrid grouped together, while its control's gDNA was the outlier (Figure 5.5). The rhizospheric gDNA from *Eucalyptus* hybrids GN018B (samples SS2 – SS10) clustered together with a similarity distance of approximately 0.42; illustrating a 42% similarity among samples based on common bands present and band intensity. Similarly, the rhizospheric gDNA from *Eucalyptus* hybrids GNPP2107 (samples SS12 – SS20) also clustered together with a similarity distance of approximately 0.55, as well as the rhizospheric gDNA from *Eucalyptus* hybrids GU111 (SS22 – SS30) with a similarity distance of approximately 0.62. The outlier, gDNA from the *Eucalyptus* hybrid control sample had a similarity distance of approximately 0.18 to the *Eucalyptus* hybrid rhizospheric samples.

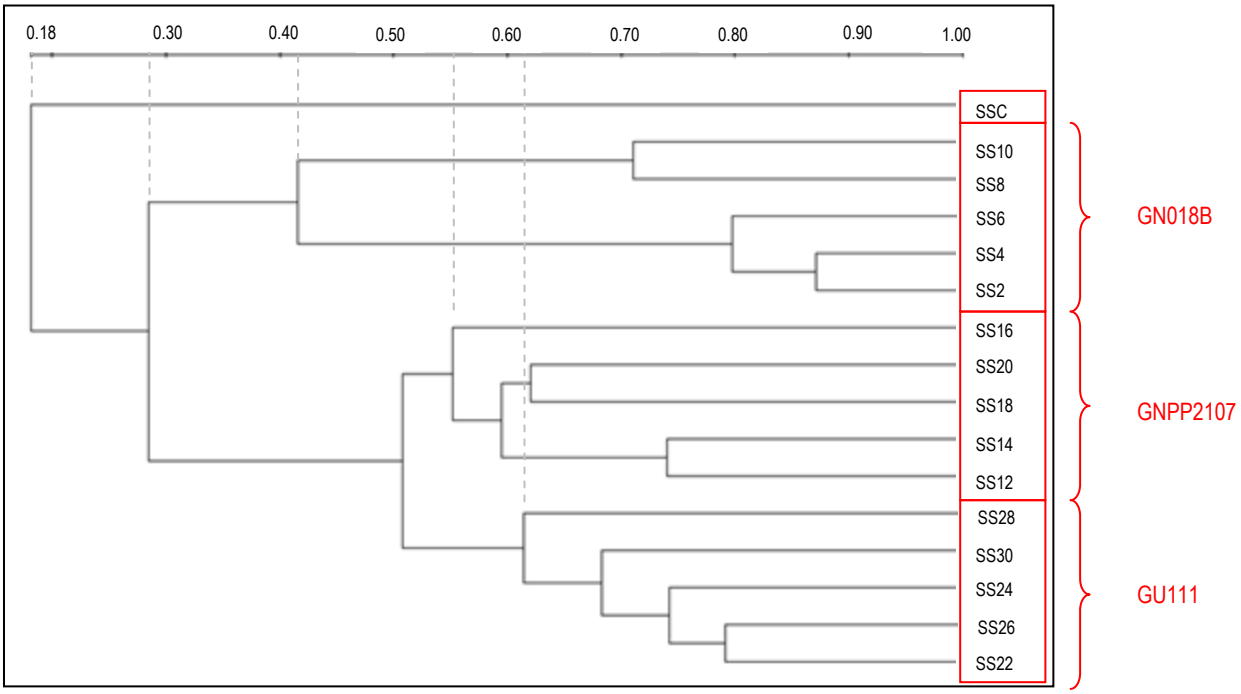


Figure 5.5: UPGMA dendrogram generated from the *Eucalyptus* hybrids DGGE profile.

The UPGMA results from the *Eucalyptus* species DGGE profile indicated different results when compared to that of the *Eucalyptus* hybrids. The rhizospheric gDNA extracted from *Eucalyptus* species samples did not demonstrate clustering, however the similarity distance of 0.36 for *Eucalyptus* species showed an overall closer relationship compared to the 0.28 of the *Eucalyptus* hybrids (Figures 5.5 and 5.6).

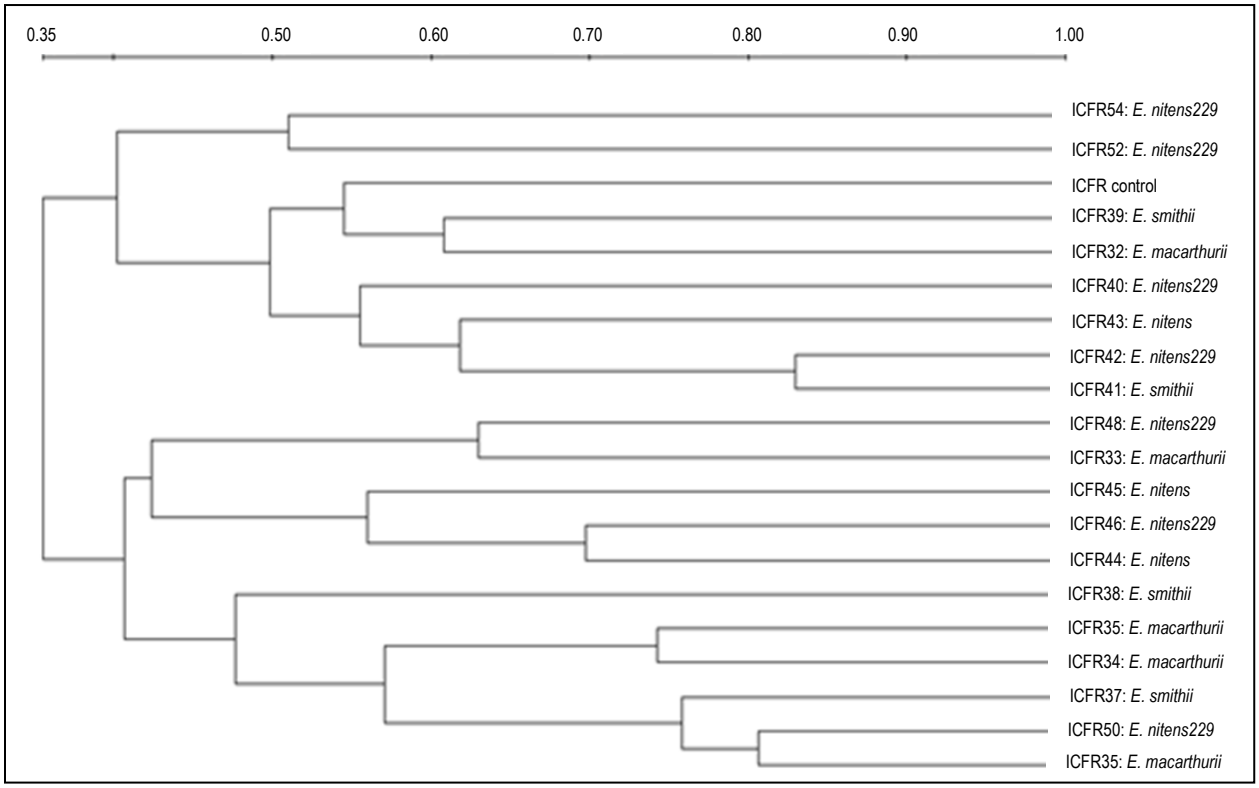


Figure 5.6: UPGMA dendrogram generated from the *Eucalyptus* species DGGE profile.

The pooled rhizospheric gDNA from the *Eucalyptus* hybrids, GN018B, GNPP2107 and GU111 clustered together on a UPGMA dendrogram (Figure 5.7). The rhizospheric gDNA of hybrids between *E. grandis* × *E. nitens* (GNPP2107 and GN018B) showed the highest similarity, with a distance of approximately 0.71. Pooled rhizospheric gDNA from the *Eucalyptus* species *E. nitens* and *E. smithii* clustered together with a similarity distance of approximately 0.55 to the pooled gDNA of its control sample. The pooled gDNA from the *Eucalyptus* hybrid control sample was the out group, with a similarity distance of 0.35.

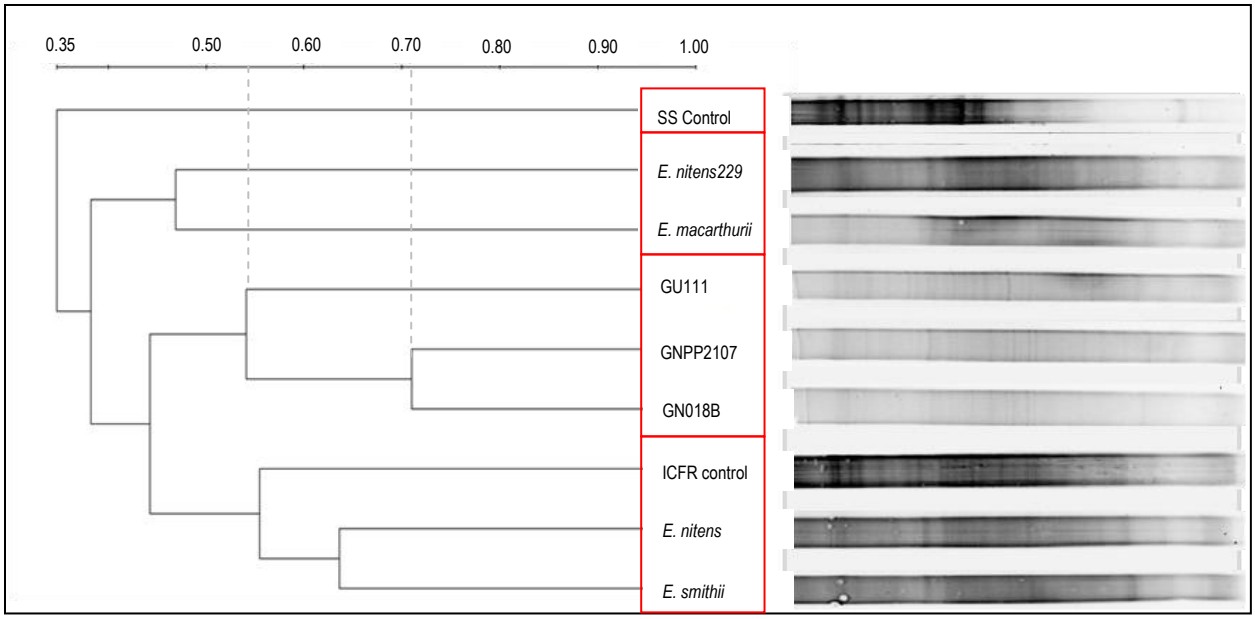


Figure 5.7: UPGMA dendrogram generated from the DGGE profile of the pooled samples of the *Eucalyptus* hybrid, species and controls soil gDNA.

5.4.4 Rhizobacterial sequences

DGGE analysis of the pooled samples resulted in detection of 201 total bands in 50 unique band positions. Of these band positions 55% (based on band intensity) were re-amplified and sequenced. The amplified gDNA from the *Eucalyptus* species control and the *Eucalyptus* hybrid GU111 rhizosphere sample had the largest number of excised bands (16) while the amplified rhizospheric gDNA from the *Eucalyptus* hybrid GN018B had the least (8). Sequences subjected to BLAST analysis showed expected values (e-values) below 0.05, with the exception of one hit, *Massilia aerilata* (Table 5.2). An e-value of less than 0.05 is considered significant (NCBI, 2013), also the smaller the e-value the better the match between sequences (NCBI, 2013). Although *Massilia aerilata* displayed an e-value higher than the significant 0.05, its maximum identity was 93%. There is no set significant cut-off for maximum identity, this is decided by the user (Integrated Breeding, 2013), resulting in *Massilia aerilata* being included on the basis of its high maximum identity. Similarly, *Paracoccus sulfuroxidans* showed low maximum identity (69%) but was included on the basis of its low e-value (4.00E-37) which was considerably lower than the significant value of 0.05. Additionally, all sequences were deposited into the NCBI database of which the accession numbers are shown in Table 5.2 (BLAST, 2013). DNA sequences showed that genetic material possible originating from

Nitrosomona eutropha, represented by band position 15, was present in all samples. Furthermore, eight unique sequences, indicated in bold, were obtained for one *Eucalyptus* hybrid, three *Eucalyptus* species and the *Eucalyptus* species control (Table 5.2).

Table 5.2: BLAST results with allocated accession numbers.

DGGE band position	Blast hit	E-value	Max identity	Number of base pairs	NCBI accession number	Representative
1	*Erythrobacter citreus*	3.00E-142	83%	496	KF038968	*Eucalyptus* hybrid and species control
2	*Paracoccus sulfuroxidans*	4.00E-37	69%	381	KF034980	***E. nitens***
3	*Flavobacterium micromati*	2.00E-147	92%	355	KF034970	GN018B, GNPP2107, GU111, *Eucalyptus* hybrid and species control
6	*Marinifilum fragile*	1.00E-74	78%	393	KF034972	GU111, *E. smithii*, *E. nitens229*, *Eucalyptus* hybrid and species control
7	*Chitinophaga niabensis*	2.00E-88	85%	334	KF034986	***E. macarthurii***
9	*Flavisolibacter ginsengisoli*	9.00E-140	91%	351	KF034969	GNPP2107, GU111, *Eucalyptus* hybrid and species control
10	*Hyphomicrobium hollandicum*	7.00E-72	76%	375	KF034979	***E. nitens229***
11	*Mucilaginibacter oryzae*	4.00E-151	94%	342	KF034975	*Eucalyptus* hybrid and species control
12	*Cytophaga fermentans*	2.00E-12	71%	187	KF034964	GU11, *E. smithii*, *E. nitens*, *E. nitens229*
13	*Adhaeribacter aquaticus*	4.00E-106	85%	358	KF034988	**GU111**
14	*Methylophilus leisingeri*	1.00E-61	76%	370	KF034974	GU111, *E. nitens*, *Eucalyptus* hybrid and species control
15	*Nitrosomonas eutropha*	2.00E-117	86%	377	KF034976	All samples
16	*Thiobacter subterraneus*	1.00E-50	71%	299	KF034987	GU111, *E. macarthurii*, *Eucalyptus* hybrid and species control
17	*Thiobacter subterraneus*	3.00E-24	71%	239	KF034977	GU111, *E. nitens229*
18	*Candidatus Solibacter usitatus*	3.00E-141	87%	415	KF034966	***Eucalyptus* species control**
19	*Massilia aerilata*	0	93%	445	KF034973	*E. smithii*, *E. nitens*, *Eucalyptus* species control
22	*Agrobacterium rhizogenes*	4.00E-132	86%	414	KF034961	***Eucalyptus* hybrid control**
23	*Sulfurivirga caldicuralii*	3.00E-94	84%	320	KF034985	*E. smithii*, *E. nitens*, *Eucalyptus* hybrid and species control
24	*Amorphus coralli*	3.00E-108	83%	406	KF034962	GN018, GNPP2107, GU111, *E. macarthurii*, *E. smithii*, *E. nitens229*, *Eucalyptus* species control
26	*Rhodospirillum centenum*	1.00E-95	80%	423	KF034983	GN018B, GNPP2107, *E. macarthurii*, *E. nitens*, *E. nitens229*
27	*Rhodothalassium salexigens*	1.00E-93	81%	370	KF034984	*Eucalyptus* hybrid and species control
28	*Prosthecomicrobium consociatum*	3.00E-83	79%	384	KF034981	***E. macarthurii***

DGGE band position	Blast hit	E-value	Max identity	Number of base pairs	NCBI accession number	Representative
29	*Methylobacterium isbiliense*	6.00E-33	78%	255	KF034978	GN018B, GNPP2107, GU111, *E. macarthurii*
31	*Blastochloris sulfoviridis*	2.00E-136	88%	399	KF034965	GN018B, GNPP2107, GU111, *E. smithii*, *E. nitens*, *E. nitens229*, *Eucalyptus* species control
36	*Rhodoplanes serenus*	4.00E-88	77%	435	KF034982	GNPP2106, GU111
43	*Holophaga foetida*	2.00E-104	79%	456	KF034971	*E. smithii, E. nitens*
44	*Desulfovibrio burkinensis*	6.00E-99	79%	456	KF034967	GN018B, GNPP2107, GU111, *E. macarthurii*
46	*Rubrobacter taiwanensis*	2.00E-22	73%	392	KF034989	***E. nitens229***
47	*Amycolatopsis rubida*	9.00E-58	73%	382	KF034963	GNPP2107, GU111, *E. smithii*

5.5 Discussion and conclusion

DGGE profiles of the respective *Eucalyptus* hybrids and species varied in rhizospheric gDNA. *Nitrosomonas eutropha* was the only bacterium present in all *Eucalyptus* hybrids and species. *N. eutopha* is known to be able to adapt to nitrogen saturated environments (Stein *et al.,* 2007). The abundance of *N. eutropha* is evidence that the *Eucalyptus* hybrids and species rhizospheric environments are probably rich in nitrogen, an essential nutrient of soil (Soil Management, 2013).

It has been shown that host genotype significantly influences its rhizospheric diversity (Berg *et al.,* 2002; Peiffer *et al.,* 2013). The *Eucalyptus* hybrids are clones from the same maternal tree, making them genetically identical. The influence of host genotype explains the clustering of each *Eucalyptus* hybrid's rhizosphere as well as the clustering of the pooled *Eucalyptus* hybrid rhizospheric communities. The genotypic effect further explains the high similarity between DGGE profiles of *Eucalyptus* hybrids GN018B and GNPP2107. These hybrids are clones from the same parent species *E. grandis* × *E. nitens*.

A tree's age can influence its rhizospheric environment (Di Cello *et al.,* 1997). According to Di Cello *et al.* the rhizospheric environment changes during different phases of plant growth (Di Cello *et al.,* 1997). As the root system develops the more favourable microorganisms, those making optimal use of rhizospheric constituents, establish themselves in the more mature rhizospheres (Di Cello *et al.,* 1997). Therefore, the lower similarity between the older *Eucalyptus* hybrid rhizospheric communities compared to the younger *Eucalyptus* species rhizospheric communities could be attributed to an aging effect. The rhizospheric communities of the younger *Eucalyptus* species are comprised mainly of first root colonisers, resulting in a broad spectrum of rhizobacteria. The broad spectrum of rhizobacteria explains the higher rhizobacterial diversity and overall similarity in the younger *Eucalyptus* species rhizospheric communities. Furthermore, the broad spectrum younger *Eucalyptus* species rhizobacterial communities could also explain why unique bacteria were mainly found in *Eucalyptus* species rhizobacterial communities.

In conclusion, molecular analysis using DGGE allowed successful characterisation of rhizobacterial communities of *Eucalyptus* hybrids and species and was able to reveal different bacterial species.

Chapter 6
Discussion and conclusion

6.1 Introduction

The aim of this study was to characterise the rhizobacterial communities of *Eucalyptus* hybrids and species. The rhizobacterial communities were firstly characterised in a broad sense using FAME analyses, and secondly in a narrow sense using DGGE analyses. The purpose of the FAME analysis was to determine to what extent the rhizobacterial communities differed in composition of fatty acid methyl esters produced by the microbial communities. DGGE, on the other hand, showed differences among and between *Eucalyptus* hybrid and species based on rhizobacterial species composition.

The FAME and DGGE profiles provided evidence of the diverse nature of the *Eucalyptus* rhizobacterial communities. These differences could be attributed to a number of factors, including the relationship between inhabitants of the rhizosphere, the age of the rhizosphere, the development of the root system of the host plant and the genotype of the host plant.

6.2 AM fungi influence

The rhizobacterial composition is influenced by AM fungi (Singh *et al.,* 2008). Only two *Eucalyptus* rhizospheric communities contained palmitoleic acid (C16:1); *E. macarthurii* and *E. nitens229*. *E. macarthurii* and *E. nitens229* also clustered together, separate from the other *Eucalyptus* species; *E. smithii* and *E. nitens* on the UPGMA dendrogram. This suggests that AM fungi influence the rhizobacterial composition because of the association between palmitoleic acid (C16:1) and AM fungi (Lynch *et al.,* 2004), supporting the findings of Singh *et al*. 2008.

6.3 *Eucalyptus* rhizospheric environment

The rhizobacterial composition of planting medium (also referred to as potting soil) used in a commercial setting is dependent on two main factors, namely, whether the medium used for planting of seeds or setting of cuttings has been treated and the age of the seedling or cutting. The potting soil investigated in this study contained an abundance of the two aerobic bacterial species *Massilia aerilata* (Weon *et al.,* 2008) and *Erythrobacter citereus* (Koblížek *et al.,* 2003) suggesting that the potting soil environment was aerobic. This study showed changes in the rhizobacterial composition over time. While the initial rhizobacterial composition suggested an aerobic environment, the young *E. nitens229* seedlings suggested a combination of aerobic and anaerobic regions, because of the presence of both aerobic and anaerobic rhizobacteria, namely, the aerobic bacterium *Rubrobacter taiwanensis* (Chen *et al.,* 2004) and the anaerobic bacterium *Anaerophaga thermohalophila* (Denger *et al.,* 2002). The more mature rhizobacterial communities demonstrated the presence of mainly anaerobic bacteria. The presence of these anaerobic bacteria which included *Rhodoplanes serenus* and *Holophaga foetida* (Liesack *et al.,* 1994; Okamura *et al.,* 2009), isolated from four *Eucalyptus* rhizospheric communities, suggested that the more mature rhizospheres were anaerobic.

A rhizobacterial community starts to develop and undergoes continual changes, depending on environmental, chemical and physical factors (McNear, 2013). An initial young rhizospheric community develops as being exclusively aerobic in nature, but as the root system develops and establishes, inhabitants use available oxygen changing the rhizospheric aerobic environment progressively to an anaerobic environment (Inglett *et al.,* 2005). This brings about changes in the composition of the rhizospheric communities as they age (Di Cello *et al.,* 1997). Further evidence of the root system causing a shift from an aerobic environment to an anaerobic environment includes less longer chain fatty acids [Behenic acid (C22:00) and lignoceric acid (C24:00)] identified in the *Eucalyptus* rhizospheric communities as long chain fatty acids tend to degrade in anaerobic conditions (Oh & Martin, 2010).

The presence of *Nitrosomona eutropha* in all rhizospheres of the *Eucalyptus* hybrids and species studied, strongly suggests that these rhizospheres are nitrogen-rich. *N. eutropha* is able to survive in high concentrations of nitrogen oxides and can adapt to nitrogen-saturated ecosystems (Stein *et al.,* 2007; Kartel *et al.,* 2012).

6.4 Genotypic effect

Research has shown that the genotype of a host plant affected the composition of the rhizospheres of poplar roots (Karliński *et al.,* 2010; Karliński *et al.,* 2013), potatoes, oilseed rape and strawberry (Berg *et al.,* 2002), as well as maize (Peiffer *et al*., 2013). A similar observation was made in this study; host genotype appeared to influence the rhizospheric community composition of *Eucalyptus* hybrids and species. DGGE analysis showed that the more similar the *Eucalyptus* genotypes were, the more similar their rhizobacterial compositions were, as demonstrated by the similar rhizobacterial community composition of *Eucalyptus* hybrids (GN018B and GNPP2107) from the same species (*E. grandis* × *E. nitens*).

6.5 Considerations

The *Eucalyptus* hybrid GU111 is known for its relatively high rooting percentages when compared to the GN hybrids. Rhizobacteria indentified from GU111 could therefore provide potential candidates for further investigation into their potential use for rooting enhancement. One of these candidates is the unique, unfamiliar rod shaped Gram-negative bacterium *Adhaenbacter aquaticus* (Rickard *et al.,* 2005) revealed by the GU111 DGGE profile. Other candidates necessitating further investigation include those rhizobacteria present in GU111 rhizospheres but absent in GN rhizospheres. These are *Marinifilum fragile*, *Cytophaga fermentans*, *Methylophilus leisingeri*, *Thiobacter subterraneus* and *Nitrosospira multiformis*. In addition, potential candidates not identified in the rhizospheres of this study, are the common plant growth promoting rhizobacteria *Bacillus, Pseudomonas* and *Agrobacterium rhizogenes*, which could also be further investigated (Thomashow, 1996; Velazquez-Selúlveda *et al.,* 2012).

6.6 Conclusion

In conclusion, this study provided some insight into the diversity of rhizobacterial communities of *Eucalyptus* hybrids and species. The results provided evidence to identify potential plant growth promoting rhizobacteria (PGPR) and also revealed that the nature of the soil environment changes with the aging of the associated host. In combination, these findings provided a better understanding of the *Eucalyptus* rhizospheric communities, allowing further investigation into the formulation of potential rhizobacterial preparations for rooting enhancement of *Eucalyptus* cuttings. Increased rooting percentages of *Eucalyptus* cuttings will mitigate the losses and alleviate the pressure created by unrooted *Eucalyptus* cuttings and limited space, which are issues currently experienced by the forestry industry.

References

Ammirato, P. V. (1986). Control of morphogenesis in culture. In: Plant Tissue Culture and its Agricultural Applications. Eds. LA Withers, PG Anderson. Butterworths, London.

AnalChemVoc. [URL: http://www.kii.ntf.uni-lj.si/analchemvoc/SPEKTRA/gascrom.htm] (September, 2012).

Andreote, F. D., Mendes, R., Dini-Andreote, F., Rossetto, P. B., Labate, C. A., Pizzirani-Kleiner, A., van Elsas, J. D., Azevedo, J. L., & Araújo, W. L. (2008). Transgenic tobacco revealing altered bacterial diversity in the rhizosphere during early plant development. *Antonie van Leeuwenhoek. 93*, 415-424.

Andreote, F. D., Carneiro, R. T., Salles, J. F., & Marcon, J. (2009). Culture-independent assessment of rhizobiales-related alphaproteobacteria and the diversity of Methylobacterium in the rhizosphere and rhizoplane of transgenic *Eucalyptus. Microbial Ecology. 57*, 82-93.

Andreote, F. D., Rossetto, P. B., Mendes, R., Avila, L. A., Labate, C. A., Pizzirani-Kleiner, A. A., Azevedo, J. L., & Araújo, W. L. (2009). Bacterial community in the rhizosphere and rhizoplane of wild type and transgenic *Eucalyptus*. *World Journal of Microbial and Biotechnology. 25*, 1065-1073.

Apsnet. [URL: http://www.apsnet.org/edcenter/illglossary/Article%20Images/rhizosphere.jpg] (July, 2012).

Armstrong, A. J., Benn, G., Bowland, A. E., Goodman, P. S., Johnson, D. N., Maddock, A. H., & Scott-Shaw, C. R. (1998). Plantation forestry in South Africa and its impact on biodiversity. *Southern African Forestry Journal*. *182*, 59-66.

Asghar, H., Zahir, A., & Arshad, M. (2004). Screening rhizobacterias for improving the growth, yield, and soil content of canola (*Brasica napus L.*). *Australian Journal of Agricultural Research. 55*, 187-194.

Baillères, H., Davrieux, F., & Ham-Pichavant, F. (2002). Near infrared analysis as a tool for rapid screening of some major wood characteristics in a *Eucalyptus* breeding program. *Annals of Forest Science. 59*, 479-490.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., & Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *The Annual Review of Plant Biology. 57*, 233-266.

Band, L. R., Úbeda-Tomás, S., Dyson, R. J., Middleton, A. M., Hodgman, T. C., Owen, M. R., Jensen, O. E., Bennett, M. O., & King, J. R. (2012). Growth-induced hormone dilution can explain the dynamics of plant root cell elongation. *Proceedings of the National Academy of Sciences Early Edition,* 1-6.

Barazani, O., & Friedman, J. (1999). Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? *Journal of Chemical Ecology. 25*(10), 2397-2407.

Barea, J. M., Pozo, M. J., Azcón, R., & Azcón-Aguilar, C. (2005). Microbial co-operation in the rhizosphere. *Journal of Experimental Botany. 56*(417), 1761-1778.

Baucher, M., Monties, B., van Montagu, M., & Boerjan, W. (1998). Biosynthesis and genetic engineering of lignin. *Critical Reviews in Plant Sciences. 17*(2), 125-197.

Bauhus, J., Paré, D., & Côté, L. (1998). Effects of tree species, stand age and soil type on soil microbial biomass and its activity in a southern boreal forest. *Soil Biology & Biochemistry. 30*(8), 1077-1089.

Barralho, N. M. G., & Wilson, P, J. (1994). Inheritance of initial survival and rooting ability in *Eucalyptus globulus labill*. Stem cuttings*. Silvae Genetics. 43*(4), 238-243.

Bashan, Y., & Holguin, G. (2002). Plant growth-promoting bacteria: a potential tool for arid mangrove reforestation. *Trees. 16*, 159-166.

Beck, S. L., & Dunlop, R. W. (2001). Micropropagation of the *Acacia* species – a review. *In Vitro Cellular and Developmental Biology Plant. 37*, 531-538.

Beck, S. L., & Fossey, A. (2007). Gamma irradiation induces sterility or seedlessdess in black wattle (*Acacia mearnsii*). *Seed Science and Technology. 35*, 351-359.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., & Smalla, K. (2002). Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. *Applied and Environmental Microbiology. 68*(7), 3328-3338.

Berg, G., & Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbial Ecology. 68*, 1-13.

Bhumibhamon, S., & Maid, M. (2009). Timor Mountain Gum improvement program in eastern Thailand. *Journal of Sustainable Development. 2*(1), 176-182.

Bisht, P., Sharma, V. K., Joshi, I., & Kapoor, M.L. (1999). Micropropagation of newly produced F_1 hybrid of *Eucalyptus* (*E. tereticornis* SM. × *E. camaldulensis* DEHN. Southern Form). *Silvae Genetica. 48*(2), 104-109.

Blakeslee, J. J., Peer, W. A., & Murphy, S. A. (2005). Auxin transport. *Current Opinion in Plant Biology. 8,* 494-500.

Bradley, W. H. (1970). Green River oil shale – concept of origin extended: an interdisciplinary problem being attacked from both ends. *Geological Society of America Bulletin. 81*(4), 985-1000.

Buée, M., De Boer, W., Martin, F., van Overbeek, L., & Jurkevitch, E. (2009). The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil. 321*, 189-212.

Cahyani, V. R., Matsuya, K., Asakawa, S., & Kimura, M. (2003). Succession and phylogenetic composition of bacterial communities responsible for the composting process of rice straw estimated by PCR-DGGE analysis. *Soil Science and Plant Nutrition. 49*(4), 619-630.

California Invasive Plant Council. [URL: http://www.cal-ipc.org/] (July, 2012).

Campbell, M. M., Brunner, A. M., Jones, H. M., & Strauss S. H. (2003). Forestry's fertile crescent: the application of biotechnology to forest trees. *Plant Biotechnology Journal. 1*, 141-154.

Carrapiso, A. I., & García, C. (2000). Development in lipid analysis: some new extraction techniques and *in situ* transesterfication. *Lipids. 35*(11), 1167-1177.

Cavigelli, M. A., Robertson, G. P., & Klug, M. J. (1995). Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. *Plant and Soil. 170,* 99-113.

Chanway, C. P., Radley, R. A., & Holl, F. B. (1991). Inoculation of conifer seed with growth promoting *Bacillus* strains causes increased seedling emergence and biomass. *Soil Biology and Biochemistry. 23*, 575-580.

Chi Square Test. [URL: http://course1.winona.edu/sberg/Equation/chi-squa.htm] (July, 2013).

Chen, M. Y., Wu, S. H., Lin, G. H., Lu, C. P., Lin, Y. T., Chang, W. C., & Tsay, S. S. (2004). *Rubrobacter taiwanensis* sp. nov., a novel thermophilic, radiation-resistant species isolated from hot springs. *International Journal of Systematic and Evolutionary Microbiology. 54*, 1849-1855.

Chowdhury, T. R., & Dick, R. P. (2012). Standardizing methylation method during phospholopid fatty acid analysis to profile soil microbial communities. *Journal of Microbiological Methods. 88*, 285-291.

Chetty, S. (2001). The development of clone-specific micropropagation protocols for three commercially important *Eucalyptus* hybrids. Dissertation, University of Natal, Durban.

Cortinas, M. N., Barnes, I., Wingfield, M. J., & Wingfield, B. D. (2010). Genetic diversity in the *Eucalyptus* stem pathogen *Tetratosphaeria zuluensis*. *Australasian Plant Pathology. 29*, 383-393.

Cupertino, F. B., Leal, J. B., Corrèa, R. X., & Gaiotto, F, A. (2011). Genetic diversity of *Eucalyptus* hybrids estimated by genomic and EST microsatellite markers. *Biologia Plantarum. 55*(2), 379-382.

DAFF. [URL: http://www.daff.gov.za/#] (March, 2012).

Davies, P. J. (2004). Plant hormones: biosynthesis, signal transduction, action! 3rd Edn. Springer, Dordrecht, the Netherlands.

Dahllöf, I. (2002). Molecular community analysis of microbial diversity. *Current Opinion in Biotechnology. 13*, 213-217.

de Almeida, M. R., Ruedell, C. M., Ricachenevsky, F. K., Sperotto, R. A., Pasquali, G., & Fett-Neto, G. (2010). Reference gene selection for quantitative reverse transcription-polymerase chain reaction normalization during *in vitro* adventitious rooting in *Eucalyptus globulus* Labill. *BMC Molecular Biology. 11*(73), 1-12.

de Assis, T. F., Fett-Neto, A. G., & Alfenas, A. C. (2004). Current techniques and prospects for the clonal propagation of hardwoods with emphasis on *Eucalyptus*. *Plantation Forest Biotechnology for the 21st Century. Research Signpost.* 303-333.

Denger, K., Warthmann, R., Ludwig, W., & Schink, B. (2002). *Anaerophaga thermohalophila* gen. nov., sp. nov., a moderately thermohalophilic, strictly anaerobic fermentative bacterium. *International Journal of Systematic and Evolutionary Microbiology. 52*(1), 173-178.

Di Cello, F., Bevivino, A., Chiarinin, L., Fani, R., Paffetti, D., Tabacchioni, S., & Dalmastri, C. (1997). Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. *Applied and Environmental Microbiology. 63*(11), 4485-4493.

Díaz, K., Valiente, C., Martínez, M., Castillo, M., & Sanfuentes, E. (2009). Root-promoting rhizobacteria in *Eucalyptus globulus* cuttings. *World Journal of Microbiology and Biotechnology. 25*, 867-873.

dos Santos, P. E. T., Geraldi, I. O., & Garcia, J. N. (2004). Estimates of genetic parameters of wood traits for sawn timber production in *Eucalyptus grandis*. *Genetics and Molecular Biology. 27*(4), 567-573.

Dubey, S. K., Tripathi, A. K., & Upadhyay, S. N. (2005). Exploration of soil bacterial communities for their potential as bioresource. *Bioresource Technology. 97*, 2217-2224.

Duineveld, B. M., Kowalchuk, G. A., Keijzer, A., van Elsas, J. D., & van Veen, J. A. (2001). Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Applied and Environmental Microbiology. 67*(1), *172-178*.

Eder, K. (1995). Gas chromatographic analysis of fatty acid methyl esters. *Journal of Chromatography Biomedical Applications. 671,* 113-131.

Eldridge, K. G., Davidson, J., Harwood, C., & van Wyk, G. (1993). Eucalypt domestication and breeding. Clarendon Press, Oxford.

Ellstrand, N. C., Whitkus, R., & Rieseberg, L. H. (1996). Distribution of spontaneous plant hybrids. *Proceedings of the National Academy of Sciences. 93*, 5090-5093.

Elster, C., & Perdomo, L. (1999). Rooting and vegetative propagation in *Languncularia racemosa*. *Aquatic Botany. 63*, 83-93.

Enebak, S. A., Wei, G., & Kloepper, J. W. (1998). Effect of plant growth promoting rhizobacteria on loblolly and slash pine seedlings. *Forest Science. 44*, 139-144.

Erturk, Y., Ercisli, S., Haznedar, A., & Cakmakci, R. (2010). Effects of plant growth promoting rhizobacteria (PGPR) on rooting and root growth of kiwifruit (*Actinidia deliciosa*) stem cuttings. *Biological Research. 43*, 91-98.

Esitken, A., Ercisli, S., Secik, S., & Sahin, F. (2003). Effect of indole-3-butyric acid and different strains of *Agrobacterium rubi* on adventives root formation from softwood and semi-hardwood wild sour cherry cuttings. *Turkish Journal of Agriculture and Forestry. 27*, 37-42.

Ezekiel, A. (2010). Viable options and factors in consideration for low cost vegetative propagation of tropical trees. *International Journal of Botany. 6*(2), 187-193.

Fantroussi, S., Verschuere, L., Verstraete, W., & Top, E. M. (1999). Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Applied and Environmental Microbiology. 65*(3), 982-1000.

Finnigan, J., & Poulton, R. (2005). Improving adoption of salinity management options in Tasmania: Lanholder Survery Report. Private Forests Tasmania, Tasmania.

Flaishman, M. A., Eyal, A., Zilberstein, C., Voisard, C., & Hass, D. (1996). Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Psedomonas putida*. *Molecular Plant-Microbe interactions*. *9*, 642-654.

Fogaça, C. M., & Fett-Neto, A, G. (2005). Role of auxin and its modulators in the adventitious rooting of *Eucalyptus* species differing in recalcitrance. *Plant Growth Regulation. 45*, 1-10.

Forestry South Africa. [URL: http://www.forestry.co.za/] (April, 2012).

Fossey, A. (2009). Breeding strategies for polyploidy *Acacia* and *Eucalyptus*. CSIR/NRE/FOR/IR/2009/0089/B, NRE, CSIR.

Furze, M. J., & Cresswell, C, F. (1985). Micropropagation of *Eucalyptus grandis* and *nitens* using tissue culture techniques. *South African Forestry Journal. 135*, 20-23.

GAP Gardens. [URL: http://www.http://www.gapphotos.com/visualfeatureresearch.asp.co.za/] (July, 2012).

García de Salamone, I. E., Hynes, R. K., & Nelson, L. M. (2006). Role of cytokinins in plant growth promotion by rhizosphere bacteria. In PGPR: Biocontrol and Biofertilization. Eds. Z.A. Siddiqui. Springer, Netherlands. (pp. 173-195).

Gardner, R. A. W. (2007). Investigating the environmental adaptability of promising subtropical and cold-tolerant eucalypt species in the warm temperate climate zone of KwaZulu-Natal, South Africa. *Southern Hemisphere Forestry Journal. 69*(1), 27-38.

Gasper, M. J., Borralho, N., & Gomes, A. L. (2005). Comparison between field performance of cuttings and seedlings of *Euclayptus globulus*. *Annals of Forest Science. 62*, 837-841.

Gene Technics. [URL: http://genetechnics.com/eucalyptus-tree-seed/] (June, 2012).

Girijasjankar, V. (2012). *In vitro* regeneration of *Eucalyptus camaldulensis*. *Physiology and Molecular Biology of Plants. 18*(1), 79-87.

Giraffa, G., & Neviani, E. (2001). DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *International Journal of Food Microbiology. 67*, 19-34.

Glucksman, A. M., Skipper, H. D., Brigmon, R. L., & Santo Domingo, J. W. (2000). Use of the MIDI-FAME technique to characterize groundwater communities. *Journal of Applied Microbiology. 88,* 711-719.

Gonzalez, N., Beemster, G. T. S., & Inzè, D. (2009), David and Goliath: what can the tiny weed *Arabidopsis* teach us to improve biomass production in crops? *Current Opinion in Plant Biology. 12*, 157-164.

Google Maps. [URL: https://maps.google.co.za/maps?hl=en] (August, 2013).

Grattapaglia, D., & Sederoff, R. (1994). Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. *Genetics. 137*, 1121-1137.

Grattapaglia, D., Bertolucci, F. L., & Sederoff, R. (1995). Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. *Theoretical and Applied Genetics. 90*, 933-947.

Grob, R. L., & Barry, E. F. (2004). Modern practice of gas chromatography. Eds. LG Robert, EF Larry. Wiley.com.

Gutierrez Mañero, F. J., Acero, N., Lucas, J. A., & Probanza, A. (1996). The influence of native rhizobacteria on European alder [*Alnus glutinosa (L.) Gaertn*.] growth. II. Characterization of growth promoting and growth inhibiting strains. *Plant Soil. 182*, 67-74.

Harfouche, A., Meilan, R., Kirst, M., Morgante, M., Boerjan, W., Sabatti, M., & Mugnozza, G. S. (2012). Accelerating the domestication of forest trees in a changing world. *Trends in Plant Science. 17*(2), 64-72.

Hartmann, H. T., Kester, D. E., & Davis, F. T. Jr. (1990). Plant propagation: Principles and Practices (5th edition), Prentice-Hall International Editions, Englewood Cliffs, New Jersey.

Hartmann, A., Rothballer, M., & Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant Soil. 312*, 7-14.

Henderson, J. E. (1967). South African pulpwood and its relation to the quality of pulp and paper. *South African Forestry Journal. 60*(1), 8-14.

Hinsinger, P., Bengough, A. G., Vetterlein, D., & Young, I. M. (2009). Rhizosphere: biophysics, biogeochemistry and ecological relevance. *Plant Soil. 321*, 117-152.

House, S. M. (1997). Reproductive biology of eucalypts. In Eucalypt Ecology: Individuals to ecosystems. Eds. J Williams, J Woinarski. Cambridge University Press.

Hu, W., Harding, S. A., Lung, J., Popko, J. L., Ralph, J., Stokke, D. D., Tsai, C., & Chiang, V. L. (1999). Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nature Biotechnology. 17*, 808-812.

Hussey, G. (1978). The application of tissue culture to vegetative propagation of plants. *Scientific Progress Oxford. 65*, 185-208.

ICFR. [URL: http://www.icfr.ukzn.ac.za/] (September, 2012).

Ichihara, K., & Fukubayashi, Y. (2010). Preparation of fatty acid methyl esters for gas-liquid chromatography. *Journal of Lipid Research. 51*, 635-640.

Inglett, P. W., Reddy, K. R., & Corstanje, R. (2005). Anaerobic soils. *Encyclopedia of soils in the environments. Elsevier Science, Oxford, UK*, 71-78.

Integrated Breeding. [www.integratedbreeding.net/courses/genomics-and-comparative-genomics] (November, 2013).

Ishii, K. (2009). DNA markers in *Eucalyptus* with emphasis on species identification. *Environment Control in Biology. 47*(1), 1-11.

Islam, M. R., Trivedi, P., Palaniappan, P., Reddy, M. S., & Sa, T. (2009). Evaluating the effect of fertilizer application on soil microbial community structure in rice based cropping system using fatty acid methyl esters (FAME) analysis. *World Journal of Microbiology and Biotechnology. 25*, 1115-1117.

Jacobs, E., & Punt, C. (2010). South African trade in 2008, focusing on agriculture, forestry and fisheries. *PROVIDE Project Background Paper 2010*:1.

Justé, A., Thomma, B. P. H. J., & Lievens, B. (2008). Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. *Food Microbiology. 25*, 745-761.

Kaneda, T. (1999). Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiology and Molecular Biology Reviews. 55*(2), 288-302.

Kao, C. M., Chen, C. S., Tsa, F. Y., Yang, K. H., Chien, C. C., Liang, S. H., Yang, C., & Chen S. C. (2009). Application of real-time PCR, DGGE fingerprinting, and culture-based method to evaluate the effectiveness of intrinsic bioremediation on the control of petroleum-hydrocarbon plume. *Journal of Hazardous Materials. 178*, 409-416.

Karliński, L., Rudawska, M., & Leski, T. (2013). The influence of host genotype and soil conditions on ectomycorrhizal community of poplar clones. *European Journal of Soil Biology*. *58*, 51-58.

Karliński, L., Rudawska, M., Kieliszewska-Rokicka, B., & Leski, T. (2010). Relationship between genotype and soil environment during colonization of poplar roots by mycorrhizal and endophytic fungi. *Mycorrhiza. 20*, 315-324.

Kartel, B., Wessels, H. J. C. T., van der Blezen, E., Francoljs, K. J., Jetten, M. S. M., Klotz, M. G., & Stein, L. Y. (2012). Effects of nitrogen dioxide and anoxia on global gene and protein expression in long-term continuous cultures of *Nitrosomonas eutropha* C91. *Applied and Environmental Microbiology. 78*(14), 4788-4794.

Kennedy, L. R., Pereg-Gerk, C., Wood, R., Deaker, K., Gilchrist, K., & Katupitiya, S. (1997). Biological nitrogen fixation in nonleguminous field crops: facilitating the evolution of an effective association between *Azospirillum* and wheat. *Plant Soil*. *194*, 65-79.

Khalid, A., Arshad, M., & Zahir, Z. A. (2004). Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology. 96,* 473-480.

Kien, N. D., Quang, T. H., Jansson, G., Harwood, C., Clapham, D., & von Arnold, S. (2009). Cellulose content as a selection trait in breeding for kraft pulp yield in *Eucalyptus urophylla*. *Annals of Forest Science. 66*, 711-719.

Kirk, J. L., Beaudette, L. A., Hart, M., Moutoglis, P., Klironomos, J. N., Lee, H., & Trevors, J. T. (2004). Methods of studying soil microbial diversity. *Journal of Microbiological Methods. 58*, 169-188.

Koblížek, M., Béjá, O., Bidigare, R. R., Christensen, S., Bentez-Nelson, B., Vetriani, C., Kolber, M. K., Falkowski, P. G., & Kolber, Z. S. (2003). Isolation and characterization of *Erythrobacter* sp. strains from the upper ocean. *Archives of Microbiology. 180*, 327-338.

Komakech, C., Swain, T., & Fossey, A. (2009). Growth potential of *Eucalyptus globules* subsp. b*icostata* provenances for mid-altitude summer rainfall regions of South Africa. *Southern Forests. 71*(1), 1-9.

Kozdrój, J., Dirk, J., & van Elsas, J. D. (2001). Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. *Journal of Microbiological Methods. 43,* 197-212.

Lambers, H., Mougel, C., Jaillard, B., & Hinsinger, P. (2009). Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. *Plant Soil. 321*, 83-115.

Lee, D. J., Debuse, V. J., Pomroy, K. J., & Nikles, D, G. (2005). Developing genetically adapted tree varieties for marginal areas of Northern Australia. Joint Venture Agroforestry Program.

Liesack, W., Bak, F., Kreft, J., & Stackebrandt, E. (1994). *Holophaga foetida* gen. nov., a new, homoacetogenic bacterium degrading methoxylated aromatic compounds. *Archives of Microbiology. 162*(1-2), 85-90.

Little, K. M., van Staden, J., Peter, G., & Clarke, Y. (2003). The relationship between vegetation management and the wood and pulping properties of a *Eucalyptus* hybrid clone. *Annals of Forest Science. 60*, 673-680.

Liu, W. T., Marsh, T. L., Cheng, H., & Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology. 63*(11), 4516-4523.

Long, H. H., Schmidt, D. D., & Baldwin, I. T. (2008). Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PLoS One*. *3*(7), 1-10.

Lovell, C. R., Piceno, Y. M., Quattro, J. M., & Bagwell, C. E. (2000). Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, *Spartina alterniflora*. *Applied and Environmental Microbiology. 66*(9), 3814-3823.

Luckman, G. A., & Menary, R. C. (2002). Increased root initiation in cutting of *Eucalyptus nitens* by delayed auxin applications. *Plant Growth Regulation. 38*, 31-35.

Lynch, J. M., Benedetti, A., Insam, H., Nuti, M. P., Smalla, K., Torsvik, V., & Nannipieri, P. (2004). Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. *Biology and Fertility of Soils. 40*, 363-385.

MacMahon, L., George, B., & Hean, R. (2010). *Eucalyptus camaldulensis*. *PRIMEFACT 1054*.

Malan, F. S., & Arbuthnot, A. L. (1995). The inter-relationships between density and fibre properties of South African grown *Euclayptus grandis*. In Eucalypt plantation: Improving fibre yield and quality. Eds. NMG Borralho, JB Reid, RN Cromer, WN Tibbits, CA Raymond. Proc. CRCTHF-IUFRO conference, Hobart (pp. 19-24).

Malave-Orengo, J., Borglin, S. E., Hazen, T. C., & Rios-Velazquez, C. (2010). A modified cell extraction method to acces microbial community structure in soil samples by phospholipid fatty acid analysis. In Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Eds. Méndez-Vilas. (pp. 1562-1569).

Malinda Morrison. [URL: http://melindamorrison.files.wordpress.com/2011/01/south-africa-map.jpg] (July, 2012).

Mallory, L. M., & Sayler, G. S. (1984). Application of FAME (Fatty Acid Methyl Ester) analysis in the numerical taxonomic determination of bacterial guild structure. *Microbial Ecology. 10,* 283-296.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., & Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology. 64*(2), 795-799.

Marschner, P. (2007). Soil microbial community structure and function assessed by FAME, PLFA and DGGE – advantages and limitations. In Advanced Techniques in Soil Microbiology. Eds. A Varma, R Oelmüller. Springer-Verlag berlon Heidelberg. (pp 181 – 200).

Martínez-Alonso, M., Escolano, J., Montesinos, E., & Gaju, N. (2010). Diversity in the surface soil of pear orchard based on 16S rRNA gene analysis. *International Microbiology*. *13*, 123-134.

McNear, D. H. (2013). The rhizosphere – roots, soil and everything in between. *Nature Education Knowledge. 4*(3), 1.

Merkle, S. A., & Dean, J. F. D. (2000). Forest tree biotechnology. *Current Opinion in Biotechnology. 11*, 298-302.

Moura, A., Tacao, M., Henriques, I., Dias, J., Ferreira, P., & Correia, A. (2009). Characterization of bacterial diversity in two aerated lagoons of wastewater treatment plant using PCR-DGGE analysis. *Microbial Research. 164*(5), 560-569.

Morgan, J. A. W., Bending, G. D., & White, P. J. (2005). Biological costs and benefits to plant-microbe interactions in the rhizosphere. *Journal of Experimental Botany. 56*(417), 1729-1739.

Muyzer, G., de Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology. 59*, 695-700.

Muyzer, G., & Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek. 73,* 127-141.

Muyzer, G., Yildirim, E., van Dongen, U., Kühl, M., & Thar, R. (2005). Identification of "*Candidatus Thioturbo danicus*," a microaerophilic bacterium that builds conspicuous veils on sulfidic sediments. *Applied and Environmental Microbiology. 7*(12), 8929-8933

Myers, R. M., Maniatis, T., & Lerman, L. S. (1987). Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods in Enzymology. 155*, 501-527.

Nakatsu, C. H. (2007). Soil microbial community analysis using denaturing gradient gel electrophoresis. *Soil Science Society of America Journal. 7,* 562-571.

NCBI. [URL: www.ncbi.nlm.nih.gov/books/NBK21097] (November, 2013).

O' Donnell, A. G. O., Seasman, M., Macrae, A., Waite, I., & Davies, J. T. (2001). Plants and fertilizers as drivers of change in microbial community structure and function in soils. *Plant and Soil. 232,* 135-145.

Oh, S. T., & Martin, A. D. (2010). Long chain fatty acids degradation in anaerobic digester: thermodynamic equilibrium consideration. *Process Biochemistry. 45*(3), 335-345.

Okamura, K., Kanbe, T., & Hiraishi, A. (2009). *Rhodoplanes serenus* sp. nov., a purple non-sulfur bacterium isolated from pond water. *International Journal of Systematic and Evolutionary Microbiology. 59*(3), 531-535.

O' Neill, G., Radley, R., & Chanway, C. (1992). Variable effects of emergence-promoting rhizobacteria on conifer seedling growth under nursery conditions. *Biology and Fertility of Soils. 13*, 45-49.

Pankhurst, C. E., Yu, S., Hawke, B. G., & Harch, B. D. (2001). Capacity of fatty acid profiles and substrate utilization patterns to describe differences in soil microbial communities associated with increase salinity or alkalinity at three locations in South Australia. *Biology and Fertility of Soils. 33*, 204-217.

Patena, L., Stutter, E. G., & Dandekar, A. M. (1988). Root induction by *Agrobacterium rhizogenes* in a difficult-to-root woody species. *Acta Horticulturae*. 227, 324-329.

Peralta, K. D., Araya, T., Valenzuela. S., Sossa, K., Martínez, M., Pena-Cortés, H., & Sanfuentes, E. (2012). Production of phytohormones, siderophores and population fluctuation of two root-promoting rhizobacteria in *Eucalyptus globulus* cuttings. *World Journal of Microbiology and Biotechnology. 28*, 2003-2014.

Pieffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., Buckler, E. S., & Ley, R. E. (2013). Diveristy and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences. 110*(16), 6548-6553.

Plant Hormones. [URL: http://www.plant-hormones.info/ethylene.htm] (September, 2012).

Pogue, T. E. (2008). A sectoral analysis of wood, paper, pulp industries in South Africa. Institute for Economic Research on Innovation.

Potts, B. M., & Dungey, H. S. (2004). Interspecific hybridization of *Eucalyptus*: key issues for breeders and geneticists. *New Forests. 27*,115-138.

Pullman, G. S., & Timmis, R. (1992). Establishment of juvenile-like shoot cultures and plantlets from 4-16 year-old Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) trees. *Plant Cell, Tissue and Organ Culture*. 29, 187-198.

Rahim, F., Jabeen, M., & Ilahi. (2003). Mass propagation in *Eucalyptus camaldulensis* Dehn. *Asian Journal of Plant Sciences. 2*(2), 184-187.

Ranjard, L., Poly, F., & Nazaret, S. (2000). Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. *Research in Microbiology. 151*, 167-177.

Rickard, A. H., Stead, A. T., O'Maly, G. A., Lindsay, S., Banner, M., Handley, P. S., & Gilbert, P. (2005). *Adhaeribacter aquaticus* gen. nov., sp. nov., a gram-negative isolate from a potable water biofilm. *International Journal of Systematic and Evolutionary Microbiology. 55*, 821-829.

Romero, C., Bellée, J. M., Vayá, J. L., Serrano, R., & Culiáñex-Maciá, F. A. (1997). Expression of the yeast *trehalose-6-phospate synthetase* gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta. 201*, 293-297.

Sasse, J., & Sands, R. (1997). Configuration and development of root systems of cutting and seedlings of *Eucalyptus globules*. *New Forests. 14*, 85-105.

Sasser, M. (2009). Microbial identification by gas chromatographic analysis of fatty acid methyl esters (GC-FAME). MIDI Technical Note 101. Newark, DE.

Shackleton, C. M., Shackleton, S. E., Buiten, E., & Bird, N. (2007). The importance of dry woodlands and forests in rural livelihoods and poverty alleviation in South Africa. *Forestry Policy and Economics. 9*(5), 558-577.

Sharma, S. L., & Ramamurthy, V. (2000). Micropropagation of 4-year old elite *Eucalyptus tereticornis* trees. *Plant Cell Reports. 19*, 511-518.

Singh, B. K., Nunan, N., Risgway, K. P., McNicol, J., Young, J. P. W., Daniell, T. J., Prosser, J. I., & Millard, P. (2008). Relationship between assemblages of mycorrhizal fungi and bacteria on grass roots. *Environmental microbiology. 10*(2), 534-541.

Soil Management. [URL: http://www.ctahr.hawaii.edu/mauisoil/c_nutrients01.aspx] (July, 2013).

Stein, L. Y., Arp, D. J., Berube, P. M., Chain, P. S. G., Hauser, L., Jetten, M. S. M., Klotz, M. G., Larimer, F. W., Norton, J. M., Op den Camp, H. J. M., Shin, M., & Wei, X. (2007). Whole-genome analysis of the ammonia-oxidizing bacterium, *Nitrosomonas Eutropha* C91: implications for niche adaptation. *Environmental Microbiology. 9*(12), 2993-3007.

Sunshine Seedlings Services. [URL: http://www.sunshineseedlings.co.za/] (June, 212).

Swain, T., & Jones, W. R. E. (2004). Full rotation measurements of *Eucalyptus badjensis* trials in the summer rainfall region of South Africa. *ICFR Bulletin Series 01/2004*. Pietermaritzburg: Institute for Commercial Forestry Reseach.

Teixeira, D. A., Alfenas, A. C., Goncalves, R., Ferreira, E. M., de Sequeira, L., Maffia, L. A., & Mounteer, A. H. (2007). Rhizobacterial promotion if eucalypt rooting and growth. *Brazilian Journal of Microbiology. 38*, 118-123.

The Wood Foundation. [URL: http://thewoodfoundation.co.za/] (April, 2012).

Thomashow, L. S. (1996). Biological control of plant pathogens. *Current Opinion in Biotechnology. 7*(3), 343-347.

Titon, M., Xavier, A., & Otoni, W. C. (2006). Clonal propagation of *Eucalyptus grandis* using the mini-cutting and micro-cutting techniques. *Scientia Forestalis. 71*, 109-117.

University of California Agriculture and Natural Resources. [URL: http://www.ipm.ucdavis.edu/PMG/r280190211.html] (June, 2013).

Velazquez-Selúlveda, I., Orozco-Mosqueda, M. C., Prieto-Barajas, C. M., & Santoyo, G. (2012). Bacterial diversity associated with the rhizosphere of wheat plants (*Triticum aestivum*): Toward a metagenomic analysis. *Phyton, International Journal of Experimental Botany. 81*, 81-87.

Virtual Chembook. [URL: http://www.elmhurst.edu/~chm/vchembook/550lipids.html] (September, 2012).

Weon, H. Y., Kim, B. Y., Son, J. A., Jang, H. B., Hong, S. K., Go., S. J., & Kwon, S. W. (2008). *Massilia aerilata* sp. nov., isolated from an air sample. *International Journal of Systematic and Evolutionary Microbiology. 58*, 1422-1425.

Werner, T., Nehnevajova, E., Köllmer, I., Novák, O., Strand, M., Krämer, U., & Schmülling, T., (2010). Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsos* and tobacco. *The Plant Cell. 22*, 3905-3920.

Whiting, D., Roll, M., & Vickerman, L. (2011). Plant growth factors: plant hormones. Colorado Master Gardener, Colorado State University Extension, *Minor revisions. 145*, 1-4.

Winton, L. (1970). Notes: Tissue culture propagation of European Aspen. *Forest Science. 17*(3), 348-350.

Wu, G., Shortt, B. J., Lawrence, E. B., Levine, E. B., Fitzsimmons, K. C., & Shah, D. M. (1995). Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. *The Plant Cell. 7*, 1357-1368.

WWF. [URL: http://wwf.panda.org/about_our_earth/about_forests/] (March, 2012).

Zhang, Y. M., & Rock, C. O. (2008). Membrane lipid homeostasis in bacteria. *Nature Reviews Microbiology. 6*(3), 222-233.

Appendices

Appendix A: **Patrick, M.A., Fossey, A., de Smidt, O. Broad characterisation of *Eucalyptus* rhzispheric communities using fatty acid methyl ester analysis. Submitted for publication to Applied Soil Ecology.**

Appendix B: **Patrick, M.A., de Smidt, O., Fossey, A. Characterising *Eucalyptus* hybrid and species rhizobacterial communities in KwaZulu-Natal, South Africa. Submitted for publication to Southern Forests.**

Appendix C: **FastDNA® Spin Kit for soil**

Appendix D: **EDTA/Ethanol precipitation protocol**

Appendix A

Broad characterisation of *Eucalyptus* rhizospheric communities using fatty acid methyl ester analysis

Melanie A. Patrick[a], Annabel Fossey[a], Olga de Smidt[a*]

[a]*Department of Life Sciences, Central University of Technology, Private Bag X20339, Bloemfontein, 9300, South Africa*

Abstract

The demand for *Eucalyptus* wood from commercial plantations for pulp and paper production is high. Hybridisation focuses on developing *Eucalyptus* genotypes with good pulp and paper quality phenotypes. *Eucalyptus* hybrids are multiplied via cuttings allowing the formation of clonal plantations. Although clonal plantations boast superior genotypes and uniformity, they suffer low rooting percentages. The forestry industry has recognised the potential of rhizobacterial preparations to enhance rooting ability. Rhizospheric samples were collected from *Eucalyptus* hybrids and species and their microbial community broadly characterised on a biochemical level using fatty acid methyl ester (FAME) analysis. FAME profiles were generated and differences among and between FAME profiles determined. FAME analysis showed that *Eucalyptus* hybrids and species varied in type and quantity of fatty acid present, suggesting a genotypic effect.

Keywords:
Rhizosphere
Eucalyptus
Rhizobacteria
Fatty acid methyl esters (FAME)

1. Introduction

South Africa has become a world leader in plantation forestry (Beck and Dunlop, 2001; Pogue, 2008; Jacobs and Punt, 2012). The province of Mpumulanga is home to most of the plantation forests (40.8%), followed by KwaZulu-Natal (39.6%) (Pogue, 2008). Plantation forests in South Africa are made up of three species, *Pinus*, *Eucalyptus* and black wattle (*Acacia mearnsii*). *Pinus* is the major contributor, comprising 52% of South African plantation forests (Pogue, 2008). *Eucalyptus* occupies 39%, while the remaining plantation forests are made up of black wattle.

Eucalyptus is of economic importance in South Africa (dos Santos et al., 2004). Their adaptability, fast growth and usefulness of their wood are characteristics contributing to their success (dos Santos et al., 2004). *Eucalyptus* wood is important for the pulp and paper industry (Pogue, 2008). Of the 700 *Eucalyptus* species, superior genotypes are identified and bred to produce phenotypes with good pulp and paper characteristics required by this industry (Grattapaglia and Sederoff, 1994; Ishii, 2009; Cupertino et al., 2011).

Eucalyptus species have low reproductive barriers, allowing them to hybridise (Fossey, 2009). The process of hybridisation allows years of evolutionary diversity to be combined into a single genotype (Fossey, 2009). The advantages of hybridisation include better-performing and better-adapted *Eucalyptus* hybrids to be bred and grown in harsh environmental conditions (Chetty, 2001; Komakech et al., 2009). The main focus of *Eucalyptus* hybridisation in South Africa is to improve timber outputs for the pulp and paper industry (Fossey, 2009).

Eucalyptus hybrids are multiplied through clonal propagation by rooting cuttings (Fossey, 2009). Breeding programmes using *Eucalyptus* cuttings produce clones that result in superior, uniform genotypes. A major drawback of *Eucalyptus* cuttings is their variety in rooting ability. The low percentage in rooting ability of *Eucalyptus* cuttings causes production and financial losses for the forestry industry. Therefore, the industry focuses on improving the rooting percentages of *Eucalyptus* cuttings.

Rooting ability has previously been enhanced using phytohormones to stimulate adventitious rooting (Whiting et al., 2011). More recently, rhizobacteria located in the area in direct contact with the root, known

as the rhizosphere have been used to promote rooting (Díaz et al., 2009). The mechanism of how these plant growth promoting rhizobacteria (PGPR) stimulate adventitious rooting is not fully understood, but it is thought that the PGPRs produce plant hormones beneficial to the root and/or siderophores that work to rid harmful microorganisms.

Several rhizobacterial preparations have been successfully applied to improve rooting percentages (Chanway et al., 1991; Enebak et al., 1998; Teixeira et al., 2007; Díaz et al., 2009). Preparations consisting of *Bacillus* and *Pseudomonas* have proven to do well in improving root formation in 'Golden Delicious' apples (Patena et al., 1988) and wild sour cherry softwood and semi-hardwood cuttings (Esitken et al., 2003). However, rhizobacterial preparations appear to be species specific, as native bacterial endophytes promote host growth in a species-specific manner (Long et al., 2008). This observation emphasises the importance of first characterising rhizobacterial communities of *Eucalyptus* species and hybrids before actual preparation of rhizobacterial preparations. In this study, rhizobacterial communities of *Eucalyptus* species and hybrids were characterised using fatty acid methyl ester (FAME) analysis to ascertain broad differences among and between the rhizobacterial communities.

2. Materials and methods

2.1 Site description

The province of KwaZulu-Natal (KZN) in South Africa was the source of sample collection. Pietermaritzburg and its surrounding areas is where many of South Africa's plantation forestry occurs (Pogue, 2008). Rhizospheric samples were obtained from two separate nurseries in the Pietermaritzburg area. One was a private commercial nursery where *Eucalyptus* hybrid rhizospheric samples were collected. The other nursery was a research nursery located at the Institute for Commercial Forestry Research (ICFR), where *Eucalyptus* species rhizospheric samples were collected.

2.2 Soil sampling

A total of 62 soil samples were collected in June 2012 from *Eucalyptus* hybrid and species rhizospheres as well as potting soil from each nursery which served as controls. Each rhizospheric sample contained

approximately 30 g of soil from the area around the rooting system and was placed in glass bottles to prevent any fatty acid contamination. After the samples were collected they were placed on ice for transportation and stored at -20°C until analysis.

Thirty of the rhizospheric samples were obtained from approximately two year old *Eucalyptus* cuttings from the commercial nursery. These rhizospheric samples originated from two hybrid genotypes, *E. grandis* × *E. nitens* (GN) and *E. grandis* × *E. urophylla* (GU). Ten rhizospheric samples were taken from the *Eucalyptus* hybrid GU111, and a further 10 rhizospheric samples from two different GN hybrids namely, GN018B and GNPP2107. An additional four control samples contained the final potting soil product that had been sprayed to eliminate pathogens before planting cuttings.

At the ICFR nursery 28 samples were collected. The ICFR rhizospheric samples came from seedlings from three different species; *E. macarthurii* (five rhizospheric samples), *E. smithii* (five rhizospheric samples) and *E. nitens* (four rhizospheric samples). These rhizospheric samples were taken from approximately six months old trees planted in bags. An additional 10 rhizospheric samples were taken from younger *E. nitens* seedlings (*E. nitens*229), still in trays. The ICFR control samples contained the final soil before planting seedlings.

2.3 Pooling of samples

Collected rhizospheric soil samples from the same *Eucalyptus* hybrids and species as well as the respective potting soils used as controls, were pooled together in such a way that a whole-soil FAME profile from each hybrid, species and control could be obtained. This resulted in a total of nine pooled samples ranging from 20 – 25 g of soil. The nine pooled samples represented nine specific microbial communities originating from four *Eucalyptus* hybrid and three *Eucalyptus* species rhizospheric samples, as well as two control soil samples.

2.4 Generation of FAME profiles

FAME analyses were performed on the nine pooled samples by SGS laboratories, Cape Town, South Africa. The AOCS Official Method Ce2-66 was used to prepare fatty acids for gas chromatography (AOCS,

1997). Following the general four-step procedure of FAME preparation which includes saponification, methylation, extraction and sample clean-up. FAMEs were then analysed by gas chromatography using the Agilent GC, model 7890A (Agilent Technologies, Santa Clara, CA, USA). FAME profiles were produced for each sample representing a specific community. The peak areas of the FAME profiles were recorded by ChemStation (Hewlett Packard, Sunnydale, CA).

2.5 Determination of significant differences among and between FAME profiles

Pearson Chi Square tests were performed to determine whether the difference among and between *Eucalyptus* hybrids and *Eucalyptus* species rhizospheric communities were of any significance. Three Pearson Chi Square statistics were calculated using the peak areas of the saturated fatty acids at a significance level of $\alpha = 0.05$, namely to test differences among *Eucalyptus* hybrids, among *Eucalyptus* species, and between *Eucalyptus* hybrids and the *Eucalyptus* species.

3. Results

3.1 FAME profiles

Gas chromatograms (FAME profiles) showed the overall fatty acid composition of each sample within a retention time of 22 minutes. The rhizospheric samples from the *Eucalyptus* hybrids GN018B, GNPP2107 and GU111 demonstrated similar FAME profiles (Fig. 1). In contrast, the FAME profile of the potting soil control had fewer peaks and therefore less fatty acids than the *Eucalyptus* hybrid samples.

The rhizospheric soils from the *Eucalyptus* species, *E. macarthurii, E. smithii*, and *E. nitens229*, as well as the potting soil used as a control showed similar FAME profiles; with the exception of *E. nitens* whose FAME profile had fewer peaks and therefore fewer fatty acids (Fig. 2). When compared to the *Eucalyptus* hybrid rhizospheric communities, the *Eucalyptus* species rhizospheric communities FAME profiles were more variable with notably more FAMEs.

A total of 18 different fatty acids were detected in the *Eucalyptus* hybrid and species rhizospheric communities, of which 13 could be identified. The concentrations of the different fatty acids were calculated

from the peak areas of the FAME profiles and expressed as a percentage. All 13 identified fatty acids had an even number of carbon atoms (indicated by the number before the colon in the abbreviation) ranging from 10 to 24 carbons (Table 1). Eight of these 13 fatty acids did not contain double bonds (indicated by the number after the colon in the abbreviation) in their structure and were therefore saturated. Four fatty acids had a single double bond, whereas one fatty acid, eicosatrienoic acid (C20:3cis11cis14cis17), had three double bonds in its structure. Sixteen of the detected fatty acids were found in soil communities from both *Eucalyptus* hybrids and species. The remaining two fatty acids, palmitoleic acid (C16:1) and lignoceric acid (C24:0) were only detected in some of the *Eucalyptus* species rhizospheric communities and in none of the *Eucalyptus* hybrid rhizospheric communities. Although all the communities contained most of the detected fatty acids, only the *Eucalyptus* species potting soil used as a control contained all 18 detected fatty acids. The *Eucalyptus* hybrid potting soil used as a control contained 13 of the identified fatty acids; this was also the community with the least number of detected fatty acids.

3.2 Comparison of FAME profiles

The Pearson Chi Square test revealed significant differences among the different *Eucalyptus* hybrid rhizospheric communities and among the different *Eucalyptus* species rhizospheric communities as well as between *Eucalyptus* hybrid and species rhizospheric communities at $\alpha = 0.05$.

4. Discussion and conclusion

The fatty acid contents of the respective *Eucalyptus* hybrids, species and control soil samples varied in type and quantity of fatty acid present. Myristic acid (C14:0) was found to be the most abundant fatty acid in all the *Eucalyptus* rhizospheric communities. In contrast, palmitoleic acid (C16:1) and lignoceric acid (24:0) were unique to the *Eucalyptus* species rhizospheric communities. The variation in fatty acids present supports the idea that rhizospheric diversity seems to be host genotype dependent (Berg et al., 2002; Peiffer et al., 2013).

Palmitic acid (C16:0) and palmitoleic acid (C16:1) differ only by a single bond, yet their quantities in the *Eucalyptus* rhizospheric communities varied greatly, where palmitic acid occurred in vastly greater concentration than palmitoleic acid. It has been shown that palmitic acid is associated with bacteria (Zhang

and Rock, 2008), while palmitoleic acid is associated with arbuscular mycorrhizal (AM) fungi (Lynch et al., 2004). Singh et al. (2008) stated that AM fungi were a major factor in determining the bacterial assemblage, but that the bacteria did not influence the AM fungi in return. Therefore, it can be argued that the presence of AM fungi in the rhizospheres of the *Eucalyptus* species influenced the particular bacterial assemblage in *Eucalyptus* species rhizospheres, while their absence in the *Eucalyptus* hybrid rhizospheres resulted in a substantially different bacterial assemblage in *Eucalyptus* hybrid rhizospheres.

Bradley reported in his studies that lauric acid (C12:0) has an inhibitory effect on bacteria that contain palmitic acid (C16:0) as a major component in their cell membrane (Bradley, 1970). However, this does not appear to be the case for *Eucalyptus* rhizospheric communities where lauric acid (C12:0) as well as palmitic acid (C16:0) occur together in abundance.

The fatty acid diversity of *Eucalyptus* hybrid and species rhizospheres supported the findings of Peiffer et al. (2013), who showed that field environments influenced rhizospheric diversity. Even though the *Eucalyptus* samples were collected from around the same town, Pietermaritzburg, there are differences that could influence the fatty acid compositions. These differences include among others, the potting soils used, the treatment of soils as well as the trees, the different genotypes, the way the trees are handled in the nurseries and the availability of water. The potting soil used for the placement of *Eucalyptus* hybrid cuttings is usually treated to eliminate pathogens and is regarded as 'clean' soil, therefore the low concentrations and diversity of bacteria in this soil sample is not surprising (Soil Management, http://www.ctahr.hawaii.edu/mauisoil/c_nutrients01.aspx). On the other hand, the untreated control soil sample used for the planting of the *Eucalyptus* species demonstrated similar fatty acid diversity and concentrations as their *Eucalyptus* species counterparts.

In conclusion, FAME analysis provided a means to rapidly identify and quantify the presence of fatty acids of rhizospheric communities of *Eucalyptus* hybrids and species.

Acknowledgements

We thank the Central University of Technology (CUT) and the National Research Foundation (NRF) for their funding to complete this study. In addition we also thank SGS who assisted with the FAME analysis.

6. References

Beck, S., Dunlop, RW., 2001. Micropropagation of the Acacia species – a review. In Vitro Cell Dev. Biol. Plant. 37, 531-538.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., Smalla, K., 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. Appl. Environ. Micro. 68(7), 3328-3338.

Bradley, W.H., 1970. Green River oil shale – concept of origin extended: an interdisciplinary problem being attacked from both ends. Geol. Soc. Am. Bull. 81(4), 985-1000.

Chanway, C.P., Radley, R.A., Holl, F.B., 1991. Inoculation of conifer seed with growth promoting *Bacillus* strains causes increased seedling emergence and biomass. Soil Biol. Biochem. 23, 575-580.

Chetty, S., 2001. The development of clone-specific micropropagation protocols for three commercially important *Eucalyptus* hybrids. Dissertation, University of Natal, Durban.

Cupertino, F.B., Leal, J.B., Corrèa, R.X., Gaiotto, F.A., 2011. Genetic diversity of *Eucalyptus* hybrids estimated by genomic and EST microsatellite markers. Biol. Plant. 55(2), 379-382.

Díaz, K., Valiente, C., Martínez, M., Castillo, M., Sanfuentes, E., 2009. Root-promoting rhizobacteria in *Eucalyptus globulus* cuttings. World J. Microb. Biot. 25, 867-873.

dos Santos, P.E.T., Geraldi, I.O., Garcia, J.N., 2004. Estimates of genetic parameters of wood traits for sawn timber production in *Eucalyptus grandis*. Int. J. Genet. Mol. Biol. 27(4), 567-573.

Enebak, S.A., Wei, G., & Kloepper, J.W., 1998. Effect of plant growth promoting rhizobacteria on loblolly and slash pine seedlings. For. Sci. 44, 139-144.

Esitken, A., Ercisli, S., Secik, S., Sahin, F., 2003. Effect of indole-3-butyric acid and different strains of *Agrobacterium rubi* on adventives root formation from softwood and semi-hardwood wild sour cherry cuttings. Turk. J. Agric. For. 27, 37-42.

Fossey, A., 2009. Breeding strategies for polyploidy *Acacia* and *Eucalyptus*. CSIR/NRE/FOR/IR/2009/0089/B, NRE, CSIR.

Grattapaglia, D., Sederoff, R., 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics. 137, 1121-1137.

Ishii, K., 2009. DNA markers in *Eucalyptus* with emphasis on species identification. Environ. Contr. Biol. 47(1), 1-11.

Jacobs, E., Punt, C., 2010. South African trade in 2008, focusing on agriculture, forestry and fisheries. *PROVIDE* 2010:1.

Komakech, C., Swain, T., Fossey, A., 2009. Growth potential of *Eucalyptus globules* subsp. b*icostata* provenances for mid-altitude summer rainfall regions of South Africa. Southern Forests. 71(1), 1-9.

Long, H.H., Schmidt, D.D., Baldwin, I.T., 2008. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS One. 3(7), 1-10.

Lynch, J.M., Benedetti, A., Insam, H., Nuti, M.P., Smalla, K., Torsvik, V., Nannipieri, P., 2004. Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. Biol. Fert. Soils. 40, 363-385.

Patena, L., Stutter, E.G., Dandekar, A.M., 1988. Root induction by *Agrobacterium rhizogenes* in a difficult-to-root woody species. Acta Hortic. 227, 324-329.

Peiffer, J.A., Spor, A., Koren, O., Jin, Z., Tringe, S.G., Dangl, J.L., Buckler, E.S., Ley, R.E., 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. 110(16), 6548-6553.

Pogue, T.E., 2008. A sectoral analysis of wood, paper, pulp industries in South Africa. Institute for Economic Research on Innovation.

Singh, B.K., Nunan, N., Risgway, K.P., McNicol, J., Young, J.P.W., Daniell, T.J., Prosser, J.I., Millard, P., 2008. Relationship between assemblages of mycorrhizal fungi and bacteria on grass roots. Environ. Microbiol. 10(2), 534-541.

Teixeira, D.A., Alfenas, A.C., Goncalves, R., Ferreira, E.M., de Sequeira, L., Maffia, L.A., Mounteer, A.H., 2007. Rhizobacterial promotion if eucalypt rooting and growth. Braz. J. Microbiol.. 38, 118-123.

Whiting, D., Roll, M., Vickerman, L., (2011). Plant growth factors: plant hormones. Colorado Master Gardener, Colorado State University Extension, Minor revisions. 145, 1-4.

Zhang, Y.M., Rock, C.O., 2008. Membrane lipid homeostasis in bacteria. Nat. Rev. Microbiol. 6(3), 222-233.

Appendix B

Characterising *Eucalyptus* hybrid and species rhizobacterial communities in KwaZulu-Natal, South Africa

Melanie A. Patrick[1], Olga de Smidt[1], Annabel Fossey[1*]

[1]Department of Life Sciences, Central University of Technology, Private Bag X20339, Bloemfontein, 9300, South Africa

**Corresponding author, e-mail: afossey@cut.ac.za*

Abstract

Eucalyptus plantations in South Africa are made up of hybrids and species. *Eucalyptus* hybrids are multiplied through cuttings. Cuttings of many *Eucalyptus* hybrid genotypes demonstrate poor rooting causing substantial production and financial losses. The application of rhizobacteria has shown improved rooting of cuttings. The aim of this study was to determine the genetic diversity of the 16S rRNA gene using denaturing gradient gel electrophoresis (DGGE) and to compare the different rhizobacterial communities of *Eucalyptus* hybrids and species. Samples were collected from the rhizosphere of three *Eucalyptus* hybrids and four *Eucalyptus* species. Phylogenetic dendrograms generated from DGGE profiles showed that there was a higher similarity among the closer related *Eucalyptus* hybrid rhizobacterial communities suggesting a host genotypic influence. Of the DNA sequences subjected to BLAST, 29 were uniquely linked to specific bacteria. The expected root enhancing rhizobacteria, *Bacillus* and *Pseudomonas* were not identified, however, Nitrosomona *eutropha* and *Adhaeribacter aquaticus* proved possible candidates for further investigation into their potential to enhance rooting of *Eucalyptus* cuttings.

Keywords: Clones, Denaturing gel gradient electrophoresis (DGGE), *Eucalyptus,* rhizobacteria, rhizosphere

Introduction

Plantation forests cover 1.3 million hectares of South Africa's land (Pogue 2008). Commercial plantation forests are major contributors to the South African economy. South Africa plays an important role in the saw timber and pulp and paper industry and is one of only two countries in Africa that significantly contributes to this industry (Shackleton et al. 2007, Pogue 2008). The South African plantation forests are made up of pine, eucalypts and black wattle (Komakech et al. 2009). Pine is the major forestry contributor comprising 52% of plantation forests (Pogue 2008). *Eucalyptus* hybrids and species are the main resource for the high demanding saw timber and pulp and paper industry, occupying 39% of plantation forests (Swain and Jones 2004, Pogue 2008). The remaining plantation forests consist of Black wattle, also a source for high quality material for the pulp and paper industry (Chetty 2001, Beck and Fossey 2007).

Eucalyptus plantations in South Africa are made up of hybrids and species. There are over 700 *Eucalyptus* species (Grattapaglia and Sederoff 1994, Ishii 2009, Cupertino et al. 2011). *Eucalyptus* species with superior genotypes are identified and bred. The superior genotypes are bred to produce phenotypes with characteristics important mostly for the pulp and paper industry. The ability of *Eucalyptus* species to hybridise allows tree breeders to rapidly combine years of evolutionary diversity into one genotype. A major advantage associated with hybridisation includes better adapted *Eucalyptus* hybrids to less favourable climatic conditions (Komakech et al. 2009).

Eucalyptus hybrids are multiplied through cuttings, producing clones. In this way uniformity is achieved and favourable genotypes are kept in the population. In contrast, a major disadvantage of cloning is the range of rooting ability that hybrids display. Cuttings of some *Eucalyptus* hybrids demonstrate poor rooting causing substantial production and financial losses. Increased production costs and the limited availability of land for forestry are important factors affecting this industry's sustainability. To meet the increasing demand, plantation forest companies need to increase their outputs. Therefore, the forestry industry has recognised the importance of improving rooting percentages of clonal cuttings.

A variety of rooting enhancing strategies are being investigated. One such strategy involves the addition of hormones to stimulate adventitious rooting (Whiting et al. 2011). More recently, a novel strategy is to use rhizobacteria to stimulate rooting (Asghar et al. 2004, Khalid et al. 2004, Díaz et al. 2009). Rhizobacteria

are found in the environment known as the rhizosphere which comprises the soil region in direct contact with the roots. The microbial diversity in the rhizosphere can be beneficial to the host plant (Asghar et al. 2004). The effects of rhizobacteria stimulation have been seen in canola (Asghar et al. 2004) and wheat (Khalid et al. 2004), where growth and yield were increased. The rhizobacteria that increase the overall well being and production of plants are referred to as plant growth promoting rhizobacteria (PGPR). The mechanisms by which PGPRs enhance rooting ability are not completely known. There are suggestions that rhizobacteria produce plant hormones and fix nitrogen asymbiotically (Gutierrez Mañero et al. 1996, Kennedy et al. 1997). Another suggestion is that the PGPRs produce siderophores against harmful microorganisms (Flaishman et al. 1996).

Recent studies confirm that treatments of cuttings with non-pathogen bacteria such as *Bacillus* and *Pseudomonas* induced root formation in some plants (Patena et al. 1988, Esitken et al. 2003). A similar effect has been seen in forestry species, including *Pinus taeda*, *Pinus elliotii*, *Pinus contorta*, *Picea glauca* and *Pseudotsuga menziessii* (Chanway et al. 1991, O'Neill et al. 1992, Enebak et al. 1998). The use of rhizobacterial treatments on *Eucalyptus* hybrids and species is limited, however in Brazil the ability of *Eucalyptus grandis* cuttings has also been increased through rhizobacterial stimulation (Teixeira et al. 2007). The rhizobacterial preparations seem to be somewhat species specific (Long et al. 2008). Therefore, to make rhizobacterial preparations for the rooting enhancement of *Eucalyptus* hybrid and species cuttings, their rhizobacterial communities first need to be studied and characterised. Therefore, the aim of this study was to determine the genetic diversity of the 16S rRNA gene using denaturing gradient gel electrophoresis (DGGE) and to compare the different rhizobacterial communities of *Eucalyptus* hybrids and species.

Materials and methods

1. Site description

This study was conducted in and around the town of Pietermaritzburg in the province of KwaZulu-Natal (KZN), South Africa. In Pietermaritzburg and its surrounding areas many of South Africa's plantation forests occur (Pogue 2008). Rhizospheric samples were obtained from two separate nurseries in the Pietermaritzburg area. One was a private commercial forestry nursery, where *Eucalyptus* hybrid

rhizospheric samples were collected. The other nursery was a research nursery located at the Institute for Commercial Forestry Research (ICFR), where *Eucalyptus* species rhizospheric samples were collected.

2. Soil sampling

A total of 62 soil samples were collected in June 2012 from *Eucalyptus* hybrid and species rhizospheres, as well as potting soil from each nursery which served as controls.

Thirty of the rhizospheric samples were obtained from the commercial nursery's *Eucalyptus* cuttings. The commercial nursery's rhizospheric samples originated from two approximately two year old hybrid genotypes, *Eucalyptus grandis* × *Eucalyptus nitens* (GN) and *E. grandis* × *Eucalyptus urophylla* (GU). Ten rhizospheric samples were taken from the *Eucalyptus* hybrid GU111, and a further 10 rhizospheric samples from two different GN hybrids namely, GN018B and GNPP2107. An additional four commercial nursery control samples were collected. These control samples contained the final potting soil product that had been sprayed to eliminate pathogens before the planting of cuttings. A further 28 samples were collected at the ICFR. The ICFR rhizospheric samples came from six month old trees in bags, established from seedlings from three different species; *E. macarthurii* (five rhizospheric samples), *E. smithii* (five rhizospheric samples) and *E. nitens* (four rhizospheric samples). An additional 10 rhizospheric samples were taken from younger (three months old) *E. nitens* seedlings (*E. nitens*229), still in trays. The ICFR control samples contained the final soil before planting seedlings.

Each sample contained approximately 30 g of rhizospheric soil. The samples were stored on ice for transportation and preserved at -20ºC until further processing.

3. DNA Extraction

The DNA extraction process requires 500 mg of soil. Genomic DNA (gDNA) was extracted from a total of 42 soil samples using the FastDNA® SPIN Kit for soil (MP Biomedicals).

4. 16S rDNA amplification

A 1 300 bp fragment of the 16S rRNA gene was amplified using the primer set 63-F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387-R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al. 1998). Each 25 µl PCR mixture contained 0.5 µl of extracted gDNA, 0.5 µM of each primer, 0.2 mM dNTPs, 2.5 µl of a 10 × PCR buffer, 1 U of SuperTherm Taq DNA polymerase (JMR Holdings) and 1 mg/ml BSA (Fermentas). Amplification was performed using the C1000™ Thermal Cycler (Bio-Rad) under the following conditions; initial denaturation at 94°C for three minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for a minute and 30 seconds. A final elongation step was incorporated at 72°C for five minutes followed by a cooling step at 4°C for five minutes. PCR product amplification was confirmed by agarose gel electrophoresis (1%).

5. *DGGE-PCR product amplification*

Once the suitability of extracted gDNA for PCR application was verified, a modified primer set was used to amplify a shorter 625 bp segment of the 16S rRNA gene for DGGE analysis. The primer set 341-F^{GC} with a 40 bp 5' GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3') and 907RM (5'-CCG TACG ATT CMT TTG AGT TT-3') were used (Muyzer et al. 2005, Martínez-Alonso et al. 2010). Each PCR mixture (50 µl) contained 1.5 µl of the extracted gDNA, 0.5 µM of each primer, 0.2 mM dNTPs, 5 µl of a 10 × PCR buffer and 1 U of SuperTherm Taq DNA polymerase (JMR Holdings). Touchdown PCR amplification was performed using the C1000™ Thermal Cycler (Bio-Rad) under the following conditions; initial denaturation at 95°C for three minutes followed by 10 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for one minute. An additional 20 cycles of denaturation at 95°C for three minutes, annealing at 55°C for 45 seconds and elongation at 72°C for one minute was performed. A final elongation step was incorporated at 72°C for six minutes followed by a cooling step at 4°C for three minutes. PCR product amplification was confirmed by agarose gel electrophoresis (1.5%). In order to reduce possible inter-sample PCR variation, two sets of PCRs were performed as independent duplicates and pooled before loading on the DGGE gel.

Initially, all 42 samples were analysed with DGGE to establish diversity, consistency or deviation among representative samples of the same *Eucalyptus* hybrid or species. Eventually, 5 µl of gDNA extracted from

all soil samples representing each *Eucalyptus* hybrid, species or control were pooled and used as template for further analyse. This resulted in a total of nine pooled samples ranging from 20 – 25 µl of gDNA. The nine pooled samples represented nine specific microbial communities originating from three *Eucalyptus* species and four *Eucalyptus* hybrid rhizospheric samples, as well as two control soil samples.

6. DGGE analysis

The bacterial diversity of all the soil samples was analysed using the DCode Universal Mutation Detection System (Bio-Rad) essentially according to the protocol described by Muyzer et al. (1993). Gels of 6% polyacrylamide with a denaturing gradient range of 40%-60% (100% denaturant is defined as a mixture of 7M urea and 40% deionised formamide) were used. Twenty five µl of the amplified PCR products were loaded *per* well. Separation was performed at 60°C at 100 V for 17 hours for single sample analysis and 130 V for five hours for pooled samples. All the gels were stained with 0.05% GelStar® (Lonza) for 10 minutes and de-stained with distilled water for five minutes. The images were visualised and captured using the Molecular Image® Gel DocTM XR system and DGGE band patterns analysed with Quantity One® 1-D Analysis imaging software (Bio-Rad). A 5% band intensity threshold was set for the band selection process. A band-matching process, based on a 1.5% position tolerance was used to obtain presence-absence matrixes, allowing the classification of individual bands according to their positions in the gel and calculation of their frequency among soil samples. The Quantity One® 1-D Analysis imaging software (Bio-Rad) also described cluster analysis pattern similarities among different soil samples using unweighted pair-group method with an arithmetic mean algorithm (UPGMA) to generate a densitometric profile (Martinez-Alsono et al. 2010). Dominant bands for further discussion were selected based on band intensity (≥ 1.892).

7. Sequencing

Selected bands were excised from the DGGE gel on a DarkReader (Clare Chemicals Research), suspended in 50 µl nanopure water and incubated at 50ºC for six hours and 30 minutes. The excised bands were re-amplified using primers 341-F (5'-CCT ACG GGA GGC AGC AG-3') and 907RM (5'-CCG TACG ATT CMT TTG AGT TT-3') (Muyzer et al. 2005). Each PCR mixture (25 µl) contained 5 µl of the excised band mixture, 0.5 µM of each primer, 0.2 mM dNTPs, 2.5 µl of a 10 × PCR buffer, 1 U of

SuperTherm Taq DNA polymerase (JMR Holdings) and 1 mg/ml BSA (Fermentas). Touchdown PCR amplification was performed using the C1000™ Thermal Cycler (Bio-Rad) and PCR product amplification confirmed as previously described for DGGE-PCR amplification (section 5).

Band positions on the DGGE gel were each assigned a unique number using Quantity One Software. At least three different representatives of each DGGE band position were sequenced. The sequences were obtained directly from 1 µl of the re-amplified product using the Big Dye® Terminator v3.1 Cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems®). Primer 341-F was used for sequencing except in cases where single (unique) bands were detected on the DGGE gel. These bands were sequenced in both directions using primer 907RM as well. The reactions were carried out using the C1000™ Thermal Cycler (Bio-Rad). Reaction conditions consisted of initial denaturation at 96ºC for one minute, followed by 25 cycles of denaturation at 96ºC for 10 seconds, annealing at 50ºC for five seconds and 60ºC for four minutes. An additional cooling step was incorporated at 4ºC for five minutes. An EDTA/Ethanol precipitation protocol was followed for sequencing clean-up. Sequences representing the same band position were aligned using Clustal Omega. Further analyses of the sequences were performed with the basic local alignment search tool BLAST program which was compared to the 16S NCBI database for identification. All sequences were deposited in the GenBank database under accession numbers indicated in Table 1 (GenBank, http://www.ncbi.nlm.nih.gov).

Results

1. DGGE analysis

Nucleic acid concentrations extracted from the soil samples ranged from 1.8 ng/µl for the *Eucalyptus* hybrid control to 72 ng/µl for the *Eucalyptus* species control, with a mean concentration of all samples of 27.8 ng/µl.

A 625 bp section of the 16S rRNA gene was successfully amplified for DGGE analysis in all the samples. The UPGMA results, based on the obtained DGGE profiles showed that rhizospheric gDNA extracted from the same *Eucalyptus* hybrid grouped together, while the potting soil's gDNA was the outlier (Figure 1). The rhizospheric gDNA from *Eucalyptus* hybrids GN018B (samples SS2 – SS8) clustered together with a

similarity distance of approximately 0.42; illustrating a 42% similarity among samples based on common bands present and band intensity. Similarly, the rhizospheric gDNA from *Eucalyptus* hybrids GNPP2107 (samples SS10 – SS20) also clustered together with a similarity distance of approximately 0.52, as well as the rhizospheric gDNA from *Eucalyptus* hybrids GU111 (SS22 – SS30) with a similarity distance of approximately 0.62. The outlier, gDNA from *Eucalyptus* hybrid control sample had a similarity distance of approximately 0.28 to the *Eucalyptus* hybrid rhizospheric samples.

The UPGMA results from the *Eucalyptus* species DGGE profile indicated different results when compared to that of the *Eucalyptus* hybrids. The rhizospheric gDNA extracted from *Eucalyptus* species samples did not demonstrate specific clustering, however the similarity distance of 0.36 for *Eucalyptus* species showed an overall closer relationship compared to the 0.18 of the *Eucalyptus* hybrids (Figure 1 and Figure 2).

The pooled rhizospheric gDNA from the *Eucalyptus* hybrids, GN018B, GNPP2107 and GU111 clustered together on a UPGMA dendrogam (Figure 3). The rhizospheric gDNA of hybrids between *E. grandis* × *E. nitens* (GNPP2107 and GN018B) showed the highest similarity, with a distance of approximately 0.70. Pooled rhizospheric gDNA from the *Eucalyptus* species *E. nitens* and *E. smithii* clustered together with a similarity distance of approximately 0.55 to the pooled gDNA from the *Eucalyptus* species control sample. The pooled gDNA from the *Eucalyptus* hybrids control sample was the out group, with a similarity distance of 0.35.

2. Rhizobacterial sequences

DGGE analysis of the pooled samples resulted in detection of 201 total bands in 50 unique band positions. Of these band positions 55% (based on band intensity) were re-amplified and sequenced. The amplified gDNA from the *Eucalyptus* hybrid GU111 rhizosphere and the *Eucalyptus* species control had the largest number of excised bands (16) while the amplified rhizospheric gDNA from the *Eucalyptus* hybrid GN018B had the least (8). Of the DNA sequences subjected to BLAST (ranging from 187-496 bp), 29 were uniquely linked to specific bacteria with identifies ranging from 71-94%. DNA sequences showed that *Nitrosomona eutropha*, represented by band 15, was present in all samples. Furthermore, eight unique sequences were obtained for one *Eucalyptus* hybrid, three *Eucalyptus* species, and both *Eucalyptus* hybrid and species control (Table 1).

Discussion

This study showed extensive variation in rhizospheric communities, with the least variation demonstrated by hybrids (GN018B and GNPP2107) from the same species (*E. grandis* × *E. nitens*) when compared to other hybrids and species. This finding supports the notion that a host genotype could significantly influence its rhizospheric diversity (Berg et al. 2002, Peiffer et al. 2013).

The rhizobacterial composition of planting medium (also referred to as potting soil) used in a commercial setting is dependent on the age of the seedling or cutting. The rhizospheric environment changes during different phases of plant growth (Di Cello et al. 1997). As the root system develops the more adapted microorganisms may be favoured (Di Cello et al. 1997). The potting soil investigated in this study contained an abundance of the two aerobic bacterial species *Massilia aerilata* (Weon et al. 2008) and *Erythrobacter citereus* (Koblížek et al. 2003) suggesting that the potting soil environment was aerobic. While the initial rhizobacterial composition suggested an aerobic environment, the young *E. nitens229* seedlings suggested a combination of aerobic and anaerobic regions, because of the presence of both aerobic and anaerobic rhizobacteria, namely, the aerobic bacterium *Rubrobacter taiwanensis* (Chen et al. 2004) and the anaerobic bacterium *Anaerophaga thermohalophila* (Denger et al. 2002). The more mature rhizobacterial communities demonstrated only the presence of anaerobic bacteria. The presence of these anaerobic bacteria which included *Rhodoplanes serenus* and *Holophaga foetida* (Liesack et al. 1994, Okamura et al. 2009), isolated from four rhizospheric communities, suggested that the more mature rhizospheres were anaerobic. Furthermore, it could be argued that the younger *Eucalyptus* species had not yet reached a more mature stage and their rhizospheric environments had not yet stabilised according to host genotype, resulting in a higher overall similarity compared to the overall similarity between *Eucalyptus* hybrids whose stabilised rhizospehric environments resulted in similarity among specific genotypes.

Bacillus and *Pseudomonas* are said to be common inhabitants of bulk soil and the rhizosphere (Thomashow 1996, Velazquez-Selúlveda et al. 2012). In this study neither of these bacteria could be identified. *N. eutropha* was the only bacterium present in all *Eucalyptus* hybrids and species rhizospheres. *N. eutopha* is known to be able to adapt to nitrogen saturated environments (Stein et al. 2007). The abundance of *N. eutropha* is evidence that the *Eucalyptus* hybrids and species rhizospheric environments

are probably rich in nitrogen, an essential nutrient of soil (Soil Management, http://www.ctahr.hawaii.edu/mauisoil/c_nutrients01.aspx) and is a candidate for further investigation into its potential use for rooting enhancement. Another potential candidate could be *A. aquaticus* isolated from the *Eucalyptus* hybrid GU111, known for its relatively high rooting percentages. Other candidates necessitating further investigation include those rhizobacteria present in GU111 rhizospheres but absent in GN rhizospheres. These are *Marinifilum fragile*, *Cytophaga fermentans*, *Methylophilus leisingeri, Thiobacter subterraneus* and *Nitrosospira multiformis*.

Conclusion

In conclusion, this study provided some insight into the diversity of rhizobacterial communities of *Eucalyptus* hybrid and species. The results provided evidence to identify potential plant growth promoting rhizobacteria (PGPR) and also revealed that the nature of the soil environment changes with the aging of the associated host. In combination, these findings provided a better understanding of the *Eucalyptus* rhizospheric communities, allowing further investigation into the formulation of potential rhizobacterial preparations for rooting enhancement of *Eucalyptus* cuttings. The next step would be to identify which of the bacterial species contribute significantly to the enhancement of rooting of cuttings.

Acknowledgements

We thank the Central University of Technology (CUT) and the National Research Foundation (NRF) for their funding to complete this study.

References

Asghar H, Zahir A, Arshad M. 2004. Screening rhizobacterias for improving the growth, yield, and soil content of canola (*Brasica napus L.*). *Australian Journal of Agricultural Research* 55: 187-194.

Beck SL, Fossey A. 2007. Gamma irradiation induces sterility or seedlessdess in black wattle (Acacia mearnsii). *Seed Science and Technology* 35: 351-359.

Berg G, Roskot N, Steidle A, Eberl L, Zock A, Smalla K. 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. *Applied and Environmental Microbiology* 68(7): 3328-3338.

Chanway CP, Radley RA, Holl FB. 1991. Inoculation of conifer seed with growth promoting *Bacillus* strains causes increased seedling emergence and biomass. *Soil Biology and Biochemistry* 23: 575-580.

Chen MY, Wu SH, Lin GH, Lu CP, Lin YT, Chang WC, Tsay SS. 2004. Rubrobacter *taiwanensis* sp. nov., a novel thermophilic, radiation-resistant species isolated from hot springs. *International Journal of Systematic and Evolutionary Microbiology* 54: 1849-1855.

Chetty S. 2001. The development of clone-specific micropropagation protocols for three commercially important *Eucalyptus* hybrids. Dissertation, University of Natal, Durban.

Cupertino FB, Leal JB, Corrèa RX, Gaiotto FA. 2011. Genetic diversity of *Eucalyptus* hybrids estimated by genomic and EST microsatellite markers. *Biologia Plantarum* 55(2): 379-382.

Denger K, Warthmann R, Ludwig W, Schink B. 2002. *Anaerophaga thermohalophila* gen. nov., sp. nov., a moderately thermohalophilic, strictly anaerobic fermentative bacterium. *International Journal of Systematic and Evolutionary Microbiology* 52(1): 173-178.

Di Cello F, Bevivino A, Chiarinin L, Fani R, Paffetti D, Tabacchioni S, Dalmastri C. 1997. Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. *Applied and Environmental Microbiology* 63(11): 4485-4493.

Díaz K, Valiente C, Martínez M, Castillo M, Sanfuentes E. 2009. Root-promoting rhizobacteria in *Eucalyptus globulus* cuttings. *World Journal of Microbiology and Biotechnology* 25: 867-873.

Enebak SA, Wei G, Kloepper JW. 1998. Effect of plant growth promoting rhizobacteria on loblolly and slash pine seedlings. *Forest Science* 44: 139-144.

Esitken A, Ercisli S, Secik S, Sahin F. 2003. Effect of indole-3-butyric acid and different strains of *Agrobacterium rubi* on adventives root formation from softwood and semi-hardwood wild sour cherry cuttings. *Turkish Journal of Agriculture and Forestry* 27: 37-42.

Flaishman MA, Eya A, Zilberstein C, Voisard C, Hass D. 1996. Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Psedomonas putida*. *Molecular Plant-Microbe interactions* 9: 642-654.

Grattapaglia D, Sederoff R. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. *Genetics* 137: 1121-1137.

Gutierre Mañero FJ, Acero N, Lucas JA, Probanza A. 1996. The influence of native rhizobacteria on European alder (*Alnus glutinosa (L.) Gaertn*. growth. II. Characterization of growth promoting and growth inhibiting strains. *Plant Soil* 182: 67-74.

Ishii K. 2009. DNA markers in *Eucalyptus* with emphasis on species identification. *Environment Control in Biology* 47(1): 1-11.

Kennedy LR, Pereg-Gerk C, Wood R, Deaker K, Gilchrist K, Katupitiya S. 1997. Biological nitrogen fixation in nonleguminous field crops: facilitating the evolution of an effective association between *Azospirillum* and wheat. *Plant Soil* 194: 65-79.

Khalid A, Arshad M, Zahir ZA. 2004. Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology* 96:473-480.

Koblížek M, Béjá O, Bidigare RR, Christensen S, Bentez-Nelson B, Vetriani C, Kolber, MK, Falkowski PG, Kolber ZS. 2003. Isolation and characterization of *Erythrobacter* sp. strains from the upper ocean. *Archives of Microbiology* 180: 327-338.

Komakech C, Swain T, Fossey A. 2009. Growth potential of *Eucalyptus* globules subsp. b*icostata* provenances for mid-altitude summer rainfall regions of South Africa. *Southern Forests* 71(1): 1-9.

Liesack W, Bak F, Kreft J, Stackebrandt E. 1994. *Holophaga foetida* gen. nov., a new, homoacetogenic bacterium degrading methoxylated aromatic compounds. *Archives of Microbiology* 162(1-2): 85-90.

Long HH, Schmidt DD, Baldwin IT. 2008. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PLoS One* 3(7): 1-10.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology* 64(2): 795-799.

Martínez-Alonso M, Escolano J, Montesinos E, Gaju N. 2010. Diversity in the surface soil of pear orchard based on 16S rRNA gene analysis. *International Microbiology* 13: 123-134.

Muyzer G, Yildirim E, van Dongen U, Küh, M, Thar R. 2005. Identification of "*Candidatus Thioturbo danicus*," a microaerophilic bacterium that builds conspicuous veils on sulfidic sediments. *Applied and Environmental Microbiology* 7(12): 8929-8933.

Okamura K, Kanbe T, Hiraishi A. 2009. *Rhodoplanes serenus* sp. nov., a purple non-sulfur bacterium isolated from pond water. *International Journal of Systematic and Evolutionary Microbiology* 59(3): 531-535.

O'Neill G, Radley R, Chanway C. 1992. Variable effects of emergence-promoting rhizobacteria on conifer seedling growth under nursery conditions. *Biology and Fertility of Soils* 13: 45-49.

Patena L, Stutter EG, Dandekar AM. 1988. Root induction by *Agrobacterium rhizogenes* in a difficult-to-root woody species. *Acta Horticulturae* 227: 324-329.

Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, Buckler ES, Ley RE. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences* 110(16): 6548-6553.

Pogue TE. 2008. A sectoral analysis of wood, paper, pulp industries in South Africa. Institute for Economic Research on Innovation.

Shackleton CM, Shackleton SE, Buiten E, Bird N. 2007. The importance of dry woodlands and forests in rural livelihoods and poverty alleviation in South Africa. *Forestry Policy and Economics* 9(5): 558-577.

Soil Management. Avaliable at: http://www.cah.hawaii.edu/mauisoil/c_nutrients01.aspx [accessed on 16 July 2013].

Stein LY, Arp DJ, Berube PM, Chain PSG, Hauser L, Jetten MSM, Klotz MG, Larimer FW, Norton JM, Op den Camp HJM, Shin M, Wei X. 2007. Whole-genome analysis of the ammonia-oxidizing bacterium, *Nitrosomonas Eutropha* C91: implications for niche adaptation. *Environmental Microbiology* 9(12): 2993-3007.

Swain T, Jones WRE. 2004. Full rotation measurements of *Eucalyptus badjensis* trials in the summer rainfall region of South Africa. *ICFR Bulletin Series 01/2004*. Pietermaritzburg: Institute for Commercial Forestry Research.

Teixeira DA, Alfenas AC, Goncalves R, Ferreira EM, de Sequeira L, Maffia LA, Mounteer AH. 2007. Rhizobacterial promotion of eucalypt rooting and growth. *Brazilian Journal of Microbiology* 38: 118-123.

Thomashow LS. 1996. Biological control of plant pathogens. *Current Opinion in Biotechnology* 7(3): 343-347.

Velazquez-Selúlveda I, Orozco-Mosqueda MC, Prieto-Barajas CM, Santoyo G. 2012. Bacterial diversity associated with the rhizosphere of wheat plants (*Triticum aestivum*): Toward a metagenomic analysis. *Phyton, International Journal of Experimental Botany* 81: 81-87.

Weon HY, Kim BY, Son JA, Jang HB, Hong SK, Go SJ, Kwon SW. 2008. *Massilia aerilata* sp. nov., isolated from an air sample. *International Journal of Systematic and Evolutionary Microbiology* 58: 1422-1425.

Whiting D, Roll M, Vickerman L. 2011. Plant growth factors: plant hormones. Colorado Master Gardener, Colorado State University Extension, *Minor revisions* 145: 1-4.

Appendix C

FastDNA® Spin Kit for Soil

Important Considerations Before Use

Preparation of SEWS-M Wash Solution

The FastDNA® SPIN Kit for Soil contains a bottle with 12 ml of Concentrated SEWS-M Wash Solution. Before using this solution, add 100 ml of 100% ethanol and mark on the bottle label the date ethanol was added. Ensure that the bottle is securely closed to prevent evaporation, mix and store at room temperature.

Sample Lysis with the FastPrep® Instrument

The fill volume in the lysing matrix tube after the addition of the Sodium Phosphate and MT Buffers to the sample should allow sufficient air space in the sample tube for efficient FastPrep® Instrument processing. MP Biomedicals recommends using 500 mg of starting material as long as there is between 250 – 500 µl of empty space in the tube. Sample loss or tube failure may result from overfilling the matrix tube. The matrix tube caps must be secure, but not over-tightened, to prevent sample leakage. If the sample is too large for processing in a single tube, divide the sample and process using multiple tubes. MP Biomedicals' Lysing Matrix particles and tubes have been rigorously tested and validated in the FastPrep® Instrument. The use of other products with the FastPrep® Instrument is not recommended and may result in sample loss or instrument failure. A single 40 second run at a speed setting of 6.0 in the FastPrep® Instrument is sufficient to lyse almost all samples. If the user experimentally determines that additional processing time is required, the sample should be incubated on ice in the Lysing Matrix E tube for at least 2 minutes between successive FastPrep® Instrument homogenizations to prevent overheating the sample and tube.

Safety Precautions

Binding Matrix contains components that, when in contact with human tissue, may cause irritation. Wear personal protective equipment to prevent contact with the skin or mucus membranes (gloves, lab coat, and eye protection). Consult the enclosed Material Safety Data Sheet for additional details.

Protocol

1. Add up to 500 mg of soil sample to a Lysing Matrix E tube. NOTE: See section 3.2 for important guidelines.

2. Add 978 µl Sodium Phosphate Buffer to sample in Lysing Matrix E tube.

3. Add 122 µl MT Buffer.

4. Homogenize in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0.

5. Centrifuge at 14,000 × g for 5-10 minutes to pellet debris.

NOTE: Extending centrifugation to 15 minutes can enhance elimination of excessive debris from large samples, or from cells with complex cell walls.

6. Transfer supernatant to a clean 2.0 ml microcentrifuge tube. Add 250 µl PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times.

7. Centrifuge at 14,000 × g for 5 minutes to pellet precipitate. Transfer supernatant to a clean 15 ml tube. NOTE: While a 2.0 ml microcentrifuge tube may be used at this step, better mixing and DNA binding will occur in a larger tube.

8. Resuspend Binding Matrix suspension and add 1.0 ml to supernatant in 15 ml tube.

9. Place on rotator or invert by hand for 2 minutes to allow binding of DNA. Place tube in a rack for 3 minutes to allow settling of silica matrix.

10. Remove and discard 500 µl of supernatant being careful to avoid settled Binding Matrix.

11. Resuspend Binding Matrix in the remaining amount of supernatant. Transfer approximately 600 µl of the mixture to a SPIN™ Filter and centrifuge at 14,000 × g for 1 minute. Empty the catch tube and add the remaining mixture to the SPIN™ Filter and centrifuge as before. Empty the catch tube again.

12. Add 500 µl prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.

NOTE: Ensure that ethanol has been added to the Concentrated SEWS-M.

13. Centrifuge at 14,000 × g for 1 minute. Empty the catch tube and replace.

14. Without any addition of liquid, centrifuge a second time at 14,000 × g for 2 minutes to “dry” the matrix of residual wash solution. Discard the catch tube and replace with a new, clean catch tube.

15. Air dry the SPIN™ Filter for 5 minutes at room temperature.

16. Gently resuspend Binding Matrix (above the SPIN filter) in 50-100 µl of DES (DNase/Pyrogen-Free Water).

NOTE: To avoid over-dilution of the purified DNA, use the smallest amount of DES required to resuspend Binding Matrix pellet.

NOTE: Yields may be increased by incubation for 5 minutes at 55˚C in a heat block or water bath.

17. Centrifuge at 14,000 × g for 1 minute to bring eluted DNA into the clean catch tube. Discard the SPIN filter. DNA is now ready for PCR and other downstream applications. Store at -20°C for extended periods or 4°C until use.

Appendix D

EDTA/Ethanol precipitation protocol for sequencing cleanup

[NOTE: Dilute a 0.5 M EDTA (pH8.0) to 125 mM EDTA with MilliQ H_2O]:

1. Adjust the sequencing reaction volume to 20 µl and transfer to a 1.5 ml eppendorf tube that contains 5 µl 125 mM EDTA and 60 µl absolute ethanol.

2. Vortex for 5 seconds to mix and precipitate at room temperature for 15 minutes.

3. Centrifuge at 4ºC for 15 minutes at 20 000 × *g*.

4. Completely aspirate the supernatant, making sure not to disturb the pellet.

5. Add 200 µl 70% ethanol to tubes and centrifuge at 4ºC for 5 minutes at 20 000 × *g*.

6. Completely aspirate the supernatant, making sure not to disturb the pellet.

7. Dry in Speed-Vac for 5 minutes.

8. Store samples in the dark at 4ºC.