Root-enhancing properties of rhizospheric bacteria on *Eucalyptus* hybrid cuttings

by

Nwigwe Chimdi Sidney

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Declaration

I, Nwigwe Chimdi Sidney, identity number (student number), do hereby declare that this research project submitted to the Central University of Technology, Free State, for the Degree Doctor of Philosophy in Environmental Health, is my own independent work. This work complies with the code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person for the attainment of a qualification.

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Nwigwe Chimdi Sidney

2018

I certify that the above statement is correct.

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Abstract

Introduction: Worldwide, the demand for hardwood from commercial plantations is rising as the consumption of forest products increases. In *Eucalyptus* plantation forestry the formation of interspecific hybrids has driven the commercial forestry industry to produce a variety of different hybrids that are often deployed in marginal areas. Superior hybrid genotypes are deployed through the application of vegetative propagation and the rooting of cuttings. However, the cuttings of some hybrid genotypes demonstrate relatively low rooting percentages, which results in financial losses experienced by many commercial forestry nurseries. This study was thus undertaken to assess the potential of rhizospheric bacteria to improve the rooting capacity of two hybrids clones of *E. grandis* × *E. nitens* (GN 018B and GN 010).

Methods: Firstly, the rhizospheric bacterial composition of *Eucalyptus* rhizospheric soils of different ages were characterised using fatty acid methyl ester (FAMEs) analyses. Thereafter, rhizospheric bacteria were isolated from *Eucalyptus* rhizospheres and characterised by sequencing the ≈1,300 base pair fragment of the 16S rRNA gene, after which the sequences were submitted to BLAST searches to identify the bacterial strains. Thereafter, the identified bacterial strains were tested for two important root promoting characteristics; the ability to produce indole-3-acetic acid and to solubilise phosphates. Bacterial inoculums were prepared and tested on cuttings of the two hybrids in the nursery. Four treatments were applied to the cuttings; the nursery standard, which acted as the control, two treatments prepared from the isolated rhizospheric bacteria and a commercial treatment containing a live fungus. Cutting survival, rooting, rooting architecture and growth were measured after eight weeks of growth.

Results: This study revealed that rhizospheric microbial communities' diversity evolved over time. Younger rhizospheres displayed significantly greater diversity when compared to the older rhizospheres. The presence of higher concentrations of saturated fatty acids in younger

rhizospheres than in the older rhizospheres was indicative of aerobic conditions, while increased proportions of polyunsaturated fatty acids in the older rhizospheres indicated anaerobic conditions. The greater proportion of polyunsaturated fatty acids in the older rhizospheric soil samples, especially linoleic (C18:2ω6c), α-linolenic (C18:3ω3c), γ-linolenic (C18:3ω3c) fatty acids, as well as the unique γ-linolenic fatty acids, suggest growing establishment of fungi as a rhizosphere ages. Both Shannon and Simpson's indexes confirmed that younger soils were more diverse. Thirty two rhizospheric bacterial strains were isolated and identified. These strains belonged to 12 unique species of eight different genera. Of the 12 rhizospheric bacterial species, seven demonstrated the ability to produce indole-3-acetic acid and to solubilise phosphates. These seven strains were used to prepare the inoculums to treat the cuttings of the two hybrids. *Bacillus aryabhattai, Pseudomonas fluorescens*, *P. koreensis* and *P. putida* were mixed into one rooting treatment, while *Brevibacterium frigoritolerans*, *Burkholderia phytofirmans* and *Chryseobacterium rhizosphaerae* were mixed into another treatment. Cuttings of both hybrid clones demonstrated relatively high survival rates and rooting percentages for all treatments, although the nursery standard treatment (indole-3-butyric acid) marginally outperformed all other treatments. The cutting growth responses of the two hybrids showed highly significant differences amongst the treatments ($P < 0.0001$). The rhizospheric rooting treatment comprising of the non-*Pseudomonas-Bacillus* bacteria, as well as fungus treatment, were closest in performance to the nursery standard, while the *Pseudomonas-Bacillus* treatment often showed lower values when compared to the other treatments. However, *Pseudomonas-Bacillus* bacterial treatment appeared to improve fibrosity of the rooting architecture of cuttings of one of the hybrid genotypes, which could be of value when rooted cuttings are planted out in plantations. A noteworthy outcome of this study was the finding that extensive genotypic differences existed between the two hybrid clones.

Conclusions: The aim of this study was to test the application of rhizospheric bacteria as root stimulating agents in the cuttings of two *Eucalyptus* hybrid genotypes. The rhizospheric bacterial

preparations used in this study did not enhance the number of rooted cuttings when compared to the nursery standard in a significant way, however, there were strong indications that the rhizospheric bacterial inoculums did promote fibrosity. These findings suggest further investigation into the formulation and application of potential rhizospheric bacterial preparations for rooting enhancement of *Eucalyptus* cuttings.

Chapter 1

Introduction

1.1 Introduction

Worldwide, the demand for hardwood from commercial plantations is rising as the consumption of forest products increases (Pijut et al., 2007), in particular for *Eucalyptus* species (FAO, 2001). In South Africa, *Eucalyptus* plantations are mostly found in the Provinces of KwaZulu-Natal and Mpumalanga. These plantations comprise of approximately 40% of the 1.3 million ha of commercial forested land (DAFF, 2009, 2015). *Eucalyptus grandis* is the most economically important species covering 55% of the total land planted to *Eucalyptus* plantations; which covers approximately 540,000 hectares (DWAF, 2005). The remaining 45% comprises of other *Eucalyptus* species and *Eucalyptus* species hybrids (DWAF, 2005, 2007).

Eucalyptus is a fast growing hardwood genus and comprises of a large number of species. These species are adapted to a wide range of soil types and climates and are mostly planted in tropical and sub-tropical regions (Turnbull, 1999; dos Santos et al., 2004; Cabral et al., 2011). *Eucalyptus* species are a source for a variety of wood and non-wood products (Turnbull, 1999; dos Santos et al., 2004; Cabral et al., 2011). These products include fuel wood; pulp for the paper industry; mining, electric and telephone poles; as well as household products such as honey, detergents, cosmetics, veterinary and dental products (Turnbull, 1999).

The demand for hard wood from commercial *Eucalyptus* plantations is increasing each year (Sharma & Ramamurthy, 2000). The success of *Eucalyptus* as plantation species is due to their large number of species, wide adaptability to soils and climates, fast growth rates, the wide knowledge and technology for their culturing and the variety of wood and non-wood products that come from them (Eldridge et al. 1993; Cabral et al. 2011; Brondani et al. 2012). Traditionally,

breeding with *Eucalyptus* species has involved traditional methods of intraspecific and interspecific hybridisation and the selection of superior genotypes (van Wyk & Verryn, 2000). The ability of *Eucalyptus* species to hybridise has been recognised as a means to combine genes from diverse sources and utilise hybrid vigour (Potts & Dungey, 2004). In an evolutionary context, interspecific hybrids provide the potential for one-step evolution by combining many years of diverse evolutionary pathways. As a result of the mixed genetic backgrounds of such hybrid genotypes, they can be deployed in regions not usually suited to pure species. Thus, the heterosis of these hybrids has driven the commercial forestry industry to produce a variety of different interspecific hybrids (Dieters et al., 1995). Therefore, mass propagation of improved genetic material has become the foundation of the commercial forestry industry and an important method for increasing competitiveness in the forestry industry.

1.2 Problem statement

Intraspecific and interspecific hybrids are traditionally multiplied through vegetative propagation, a method widely used for the establishment of clonal plantations (de Assis et al., 2004). The most attractive advantage of clonal multiplication of hybrid genotypes and a number of pure species genotypes, is the uniformity of clonal plantations. In contrast, a major drawback that is faced by the commercial forestry industry, is the manifestation of poor rooting by some genotypes because of endogenous and exogenous factors resulting in considerable losses. For example, the species *Eucalyptus nitens* and hybrids of this species present great variability in their rooting capacity and are considered to be a recalcitrant species (Martellet & Fett-Neto, 2005). Thus, clonal plants cost substantially more than seedlings, partially because of the lower survival frequencies and labour intensive practice. The improvement of rooting percentages of clonal cuttings that mitigate losses through poor survival and low rooting percentages will contribute to more efficient and cost effective

clonal forestry practices. Research is thus required to develop improved *Eucalyptus* rooting regimes that can be applied in commercial forestry nurseries.

1.3 Motivation for the study

At the core of clonal propagation operations lies the process of adventitious rooting (de Assis et al., 2004). *Eucalyptus* hybrids are commercially multiplied by rooting of cuttings, which in turn provide young clonal plants (Ruaud et al., 1999). Nursery growers accomplish clonal propagation mostly through the rooting of juvenile stem-cuttings (macro-cuttings) obtained from macro-hedges in outdoor clone gardens or mini- and micro-cuttings from indoor mini-hedge clone gardens, with varied success (de Assis et al., 2004). Thompson (2009) also refers to mini-hedges as micro-hedges. Typically, stem-cuttings are used as a source of propagules for the establishment of mini-hedge clone gardens. Generally, clones that are difficult to root with stem-cuttings perform better with mini- and micro-cuttings (Stape et al., 2001; Titon et al., 2002; de Assis et al., 2004). In South Africa, many clonal propagation operations employ stem-cuttings, although indoor operations are rapidly gaining popularity.

Research to improve rooting of especially recalcitrant genotypes, most often addresses investigations to stimulate adventitious rooting or to find the best hedge configuration for a particular genotype. One of the strategies to improve rooting capacity, which has not been explored extensively in tree species is that of using rhizospheric bacteria to stimulate rooting (Diaz et al., 2009). Studies have shown that rhizospheric bacteria can stimulate plant growth, and more recently have also shown to increase rooting of stem-cuttings (Teixeira et al., 2007; Diaz et al., 2009; Erturk et al., 2010; Zafar et al., 2012).

1.4 Aim and objectives

Because of financial losses experienced by many commercial forestry nurseries as a result of the poor rooting capacity of some superior *Eucalyptus* genotypes, this study was undertaken to assess the potential of rhizospheric bacteria to improve the rooting capacity of some recalcitrant *Eucalyptus* hybrid genotypes.

To meet this aim, the following objectives were devised:

- To characterise the rhizospheric bacterial composition of *Eucalyptus* rhizospheric soils using fatty acid methyl ester (FAMEs) analyses;
- To isolate, identify and preserve rhizospheric bacteria isolated from *Eucalyptus* rhizospheric soils;
- To screen rhizospheric bacteria for their plant growth promoting properties; namely, the ability to produce indole-3-acetic acid and the ability of solubilise phosphates;
- To prepare rhizospheric bacterial inoculums and to test these inoculums on cuttings of two *Eucalyptus* hybrid genotypes that display low rooting percentages; and
- To analyse the data and determine whether the isolated rhizospheric bacterial inoculums enhanced rooting sufficiently enough to be implemented in a commercial nursery.

1.5 Thesis layout

This thesis has been partitioned into seven chapters. Briefly, these chapters cover the following topics:

Chapter 1: Introduction

This chapter provides a brief synopsis of the study, including the problem statement, motivation of the study, aim and the objectives.

Chapter 2: Literature review

This chapter reviews previous studies on *Eucalyptus* as a plantation genus, *Eucalyptus* breeding strategies, clonal propagation and rhizospheric bacteria as an alternative source for improving growth and development of plants, as well as the stimulation of adventitious roots in cuttings.

Chapter 3: Study design

This chapter presents the study design and a description of the different components of this research project.

Chapter 4: Characterisation of *Eucalyptus* rhizospheric communities using FAME profile analysis

This chapter presents the characterisation of rhizospheric soils of different ages of *Eucalyptus* hybrid clones using fatty acid methyl esters (FAMEs). FAME analysis was used to compare the effect of ageing on the microbial composition of three-month and five-year old *Eucalyptus* rhizospheres. The materials and methods are presented together with the results of the analyses of the data that includes the composition and comparison of fatty acids found in the different rhizospheric soils.

Chapter 5: Isolation, identification and screening of rhizospheric bacteria for rooting promoting properties

This chapter presents the rhizospheric bacteria that were characterised by sequencing of the ≈1,300 base pair fragment of the 16S rRNA gene. The identified bacteria were also screened for their ability to produce plant growth promoting hormones and other properties necessary to stimulate adventitious rooting. The materials and methods are presented together with the results of the analyses of the data that includes the identification of rhizospheric bacteria that could be employed in field tests to promote rooting of cuttings.

Chapter 6: Rooting enhancement of rhizospheric bacteria on *Eucalyptus* hybrid cuttings

This chapter presents the preparation of rhizospheric bacterial inoculums that were tested in a commercial nursery setting for their ability to stimulate the rooting of cuttings of two *Eucalyptus* hybrids. The materials and methods are presented together with the results of the analyses of the data, which includes growth characteristics of the identified rhizospheric bacteria, preparation of two rhizospheric bacterial inoculums, and the testing of the rhizospheric bacterial inoculums in field experiments, together with the nursery standard and a commercial biological rooting agent.

Chapter 7: Discussion and conclusions

This concluding chapter presents the key findings of the study and also integrates these findings into existing knowledge. A discussion on the challenges and further prospects in clonal forestry is also presented.

References: The references of this thesis were generated with the reference manager Mendeley.

Appendix: The appendix contains the raw data of the FAME results.

Chapter 2

Literature review

2.1 Introduction

Forests are of global importance providing numerous benefits. Forests could be in a natural or in a plantation (commercial) form. Natural and plantation forests play an important role in the livelihood of many people throughout the world providing shelter, firewood, furniture and many other forest related products (Poyton, 1979; Palo & Uusivuori, 1999). Natural and plantation forests also play an important role in the preservation of ecosystems; providing shelter and food for a diverse range of organisms. They also protect soils and store carbon, thereby mitigating the effects of climate change that threatens sustainable development (Siry et al., 2005). They are also known to increase the diversity and geographic spread of forest birds (Oatley, 1984). Furthermore, plantation forestry contributes to the expansion and recovery of the natural forests. They may act as protectors of natural forests by deterring and reducing the effects of the destructive nature of fires in the vicinity of natural forests (Geldenhuys et al., 1986).

Plantation forestry is a major role player in the global economy. These forests provide employment and generate income for many nations of the world (Tewari, 2001). In South Africa, more than 22 million cubic metres of round wood is produced annually from plantation forests, generating between 9.5 billion to 15 billion rands for the economy each year (DAFF, 2015). Plantation forests and associated downstream processing plants, contribute about 1% to the country's gross domestic product (GDP). In terms of regional GDP, plantation forestry in KwaZulu-Natal contributes 4.4%; Mpumalanga 3.7%; Eastern Cape 0.6%; and Limpopo about 0.6% (SA GOV, n.d.).

The plantation forestry industry employs just over 201,000 people with 77,000 directly employed by the forestry subsector. The total number of people dependent on the forestry industry for their livelihood is estimated at 2.3 million with 1.7 million people being the dependents of those people working in the industry (DAFF, 2009). Plantation forestry and downstream industry products earn valuable foreign exchange when exported (Dlamini, 2002). In South Africa, these products include mostly paper, pulp, as well as tannins for leather industries (Poyton, 1979; Beck & Dunlop, 2001; Pogue, 2008; Jacobs & Punt, 2010).

2.2 Plantation forestry in South Africa

Plantation forests were established in South Africa at the turn of the 20th century (Owen & van der Zel, 2000; FAO, 2001). The afforested area is about 1.27 million ha or about 1% of the total South African land area of 122.3 million ha. Over 80% of plantation forests occur in the provinces of Mpumalanga, Eastern Cape and KwaZulu-Natal. Approximately 68% of the area covered by plantations is planted with exotic tree species (DAFF, 2009). The balance of these estates contains natural vegetation, including natural forests.

The cultivated exotic trees comprise of mainly three genera. These genera include species of the genus *Pinus* that produce softwood and hardwood species of the genus *Eucalyptus*, as well as the hardwood species *Acacia meansii,* black wattle (Pogue, 2008). The most widely cultivated exotic tree genus in South Africa is *Pinus*. *Pinus* plantations cover approximately 671,000 ha, mostly of species *Pinus patula*, *Pinus elliotti*, *Pinus taeda* and *Pinus gregii* (SA Forestry, n.d.). *Eucalyptus* is the second most cultivated plantation genus covering a land area of approximately 540,000 ha (DWAF, 2005). These forests comprise of a number of *Eucalyptus* species, of which *Eucalyptus grandis* is the most prevalent species. Hybrids of these species, mostly with *E. grandis*, have become a prominent feature in South African *Eucalyptus* forestry. Black wattle covers the smallest land area of approximately 104,000 ha (Beck & Dunlop, 2001; Pogue, 2008).

2.3 The genus *Eucalyptus*

The genus *Eucalyptus* belongs to the family Myrtaceae and the subfamily Leptospermoideae. This subfamily is currently classified into three genera, namely, *Angosphora* consisting of 14 species, *Corymbia* with 113 species, and *Eucalyptus* consisting of more than 740 species (Grattapaglia & Sederoff, 1994; Delaport et al., 2001; Potts, 2004; Ishii, 2009; Cupertino et al., 2011; Richardson & Rejmánek, 2011). These three genera are native to Australia and a few pacific islands (Delaport et al., 2001; Potts, 2004; Cupertino et al., 2011). *Angosphora* and *Corymbia* are often treated as subgenera of *Eucalyptus sensu lato*. The genus *Eucalyptus sensu stricto* is currently divided into ten subgenera, six of which are monotypic containing only one species (Delaport et al., 2001; Richardson & Rejmánek, 2011).

Eucalyptus species ranges from small shrubs to large trees, growing up to 110 m in height (Turnbull, 1999). The first *Eucalyptus* species to be cultivated from seeds outside its native range was *Eucalyptus obliqua* in the Royal Botanic Gardens at Kew, United Kingdom, in 1774 (Richardson & Rejmánek, 2011). Other species were soon cultivated in botanical gardens and arboreta in Europe as botanical curiosities and ornamentals.

The fast growth of *Eucalyptus*, its ability to thrive on nutrient-poor soils and its potential as a timber genus appealed to foresters. Today, the genus *Eucalyptus* is one of the most widely cultivated hardwood genus in the tropical and subtropical regions of the world, especially across the southern hemisphere, notably in Brazil and South Africa (dos Santos et al., 2004).

In the southern hemisphere the shorter timber growth cycle of *Eucalyptus* is an added advantage (Grattapaglia & Sederoff, 1994; Pogue, 2008). *Eucalyptus* takes approximately nine years to reach a reasonable size for pulping in the southern hemisphere, which is considerably shorter when compared to the northern hemisphere (Pogue, 2008). *Eucalyptus* cultivation successes in these

regions reflect largely the adaptability of this genus to a variety of climatic and edaphic conditions, its fast growth, and its versatility and economic importance (dos Santos et al., 2004).

2.4 *Eucalyptus* forestry in South Africa

South Africa was one of the first countries in the southern hemisphere to cultivate *Eucalyptus* at a commercial scale (Owen & van der Zel, 2000). South African *Eucalyptus* plantations are located in different climatic areas; however, humid, warm temperate and subtropical regions are preferred (Komakech et al., 2013). Traditionally, *E. grandis* and its hybrids make up 51% of the total *Eucalyptus* plantations in South Africa and have been favoured in these climatic regions because of their rapid growth and provision of desired wood properties (Poyton, 1979; Malan & Arbuthnot, 1995; Chetty, 2001; DWAF, 2008; Komakech et al., 2009; McMahon et al., 2010).

During the late 1980s and early 1990s, the major South African forestry companies expanded their forestry land-bases into low productivity areas in order to meet the increasing demands. These areas included mid- and high-altitude summer rainfall areas that are drier and warmer, or drier and colder (Swain & Gardner, 2004). In these areas, cold tolerant species such as *Eucalyptus dunnii*, *Eucalyptus macarthurii, Eucalyptus nitens* and *Eucalyptus smithii* are mostly planted (Swain & Gardner, 2004). Today, most of the *Eucalyptus* plantations are located in the eastern part of the country with 40% located in the Mpumalanga province covering 8% of the province's land surface (Scholes et al., 1995; Pogue, 2008). The remainder of the *Eucalyptus* plantations occurs in the KwaZulu-Natal (39.6%) and other provinces, mainly the Eastern Cape.

In South Africa, *Eucalyptus* is utilised for a wide range of products. *Eucalyptus* is mostly used in pulp and paper production, as construction and building material, for transmission poles, mining supports, furniture wood, fibre board, extractable tannins, as well as for essential oil, honey and bio-fuel production (Turnbull, 1999; Kataria et al., 2013). *Eucalyptus* can also be planted as windbreaks and

is becoming increasingly desirable for its ornamental value. Furthermore, *Eucalyptus* contributes to phytoremediation of soils and heavy metal polluted water (Owen & van der Zel, 2000). Pulp production, the most important industry that *Eucalyptus* participates in, amounts to 2.4 million tons of pulp per year (FAO, 2001). Pulp production is mostly used for the production of packaging papers, printing and writing papers, as well as for the production of soft tissue paper (FAO, 2001).

2.5 Breeding of *Eucalyptus*

2.5.1 Introduction

Successful breeding programmes are a top priority of the forestry industry (Little et al., 2003). Traditionally, *Eucalyptus* domestication has evolved through the application of conventional breeding strategies (Potts, 2004). In conventional breeding, repeated cycles of breeding and selection are performed on initial populations. *Eucalyptus* breeding programmes also include intraspecific and interspecific hybridisation and the selection of superior genotypes (Raymond, 2000).

In South Africa, *Eucalyptus* breeding started in the early 1960's with *E. grandis*. Breeding at that time focused mainly on good growth characteristics, resistance to pest and disease, adaptability, and low levels of growth stress, spirality and brittle heart (Malan, 1994). As breeding programmes progressed, the range of traits assessed increased to include fitness, which relate to the ability of trees to survive environmental threats, and to quality, of which those pertaining to wood quality were amongst the most important (Malan, 1994).

With the market demands for forestry products constantly changing, breeders have to recognise these changes and continually revise breeding strategies. This has resulted in the addition of numerous breeding characteristics to take care for the ever increasing market demands (Table 2.1).

Table 2.1 Key tree and wood properties for a range of product classes (adapted from Raymond, 2002)

Product class		
Pulp and paper	**Sawn timber**	**Composites**
Basic density	Basic density and gradient	Basic density
Pulp yield/cellulose content	Microfibril angle	Lignin content
Fibre length	Strength and stiffness	Extractive content
	Dimensional stability	Cellulose content
	Shrinkage and collapse	
	Tension wood	
	Knot size	
	Incidence of decay, spiral grain and end splits	

Conventional breeding is usually slow in long rotational crops and may take decades before improvement in a particular trait manifests (Fossey, 2005). Incorporation of new precise methods often complements conventional breeding programmes to aid in the improvement of a particular species (van Wyk & Verryn, 2000). Thus, molecular technologies, such as the use of transformation (Potts, 2004), and the use of molecular markers in selection (marker assisted selection), have in recent times gained prominence in tree breeding (Potts, 2004). Although many genetically modified varieties have been produced through transformation in agriculture (Fossey, 2005), this type of genetic improvement has been relatively sluggish in *Eucalyptus* (Potts, 2004). Transgenic *Eucalyptus* species such as *E. grandis*, *Eucalyptus saligna* and *Eucalyptus urophylla* have been

tested in field trials in a number of countries, including South Africa, Spain and United Kingdom (Potts, 2004). However, it will take over a decade until genetically modified clones are planted on a large scale. This has resulted in an increased interest in the use of molecular breeding of *Eucalyptus* (Potts, 2004).

Complementary to successful tree breeding programmes is silvicultural practices. An estimated 40% increase in timber yields in South Africa could be achieved through the consolidation and improvement in present site-species matching and the breeding of superior trees (Schönau, 1990). Of the most important silvicultural management practices, which have been shown to increase the potential volume obtained at harvest, include combinations of appropriate site preparation, as well as fertilisation and weed control (Cromer et al., 1977; Squire, 1977; Flinn, 1978; Cogliastro et al., 1990; Neary et al., 1990; Turvey, 1996; Cromer et al., 1998). These silvicultural practices have also been shown to have an influence on the rate of growth and hence the pulping properties of trees (Wilkins, 1990; Wilkins & Kitahara, 1991).

2.5.2 Breeding strategies

Breeding strategies and mating designs range from simple to the more complex. All *Eucalyptus* breeding strategies have the common goal of reducing generation time to increase returns on investment (White, 2001). Developing breeding strategies is not an exact science but rather an art (White, 2001). Shelbourne et al. (1986) stated that “because intuition and subjective judgement play no small part in strategy development, thus, no two tree breeding strategies are or should be identical”. However, breeding strategies are usually developed around a common conceptual framework (Figure 2.1). A breeding strategy embraces the overall concept of how to go about breeding for particular market demands. A strategy usually focuses on the genetic improvement of a population through the combination of particular mating types and specific selection options, generally starting from a well-adapted and broad genetic base (Eldridge et al., 1993). A breeding

strategy is an on-going and recurrent process. It involves the selection of forest trees (provenances) continuing past the first generation, and includes re-selection generation after generation with inbreeding of selections to provide for recombination (McKinley & van Buijtenen, 1998).

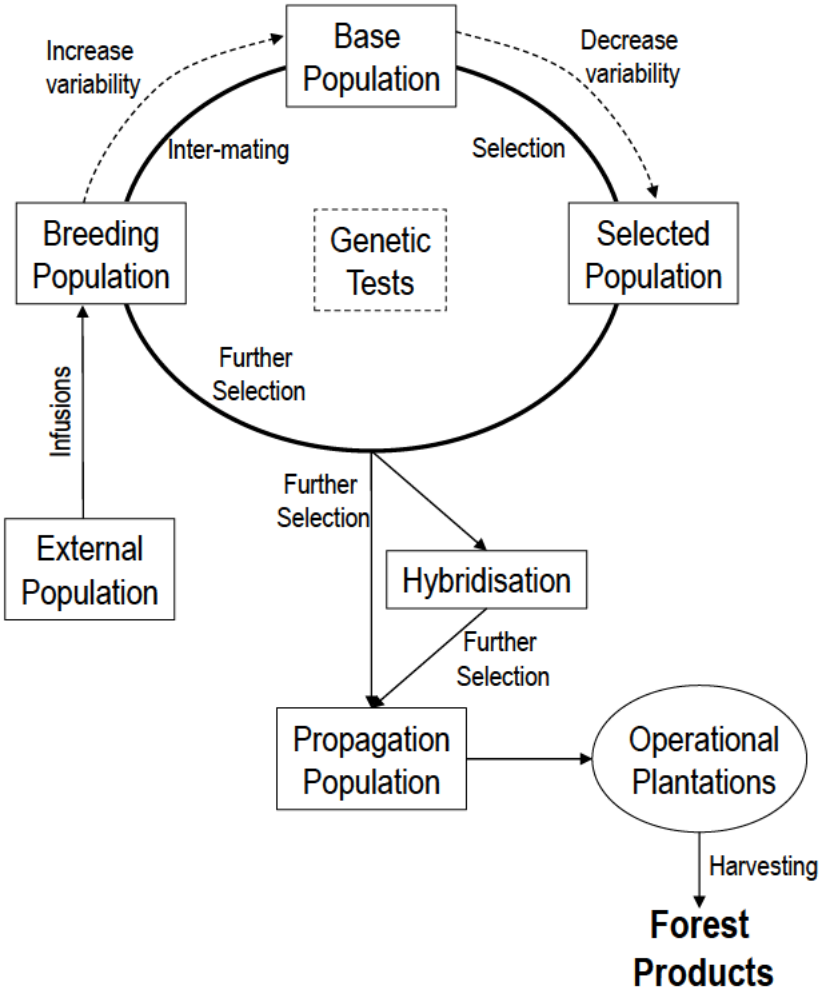

Figure 2.1 Tree breeding cycle (modified from White, 1987)

An effective breeding strategy ensures the maintenance of three population types, namely, base, breeding and production populations. A base population serves as a resource of genetic diversity from which breeding populations and production populations are developed (van Wyk & Verryn, 2000). The breeding population comprises of progeny trials and clonal archives in which the breeding cycle of selection and mating is repeated over many generations, and it is this population

that is the breeder's main focus. The production population, for a given generation, is composed of selected individuals from the breeding population. The function of the production population is to produce genetically improved offspring for operational forestation. The increased yield realised from harvesting plantations established with these genetically superior trees is the primary benefit of most tree improvement programmes (White, 1987).

Selection and hybridisation in breeding is the basis for making genetic gains in a breeding programme. Cotteril and Dean (1990) stated that "maximising gains from advanced generation breeding is largely a matter of efficient selection". Both forward and backward selections are made in breeding programmes (Hamilton & Potts, 2008). Forward selection involves the selection of the best individuals within the best families, based on information gathered from the individual and its family (siblings). Backward selection has been described as the selection of superior parents based on the performance of their progeny (Shelbourne et al., 1989). Superior genotypes are identified and are used in crossing and testing to develop advanced generation breeding populations, including specific hybrids. Therefore, it is the function of well-planned, long-term *Eucalyptus* breeding strategies to manage broadly-based and constantly improving breeding populations in which inbreeding is minimised (Eldridge et al., 1993).

Recurrent selection is a way of making stepwise changes in gene frequency within a population, while maintaining sufficient genetic variability for continued selection (Otegbeye, 1998). In most tree improvement programmes, first generation selection is generally through mass selection, whereas for second or advanced generation selections, both between- and within-family (combined) selection occurs. The primary difference between first and advanced generation procedures/techniques is the amount of information available on which to base selection decisions (McKinley & van Buijtenen, 1998).

Another important aspect of a breeding programme involves progeny testing. Progeny testing permits the evaluation of important genetic parameters that are central to a selection process (Fins et al., 1992). These parameters include the determination of variance components which are used to calculate individual breeding values, heritabilities for certain traits in a population for a particular site. Heritability (h^2) is measured as the ratio of genetic variation (V_g) and total or phenotypic variation (V_p). Correlations are useful in calculating genotype by environmental (*GE*) interaction. It is intuitive that if environmental variation is minimised, the ability to discriminate among better candidate trees is easier (Yanchuk, 2010).

2.5.3 Hybridisation

The cytologically stable nature of *Eucalyptus* has played a major role in its breeding and has allowed for the combination of diverse traits through hybridisation. Hybridisation allows for the combination of years of evolutionary diversity in one step (Ellstrand et al., 1996). *Eucalyptus* species have the ability to hybridise because of their low reproductive barriers (Fossey, 2009). Also, the chromosome complement that exists between *Eucalyptus* species allows for the combination of diverse genotypes through interspecific hybridisation (Zobel and Talbert 1984). Thus, the process of hybridisation combines desirable traits of two selected species and captures its benefits in the off-spring (Bisht et al., 1999). Furthermore, the intraspecific hybridisation of provenances within particular species has also brought about desirable combinations of traits (Zobel and Talbert 1984).

The process of either intraspecific or interspecific hybridisation involves pollination either through controlled pollination or open pollination. Controlled pollination is important in many *Eucalyptus* breeding programmes (House, 1997). It involves the crossing of two pre-selected genotypes for their desired traits (Wright, 1976). The first step in the process of controlled pollination involves the collection of pollen from selected pollen donor trees. Pollen is collected from a bud during the operculum lift stage. Pollen collection involves the emasculation of the bud by removing the anthers

below the operculum, without damaging the style and stigma. The collected anthers containing pollen is then sieved and the pollen collected in a vial (House, 1997).

For the successful application of controlled pollination in breeding programmes, extensive pedigree information should be available. This makes it possible to identify and select good specific combiners that can be used in pre-determined mating designs and thereby exploiting specific combining ability. These combinations create structured pedigree families suitable for testing. Furthermore, this also allows for the provision of more accurate assessments of genetic parameters and the selection of superior genotypes for advanced breeding and for the establishment of seed orchards (Otegbeye, 1998; Hamilton & Potts, 2008).

To obtain successful combinations of genotypes, a large number of crosses are necessary. These mating schemes can be rather complex and create logistical difficulties. They can take many years to complete, with a large cost implication. In many instances, the authenticity of the parents of the resultant offspring may be doubtful (El-Kasssaby & Lstibürek, 2009).

In South Africa, many hybrids have been constituted with *E. grandis* as one of the parent species, because of its good form and desirable fast growth (Eldridge et al., 1993). The most common *Eucalyptus* hybrids cultivated in South Africa, include, *E. grandis* × *Eucalyptus camaldulensis*, *E. grandis* × *E. urophylla* and *E. grandis* × *Eucalyptus tereticomis* (Chetty, 2001). *E. grandis* and its hybrids with *E. camaldulensis* and *E. urophylla* are predominant along the coastal plains of South Africa (Gardner, 2007). Additionally, a host of hybrids have been produced to improve economically important traits and to broaden the site planting range of *Eucalyptus* (Potts & Dungey, 2004). Also, controlled pollinated crosses of *E. camaldulensis* with *E. grandis* and *E. globulus* have been tested in Australia and other countries (Silva et al., 2011; Madhibha et al., 2013).

Open pollination allows for trees to receive pollen from a wide range of different trees establishing wide-ranging genetic combinations. With open pollination, only the maternal pedigree is known and there is no control of inbreeding, which may result in decreased genetic variation due to increased relatedness in the offspring. However, open pollination is suitable for both family and within-family selection, and can be used to evaluate general combining ability of parents/families (Otegbeye, 1998).

2.6 Clonal forestry

2.6.1 Introduction

Clonal forestry plays a significant role in many forestry plantation operations around the world, and is now common practice, not only with *Eucalyptus*, but also with *Pinus* and *Acacia*. The South African forestry industry recognised the benefits of clonal propagation as far back as the 1970's (Sunshine Seedlings, n.d.). Clonal forestry can be defined as the deployment of tested clones (Antony & Lal, 2013). The development of clonal propagation techniques has allowed for the preservation of superior genotypes of selected trees through vegetative propagation. It also offers a chance to propagate a superior genotype that would be lost through sexual reproduction (Antony & Lal, 2013). In clonal propagation the original tree from which parts were taken for multiplication are referred to as the ortet, while the plants propagated from an ortet are referred to as ramets. Members of a clone have the same genetic constitution except for mutations that may have occurred during propagation. The clones of a selected tree (ortet), when planted, result in uniform clonal forests of the same genotype as the parental selected tree. Clonal forests are phenotypically relatively uniform and produce homogenous raw materials (Ferreira et al., 2004). Also, clonal forests allow for more accurate predictions of yield and, therefore, of profits (Brondani et al., 2012). Clonal propagation is thus extensively used today to multiply genotypes with desirable properties and to test and assess many different forestry problems, such as disease resistance (de Assis et al., 2004).

The clonal approach in commercial forestry is firstly to identify individual trees with desired properties. Usually these trees have been tested in a number of environments suited to their genetic make-up. They may also demonstrate superior qualities such as good tree form, wood quality, speed of growth, tolerance to salinity and disease resistance. After identifying superior trees, they can then be multiplied vegetatively to create plantations of trees with identical genotypes (de Assis et al., 2004). Methods of vegetative propagation can be broadly classified as macro-propagation and micro-propagation techniques. In macro-propagation in clonal forestry cuttings are mainly used (Thompson, 2009). In micro-propagation, on the other hand, tissue culturing techniques are applied.

2.6.2 Macro-propagation

Macro-propagation involves removing cuttings from ramet hedge plants. Therefore, for clonal propagation hedge gardens have to be established for mass production of vegetative propagules. Over the years, a number of different types of hedge gardens have been developed. These include macro- and mini-hedges (micro-hedges). Macro-hedges are managed outside at an escapement of approximately 36 stems per m^2 and kept at a height of 30 cm (Thompson, 2009). These hedges are exposed to climate fluctuation, thus making it difficult to maintain the nutritional status and to manage diseases of these hedges, especially during winter (de Assis et al., 2004). Some of the main problems of managing macro-hedge systems include reduced photosynthesis, reduced nutrient uptake and high levels of nutrient loss through leaching during periods of excessive rainfall, or even during irrigation (de Assis et al., 2004). These problems have led to the development of indoor hedge systems. The indoor hedges, referred to as mini-hedges (de Assis et al., 2004), or as micro-hedges (Thompson, 2009) are either planted in soil or managed hydroponically. In soil, mini-hedges are grown inside plastic-covered tunnels at a spacing of approximately 80 stems per m^2. These mini-hedges are kept as low as possible and are planted into holes made in weed matting (Thompson, 2009). The major advantages of indoor systems lie in the realisation of higher productivity of cuttings often with lower labour demands and lower consumption of chemicals and water (de Assis et al.,

2004). This brings about greater economic gains for the industry (de Assis et al., 2004). In addition, the improved nutritional status of mini-hedges results in the realisation of higher rooting percentages than their macro-hedge counterparts (de Assis et al., 2004).

Cuttings taken from the different hedge types are treated with plant growth regulators and grown under controlled environmental conditions (Ezekiel, 2010). Cuttings taken from macro-hedges are regarded as the traditional type of cutting derived from lignified stem portions, usually with one pair of trimmed leaves. They are also referred to as stem cuttings and have been successfully used in *Eucalyptus* propagation (Titon et al., 2006). Traditionally these cuttings are collected from outdoor hedge gardens. On the other hand, cuttings taken from mini- and micro-hedges are referred to as mini- and micro-cuttings. These cuttings are very similar in both concept and operational procedures, but differ in their source of propagule (de Assis et al., 2004). They are also generally smaller than macro-cuttings. Mini-cuttings comprise mostly of axillary sprouts sourced from juvenile parts of plants, while micro-cuttings are obtained from shoot apices originating from micro-propagated plants. The presence of the shoot apex is important for the development of a taproot-like system. The actual length of *Eucalyptus* micro-cuttings is about 3 cm with two to three leaf-pairs (de Assis et al., 2004). Mini- and micro-cuttings require well-equipped green houses with temperature and humidity control. Micro-stumps left after micro-cutting harvest, sprout rapidly producing new micro-propagules, which can be harvested for use within a period of 15 days in the summer and 30 days in the winter (de Assis et al., 2004). Mini- and micro-cuttings can also provide the genetic material for the micro-propagation process (Chinnaraj & Malimuthu, 2011).

2.6.3 Micro-propagation

Micro-propagation involves the production of plant material under *in vitro* conditions, using various media and plant growth regulators to manipulate the development process for optimal growth (Kataria et al., 2013). Micro-propagation utilises small quantities of axenic plant material ranging

from single cells to tissue segments (Alistock & Shafer, 2006). Micro-propagation is expensive but it captures genetic gains quicker than macro-propagation (Ezekiel, 2010). The micro-propagation process can occur along two routes; namely through somatic embryogenesis or through organogenesis. Somatic embryogenesis involves the formation of embryos from somatic or haploid cells without gametic fusion (de Assis et al., 2004). This *in vitro* technique is considered useful for large-scale propagation of elite plants. Somatic embryos (or embryoids) are similar to zygotic embryos, as both present polarity and undergo similar morphological developmental stages. However, somatic embryos lack an endosperm and may display anomalous development, for example an extra cotyledon or trumpet-like cotyledons (Open University and University of Greenwich, 1994).

Somatic embryogenesis has been achieved for many species including *Eucalyptus* (de Assis et al., 2004). In *Eucalyptus*, somatic embryogenesis has been reported for several species, including *Corymbia citriodora* (Muralidharan & Mascarenhas, 1987; Watt et al., 1991), *Eucalyptus dunnii* (Termignoni et al., 1996), *E. nitens* and *E. globulus* (Bandyopadhyay et al., 1999), with varied success. The high production cost, genetic variation in the starting material and low regeneration frequencies in soughtt-after clones, currently limit its application for commercial production (de Assis et al., 2004).

The second route of micro-propagation is via organogenesis. This is also an *in vitro* process that allows for the *de novo* generation of organised structures such as shoots, roots and leaves (Open University and University of Greenwich, 1994). Organogenesis can be direct, whereby organs are generated directly from the explant, or it may be indirect, where organs are generated via callus formation. This method of propagation is advantageous in species that may be difficult to propagate under conventional macro-propagation conditions. The resultant plantlets are acclimatised to the external environment and planted in the field (George, 1993; Kataria et al., 2013).

2.6.4 Adventitious rooting of cuttings

Adventitious rooting is a key step in the process of clonal propagation. The rooting of a cutting facilitates the establishment of a cutting in planting medium (substrate/soil), which allows for the uptake of water and nutrients from the medium (Da Rocha Corrêa & Fett-Neto, 2004). The fundamental mechanisms that trigger or regulate the initiation and development of adventitious roots on cuttings are complex physiological, genetic, and environmental processes, which are still not well understood (Fogaça & Fett-Neto, 2005).

Adventitious rooting can be divided into three phases (Fogaça & Fett-Neto, 2005). These phases include the induction, initiation and expression of rooting (Kevers et al., 1997; de Assis et al., 2004; Fogaça & Fett-Neto, 2005). Induction is the first step in the process of adventitious rooting and involves molecular and biochemical events that precede morphological modifications (Da Rocha Corrêa & Fett-Neto, 2004). In practical terms, the initiation and expression phases are often considered together as the root formation phase (Fett-Neto et al., 2001). The formation of a root is characterised by cell divisions, root primordial organisation and root growth. The formation of a root primordial is generally not synchronised, hence, these root formation events may overlap in time within a given cutting that is undergoing adventitious rooting (de Assis et al., 2004).

Each of the three adventitious rooting phases have specific requirements, each being controlled by exogenous (environmental) and endogenous factors. Exogenous factors include temperature, light and nutrition. Temperature can potentially influence rooting capacity in many aspects, such as, interfering with water and nutrient uptake. Temperature can also affect metabolism in general by promoting or inhibiting enzymatic actions (Taiz & Zeiger, 2002). On the other hand, light intensity can strongly influence cutting productivity and rooting by reducing or increasing endogenous phenolic substances that can act as rooting inhibitors or promoters depending upon the concentration in the tissues and the species involved (Zobel & Ikemori, 1983). Light can also affect

the concentration of endogenous cytokinins, which can function as rooting inhibitors (Bollmark & Eliasson, 1990). Other effects of light include changes in either nutrient or auxin uptake and induction of photochemical or metabolic degradation of auxins (Jarvis & Shaheed, 1987).

Provision of well-balanced nutrients to cuttings is another key factor affecting rooting predisposition. Both macro- and micronutrients are important for root initiation, growth and lateral elongation of adventitious roots (de Assis et al., 2004). For example, the percentage rooting of *Eucalyptus* cuttings decreased considerably when the calcium concentration in the growth medium was dropped to 0.7% causing shoot apex necrosis (de Assis et al., 2004). Micronutrients affect rooting in a number of different ways and vary from species to species. For example, the micronutrient zinc, increases the endogenous content of auxins, presumably by increasing tryptophan pools (de Assis et al., 2004), while the removal of boron from the growth medium resulted in improved rooting of micro-propagated *E. globulus* cuttings by 10% (Trindade & Pais, 1997).

Endogenous factors such as phytohormones, also known as plant hormones, play a complex role in rooting of plants. Phytohormones are chemical messengers that affect a plant's ability to respond to its environment. Phytohormones interact with specific target tissues to cause physiological responses, such as growth or fruit ripening (Davies, 2004). Each response is often the result of two or more hormones acting together (de Assis et al., 2004). Five major groups of plant hormones are recognised, namely, auxins, gibberellins, ethylene, cytokinins, and abscisic acid (de Assis et al., 2004).

Among the different plant hormones, auxins have been recognised to play a major role in rooting of cuttings (De Klerk et al., 1999; de Assis et al., 2004; Erturk et al., 2010). Auxins are usually synthesised in the stem tips and tender leaves of plants and then transported to areas in the plant where they exert their effects (Ljung et al., 2001). There are different types of auxins that are used

to stimulate rooting, growth and yield in plants (de Assis et al., 2004). The most commonly used auxins in commercial propagation are indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA). IAA was the first to be used to stimulate rooting of cuttings (Cooper, 1935), followed by IBA. Although IAA is the main endogenous auxin, in rooting of cuttings IBA is preferred because IBA has been considered to be a better rooting stimulant than IAA (Zimmerman & Wilcoxon, 1935; Brondani et al., 2012; Gehlot et al., 2014). Application of IBA to cuttings of many plant species results in the induction of adventitious roots, in many cases more efficiently than IAA (Epstein & Ludwig-Müller, 1993). For example, in *Vigna radiata* the induction of adventitious roots was observed after IBA application, but not with IAA application (Riov & Yang, 1989). Also, IBA but not IAA, efficiently induced adventitious rooting in *Arabidopsis* stem cuttings at a concentration of 10 µM (Ludwig-Müller et al., 2015). The greater ability of IBA to promote adventitious root formation when compared to IAA has been attributed to the higher stability of IBA than IAA, both in solution and in plant tissue (Nordström et al., 1991). The acropetal (towards the root apex) transport of IBA is also better than that of IAA (Ludwig-Müller, 2000). IBA is applied as rooting stimulant in the form of a powder, liquid (Almeida et al., 2007; Brondani et al., 2010a; Brondani et al., 2010b) and more recently, as a gel (Brondani et al., 2008). Although IBA has been shown to outperform IAA in adventitious root formation in cuttings, its efficacy in adventitious root development in cuttings is plant species specific. According to Spassin and Garcia (2016), IBA had no effect on the survival and rooting of the mini-cuttings of *E. dunnii*.

The efficacy of the type of auxin used in the rooting of cuttings depends on a number of factors. These factors include the affinity of the auxin for the receptors involved in rooting, on the concentration of free auxin that reaches target competent cells, the amount of endogenous auxin, as well as the metabolic stability of the auxin (De Klerk et al., 1999). Metabolic stability is relatively low in IAA, intermediate in IBA and high in NAA (De Klerk et al., 1999). The acropetal pathway is the main pathway of exogenous auxin intake.

Auxins have been shown to regulate different aspects of adventitious rooting of cuttings. They are involved in bringing about metabolic changes during the initiation, emergence and root primordial development (de Assis et al., 2004; Husen, 2008). de Assis et al. (2004) has produced experimental evidence confirming some of the key roles of endogenous and exogenous auxins in rooting. This research has shown that auxins are the only chemicals that consistently enhance root formation in naturally responsive cuttings. The presence of endogenous auxin in buds and young leaves support the notion that these organs are a source of auxin, which is transported in a basipetal manner to the roots. When auxin transport inhibitors are applied, root generation is blocked. Exogenous auxins can be supplied as a substitute for natural auxin sources. Auxins also play an important role in the mobilisation of carbohydrates in leaves and upper stems, and increases carbohydrate transport to the rooting zone of cuttings during adventitious root formation (Altman, 1972; Altman & Wareing, 1975; Haissig, 1986; Husen & Pal, 2007). Mobilisation of carbohydrates is through increased activity of hydrolysing enzymes initiated by auxin (Altman, 1972; Husen, 2008).

2.6.5 Root architecture of cuttings

Adventitious roots develop from places other than at a shoot apical meristem. In cuttings they develop at the cutting edge of a cutting. The adventitious root formation in cuttings occurs in different configurations, which are termed root architecture. Root architecture has been defined as the "spatial configuration of a root system in the soil" (Yamauchi et al., 1996; Gregory, 2006). The plant root system consists of roots that differ in morphology, including length and radius, as well as function (Wang et al., 2006). Several factors contribute to the formation of different root architectures. Soil characteristics, plant nutrition and plant genetics contribute to the formation of particular root architectures (Robinson, 1994). Root morphology and architecture influence the acquisition of nutrients and is also influenced by the resource distribution within the rooting environment (Yamauchi et al., 1996; Yamauchi, 2001; Da Silva et al., 2011). In the root system architecture of *E. grandis* it was found that nitrogen, potassium and calcium tracers, at varying soil

depths, yielded different uptake rates related to the depth of the nutrient (Da Silva et al., 2011). Therefore, it could be concluded that growth and development of a plant is in part dependent on a well-developed root architecture system. Therefore, the differences in root architecture may possibly have an impact on plant growth and development (Rockwood & Warrang, 1994).

2.7 Rooting of *Eucalyptus* cuttings

2.7.1 Introduction

At the core of clonal propagation lies the process of adventitious rooting (de Assis et al., 2004). However, the rooting percentages of especially macro-cuttings of a number of economically important *Eucalyptus* species, including *C. citriodora*, *E. maculata*, *E. paniculata, E. coleziana*, are relatively low, making this technique problematic for the high demanding forestry industry (Sasse & Sands, 1997; Luckman & Menary, 2002; de Assis et al., 2004). Problems associated with low rooting rates with macro-cuttings have been attributed to the predisposition of an accelerated maturation process causing rapid loss of rooting and the manifestation of topophysis. Topophysis is a phenomenon whereby cuttings maintain their growth characteristics from where they originated (Haissig & Riemenschneider, 1988). Topophysis affects different clones in different intensities, and is probably one of the main causes of intra-clonal differences in growth and reduction of rooting ability (de Assis et al., 2004). Another limitation of rooting macro-cuttings has been attributed to alterations of the root architecture, leading to root deformities. Such root deformities reduce the available number of species or hybrids that can be effectively cloned using macro-cuttings (de Assis et al., 2004).

Owing to the limitations of rooting macro-cuttings, alternative and more effective methods have been developed to counteract these limitations. The use of mini- and micro-cutting techniques that use more juvenile plant material have demonstrated improved nutritional content of a cutting and increased rooting (Gehlot et al., 2014). Although the rooting of mini-and micro-cuttings have shown

impressive results over macro-cuttings, many valuable *Eucalyptus* species and hybrid genotypes still show variation in their rooting capacity (Barralho & Wilson, 1994; Martellet & Fett-Neto, 2005; Diaz et al., 2009; Peralta et al., 2012). Furthermore, mini- and micro-cuttings also tend to lose their ability to form adventitious roots as the hedge plant ages, because of morphological, anatomical and biochemical changes that occur as the plant matures (Ezekiel, 2010). The morphological, anatomical and biochemical changes may be as a result of stress responses that results from wounding, changes in plant water relations, and separation from original root system (de Assis et al., 2004; Peralta et al., 2012). In difficult-to-root *Eucalyptus* species and hybrids, tissue dehydration of cuttings are often responsible for the low rooting percentages (de Assis et al., 2004). Other contributing factors to poor rooting are genetic characteristics (Fett-Neto et al., 2001; de Assis et al., 2004), loss or an inability to photosynthesise new carbohydrates (Haissig, 1984), as well as limited response to exogenous auxins (Epstein & Ludwig-Müller, 1993). In the nursery, the application of auxin of varying kinds and concentrations, well controlled nutrition, temperature control, watering regimes have been used to promote rooting of cuttings with varied success (Davies, 2004; Werner et al., 2010; Whiting et al., 2011; Brondani et al., 2012). Thus, alternative mechanisms that stimulate rooting of *Eucalyptus* cuttings are constantly being sourced.

2.7.2 Rhizospheric microbial communities as rooting agents

In recent times, microorganisms that have been extracted from the rhizosphere of plants have been shown to promote rooting though various means. The rhizosphere of a plant root is the area in the immediate vicinity of a rooting system and is referred to as the rhizospheric zone. This zone is about 1 mm wide, but has no distinct edge. A large number of microorganisms, such as bacteria, fungi, protozoa and algae coexist in the rhizospheric zone of a plant root system. These groups of microorganisms constitute the rhizospheric microbial community, with bacteria being the most abundant (Malave-Orengo et al., 2010; Saharan & Nehra, 2011; Chowdhury & Dick, 2012) (Figure 2.2). These bacteria are referred to as rhizospheric bacteria and their abundance can be attributed

to their rapid growth and ability to utilise a large range of substances as carbon and nitrogen sources (Glick, 1995). Plant roots release organic compounds through exudates, which act as a selective medium for specific group of rhizospheric bacteria (Marilley & Aragno, 1999; Garcia et al., 2001). As a result, plants select rhizospheric bacteria that are competitively fit to occupy compatible niches without causing pathological stress on them, and are in symbiotic association with the host plant (Etesami et al., 2015). The rhizospheric bacteria that are in symbiotic association with plants are referred to as plant growth promoting rhizospheric bacteria (PGPB) or yield increasing bacteria (YIB) (Chen et al., 1994). Saharan and Nehra (2011) stated that rhizospheric bacteria probably have a greater influence on plant physiology than other rhizospheric microorganisms, because of their abundance.

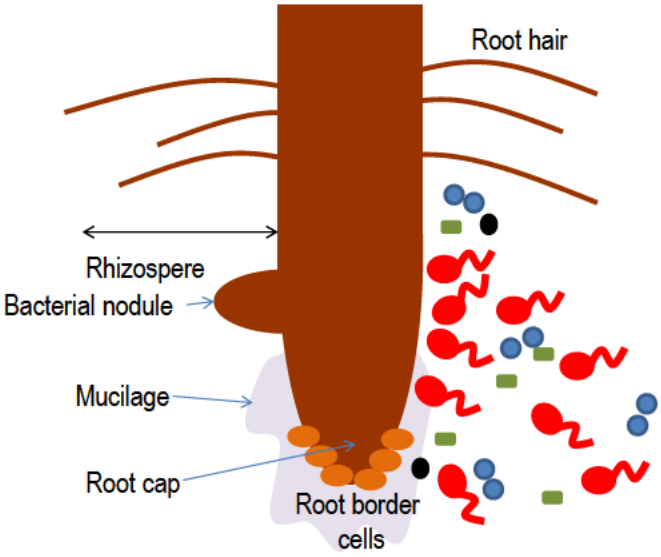

Figure 2.2 Plant root showing the rhizospheric zone with its associated microorganisms

Rhizospheric bacteria can be prejudicial, beneficial or innocuous to the host plant (Lazarovits & Nowak, 1997; Asghar et al., 2004; Chauhan et al., 2015; Halder & Sengupta, 2015; De La Torre-Ruiz et al., 2016). PGPB have been shown to stimulate plant growth and adventitious root formation in cuttings (Teixeira et al., 2007; Diaz et al., 2009; Erturk et al., 2010; Zafar et al., 2012). Recent

studies confirm that the treatments of cuttings or seeds with rhizospheric bacteria, such as *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Pseudomonas* and *Streptomyces* induced root formation in some plants because of natural auxin production by these bacteria (Patena et al., 1988; Esitken et al., 2003). The growth promoting effect of rhizospheric microorganisms has been demonstrated in a number of forest species, such as *Picea glauca* and *Pseudotsuga menziessii* (Chanway et al., 1991; O'Neill et al., 1992; Enebak et al., 1998). In *E. grandis*, rhizospheric bacteria have been shown to increase the rooting and root biomass of macro-cuttings and mini-cuttings (Teixeira et al., 2007). Therefore, one of the strategies to stimulate the rooting of cuttings involves the use of rhizospheric bacteria that inhabit the rhizosphere of plant roots.

The mechanisms by which PGPR effect rooting are not completely understood. It is accepted that root induction by PGPR is the result of the actions of phytohormones, such as auxin production, inhibition of ethylene synthesis, as well as through the mineralisation of nutrients (Goto, 1990; Steenhoudt & Vanderleyden, 2000). Considering the numerous interactions between the different hormonal signaling pathways in plants, it is difficult to assess which of these pathways is the primary target of PGPR (Ertuk et al., 2010). More likely, PGPR alter several hormonal pathways, which results in an increased lateral root elongation, and perhaps an increased initiation rate of lateral roots resulting in a more branched root system architecture (Kapulnik et al., 1985; Lifshitz et al., 1987). However, Saharan and Nehra (2011) broadly classified three main attributes, which are thought to be the roles by which PGPR could contribute to rooting in plants. These attributes include their abilities to act as biological protectants, as biological stimulants and as biological fertilisers (Saharan and Nehra, 2011).

PGPR can promote rooting and growth of plants indirectly through their ability to act as biological protectants (Ahmad et al., 2008; Saharan & Nehra, 2011). This occurs when PGPR prevent or lessen deleterious effects of phytopathogenic microorganisms in plants (Hoon et al., 2007; Ahmad et

al., 2008; Lugtenberg & Kamilova, 2009; Saharan & Nehra, 2011; Bach et al., 2016). For example, rhizospheric bacteria have been reported to have induced the control of rust (*Pucinia psidii*) and cutting rot (*Botrytis*, *Cyllindrocladium*, *Rhizoctonia* and *Sporothrix*) in *Eucalyptus* cuttings (de Assis et al., 2004). PGPR are also capable of inducing systemic resistance to pathogens in plants. Siderophore producing rhizospheric bacteria have a high affinity for iron, which deprives pathogens of iron and consequently affects their growth (Scher & Baker, 1982; Alexander & Zuberer, 1991; Subba Rao, 1999). Other mechanisms that have been suggested to be involved as bio-protectants include the suppression or antagonism against deleterious microorganisms through the production of β–1,3 glucanase, chitinases, antibiotics (Shanahan et al., 1992), and cyanide (Flaishman et al., 1996). However, the role of cyanide production is contradictory, as it may be associated with deleterious, as well as with beneficial effects (Baker & Schippers, 1987; Alstrom & Burns, 1989).

PGPR as biological stimulant involves the synthesis of auxin hormones, similar to those produced by plants that are able to stimulate rooting of cuttings (Gaudin et al., 1994). Studies have shown that the treatment of cuttings of *E. globulus* with PGPR such as, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Comamonas*, *Paenibacillus*, *Pseudomonas* and *Streptomyces* induced root formation because of auxin production and the inhibition of ethylene synthesis (Tripp & Stomp, 1997; Esitken et al., 2003; Teixeira et al., 2007). The application of *Agrobacterium rhizogenes* to cuttings of difficult-to-root species of apple (Zhu et al., 2001), Plum, *Pyrus pyraster*, almonds (Damiano & Moonticelli, 1998) and walnuts (Caboni et al., 1996) has been generally successful, with the development of improved rooting and root systems without morphological abnormalities. Furthermore, the PGPR belonging to the genera *Bacillus*, *Comamonas* and *Paenibacillus* have been shown to produce IAA that promoted root formation in kiwifruit cuttings in mass clonal propagation (Erturk et al., 2010).

Several strains of PGPR have been shown to stimulate rooting and pant growth through their fertilising ability. These beneficial PGPR strains are mostly from genera of *Azospirillum*, *Azotobacter*,

Bacillus, *Burkholdaria*, *Enterobacter*, *Erwinia*, *Flavabacterium*, *Herbaspirillum*, *Pseudomonas* and *Rhizobium* (Rodriguez & Fraga, 1999; Prathibha & Siddalingeshwara, 2013; Neiverth et al., 2014; Gontia–Mishra et al., 2017). PGPR, as biological fertilisers, assist in the mineralisation of nutrients in the soil and uptake of nutrients by plants (Kucey, 1983; de Freitas et al., 1997). Micronutrients such as zinc, copper, iron, molybdenum, boron and manganese play a vital role in plant growth at different stages (Uchida, 2000). Zinc is an essential micronutrient required by plants for better growth and nutrition. In plants, zinc has been found to be an important component in several enzymatic reactions, carbohydrate metabolism, maintenance of the integrity of cellular membranes, protein synthesis and in auxin synthesis. Zinc also plays a vital role in the regulation of the gene expression needed for the tolerance of environmental stresses in plants (Cakmak, 2000). Several zinc solubilising bacteria have been isolated from the rhizosphere of rice (Gontia–Mishra et al., 2017). These zinc solubilising bacteria include the species *Burkholderia cepacia*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Ralstonia picketti*, which have been shown to enhance and promote the growth of rice seedlings (Gontia–Mishra et al., 2017). Most phosphorus occurs in insoluble compounds and is unavailable to plants in that form; hence, phosphorus solubilising bacteria convert insoluble phosphorus into a form that plants can absorb (Gaur, 1990; Çakmakçi et al., 2007; Beneduzi et al., 2013; Armada et al., 2016). Several PGPR have been reported to stimulate rooting of cuttings, as well as to improve growth and yield of plants directly by asymptomatic production and uptake of nitrogen (Glick et al., 2007).

When attempting to use PGPR as biological rooting agents, an understanding of the diversity, abundance and attributes of rhizospheric microorganisms are necessary, because the diversity of rhizospheric microorganisms are highly plant genotype specific (Malave-Orengo et al., 2010). The diverse composition of rhizospheric microorganisms determines a particular rhizospheric community's structure and function (Malave-Orengo et al., 2010; Chowdhury & Dick, 2012). The characteristics of rhizospheric microorganisms are used as indicators of the relationship between a

particular host plant and its rhizospheric microorganism community; the quality of the ecosystem; as well as soil health (Hill et al., 2000).

Traditionally, the diversity and abundance of soil microorganisms have been studied by culturing techniques using specific media and conditions in the laboratory (Leckie, 2005). However, such culturing techniques only allow the estimation of a small fraction of the different microbial species and thus underestimate the extent of microbial diversity (Leckie, 2005). This has brought about the development of methods that are culture-independent. These methods are mainly molecular and biochemical in nature and are able to describe soil microbial species more extensively. These newer techniques are now being used to estimate the diversity of different soil microbial communities, compare the composition of these communities, and begin to describe differences in the functioning of the microbial communities (Leckie, 2005). The molecular techniques are genotype-DNA–based techniques that rely on sequencing of DNA or the visualisation of DNA bands and various profiling methods (Fakruddin et al., 2013), while biochemical techniques are based on biochemical pathways and substances. One of the more popular biochemical methods that are used to describe soil microbial diversity involves profiling microbial communities by their phospholipid fatty acid (PLFA) composition. PLFAs are components of the cell membranes of microorganisms living in the soil and have been used as informative biological markers for the profiling of soil microbial communities. PLFAs are easily extracted from soil and then converted to fatty acid methyl esters (FAMEs) by alkaline methanolysis, after which they are identified by gas chromatography. These FAMEs can then be used to describe the diversity of a microbial community resident in the soil (Hill et al., 2000).

2.8 Conclusion

Studies gained from rhizosphere research have improved our ability to steer the knowledge into technological applications in agriculture (Halder & Sengupta, 2015). The applicability of PGPR in agriculture has steadily increased as these regulators offer the opportunity to replace chemical

fertilisers, pesticides and mineral nutrients (Bhattacharyya & Jha, 2012). The use of chemicals in plant propagation can be detrimental to the environment and may also increase the cost of propagation in nurseries (Erturk et al., 2010; Chauhan et al., 2015).

Environmentally friendly applications in agriculture have gained more importance, in particular in horticulture and nursery production. The use of PGPR for nursery material multiplication may be important for obtaining organic nursery material because the use of formulations of synthetic plant growth regulators, such as, IBA, is prohibited in organic agriculture throughout the world (Erturk et al., 2010). Furthermore, the use of PGPR is well established for agricultural crops, but little is known about their potential contribution to promoting growth in forestry clones (Teixeira et al., 2007). Few genera of PGPR have been used as root inducers in forest trees, particularly in *Eucalyptus* cuttings of *E. globulus*, *E. saligna and* mini-cuttings of a hybrid *E. grandis* × *E. urophylla clone* (Teixeira et al., 2007; Diaz et al., 2009). Thus, testing different PGPR strains as potential rooting enhancers for commercially important *Eucalyptus* species and hybrids may be advantageous to clonal forestry.

Chapter 3
Study design

3.1 Introduction

Eucalyptus hybrids are highly valued in the forestry industry in South Africa. This is mainly attributed to their hybrid vigour, growth and wood properties. The hybrids of *E. grandis* and *E. urophylla* (GU), as well as the hybrids of *E. grandis* and *E. nitens* (GN), are some of these sought after hybrids in South Africa. *Eucalyptus* hybrids are commercially multiplied by the rooting of cuttings and producing young clonal plants in nurseries. However, some of the sought after hybrid genotypes vary greatly in their rooting capacity; some demonstrating relatively low rooting percentages, thereby bringing about substantial losses for commercial nursery growers.

Because of the close symbiotic association between rhizospheric bacteria and a host plant; rhizospheric bacteria contribute to a number of beneficial plant growth properties. Within the forestry industry, interest in these bacteria lies in their potential to enhance the rooting of cuttings, particularly of those genotypes that are deemed difficult-to-root.

In this chapter, the study design and methods that pertain to the whole research project are reported. However, specific methods that pertain to a specific part of the study have been reported in the chapter of interest.

3.2 Study design

A number of smaller projects within the large project was undertaken in preparation for the testing of the root stimulating effect of rhizospheric bacteria. Firstly, rhizospheric soils were collected from *Eucalyptus* hedge plants participating in this project. Thereafter, the rhizospheric microbial communities were characterised using fatty acid methyl esters (FAMEs). Also, rhizospheric bacteria

were isolated and characterised by sequencing the 16S rRNA genes. The identified rhizospheric bacteria were then screened for rooting enhancing properties, which included their ability to produce indole-3-acetic acid (IAA) and to solubilise phosphates. Once rhizospheric bacteria had been characterised, inoculums of bacteria were prepared and then tested for their ability to stimulate rooting of cuttings. Figure 3.1 provides a flow diagram of the different components of the study and also mentions the chapters in which the results have been reported.

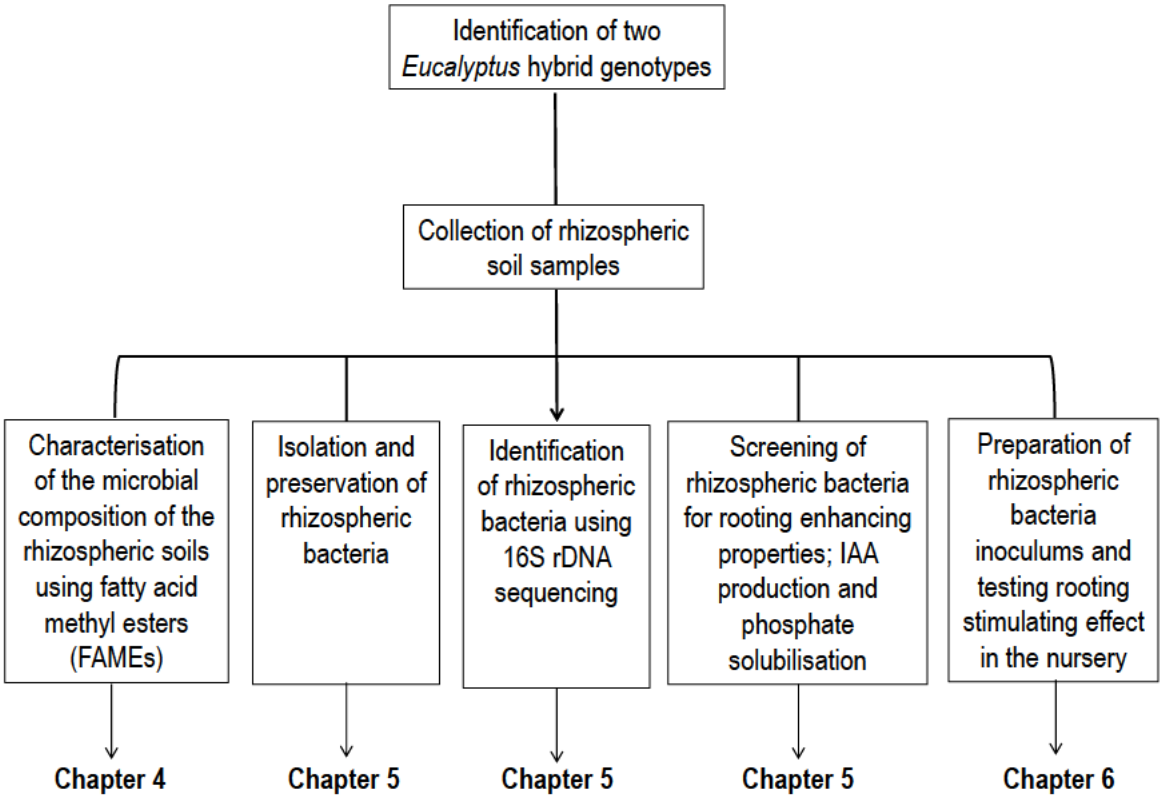

Figure 3.1 Study design and presentation in chapters

3.3 *Eucalyptus* genotypes used in this study

In South Africa, *E. grandis* and its hybrids are by far the most widely cultivated *Eucalyptus* genotypes (Poyton, 1979; Malan & Arbuthnot, 1995; Chetty, 2001; Komakech et al., 2009; McMahon et al., 2010). *E. grandis* is relatively fast growing and most commonly used as a source of pulpwood, fuel and timber. The cuttings of this species root relatively well, making it suited for cloning.

However, the cuttings of some of the sought after hybrids of *E. grandis*, particularly with *E. nitens*, have demonstrated relatively poor rooting (Personal communication, CSIR, South Africa). Thus, two *E. grandis* × *E. nitens* (GN) hybrid genotypes were selected for this project, namely GN 018B and GN 010.

Chapter 4

Characterisation of *Eucalyptus* rhizospheric communities using FAME profile analysis

This chapter has been summited to the international accredited journal, the *Journal of Soil Science and Plant Nutrition* for publication.

Presented in Chapter 4 is a copy of the article that was sent to the journal, excluding the references. The copy of the article presented in Chapter 4 has also been numbered to keep the formatting of the thesis intact. The references of this article have been taken up with the references of the entire thesis.

The raw data of the FAME profile analyses have been taken up in the appendix.

Abstract

Hybrid vigour is exploited in *Eucalyptus* forestry through producing interspecific hybrids. Hybrids are deployed via cuttings that often suffer poor rooting. Rhizospheric microorganisms have been associated with rooting enhancement, therefore knowledge of changes in rhizospheric microorganism composition may provide ways to manipulate soil to improve rooting. Fatty acid methyl ester profiles were prepared for 3-month and 5-year old soil samples collected from rhizospheres of *Eucalyptus grandis* × *Eucalyptus nitens* clones to characterise microbial diversity. Saturated, mono-unsaturated and poly-unsaturated fatty acids were present in both soil age groups, although in different proportions. Proportions of saturated fatty acids in the younger samples were greater than in the older samples, while proportions of unsaturated fatty acids was less in the younger than the older samples. Palmitic (C16:0) and stearic (C18:0) fatty acids were the most prevalent. Both Shannon and Simpson's indexes confirmed that younger soils were more diverse. Reduced proportions of saturated fatty acids in the older samples are associated with increasing anaerobic conditions. The greater proportion of polyunsaturated fatty acids in older samples, especially linoleic, α-linolenic and γ-linolenic fatty acids, suggests growing establishment of fungi. This study confirmed that the microbial composition in younger rhizospheric soils was more diverse than in the older soils.

Keywords: Microbial community, rhizospheric microorganisms, soil quality, fatty acid methyl esters, diversity index, FAME

4.1 Introduction

Eucalyptus is one of the most widely cultivated hardwood genera in the tropical and subtropical regions of the world. *Eucalyptus* plantation forestry is practiced predominantly across the southern hemisphere, notably in Brazil and South Africa (dos Santos et al., 2004). *Eucalyptus* species are fast growing trees that are tolerant of different soil and climatic conditions (dos Santos et al., 2004). Breeding of *Eucalyptus* species through intraspecific and interspecific hybridisation has led to the selection of a great variety of superior genotypes (van Wyk & Verryn, 2000).

The ability of *Eucalyptus* species to hybridise has been recognised as a means to combine genes from diverse sources and to utilise hybrid vigour (Potts & Dungey, 2004). In an evolutionary context, interspecific hybrids provide the potential for one-step evolution by combining many years of diverse evolutionary pathways. As a result of the mixed genetic backgrounds of such hybrid genotypes, hybrids can be deployed in regions not usually suited to the pure species. Thus, the exploitation of hybrid vigour of these hybrids has driven the commercial forestry industry to produce a variety of different interspecific hybrids (Dieters et al., 1995).

Mass vegetative propagation complements hybridisation when producing clonal forests. The cloning of hybrids is based on the rooting of cuttings. However, a major drawback in *Eucalyptus* clonal forestry is the manifestation of poor rooting by some valued hybrid genotypes (Diaz et al., 2009; Peralta et al., 2012). One of the strategies that have been shown to improve rooting of cuttings is through the application of rhizospheric microorganisms (Diaz et al., 2009). Rhizospheric microorganisms inhabit the rhizosphere of plant roots where most of them are in a symbiotic relationship with the host plant. These rhizospheric microorganisms feed on sloughed-off cells and root exudates. In recent years the interest in these soil microorganisms has increased, as they are key contributors to nutrient cycling and maintenance of soil fertility. Many of the beneficial soil microorganisms synthesise plant growth hormones and induce the host plant to synthesise plant

growth hormones, such as indole-3-acetic acid (IAA). IAA is influential, not only to plant growth and development, but also in the formation of adventitious roots in cuttings (Teixeira et al., 2007; Erturk et al., 2010) .

The quantity and composition of the root exudates are influenced by a variety of factors. Two of the more important factors that influence and may change the composition of root exudates include the genotype of the plant and the plant's age. Changes in the composition of plant root exudates have an impact on the microbial community succession in a rhizosphere (Rasch et al., 2006; Micallef et al., 2009; Halder & Sengupta, 2015). Plants of different cultivars of *Arabidopsis thaliana* that were grown simultaneously under uniform conditions were found to produce unique combinations of root exudates (Micallef et al., 2009). However, within six weeks post germination, genotype-specific rhizospheric microorganism communities could be identified for the different plant genotypes, indicating that root exudate composition is under genetic control (Halder & Sengupta, 2015). The differences in genotype-specific root exudates, therefore, explain the plant-specific diversity of rhizospheric microorganism communities that have been observed for different plant species grown under similar conditions (Rasch et al., 2006).

As plants age, so do the compositions of root exudates that they produce also change. These changes in the compositions of root exudates thus bring about changes in the composition of the rhizospheric microbial communities (Di Cello et al., 1997; Hai et al., 2009; Hayden et al., 2010; Musyoki et al., 2016). The rhizospheric environment changes during different phases of plant growth. As the plant grows and the root system develops, the more favourable microorganisms that make optimal use of the rhizospheric constituents establish themselves (Di Cello et al., 1997; Musyoki et al., 2016). The rhizospheric environment of young plants accommodates mainly first root colonisers, resulting in a more diverse rhizospheric microorganism community, however, this diversity decreases as the plant ages (Di Cello et al., 1997). Therefore, when attempting to exploit

rhizospheric microorganisms for their properties in biotechnological applications in clonal forestry, such as the enhancement of the rooting of cuttings, it is necessary to have some understanding of the composition and abundance of rhizospheric microorganism communities, and change over time.

A number of methods have been applied to study the diversity of rhizospheric microbial communities and how their compositions change over time. The traditional culturing methods that have been applied to describe the diversity of microbial communities pose a number of drawbacks. Besides being labour intensive, these methods can only reveal a limited number of microbial strains. The number of culturable soil microorganisms represents only a small fraction of the overall diversity of microorganisms that are present in most soils; of which many may be ecologically unimportant strains (Torsvik et al., 1998; Trevors, 1998; Anderson & Cairney, 2004; Leckie, 2005). Many fungal species also elude culturing in the laboratory (van Elsas et al., 2000). A DNA association kinetics study revealed that at least 4,000 bacterial strains occurred in 30 g of forest soil collected in southern Norway. The number of culturable stains represented less than 1% of the total number of these strains (Torsvik et al., 1990a; Torsvik et al., 1990b; Atlas & Bartha, 1998). This finding is consistent with another study where 100 times more soil bacteria were seen with direct microscopy than what were found using selective culturing methods (Cavigelli et al., 1995). Thus, when culturing methods are used to study soil microbial community diversity and structure, they appear to severely underestimate the number of microbial strains present in the soil.

Recently, biochemical methods have been used to characterise soil microbial communities. The profiling of phospholipid fatty acids (PLFAs) is one of the more popular biochemical methods that have been used to characterise soil microbial communities. PLFAs are components of the cell membranes of microorganisms living in the soil. These PLFAs have been used as informative biological markers for the profiling of soil microbial communities. PLFAs are easily extracted from soil and then converted to fatty acid methyl esters (FAMEs) by alkaline methanolysis. These FAMEs

can then be used to describe the diversity of a microbial community resident in the soil (Hill et al., 2000). This diversity profiling method is able to describe the soil microbial community more extensively than would otherwise be possible when using culture-dependent methods (Hill et al., 2000).

PLFAs make up a relatively constant proportion of cell membranes of microorganisms. The presence of specific and unique PLFAs that exist in some microorganisms can be used to differentiate between major taxonomic groups within a microbial community (Kirk et al., 2004). Therefore, profiling PLFAs through FAME analysis provides information on a microbial community composition based on groupings of PLFAs, because unique PLFAs are indicative of specific groups of microorganisms (Hill et al., 2000). A large number of bacterial species, more than 1,500, can be identified based on their unique fatty acid profiles (Sasser, 2009).

A FAME profile, therefore, describes soil microbial diversity in terms of the PLFA chain length, double-bond position, and substituent groups. Changes in a FAME profile would represent changes in a microbial population's composition. Therefore, the aim of this study was to describe the diversity of rhizospheric microbial communities of different ages for the *Eucalyptus* hybrid; *Eucalyptus grandis* × *Eucalyptus nitens* (GN hybrid). The rhizospheric microbial communities of 3-month old and 5-year old clones of the GN hybrid were profiled and compared using FAME profile analysis.

4.2 Materials and methods

4.2.1 Collection of rhizospheric soil samples

The rhizospheric soil samples were collected from 3-month old and 5-year old GN hybrid clones at a commercial forestry nursery in the midlands of the KwaZulu-Natal Province in South Africa. Approximately 60 ml of soil was collected from six rhizospheric soils of each age group. These soil samples were kept on ice while in transport and then refrigerated in the laboratory at -80°C till use.

4.2.2 Generation of FAME profiles

FAME profile analyses of the soil samples were performed by SGS Agricultural Food Laboratory in Cape Town, South Africa. The AOCS Official Method Ce2-66 was used to prepare the PLFA of each soil sample for gas chromatography (Quideau et al., 2016). The general four-step procedure of saponification, methylation, extraction and sample clean-up was used to prepare the FAMEs for analysis. In general terms, FAME profiles were generated by adding sodium hydroxide in methanol to a soil sample and then heated to saponify the PLFAs in the soil sample. Thereafter, methylation-formation of methyl esters of the fatty acids (FAMEs) was achieved by adding hydrochloric acid in methyl alcohol. At this point in the method, the FAMEs are poorly soluble in the aqueous phase and therefore hexane and methyl tert-butyl ether was added to solubilise the FAMEs. These FAMEs were then extracted into the organic phase of the solution, after which the FAME containing fraction was cleaned. After the cleaning-up, the organic phase was then transferred to a gas chromatography vial and then placed into the Agilent 7890A gas chromatograph for FAME analysis and the results recorded by ChemStation software. A gas chromatogram of a FAME profile was generated for each soil sample. These FAME profiles were used to indicate which PLFAs were present in a particular soil sample, as well as their abundance.

4.2.3 Comparison of FAME profiles

In an attempt to compare the PLFA compositions of the 3-month old and the 5-year old rhizospheric microbial communities, three different diversity indexes were calculated. The first diversity index, PLFA richness, describes the number of different PLFAs that was present in the different rhizospheric soil samples. PLFA richness (R) is represented by the following equation:

Richness (R) = *Number of different fatty acids* *Equation 1*

The Shannon index (H) is based on the proportions of the respective PLFAs and their evenness (abundance) in a rhizospheric soil sample. This index assumes that all PLFAs were randomly sampled. In the Shannon index p_i represents the proportion of the i^{th} PLFA, while *s* represents the total number of PLFA. In this study the natural logarithm was calculated, although the base of the logarithm can be chosen freely. The Shannon index is represented by the following equation.

Shannon index (H) = $-\sum_{i=1}^{s} p_i \ln p_i$ *Equation 2*

The Simpson index (D) was also calculated. This index is a dominance index, because it gives more weight to common or more abundant PLFAs that are present in the rhizospheric soil sample. In the Simpson index a few rare PLFAs with only a few representatives will not affect the diversity. In the Simpson index p_i represents the proportion of the i^{th} PLFA, while *s* represents the number of fatty acids. The Simpson index is represented by the following equation.

Simpson index (D) = $\frac{1}{\sum_{i=1}^{s} p_i^2}$ *Equation 3*

A number of different statistical procedures of the software program Statistica 13.0 (TIBCO Software, 2015) were used to determine the effect of age on the diversity of the rhizospheric microbial communities of the different soil samples. The FAME profiles of the rhizospheric microbial communities of the 3-month old and 5-year old rhizospheric soil sample groups were compared through an analysis of variance (ANOVA), Wilks test, principal component analysis and factor analysis.

4.3 Results

4.3.1 FAME composition of 3-month old rhizospheres

The FAME profiles of the 3-month old rhizospheric soil samples demonstrated the presence of saturated, mono-unsaturated and poly-unsaturated fatty acids with the saturated fatty acids being the most prevalent in terms of the number of different fatty acids, as well as their abundance (mean area %). When considering the composition of fatty acids, 35% were identified saturated fatty acids, 44.5% identified unsaturated fatty acids and 22.5% unknown fatty acids. In the FAME profiles, 31 different fatty acids were identified in the rhizospheric soil samples of which 45.1% were saturated fatty acids, 25.8% mono-unsaturated fatty acids and 29.0% poly-unsaturated fatty acids, representing a ratio of approximately 2 : 1 : 1 of saturated fatty acids : monounsaturated fatty acids : poly-unsaturated fatty acids (Table 4.1). Forty percent of the fatty acids (including unknown fatty acids) occurred in all six samples. Within the saturated fatty acid class, 50% of the fatty acids occurred in all six samples, whereas of the mono-unsaturated fatty acids 37.5% and of the poly-unsaturated fatty acids, 11% occurred in all six samples. The most prevalent fatty acid in the saturated fatty acid class was stearic, a 16-carbon fatty acid, followed by palmitic, an 18-carbon fatty acid. In the mono-unsaturated fatty acid class, the 18-carbon fatty acid oleic was the most prevalent. When considering the abundance of saturated, mono-unsaturated and poly-unsaturated fatty acids; excluding the unidentified fatty acids, they occurred in an approximate ratio of 6 : 2 : 1.

Table 4.1 FAME profile of 3-month old rhizospheres

Fatty acid class	Fatty acid name	Symbolic name of fatty acids	*n*	Mean area % composition	Total mean area % composition
Saturated FA	Caproic acid	C6: 0	2	0.07	56.64
	Caprylic acid	C8: 0	3	0.17	
	Capric acid	C10: 0	1	0.07	
	Lauric acid	C12: 0	6	0.63	
	Tridecanoic acid	C13: 0	2	0.35	

Fatty acid class	Fatty acid name	Symbolic name of fatty acids	*n*	Mean area % composition	Total mean area % composition
	Myristic acid	C14: 0	6	2.98	
	Pentadecanoic acid	C15: 0	4	0.47	
	Palmitic acid	C16: 0	6	14.55	
	Heptadecanoic acid	C17: 0	6	1.48	
	Stearic acid	C18: 0	6	24.43	
	Arachidic acid	C20: 0	6	3.03	
	Behenic acid	C22: 0	6	3.00	
	Tricosanoic acid	C23: 0	2	0.38	
	Lignoceric acid	C24: 0	6	5.03	
Mono-unsaturated FA	Myristoleic acid	C14: 1ω5c	5	0.65	18.64
	Pentadecenoic acid	C15: 1ω10c	1	0.07	
	Palmitoleic acid	C16: 1ω7c	6	1.73	
	Heptadecenoic acid	C17: 1ω10c	1	0.27	
	Elaidic acid	C18: 1ω9t	6	2.22	
	Oleic acid	C18: 1ω9c	6	12.60	
	Erucic acid	C22: 1ω9c	1	0.35	
	Nervonic acid	C24: 1ω9c	3	0.75	
Poly-unsaturated FA	Linolelaidic acid	C18: 2ω6t	3	0.18	9.47
	Linoleic acid	C18: 2ω6c	6	3.79	
	α-Linolenic acid (ALA)	C18: 3ω3c	4	0.60	
	Eicosadienoic acid	C20: 2ω6c	5	1.10	
	Arachidonic acid	C20: 4ω6c	3	0.37	
	Eicosapentaenoic acid (EPA)	C20: 5ω3c	3	0.68	
	Docosadienoic acid	C22: 2ω6c	4	2.15	
	Docosapentaenoic acid (DPA)	C22: 5ω3c	1	0.12	
	Docosahexaenoic acid (DHA)	C22: 6ω3c	3	0.48	
Unidentified			2	0.10	9.48
			1	0.15	
			1	0.02	
			4	0.68	
			6	1.77	
			4	0.73	
			6	1.93	
			6	1.30	
			6	2.80	
Total number of different fatty acids detected (excluding unidentified):			31		
Total number of different fatty acids detected (including unidentified):			40		

FA = fatty acid; n = number of samples containing the fatty acid; ω = omega; c = *cis* fatty acid; t = *trans* fatty acid

4.3.2 FAME composition of 5-year old rhizospheres

Similarly to the 3-month old rhizospheric soil samples, the FAME profiles of the 5-year old rhizospheric soil samples also demonstrated all classes of fatty acids, although fewer fatty acids were identified in the 5-year old samples than in the 3-month old samples. When considering the composition of fatty acids, 33.3% were identified saturated fatty acids, 38.9% identified unsaturated fatty acids and 27.8% unknown fatty acids. The unknown fatty acids in these soil samples were slightly more than those of the 3-month old soil samples. In the FAME profiles, 26 different fatty acids were detected of which 46.2% were saturated fatty acids, 23.1% mono-unsaturated fatty acids and 30.8% poly-unsaturated fatty acids, representing a ratio of approximately 4 : 2 : 3 of saturated fatty acids : monounsaturated fatty acids : poly-unsaturated fatty acids (Table 4.2). Similarly to the 3-month old rhizospheric soil samples, approximately one third of the fatty acids (including unknown fatty acids) occurred on all six samples. Within the saturated fatty acid class, 25% of the fatty acids occurred in all six samples, whereas 50% of the mono-unsaturated fatty acids and 25% of the poly-unsaturated fatty acids occurred in all six samples. Similarly to the 3-month old rhizospheric soil samples, the same saturated fatty acids were the most prevalent; although palmitic fatty acid was the most abundant, followed by stearic fatty acid, while oleic fatty acid was the most abundant in the mono-unsaturated fatty acid class. When considering the abundance of saturated, mono-unsaturated and poly-unsaturated fatty acids; excluding the unidentified fatty acids, they occurred in an approximate ratio of 7 : 3 : 2.

Table 4.2 FAME profile of 5-year old rhizospheres

Fatty acid class	Fatty acid name	Symbolic name of fatty acids	*n*	Mean area % composition	Total mean area % composition
Saturated FA	Caproic acid	C6: 0	1	0.07	51.12
	Caprylic acid	C8: 0	1	0.08	
	Capric acid	C10: 0	2	0.22	
	Lauric acid	C12: 0	3	0.53	
	Tridecanoic acid	C13: 0	3	0.87	
	Myristic acid	C14: 0	6	2.35	
	Pentadecanoic acid	C15: 0	4	0.70	
	Palmitic acid	C16: 0	6	25.27	
	Stearic acid	C18: 0	6	15.25	
	Arachidic acid	C20: 0	4	1.92	
	Behenic acid	C22: 0	3	1.75	
	Lignoceric acid	C24: 0	4	2.12	
Mono-unsaturated FA	Myristoleic acid	C14: 1ω5c	2	0.10	21.25
	Pentadecenoic acid	C15: 1ω10c	1	0.07	
	Palmitoleic acid	C16: 1ω7c	6	1.97	
	Elaidic acid	C18: 1ω9t	6	3.90	
	Oleic acid	C18: 1ω9c	6	14.63	
	Erucic acid	C22: 1ω9c	1	0.58	
Poly-unsaturated FA	Linoleicacid	C18: 2ω6c	6	8.1	14.55
	γ-Linolenic (GLA)	C18: 3ω6c	1	0.58	
	α-Linolenic acid (ALA)	C18: 3ωc3	6	2.57	
	Eicosadienoic acid	C20: 2ω6c	5	2.07	
	Arachidonic acid	C20: 4ω6c	1	0.43	
	Eicosapentaenoic acid (EPA)	C20: 5ω3c	1	0.35	
	Docosadienoic acid	C22: 2ω6c	1	0.33	
	Docosapentaenoic acid (DPA)	C22: 5ω3c	1	0.12	11.13
Unidentified			3	1.22	
			2	1.1	
			2	0.63	
			2	0.27	
			5	1.87	
			6	1.85	
			2	0.28	
			6	2.32	
			3	1.17	
			1	0.42	
Total number of different fatty acids detected (excluding unidentified):			26		
Total number of different fatty acids detected (including unidentified):			36		

FA = fatty acid; n = number of samples containing the fatty acid; ω = omega; c = *cis* fatty acid; t = *trans* fatty acid

4.3.3 Comparison of 3-month old and 5-year old rhizospheres

Unique fatty acids

Besides the 25 fatty acids that occurred in both rhizospheric soil sample age groups, a number of unique fatty acids occurred in the 3-month old and 5-year old rhizospheric soil sample age groups. The number of unique fatty acids that were detected in the 3-month old rhizospheric soil samples was greater than in the 5-year old rhizospheric soil samples, suggesting a greater fatty acid diversity. In the 3-month old rhizospheric soil samples, six unique fatty acids were detected as compared to one unique fatty acid in the 5-year old rhizospheric soil samples. The unique fatty acids in the 3-month old rhizospheric soil samples were two saturated, two mono-unsaturated and two poly-unsaturated fatty acids. The 5-year old rhizospheric soil samples contained one unique poly-unsaturated fatty acid (Table 4.3).

Table 4.3 Unique fatty acids present in the 3-month and 5-year old rhizospheric soil sample groups

3-month old rhizospheric soil sample group		5-year old rhizospheric soil sample group	
Fatty acid	**Fatty acid class**	**Fatty acid**	**Fatty acid class**
Heptadecanoic acid	S	γ-Linolenic acid	PU
Tricosanoic acid	S		
Heptadecenoic acid	MU		
Nervonic acid	MU		
Linolelaidic acid	PU		
Decosahexaenoic acid	PU		
Number of unique fatty acids	6		1

S = saturated fatty acid; MU = mono-unsaturated fatty acid; PU = poly-unsaturated fatty acid

Diversity analysis of fatty acids

The 3-month old rhizospheric soil samples were more diverse in terms of the number of fatty acids as revealed by the greater value of the richness index (Table 4.4). In terms of the relative proportions of fatty acids, the Shannon index also supported this notion. This is also the case with the Simpson's index that revealed that the 3-month old rhizospheric soil samples were more diverse than the 5-year old rhizospheric soil samples when more weight was given to the common fatty acids.

Table 4.4 Fatty acid diversity indexes of the 3-month and 5-year old rhizospheric soils

Soil type	Richness	Shannon's index	Simpson's index
3-month old	40	-176.2	0.000934
5-year old	36	-195.367	0.000821

Comparison of fatty acid diversity between the rhizospheric soil sample age groups

In an attempt to compare the effect of plant age on rhizospheric soil, FAME profiles of the rhizospheric soil samples of the 3-month and 5-year old soil samples were subjected to a number of statistical procedures. Firstly, the original data set was scrutinized and the following concluded:

- In some incidences only one or two measurements of a particular fatty acid out of a possible six occurred in a rhizospheric soil sample age group; and
- Certain of the fatty acids were not present in both the rhizospheric soil sample age groups.

The data were subsequently cleaned to contain only fatty acids that occurred in both rhizospheric soil sample age groups with four or more measurements out of a possible six. This resulted in the reduction of the overall 32 fatty acids to 14 that could be compared between the two rhizospheric soil sample age groups (Table 4.5).

Table 4.5 Original group of fatty acids identified in the rhizospheric soil samples age groups and the resulting fatty acids after fatty acid reduction

Fatty acids identified in the rhizospheric soil samples	Fatty acid present in rhizospheric soil sample group	Fatty acid after data clean-up
Caproic acid	Both	Pentadecanoic acid
Caprylic acid	Both	α-Linolenic acid (ALA)
Capric acid	Both	Eicosadienoic acid
Lauric acid	Both	Lauric acid
Tridecanoic acid	Both	Behenic acid
Myristic acid	Both	Arachidic acid
Pentadecanoic acid	Both	Lignoceric acid
Palmitic acid	Both	Myristic acid
Heptadecanoic acid	3-M	Palmitic acid
Stearic acid	Both	Palmiloleic acid
Arachidic acid	Both	Stearic acid
Behenic acid	Both	Elaidic acid
Tricosanoic acid	3-M	Oleic acid
Lignoceric acid	Both	Linoleic acid
Myristoleic acid	Both	
Pentadecenoic acid	Both	
Palmitoleic acid	Both	
Heptadecenoic acid	3-M	
Elaidic acid	Both	
Oleic acid	Both	
Erucic acid	Both	
Nervonic acid	3-M	
Linolelaidic acid	3-M	
Linoleic acid	Both	
α-Linolenic acid (ALA)	Both	
Eicosadienoic acid	Both	
Arachidonic acid	Both	
Eicosapentaenoic acid (EPA)	Both	
Docosadienoic acid	Both	
Docosapentaenoic acid (DPA)	Both	
Docosahexaenoic acid (DHA)	3-M	
γ-Linolenic acid (GLA)	5-Y	

Both = present in 3-month old and 5-year old rhizospheric soil samples; 3-M = present only in 3-month old rhizospheric soil samples; 5-Y = present only in 5-year old rhizospheric soil samples.

A one-way multivariate analysis of variance (MANOVA) was conducted to determine the effect of the two rhizospheric soil sample age groups on 14 dependent variables (fatty acids) using the Wilks' lambda test. After transforming the Wilks' statistic to approximate an *F* distribution, no difference (*P* = 0.159) could be identified at an α = 0.05 between the means of the rhizospheric soil sample age groups.

ANOVA tests were performed between the means of each of the 14 fatty acids individually in the two rhizospheric soil sample age groups. Six of these ANOVA tests demonstrated significant differences at α = 0.05, which were included in further analyses (Table 4.6). Although the two ubiquitous fatty acids, palmitic acid and stearic acid, demonstrated the highest mean area percentage composition in the two rhizospheric soil sample age groups, the differences between the two rhizospheric soil sample age groups for these fatty acids were highly significant.

Table 4.6 ANOVA tests of the means of individual fatty acids in the two rhizospheric soil sample age groups

Fatty acid	Symbolic name of fatty acid	*P*-value
Pentadecanoic acid	C15: 0	0.079
α-Linolenic acid (ALA)	C18: 3ω3c	**0.031**
Eicosadienoic acid	C20: 2ω6c	**0.011**
Lauric acid	C12: 0	0.156
Behenic acid	C22: 0	0.434
Arachidic acid	C20: 0	0.817
Lignoceric acid	C24: 0	0.158
Myristic acid	C14: 0	0.514
Palmitic acid	C16: 0	**0.0001**
Palmitoleic acid	C16: 1ω7c	0.646
Stearic acid	C18: 0	**0.0042**
Elaidic acid	C18: 1ω9t	**0.0217**
Oleic acid	C18: 1ω9c	0.2694
Linoleic acid	C18: 2ω6c	**0.0004**

Bold = significant differences at α = 0.05; ω = omega; c = *cis* fatty acid; t = *trans* fatty acid

A visual perspective of the mean area percentage compositions of the six significantly different fatty acids indicated that some fatty acids in the two rhizospheric soil sample age groups showed marked differences (Figure 4.1). When the two rhizospheric soil sample age groups were compared, two of the fatty acids, palmitic acid and stearic acid, revealed opposite mean area percentage compositions. Whereas, the mean area percentage compositions of the remaining four fatty acids occurred in lesser amounts in the 3-month old rhizospheric soil sample age group when compared to the 5-year old rhizospheric soil sample age group.

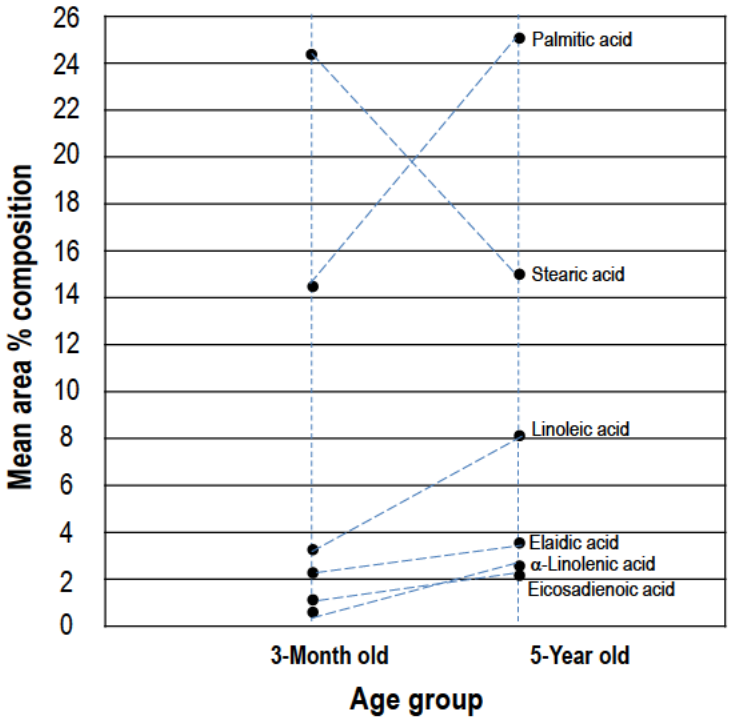

Figure 4.1 Mean area percentage composition of the six significantly different fatty acids indicating differences between the rhizospheric soil sample age groups

Multivariate analyses were carried out on the six significant fatty acids with the aim of reducing the number of variables to one or two linear combinations, without much loss of inherent variance. Therefore, a principal component and factor analyses were conducted to ascertain if there was

separation between the fatty acid composition of the 3-month old and 5-year old rhizospheric soil sample age groups.

In the factor analysis the eigenvalues were determined to ascertain how much of the total variance each of the six significant fatty acids (factors) explained. These calculations were based upon a principal component extraction method for the extraction of unrotated factors. Two of the factors explained together 93% of the total variance, of which factor 1 (α-linolenic acid) explained more than 80% of the total variance. Factor 2 (eicosadienoic acid) explained 10.9% of the total variance. The remaining four factors explained little of the total variance; only 6.98% (Table 4.7). Thus, the remainder of the factor analysis was based upon the two factors explaining most of the variance.

Table 4.7 Eigenvalues of the correlation matrix and total and cumulative variances

Fatty acid variable	Eigenvalue	% Total variance	Cumulative Eigenvalue	% Cumulative variance
α-Linolenic acid (ALA)	4.927	82.12	4.93	82.12
Eicosadienoic acid	0.654	10.90	5.58	93.02
Palmitic acid	0.232	3.86	5.81	96.88
Stearic acid	0.109	1.82	5.92	98.70
Elaidic acid	0.071	1.18	5.99	99.88
Linoleic acid	0.006	0.11	6.00	100.00

A scree plot was constructed to visualize the amount of variance that each fatty acid, or factor, contributed to the total variance. The scree plot shows the eigenvalues of the correlation matrix in descending order of magnitude (Figure 4.2). The scree plot clearly indicates that factor 1 was responsible for most of the total variance.

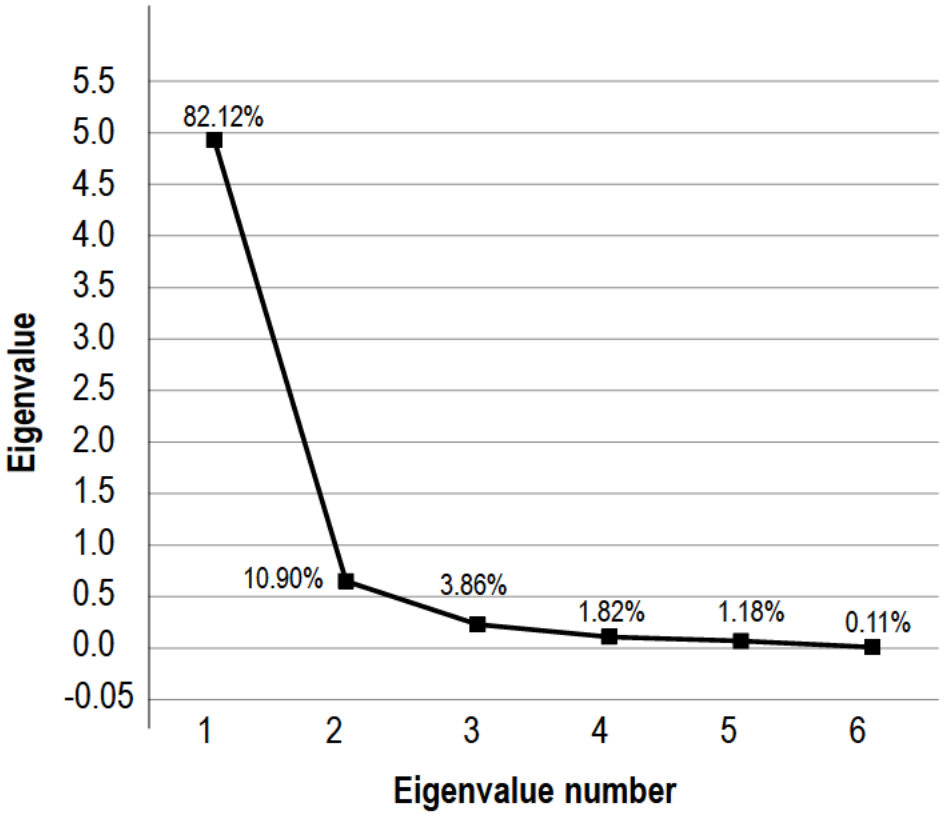

Figure 4.2 **Scree plot showing how much of the total variance each fatty acid (factor) explained**

The factor loadings of the two main factors (α-linolenic acid (ALA) and eicosadienoic acid) were then determined. These factor loadings are the correlations between the factors and the six significant fatty acids (variables). Factor 1 showed a strong positive correlation with stearic acid and strong negative correlations with the other fatty acids (Table 4.8). In contrast, factor 2 showed a weak positive correlation with α-linolenic (-0.253) and elaidic acid (0.200), and a negative correlation with the remainder of the fatty acids, of which the strongest negative correlation was with palmitic acid (0.704).

Table 4.8 Factor coordinates of the variables based on correlations

Fatty acid variable	Factor 1	Factor 2
α-Linolenic acid (ALA)	-0.929	0.253
Eicosadienoic acid	-0.946	0.143
Palmitic acid	-0.699	-0.704
Stearic acid	0.953	-0.092
Elaidic acid	-0.932	0.200
Linoleic acid	-0.951	-0.159

The factor scores were calculated for each soil sample for the two main factors. Nine of the twelve soil samples contained all six fatty acids used in the factor analysis and were included in the factor score estimation. A factor score is an estimated value that a given sample would have scored taking the factor loadings into account. Table 4.9 presents the factor scores of the two main factors for the nine rhizospheric soil samples.

Table 4.9 Factor scores of the two main factors for the nine rhizospheric soil samples

Soil sample	Rhizospheric soil sample age group	Factor scores	
		1	2
1	Tree 3M	0.509	0.605
3	Tree 3M	2.805	0.331
4	Tree 3M	3.031	-0.225
6	Tree 3M	1.771	0.483
8	Tree 5Y	-3.127	0.938
9	Tree 5Y	-2.587	0.164
10	Tree 5Y	-0.236	-1.113
11	Tree 5Y	-1.305	-1.494
12	Tree 5Y	-0.863	0.311

3M = 3-month old rhizospheric soil sample; 5Y = 5-year old rhizospheric soil sample

A plot of the factor scores clearly demonstrates the difference between the rhizospheric soil sample age groups. For factor 1, the 3-month old rhizospheric soil samples were all grouped in the positive quadrants, while the 5-year old rhizospheric soil samples were all grouped in the negative quadrants. In contrast for factor 2, the factor scores of the rhizospheric soil sample age groups were more randomly displayed, which is expected as this factor only explains 10.9% of the total variance (Figure 4.3).

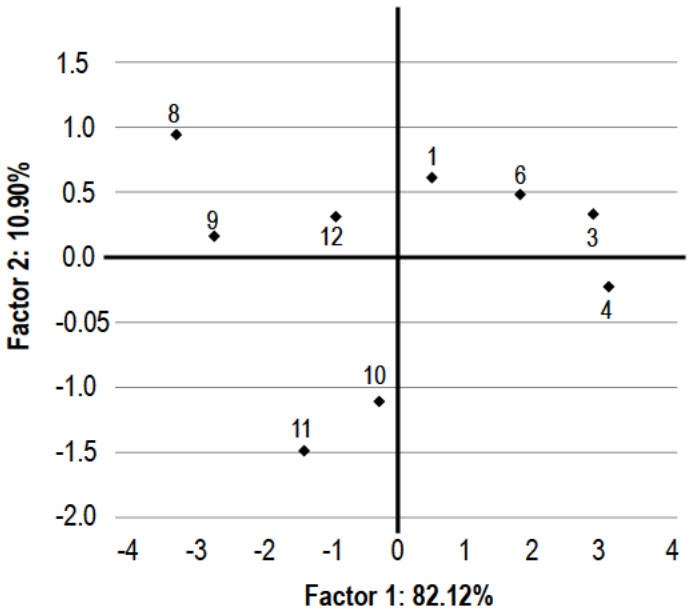

Figure 4.3 **Plot of the factor scores for nine rhizospheric soil samples. Numbers 1 to 6 refer to 3-month old soil samples and numbers 8 to 12 to 5-year old soil samples**

4.4 Discussion

The FAME profile analyses and diversity indexes showed that the rhizospheric microbial communities of the 3-month old rhizospheric soil samples were more diverse that those of the 5-year old rhizospheric soil samples. This outcome confirms that the diversity of rhizospheric microbial communities becomes less as plants age (Di Cello et al., 1997). This change in composition of the

rhizospheric microbial communities can mainly be attributed to the changes in exudation patterns of roots of the aging plants, as a consequence, more adapted rhizospheric microorganisms are favoured (Di Cello et al., 1997).

The proportions of saturated and unsaturated (mono- and poly-unsaturated) fatty acids, in particular, have been proposed as important indicators when profiling soil microbial communities (Larkin, 2003). Straight chain saturated fatty acids represented the greatest proportion of fatty acids in both rhizospheric soil age groups, which is not surprising as saturated fatty acids form the greatest proportion in soil (Larkin, 2003). When the rhizospheric soil age groups were compared, the proportions of the saturated fatty acids relative to the proportions of the unsaturated fatty acids were different. The proportion of saturated fatty acids in the 3-month old rhizospheric soil samples was greater than the proportions in the 5-year old rhizospheric soil samples, while the proportions of the unsaturated fatty acids was less in the 3-month old than the 5-year old rhizospheric soil samples. The reduced proportions of saturated fatty acids in the 5-year old rhizospheric soil samples can be associated with increasing anaerobic conditions in the rhizospheric soil as the plant ages, because saturated fatty acids have been associated with aerobic bacteria (Quezada et al., 2016). Furthermore, the greater proportions of unsaturated fatty acids in the 5-year old rhizospheric soil samples also represent an increase in the presence of anaerobic bacteria. The monounsaturated fatty acids are mainly associated with gram negative bacteria, but some are associated with both bacteria and fungi (Larkin, 2003; Kaur et al., 2005). The polyunsaturated fatty acids, on the other hand, are primarily associated with fungi (Larkin, 2003).

As expected, the most common saturated fatty acid in animals, plants and microorganisms, palmitic acid (16:0), occurred in the greatest concentration in the 3-month old soil samples and second most in the 5-year old soil samples (Rustan & Drevon, 2005). Stearic (18:0), also a common saturated fatty acid, occurred in high concentrations in both rhizospheric soil sample age groups. The

presence of the greater proportion of polyunsaturated fatty acids in the 5-year old rhizospheric soil samples, especially linoleic (C18:2ω6c), α-linolenic (C18:3ω3c) and γ-linolenic (C18:3ω3c) fatty acids, suggests growing establishment of fungi in the older rhizospheric soils. The presence of the unique polyunsaturated fatty acid, γ-linolenic, further supports this notion.

Similarly, the poly-unsaturated fatty acids, especially linoleic (C18:2ω6c), α-linolenic (C18:3ω3c) and γ-linolenic (C18:3ω3c) fatty acids, which are associated primarily with fungi (Larkin, 2003), were more in abundance in the 5-year old rhizospheric soil samples than in the 3-month old rhizospheric soil samples. The γ-linolenic acid is unique in the 5-year old rhizospheric soil samples. Therefore, it could be concluded that the increase in abundance, of especially fungi in the 5-year old rhizospheric soil samples, could be attributed to an aging effect.

4.5 Conclusions

The FAME analyses revealed that as plants age, the rhizospheric microbial communities' composition change. These changes are influenced by numerous plant-related and environmentally-related factors. Knowledge of changes in these microbial communities may provide practical ways to manipulate the soil microbial environment in commercial forestry and possibly improve the rooting when establishing clonal forests.

Chapter 5

Isolation, identification and screening of rhizospheric bacteria for rooting promoting properties

Abstract

Plant growth promoting rhizospheric bacteria are known to enhance plant growth and stimulate adventitious rooting of cuttings. The aim of this study was to isolate and identify rhizospheric bacteria, which are able to produce indole-3-acetic acid and solubilise phosphate. Rhizospheric soil samples were collected from *Eucalyptus grandis* × *E. urophylla* hybrid. Thirty-two bacterial strains were isolated from the soil using the potato dextrose and R-2A agar. The 16S rRNA gene of the 32 bacterial isolates were amplified and sequenced. The bacterial strains were identified through sequence homology searches using the BLAST algorithm. A total of 12 unique bacterial species were identified in this study belonging to eight genera. Two of the 12 species, *Aeromicrobium ginsengisoli* and *Curtobacterium oceanosedimentum*, tested negative for both indole-3-acetic acid production and phosphate solubilisation. The remaining 10 species tested positive for indole-3-acetic acid production, of which seven were able to also solubilise phosphate. These seven species, *Bacillus aryhabattai*, *Brevibacterium frigoritolerans*, *Burkholderia phytofirmans, Chryseobacterium rhizospharae*, as well as three *Pseudomonas* species were selected for the production of rhizospheric inoculums for the field trials. The three *Pseudomonas* species were *P. fluorescens*, *P. koreensis* and *P. putida.* The species *Burkholderia phytofirmans* is also known for its ability to solubilise zinc into a form that plants can absorb, thereby improving plant health and growth.

Keywords: Indole-3-acetic acid, phosphate solubilisation, rhizospheric bacteria, 16S rRNA gene, *Bacillus*, *Pseudomonas*

5.1 Introduction

Plant growth promoting rhizobacteria (PGPR) are a varied group of rhizospheric bacteria that reside in rhizospheres, and at root surfaces of plants (Ahmad et al., 2008). PGPR are in symbiotic association with their host plants, and feed on sloughed-off cells and root exudates, while in return improve the quality of plant growth either directly and or indirectly (Ahmad et al., 2008). PGPR contribute directly to plant growth either through providing the plant with plant growth promoting substances or by facilitating the uptake of certain plant nutrients from the soil. PGPR may also promote plant growth indirectly by mitigating or preventing the damaging effect of one or more phytopathogenic microorganisms. A number of non-pathogenous PGPR belonging to the genera *Agrobacterium*, *Alcaligens*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Brassica*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas, Serratia* and *Streptomyces* have been reported to enhance plant growth either directly or indirectly (Teixeira et al., 2007; Hye et al., 2014; Ma et al., 2016).

The exact mechanisms by which PGPR promote plant growth are not fully understood. However, studies have shown that a number of PGPR traits are involved in the promotion of plant growth. One of the more important traits is their ability to produce or change the concentrations of plant growth regulators, such as for auxin, gibberellic acid, cytokinins and ethylene (Arshad & Frankenberger, 1993; Glick, 1995). Another important trait is their ability to solubilise mineral phosphate and other nutrients (Gaur, 1990; de Freitas et al., 1997).

Auxins are a group of tryptophan-derived plant growth regulators, which are involved in many aspects of plant development (Woodward & Bartel, 2005). Auxins control early plant development, particularly the early stages of embryogenesis. These regulators also play a role in the organisation of apical meristem (phyllotaxy), the branching of shoots (apical dominance), formation of the main root, as well as the initiation of lateral and adventitious roots (Went & Thimann, 1937). These regulators control plant development by influencing cell division or cell growth directly. They also

affect a plant indirectly through their interactions with other hormones or signalling molecules (Gehlot et al., 2014).

Plants produce different types of endogenous auxins. However, indole-3-acetic acid (IAA) is the most commonly produced auxin in nature (Teixeira et al., 2007; Egamberdieva, 2011). Endogenous IAA is synthesized in the stem and tender leaves of a plant. It is then transported from the areas of synthesis to an action site where it exerts its influence. Similarly, PGPR in the rhizosphere also synthesizes IAA as exogenous auxin (Ljung et al., 2001; Teixeira et al., 2007). It is known that PGPR-produced exogenous auxin also enhances IAA production in the host plant (Teixeira et al., 2007). A high endogenous IAA concentration is normally associated with a high rooting rate at the beginning of the rooting process (Caboni et al., 1996; Blazkova et al., 1997). IAA thus stimulates lateral and adventitious root development, thereby increasing the nutrient absorbing surfaces, which results in better assimilation of water and nutrients from the soil (Egamberdieva, 2011).

The ability of some PGPRs to solubilise phosphate allows these regulators to promote plant growth. Phosphate solubilising rhizospheric bacteria increase available phosphorus for a plant, especially in soils with large amounts of precipitated phosphate (Pikovskaya, 1948; Gaur, 1990; de Freitas et al., 1997; Ma et al., 2009). These rhizospheric bacteria release bound phosphate by secreting a number of organic acids (de Freitas et al., 1997; Ma et al., 2009).

The role that PGPRs plays in the natural environment, particularly their ability to stimulate root growth and facilitate nutrient absorption, is of great value to the commercial forestry industry. It has been shown that exogenous IAA that is synthesised by PGPRs in the rhizosphere complements the endogenous IAA. This results in a peak IAA concentration that initiates the rooting process in a cutting (Gaspar et al., 1996; Gatineau et al., 1997). Through the application of PGPRs to stimulate the rooting of *Eucalyptus* cuttings, substantial savings could be brought about for commercial

forestry nursery growers. However, not all rhizospheric bacteria act as PGPRs, therefore to exploit the attributes of rhizospheric bacteria, specific strains need to be isolated and screened for their potential rooting promoting abilities. Therefore, the aim of this part of the study was to isolate and identify rhizospheric bacteria, which were then screened for their ability to produce IAA and solubilise phosphates.

5.2 Materials and methods

5.2.1 Collection of rhizospheric soil

Rhizospheric soil samples were collected from the *Eucalyptus grandis* × *Eucalyptus urophylla* clone, GU III, at Sunshine Seedlings, Pietermaritzburg, KwaZulu-Natal. The collection of soil samples from GU hedges was because GU clones root far better than GN clones. The soil samples were collected from the soil surrounding the roots using a teaspoon and placed in a sterile plastic bag. Approximately 60 ml (4 scoops) of soil were collected from each plant. These soil samples were kept on ice while in transport. In the laboratory, the soil samples were transferred to sterile falcon tubes and kept in a freezer at -80°C until used.

5.2.2 Isolation of rhizospheric bacteria

Rhizospheric bacterial isolates were prepared from the soil samples collected from the rhizospheres of the *Eucalyptus* hybrid clone. An initial stock solution was prepared for each soil sample by adding 3.5 g of soil to 100 ml of sterile distilled water in a conical flask. Each initial stock solution was then placed on a shaker at 120 rpm for 20 minutes at room temperature. Thereafter, serial saline dilutions of 10^{-1}, 10^{-2} and 10^{-3} were prepared for each initial stock solution. From each of these dilutions, 100 µl aliquots were plated onto two different agar preparations, namely, potato dextrose agar (PDA; Difco, Detroit, MI) and R-2A (Difco) agar. Thereafter the plates were incubated at 25°C for 48 h. After incubation, representatives of bacterial colonies presenting different morphological variations

(colour, shape and size) were isolated and transferred to freshly prepared agar plates and incubated for a further 48 h at 25°C. Cells from each isolate were then cryo-preserved at -80°C in the Microbank™ (Pro-Lab Diagnostics) according to the manufacturer's instructions. Thirty-two bacterial isolates were preserved in the Microbank™.

5.2.3 DNA Extraction

Each of the 32 cryo-preserved bacterial isolates was prepared for DNA extraction. The bacterial isolates were first thawed and then plated on freshly prepared PDA, after which they were incubated at 25°C for 24 h. After incubation, the colonies were prepared for whole cell amplification of DNA.

DNA (template) for whole cell amplifications was prepared by suspending a touch volume (yellow tip) of cells from the preserved bacterial isolates in 20 µl nanopure water. The suspended cells were incubated at 95°C for 10 min and then cooled on ice for 5 min. The cell suspensions were centrifuged, after which 10 µl of each cell suspension were added as template to a PCR tube containing the components for the polymerase chain reaction (PCR).

In cases where whole cell PCR were unsuccessful, genomic DNA (gDNA) was extracted from cells using the harsh lysis extraction method (Labuschagne & Albertyn, 2007). This lysis method involves the addition of 500 µl lysis solution (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 1% SDS) and 200 µl glass beads (425-600 µm diameter) to 0.4 g wet cells. This was followed by vortexing the preparation for 4 min, cooling on ice and the addition of 275 µl ammonium acetate (7 M, pH 7.0). The preparation was then incubated at 65ºC for 5 min followed by cooling on ice for 5 min. Thereafter, 500 µl of chloroform was added, vortexed and centrifuged (17,000 × *g*; 2 min at 4ºC). The supernatant was then transferred and the DNA precipitated in 1 volume isopropanol and centrifuged (17,000 × *g*; 5 min at 4ºC). The pellet was washed with 70% (v/v) ethanol, dried and resuspended in 100 µl TE (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

5.2.4 16S rDNA amplification

After gDNA was extracted from the different bacterial isolates, a ≈1,300 base pair (bp) fragment of the 16S rRNA gene was amplified. 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') was used to amplify the 16S rRNA gene (Marchesi et al., 1998). Amplification was performed using the C1000™Thermal Cycler (Bio-Rad) with either whole cell preparation or 1 µl of gDNA as template. Amplification reactions were performed in 25 µl containing 1× ThermoPol® Reaction buffer [20 mM Tris-HCl; 10 mM $(NH_4)_2SO_4$; 10 mM KCl; 2 mM $MgSO_4$; 0.1% Triton®X-100, pH 8.8], 0.2 mM dNTPs, 0.5 µM of each primer, 0.1 µg/ml BSA, 1 U of *Taq* DNA Polymerase (New England BioLabs). The following PCR conditions were applied; initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 68°C for 1 min and 30 sec. A final elongation step was incorporated at 68°C for 5 min. Successful amplification was confirmed by electrophoresis on a 1% agarose gel stained with 0.05% Gold View (Guangzhou Geneshun Biotech). The appropriate bands (≈1,300 bp) were excised from the gel, the DNA purified using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) and used as template for sequencing.

5.2.5 Sanger sequencing

Sequencing was performed in single reactions using primers 63-F, 1387-R, as well as internal primers 533-F (5'- GTG CCA GCM GCC GCG GTA A-3') and 805-R (5'- GAC TAC CAG GGT ATC TAA TC-3') on the ABI Prism 3130 XL genetic analyser using the BigDye® Terminator V3.1 Cycle Sequencing Kit (Life Technologies). DNA was prepared for sequencing by precipitation with EDTA and ethanol. Sequences were assembled using DNA Baser sequence assembly software and the contig construct, representative of the partial 16S rRNA gene sequence of each bacterial isolate, was used to determine sequence homology. Sequence homology searches were undertaken by

comparing each of the contig constructs (query sequences) against nucleotide sequence databases (Nucleotide collection/16S ribosomal RNA sequences). The BLAST server of the National Centre for Biotechnology Information (NCBI) was used for the respective sequence homology searches using the BLAST algorithm (megablast). Only similarities with a BLAST index of 90% and above were considered for identification. Successful searches provided an NCBI accession number, the number of base pairs of the amplified gene, as well as the taxonomic classification of the bacterial strain to species level.

5.2.6 Screening for IAA production

The respective bacterial isolates were also screened for their ability to produce IAA. A qualitative approach was applied to determine if a bacterial isolate possessed this attribute. The bacterial strains were first revived from the cryo-preservation by plating on potato dextrose agar (PDA) and incubated for 24 hours at 25°C. Thereafter, the bacteria strains were spot plated onto fresh PDA and incubated for 48 hours at 25°C. After incubation, 50 ml of Salkowaski reagent were prepared, containing 0.5 M $FeCl_3$ in 35% $HClO_4$ in a proportion of 1:5 (v/v). One millilitre of the Salkowaski reagent was poured in a thin layer over the bacterial growth in a Petri dish. Bacterial strains with the ability to produce IAA reacted with the Salkowaski reagent and showed a characteristic pink colouration.

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5.2.7 Screening for phosphate solubilisation

The respective bacterial isolates were also screened for their ability to solubilise phosphate. Similarly to the test for IAA production, this test was also a qualitative test. The medium for this test was prepared by adding 5 g tribasic phosphate ($Ca_5HO_{13}P_3$), 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl_2, 0.1 g $MgSO_4.7H_2O$, 0.5 g yeast extract and 15 g PDA to 1,000 ml distilled water. The mixture was then

autoclaved, left in a water bath for 25 min at 45°C. This mixture was then poured into Petri dishes to solidify. Thereafter, individual bacterial strains were plated onto the prepared agar plates and incubated at 25°C for seven days. A clear halo formed around a colony if the bacterial strain was able to solubilise phosphates.

5.3 Results

5.3.1 Rhizobacterial 16S rRNA gene sequences

The 16S rRNA gene sequences (query sequences) of the 32 bacterial strains were submitted to a BLAST search to compare their DNA homologies to those of known (subject) sequences. The BLAST search returned unique identities (hits) for all 32 query sequences. The BLAST algorithm also calculated similarity scores for local alignment between the 32 query sequences and the subject sequences. The Expect value, E, was zero for all the hits indicating that all matches were significant and not due to chance. Furthermore, most of the maximum percentage identify scores of the nucleotide-to-nucleotide alignments were close to 100%. Table 5.1 shows the results of the BLAST search. Eight different bacterial genera were identified with *Pseudomonas* being the most prevalent, containing four different species. *Pseudomonas* was followed by *Bacillus* with two different species. The remaining six genera, *Aeromicrobium*, *Arthrobacter*, *Brevibacterium*, *Burkholderia*, *Chryseobacterium* and *Curtobacterium*, were each represented by a single species. A total of 12 unique species were thus identified in this study.

Table 5.1 BLAST search results of the 32 query sequences

Number	Caption isolate identity	Description	NCBI accession number	Number of base pairs	Max. % identity
1	R12	*Brevibacterium frigoritolerans* strain DSM 8801 16S ribosomal RNA gene, partial sequence	117474.1	1062	99
2	R13	*Brevibacterium frigoritolerans* strain DSM 8801 16S ribosomal RNA gene, partial sequence	117474.1	1068	100
3	R15	*Aeromicrobium ginsengisoli* strain Gsoil 098 16S ribosomal RNA gene, partial sequence	041384.1	1033	99
4	R1	*Arthrobacter oryzae* strain KV-651 16S ribosomal RNA gene, partial sequence	041545.1	1045	98
5	R2	*Arthrobacter oryzae* strain KV-651 16S ribosomal RNA gene, partial sequence	041545.1	1035	98
6	R47	*Arthrobacter oryzae* strain KV-651 16S ribosomal RNA gene, partial sequence	041545.1	1157	98
7	R23	*Bacillus acidiceler* strain CBD 119 16S ribosomal RNA gene, partial sequence	043774.1	1087	91
8	R25	*Bacillus acidiceler* strain CBD 119 16S ribosomal RNA gene, partial sequence	043774.1	1245	99
9	R65	*Bacillus aryabhattai* strain B8W22 16S ribosomal RNA gene, partial sequence	115953.1	1301	100
10	P10	*Burkholderia phytofirmans* strain PsJN 16S ribosomal RNA gene, partial sequence	042931.1	1261	99
11	P11	*Burkholderia phytofirmans* strain PsJN 16S ribosomal RNA gene, partial sequence	042931.1	1117	99
12	P12	*Burkholderia phytofirmans* strain PsJN 16S ribosomal RNA gene, partial sequence	042931.1	1259	99
13	P16	*Burkholderia phytofirmans* strain PsJN 16S ribosomal RNA gene, partial sequence	042931.1	911	99
14	R9	*Chryseobacterium rhizosphaerae* strain RSB3-1 16Srhibosomal RNA gene, partial sequence	125812.1	1147	99
15	R17	*Chryseobacterium rhizosphaerae* strain RSB3-1 16S ribosomal RNA gene, partial sequence	125812.1	1258	99
16	R44	*Chryseobacterium rhizosphaerae* strain RSB3-1 16S ribosomal RNA gene, partial sequence	125812.1	1122	99

Number	Caption isolate identity	Description	NCBI accession number	Number of base pairs	Max. % identity
17	R42	*Chryseobacterium rhizosphaerae* strain RSB3-1 16S ribosomal RNA gene, partial sequence	125812.1	1107	99
18	P13	*Curtobacterium oceanosedimentum* strain ATCC 31317 16S ribosomal RNA gene, partial sequence	104839.1	1107	99
19	R34	*Pseudomonas fluorescens* Pf0-1 strain Pf0-1 16S ribosomal RNA, complete sequence	102835.1	1172	99
20	R32	*Pseudomonas koreensis* strain Ps 9-14 16S ribosomal RNA gene, partial sequence	025228.1	1076	99
21	R35	*Pseudomonas koreensis* strain Ps 9-14 16S ribosomal RNA gene, partial sequence	025228.1	1137	99
22	R62	*Pseudomonas koreensis* strain Ps 9-14 16S ribosomal RNA gene, partial sequence	025228.1	1125	99
23	R63	*Pseudomonas koreensis* strain Ps 9-14 16S ribosomal RNA gene, partial sequence	025228.1	1101	99
24	R64	*Pseudomonas koreensis* strain Ps 9-14 16S ribosomal RNA gene, partial sequence	025228.1	1181	100
25	R39	*Pseudomonas graminis* strain DSM 11363 16S ribosomal RNA gene, complete sequence	026395.1	1007	98
26	R5	*Pseudomonas putida* F1 strain F1 16S ribosomal RNA, complete sequence	074739.1	1121	99
27	R6	*Pseudomonas putida* F1 strain F1 16S ribosomal RNA, complete sequence	074739.1	1049	99
28	R45	*Pseudomonas putida* F1 strain F1 16S ribosomal RNA, complete sequence	074739.1	971	99
29	R14	*Pseudomonas putida* KT2440 strain KT2440 16S ribosomal RNA, complete sequence	074596.1	1076	99
30	R46	*Pseudomonas putida* KT2440 strain KT2440 16S ribosomal RNA, complete sequence	074596.1	1200	100
31	R48	*Pseudomonas putida* KT2440 strain KT2440 16S ribosomal RNA, complete sequence	074596.1	1234	100
32	R67	*Pseudomonas putida* F1 strain F1 16S ribosomal RNA, complete sequence	074739.1	1180	99

5.3.2 Bacterial species screened for IAA production and phosphate solubilisation

The 12 unique bacterial species were screened for their ability to produce IAA and solubilise phosphate. All the bacteria species, except for *Aeromicrobium ginsengisoli* and *Curtobacterium oceanosedimentum* tested positive for IAA production (Table 5.2). Seven of the screened bacterial species demonstrated an ability to solubilise phosphate. All the *Pseudomonas* bacterial species, except for *P. graminis,* could solubilise phosphate. The other species that were able to solubilise phosphate were *Bacillus aryhabattai*, *Brevibacterium frigoritolerans*, *Burkholderia phytofirmans* and *Chryseobacterium rhizospharae*.

Table 5.2 IAA production and phosphate solubilisation abilities of the twelve unique bacterial species

Genus	Species	IAA production ability	Phosphate solubilisation ability
Aeromicrobium	*ginsengisoli*	-	-
Arthrobacter	*oryzae*	+	-
Bacillus	*acidiceler*	+	-
	aryabhattai	+	+
Brevibacterium	*frigoritolerans*	+	+
Burkholderia	*phytofirmans*	+	+
Chryseobacterium	*rhizosphaerae*	+	+
Curtobacterium	*oceanosedimentum*	-	-
	koreensis	+	+
Pseudomonas	*fluorescens*	+	+
	putida	+	+
	graminis	+	-

+ = Positive for IAA production or phosphate solubilisation, - = Negative for IAA production or phosphate solubilisation

5.4 Discussion

Several bacterial genera in this study demonstrated the two traits that are important in plant growth enhancement and for the stimulation of adventitious rooting of cuttings (Diaz et al., 2009). In this investigation seven of the 12 unique bacterial species that were identified were able to produce IAA and to solubilise phosphate. Species of two of these genera, *Bacillus* and *Pseudomonas*, are commonly used as biological stimulants to improve crop yield and to stimulate adventitious root formation in cuttings (Gonzalez-Lopez et al., 1986; Patena et al., 1988; Nieto & Frankenberger, 1989; Esitken et al., 2003; Ahmad et al., 2008). Species of these genera are of particular importance, because it has been shown that they are able to improve adventitious root formation and increase root biomass of *Eucalyptus* cuttings (Teixeira et al., 2007; Diaz et al., 2009). Besides the ability to produce IAA and to solubilise phosphate, species of the genus *Burkholderia,* are also able to solubilise zinc into a form so that plants can absorb this mineral (Ahmad et al., 2008; Armada et al., 2016; Gontia–Mishra et al., 2017). Therefore, the seven species that demonstrated the ability to produce IAA and to solubilise phosphate were selected for the production of rhizospheric inoculums for field trials.

Chapter 6

Rooting enhancement of rhizospheric bacteria on *Eucalyptus* hybrid cuttings

Abstract

The aim of this investigation was to assess the rooting capacity of cuttings taken from two *Eucalyptus* hybrid clones of *E. grandis* × *E. nitens*, known for their relatively low rooting ability, through the application of plant growth promoting bacteria. These hybrid clones were GN 018B (Experimental Trial 1) and GN 010 (Experimental Trial 2). Seven rhizospheric bacterial species that demonstrated the ability to produce indole-3-acetic acid and to solubilise phosphate were used to prepare two rhizospheric bacterial inoculums. These inoculums, together with a commercial biological rooting agent, were tested for their rooting stimulating capacity on cuttings of the two hybrids and compared to the nursery standard (control) indole-3-butyric acid. Each of the four treatments was applied to 32 cuttings in Unigrow trays. A total of ten trays were prepared for each experiment, making up a total of 320 cuttings treated with each treatment. The ten trays represent ten replications in this study. Both hybrid clones demonstrated significant ($P < 0.0001$) genotypic differences for all three growth responses, total length, root length and shoot length. Cuttings of both hybrids demonstrated relatively high survival rates and rooting percentage for all treatments. Although several rooting architectural configurations were prevalent in this study, there were indications that some of the treatments promoted adventitious root development and fibrosity, particularly in cuttings of the GN 018B clone. Both experimental trials showed highly significant differences amongst the treatments in both experimental trials ($P < 0.0001$).

6.1 Introduction

Clonal plantation forestry involves the rooting of cuttings from superior genotypes selected for their hybrid vigour and desired qualities. However, the cuttings of some *Eucalyptus* species and their hybrid genotypes present difficulties in their rooting capacity (Diaz et al., 2009). This results in a reduced number of rooted cuttings leading to financial losses for nursery growers. Some of the *Eucalyptus* species and the hybrids of these species that present poor rooting include *Eucalyptus nitens*, *C. citriodora*, *C. maculata*, *Eucalyptus paniculata*, *Eucalyptus cloeziana* and *Eucalyptus globulus* (Sasse & Sands, 1997; Luckman & Menary, 2002; de Assis et al., 2004; Martellet & Fett-Neto, 2005; Diaz et al., 2009; Peralta et al., 2012).

In the nursery, several methods have been applied to improve the rooting of cuttings of hybrid genotypes that demonstrate low rooting percentages. These methods include the use of plant growth regulators, different watering regimes and temperature control (Brondani et al., 2012). The application of plant growth promoting rhizospheric bacteria (PGPB) to the cutting growth medium as root stimulating agent, has not been extensively studied for forestry tree species (Teixeira et al., 2007). The root growth promoting effect of PGPB has been demonstrated in a number of forest species, such as *Pinus contorta*, *Pinus elata*, *Pinus elliotti*, *Pica glauca* and *Pseudotsuga menziessii* (Enebak et al., 1998). PGPB, such as *Agrobacterium*, *Bacillus* and *Pseudomonas*, have been shown to promote the root development and growth, as well as, root architecture in conifers and deciduous trees (*Larix*, *Picea Quercus*, *Taxus* and *Thuja*) that have been propagated vegetatively (Zaspel & Ewald, 2001). Studies performed in Brazil demonstrated that PGPB from the rhizosphere of *Eucalyptus* clones increased rooting and rooting biomass in cuttings of *E. grandis* (Teixeira et al., 2007; Diaz et al., 2009). PGPB isolated from the rhizosphere of *E. globulus* and *E. nitens* stimulated the development of adventitious roots and increased the root biomass of *E. globulus* cuttings (Diaz et al., 2009). However, extensive information about the role of PGPB in promoting rooting of cuttings

in *Eucalyptus* plantation forestry is limited. Thus, the enhancement of rooting percentages of valued *Eucalyptus* species and hybrid cuttings demands further investigation. In this context, the aim of this investigation was to assess the rooting capacity of two *Eucalyptus* hybrid clones (GN 018B and GN 010), known for their relatively low rooting ability, through the application of PGPB to the cutting growth medium.

6.2 Materials and methods: Microbiology

6.2.1 Introduction

Of the bacterial species that were isolated from the rhizosphere, seven demonstrated the ability to produce IAA and to solubilise phosphate (Chapter 5). These seven species were used to prepare inoculums that were tested in the nursery for their ability to enhance the rooting of *Eucalyptus* hybrid cuttings. Table 6.1 provides the genera and species names of the bacterial species that were used for the preparation of inoculums.

Table 6.1 Rhizospheric bacterial species used for the preparation of inoculums

Isolate number	Genus	Species
CSN R65	*Bacillus*	*aryabhattai*
CSN R12	*Brevibacterium*	*frigoritolerans*
CSN P16	*Burkholderia*	*phytofirmans*
CSN R44	*Chryseobacterium*	*rhizosphaerae*
CSN R51	*Pseudomonas*	*fluorescens*
CSN R49		*koreensis*
CSN R5		*putida*

Four rooting treatments were prepared and tested in the nursery setting. The nursery standard (T1) was applied as the control treatment in this study. The nursery standard comprised of the application of *Seradix 2* to cuttings prior to their setting in media. *Seradix 2* contains one active ingredient, indole-3-butyric acid (IBA). It is applied by dipping a cutting's cut edge into *Seradix 2* powder before the cutting is set. A commercial biological rooting agent, *Eco-T*, was also included as a treatment (T2). This biological rooting agent contains live fungus as active ingredient. Two treatments were prepared from the seven species that had the potential to stimulate the rooting of *Eucalyptus* hybrid cuttings. Treatment 3 (T3) comprised mainly of species that were known for their rooting enhancement abilities. Treatment 4 (T4), on the other hand, consisted of the remaining three species. Treatments T2, T3 and T4 a[so received the IBA rooting powder. Table 6.2 shows the compositions of the different treatments that were applied in this study. Besides the control treatment (T1) that was applied at the setting of the cuttings, the other treatments were applied two days after the cuttings were set in media.

Table 6.2 Description of cutting treatments

Treatment number	Treatment description	Composition
T1	NS	IBA
T2	Commercial rooting agent + NS	*Trichoderma harzianum* (fungus)
T3	Bacterial inoculum mix in 1 : 1 : 1 : 1 ratio + NS	*Bacillus aryabhattai* *Pseudomonas fluorescens* *P. koreensis* *P. putida*
T4	Bacterial inoculum mix in 1 : 1 : 1 ratio + NS	*Brevibacterium frigoritolerans* *Burkholderia phytofirmans* *Chryseobacterium rhizosphaerae*

NS = nursery standard = treatment with *Seradix 2*

6.2.2 Preparation of bacterial inoculum

Bacterial growth curves were prepared for each of the bacterial species so that the time when to harvest bacteria at the preferred concentrations (cfu/ml) could be determined. Figure 6.1 demonstrates the process to produce bacterial inoculums at the preferred concentration.

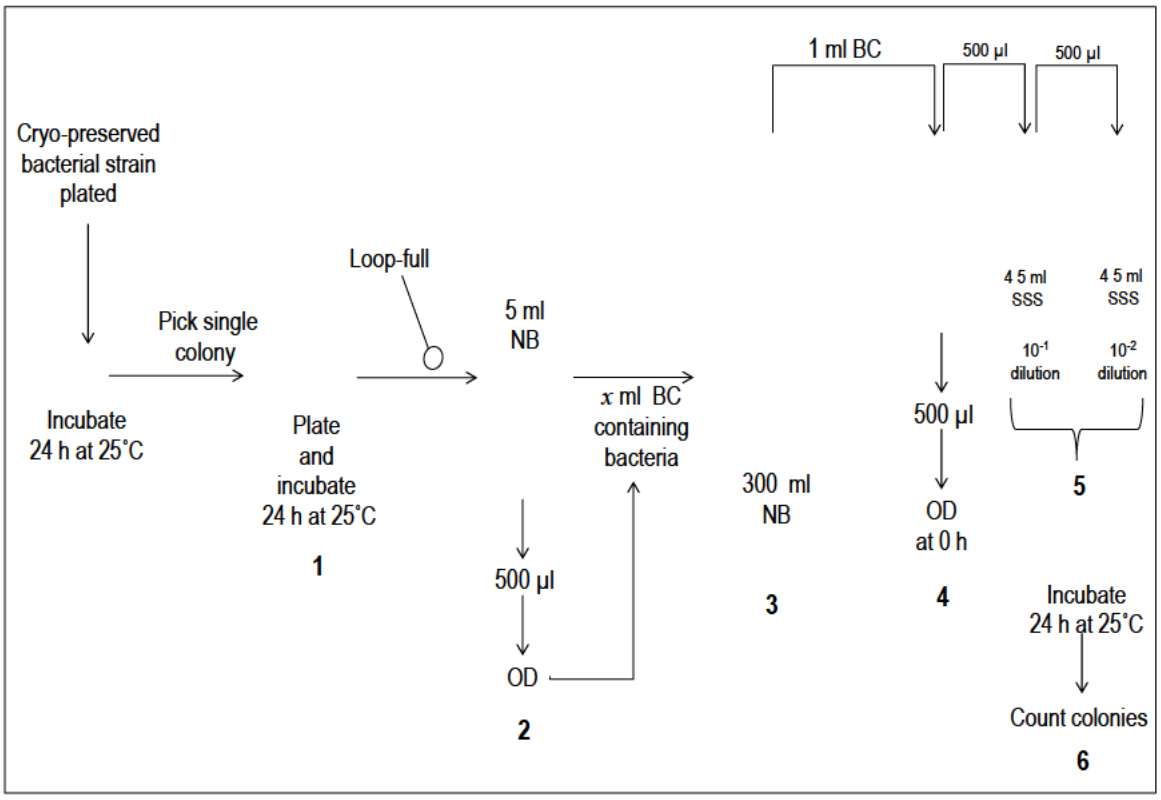


Figure 6.1 Demonstration of the process to produce bacterial inoculums at preferred concentrations (NB = nutrient broth; OD = optical density; BC = broth culture; SSS = sterile saline solution)

Each bacterial species was revived from cryo-preservation by plating onto nutrient agar (NA) plates and incubating for 24 hours at 25°C. The different steps to prepare the inoculums were as follows:

1. A single colony was picked, transferred to a fresh plate and incubated for 24 hours at 25°C.
2. After incubation, a loop-full of the bacterial cells was transferred into a sterile McCartney bottle containing 5 ml sterile nutrient broth (NB), the broth culture was vortexed and optical

density (OD) determined at 620 nm. In order to adjust the OD of the inoculum to a standard value (0.01) the following formula was used: OD1V1 = OD2V2, where OD1 = OD of the 5 ml broth culture prepared in step 2; V1 = volume of this broth culture to be added as inoculum (x ml); OD2 = OD of the inoculum in the conical flask (as a standard, this value was adjusted to 0.01); V2 = volume of the media in the conical flask (300 ml NB).

3. After inoculation, the conical flask was incubated at 25ºC, with shaking (150 rpm) on a Stuart S1500 orbital shaker, and monitored on an hourly basis for up to 11 hours.

4, 5. For each sample, OD was measured at 620 nm, viability determined by plating selected vials of a serial dilution (up to 10^{-4}) on NB plates using the exponential setting on the easySpiral®Pro, which automatically diluted each sample further to 10^{-4}. Plates were then incubated for 24 hours at 25°C.

6. The colonies on each plate were enumerated using the high resolution Scan® 1200 automatic colony counter. Concentrations were expressed as cfu/ml.

The generated data were used to construct growth curves for each bacterial species displaying the OD and cfu/ml against time. These graphs were used to determine the appropriate time interval and OD value for preparation of inoculums for field trials.

6.2.3 Preparation and preservation of inoculums for field trials

Separate inoculums were prepared for each bacterial species (described in 6.2.2), which were mixed prior to the application of the treatments in the field trials. Four 300 ml cultures were prepared for each species with cell densities of $\approx 10^7$ cfu/ml. Cell suspensions of each species were harvested at different time intervals, based on growth data following OD. The content of each flask was aseptically transferred to a 500 ml sterile Schott bottle, which contained 45 g glycerol (15% final concentration), then mixed gently and stored at -80°C. Viability, indole-3-acetic acid production and

phosphate solubilisation of the bacterial colonies were determined before freezing and after thawing (before use in field trials). Schott bottles containing the bacterial inoculums were transported to the field in a mobile freezer at -25°C and allowed to thaw overnight before application as treatment in field trials.

Prior to the application of rhizospheric bacterial treatments to cuttings, the bacterial inoculums for treatments 3 and 4 were mixed in a sterile 2 L Schott bottles in the ratio of 1 : 1 : 1 : 1 for T3 and 1 : 1 : 1 for T4. Also, treatment 2 (commercial biological rooting agent) was prepared according to specifications. A dispenser was then used to aliquot inoculum to each cutting in a cell of a Unigrow tray (Figure 6.2).

Figure 6.2 Dispenser used to aliquot mixed bacterial suspensions as treatment in field trails

6.3 Materials and methods: Field trials

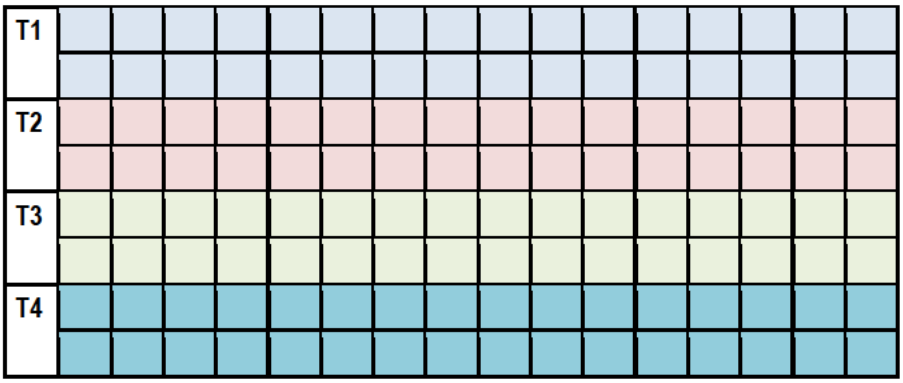
6.3.1 Preparation of cuttings

Cuttings were collected from mini-hedge ramets of the two *Eucalyptus* hybrid clones grown in nursery tunnels at Sunshine Seedlings near Pietermaritzburg. Shoots with a length of approximately 7 cm were carefully removed from the mini-hedges with sharp secateurs and placed in a 20-litre bucket, half filled with water. Thereafter, the buckets containing the cuttings were taken to the cutting room. The cutting room was constantly exposed to misting irrigation for 30 seconds every five minutes. The cuttings were prepared by trimming the shoots and leaves with a sharp scissors. The leaves were cut to a size of approximately one third of the original leaf surface area to reduce transpiration and to conserve energy. Finally, cuttings were stored in a water bath until setting in rooting medium.

6.3.2 Setting and treatment of the cuttings

Prior to the setting of the cuttings, the cuttings were washed in dilute Vapour Gard with Di-1-p-Menthene to reduce moisture stress in the cuttings. The cutting bases were then dipped into the *Seradix 2* according to specifications. Thereafter, the cuttings were placed in the centre of Unigrow cells filled with rooting media at a depth of approximately 2 cm.

The three potential root enhancing treatments (T2, T3 and T4) were applied to cuttings that had already been treated with *Seradix 2* (control treatment 1). In Experiment Trial 1, the treatments were applied to the hybrid clone GN 018B, and in Experiment Trial 2 the treatments were applied to the hybrid clone GN 010. The four treatments (T1, T2, T3 and T4) were applied to the respective *Eucalyptus* hybrid clone cuttings in a Unigrow tray that comprises of 128 cells arranged in 16 columns and 8 rows. Each of the four treatments was applied along two rows of 16 cells delineated with plastic markers. Each treatment was, therefore, applied to 32 cuttings per tray (Figure 6.3 a, b).



a.

b.

Figure 6.3 **Layout of treatments in a Unigrow tray. a. Example of an arrangement of the four treatments applied to a single Unigrow tray: T1 = control; T2 = commercial biological mixture + NS; T3 = bacterial inoculum mixture + NS; T4 = bacterial inoculum mixture + NS; and b. Treatments applied to cuttings in a Unigrow tray showing plastic markers as delineators.**

The application of the four treatments was applied in a random manner. The allocation of the position of a particular treatment in a Unigrow tray was accomplished by generating random

numbers using Microsoft Excel. Figure 6.4 shows the respective positions of the treatments for two Experimental Trials 1 and 2.

Tray 1	Tray 2	Tray 3	Tray 4	Tray 5	Tray 6	Tray 7	Tray 8	Tray 9	Tray 10
3	C	3	N	4	N	3	3	3	N
C	N	C	4	N	4	4	4	4	C
N	3	N	C	C	C	N	C	N	4
4	4	4	3	3	3	C	N	C	3

a.

Tray 1	Tray 2	Tray 3	Tray 4	Tray 5	Tray 6	Tray 7	Tray 8	Tray 9	Tray 10
3	C	3	N	4	N	3	3	3	N
C	N	C	4	N	4	4	4	4	C
N	3	N	C	C	C	N	C	N	4
4	4	4	3	3	3	C	N	C	3

Green (3) = T3; Blue (C) = T2; Yellow (N) = T1 and Red (4) = T4

b.

Figure 6.4 Treatment position in the Unigrow trays. a. Experimental Trial 1 and b. Experimental Trial 2

The four treatments were applied using the treatment dispenser. The treatments were applied at a rate of 5 ml per cutting. After the treatments were applied to the cuttings in the 10 Unigrow trays of each experimental trial, the trays were arranged across the nursery floor from one side to the other. The 10 Unigrow trays represent 10 replications. This arrangement allowed both experiments to be exposed to similar climatic variation in the nursery. The treated cuttings were left in the nursery for

eight weeks, at which time the growth measurements were taken. Figure 6.5 shows the arrangement of the two rows of 10 trays of each experiment across the nursery floor. The plastic delineators are clearly visible (knives, forks and spoons).

Figure 6.5 Arrangement of Unigrow trays of Experimental Trials 1 and 2 inside the nursery

6.4 Materials and methods: Measurement of treatment outcomes

At eight weeks of growth, several measurements were taken for each cutting. Firstly, it was noted whether the cutting had survived. If a cutting had survived, growth measurements were made and

the rooting architecture configuration described. Three growth measurements were made for each surviving cutting, namely, total length of cutting, root length and shoot length.

The rooting architecture of each rooted cutting was described by using six different descriptive configurations (Figure 6.6). The six different rooting configurations comprised of the number of primary adventitious roots with secondary and tertiary roots (R^+), or without secondary and tertiary roots (R^-). Separate categories were allocated to the number of primary adventitious roots until five roots. Cuttings with more than five adventitious roots were placed into a category called “many roots (RM).”

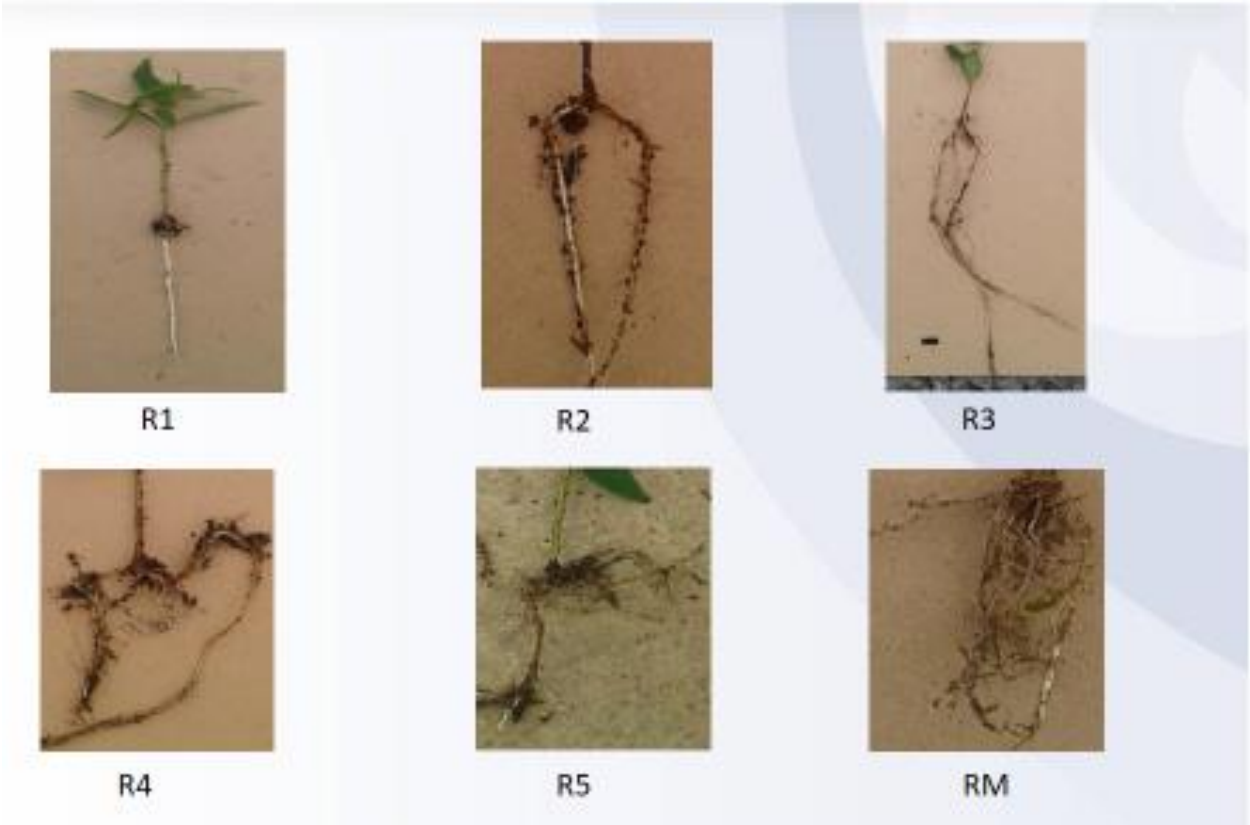


Figure 6.6 Key for the identification of rooting architectural configurations

6.4.1 Statistical analyses of treatment outcomes

Several analyses were undertaken for the two experiments. Summary statistics were calculated for the survival of the cuttings, growth properties of the cuttings, as well as for the rooting architectural configurations of the cuttings. Several analyses of variance (ANOVA) tests were performed on the data to ascertain if significant differences existed between the treatments of the two experiments. In instances where an ANOVA test produced a significant result, a Tukey test was performed to determine which treatments differed significantly. Chi-square tests of independence were performed to test if the different rooting architectural configurations were independent of treatment. Finally, the two experiments were compared by applying a two-way ANOVA to compare the different growth properties of the two hybrid clones.

In an attempt to ascertain if the treatments in this investigation promoted adventitious root formation, the rooting architecture data were re-categorised into three levels of fibrosity (Diaz et al., 2009). Level 1, which referred to the lowest level of fibrosity, included the rooting architectural types R1 and R2, including both plus and minus types. Level 2, referred to a medium level of fibrosity, and included the rooting architectural types R3 and R4, also inclusive of both plus and minus types. Level 3, referred to the highest level of fibrosity, and included the rooting architectural types R5 and RM, also inclusive of both plus and minus types. Goodness of fit chi-square tests were performed to test the three treatments (T2, T3 and T4) against the control (T1), which provided the expected values in the tests.

6.5 Result of bacterial growth investigation and inoculum preparation

Bacterial growth curves were prepared to determine at which time the bacterial cultures of the different rhizospheric bacterial species had attained their maximum population density while still in

exponential growth phase as demonstrated by the OD. Maximum optical densities were achieved within seven to eight hours for some species, while other achieved maximum density within eight to 10 hours (Figure 6.7). The ODs of the different bacterial cultures further revealed that, for the most, the maximum population densities of the respective rhizospheric bacterial species was in the order of $\approx 10^7$ cfu/ml.

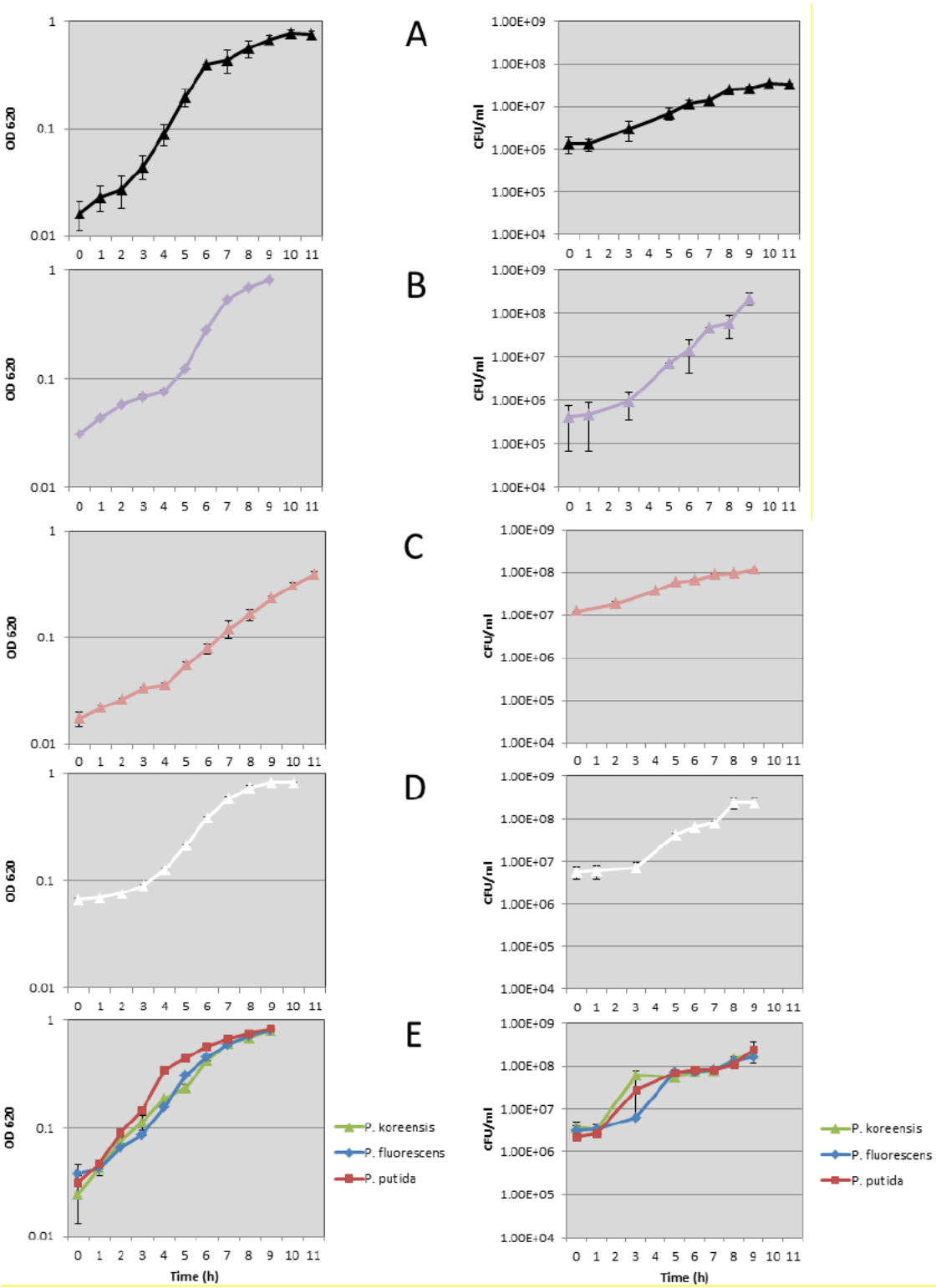


Figure 6.7 **Growth curves plotting OD against time and concentration against time for A. *Brevibacterium frigoritolerans*, B. *Bacillus aryabhattai*, C. *Burkholderia phytofirmans*, D. *Chryseobacterium rhizosphaerae*, E. *Pseudomonas* species.**

Average values of independent biological repeats were plotted, error bars represent standard deviations.

The growth investigation showed that not all of the seven bacterial species could attain a growth concentration of ≈10^8 cfu/ml as suggested by Teixeira et al. (2007) and Diaz et al. (2009), however, all of the species attained a concentration of ≈10^7 cfu/ml within 7 to 10 hours of incubation (Table 6.3). The study also revealed that the IAA activity and the ability to solubilise phosphate was not affected by storage of bacterial cultures in glycerol and the thawing of the cultures.

Table 6.3 Harvesting time intervals, OD values and viability and activity screening before and after freezing of inoculums for field trial

Species	Time (h)	Mean OD ± SD	Mean population density in cfu/ml × 10^7 ± SD		IAA activity		Phosphate solubilising activity	
			Before freezing	Before field trial	Before freezing	Before field trial	Before freezing	Before field trial
Bacillus aryabhattai	9	0.66±0.06	8.50±0.38	8.02±0.42	+	+	+	+
Brevibacterium frigoritolerans	10	0.76±0.004	1.93±0.16	1.87±0.13	+	+	+	+
Burkholderia phytofirmans	8	0.22±0.01	2.27±0.16	2.33±0.23	+	+	+	+
Chryseobacterium rhizosphaerae	7	0.62±0.03	1.72±0.19	1.68±0.24	+	+	+	+
Pseudomonas fluorescens	7	0.64±0.06	3.00±0.07	3.40±0.60	+	+	+	+
Pseudomonas koreensis	7	0.59±0.007	2.92±0.11	2.40±0.27	+	+	+	+
Pseudomonas putida	8	0.71±0.02	3.91±0.30	3.73±0.46	+	+	+	+

OD = optical density; cfu/ml = colony forming units per ml indicating bacterial population density; + = tested positively

6.6 Results of Experiment Trial 1: Hybrid GN 018B

6.6.1 Survival and rooting of cuttings

The number of cuttings that survived after eight weeks of growth was counted. Because some of the survived cuttings did not produce roots, the number of rooted cuttings was also calculated. For all treatments, more than 90% of the cuttings survived, although treatments 1 and 4 performed better than treatments 2 and 3. For most trays the number of survived rooted cuttings exceeded 20, however trays T1.3, T2.2 and T6.3 had less than 20 surviving rooted cuttings. For all treatments, the total mean percentage of survived rooted cuttings was greater than 75% with the control treatment (T1) outperforming the other treatments (Table 6.4).

Table 6.4 Survival and rooted number (%) of cuttings per tray per treatment

Tray (T) and treatment (.x)	Number of cuttings that survived (%)	Number of cuttings that died (%)	Number of cuttings that survived and rooted (%)
T1.1	27 (84.4)	5 (15.6)	25 (92.6)
T1.2	27 (84.4)	5 (15.6)	26 (96.3)
T1.3	21 (65.6)	11 (34.4)	12 (57.1)
T1.4	32 (100)	0 (0.0)	24 (75.0)
T2.1	29 (90.6)	3 (9.4)	28 (96.6)
T2.2	26 (81.3)	6 (18.8)	19 (73.1)
T2.3	31 (96.9)	1 (3.1)	28 (90.3)
T2.4	25 (78.1)	7 (21.9)	24 (96.0)
T3.1	28 (87.5)	4 (12.5)	27 (96.4)
T3.2	29 (90.6)	3 (9.4)	29 (100.0)
T3.3	28 (87.5)	4 (12.5)	26 (92.9)
T3.4	32 (100.0)	0 (0.0)	31 (96.9)
T4.1	27 (84.4)	5 (15.6)	23 (85.2)
T4.2	30 (93.8)	2 (6.3)	27 (90.0)

Tray (T) and treatment (.x)	Number of cuttings that survived (%)	Number of cuttings that died (%)	Number of cuttings that survived and rooted (%)
T4.3	31 (96.9)	1 (3.1)	23 (74.2)
T4.4	25 (78.1)	7 (21.1)	22 (88.0)
T5.1	32 (100.0)	0 (0.0)	30 (93.8)
T5.2	30 (93.8)	2 (6.3)	28 (93.3)
T5.3	30 (93.8)	2 (6.3)	22 (73.8)
T5.4	30 (93.8)	2 (6.3)	28 (93.3)
T6.1	32 (100.0)	0 (0.0)	29 (90.6)
T6.2	27 (84.4)	5 (15.6)	27 (100.0)
T6.3	29 (90.6)	3 (9.4)	18 (62.1)
T6.4	31 (96.9)	1 (3.1)	25 (80.6)
T7.1	31 (96.9)	1 (3.1)	29 (93.5)
T7.2	32 (100.0)	0 (0.0)	28 (87.5)
T7.3	32 (100.0)	0 (0.0)	21 (65.6)
T7.4	32 (100.0)	0 (0.0)	31 (96.9)
T8.1	31 (96.9)	1 (3.1)	31 (100.0)
T8.2	31 (96.9)	1 (3.1)	28 (90.3)
T8.3	31 (96.9)	1 (3.1)	27 (87.1)
T8.4	21 (100.0)	0 (0.0)	28 (87.5)
T9.1	32 (100.0)	0 (0.0)	28 (87.5)
T9.2	31 (96.9)	1 (3.1)	23 (74.2)
T9.3	32 (100.0)	0 (0.0)	25 (78.1)
T9.4	31 (96.9)	1 (3.1)	22 (71.0)
T10.1	32 (100.0)	0 (0.0)	30 (93.8)
T10.2	32 (100.0)	0 (0.0)	28 (87.5)
T10.3	31 (96.9)	1 (3.1)	27 (87.1)
T10.4	32 (100.0)	0 (0.0)	26 (81.3)
T1 Total mean %	94.06	5.94	93.02
T2 Total mean %	92.19	7.81	89.15
T3 Total mean %	92.50	7.50	77.36
T4 Total mean %	94.38	5.62	86.42

A visual perspective of the total mean percentages of the cuttings that survived and total mean percentages of the surviving cuttings that rooted, shows clearly that more than 90% of the cuttings survived. The mean percentage of cuttings that survived and had rooted in response to the four treatments were more than 75%. The control treatment (T1) showed the highest mean percentage of cuttings that rooted. Treatment T1, T2 and T4 produced more than 80% rooted cutting, while T3 showed the least number of rooted cuttings, less than 80% (Figure 6.8).

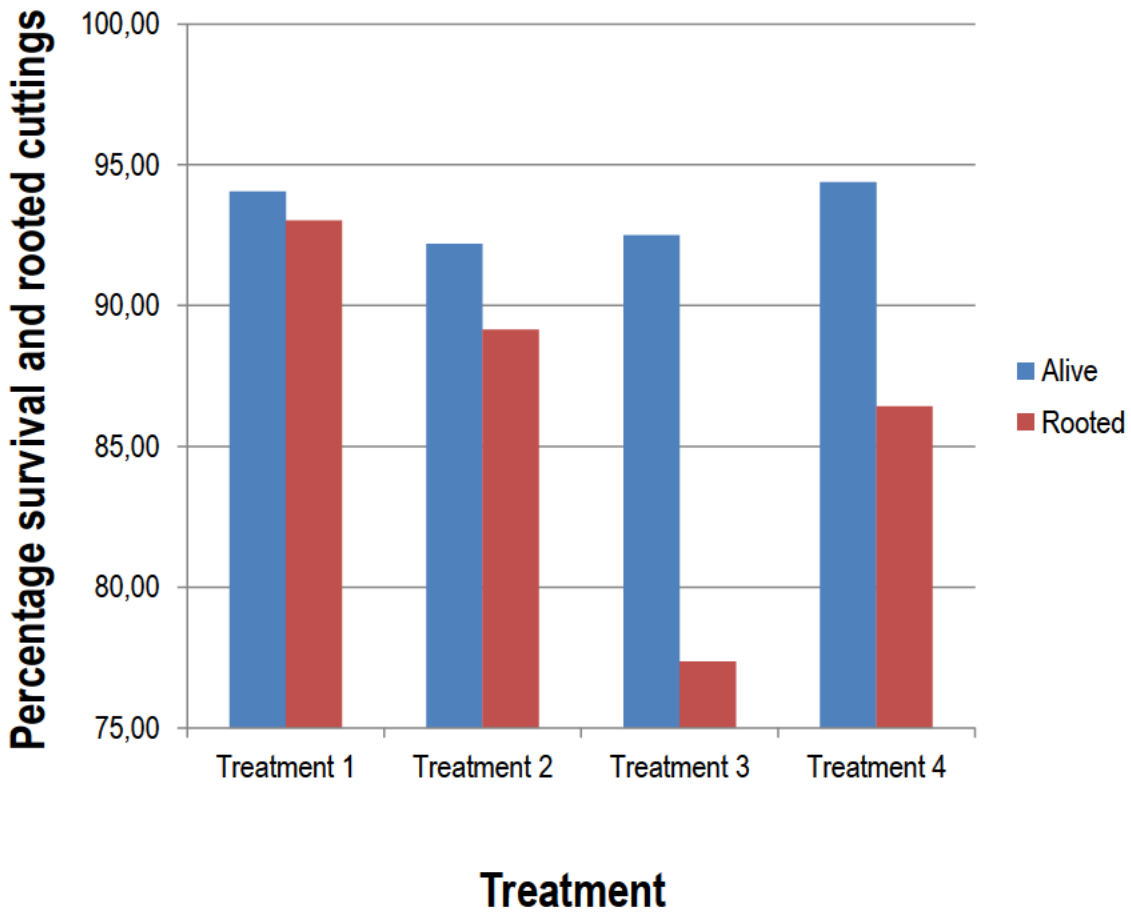


Figure 6.8 **Mean percentage of cuttings that survived and mean percentage of cuttings that rooted**

6.6.2 Rooting architecture of cuttings

The rooting architecture of the roots of the cuttings, in response to the different treatments, showed different presentations of the number of primary adventitious roots (R) with (R^+) or without (R^-) secondary and tertiary roots (Table 6.5). When the R^+ and R^- were grouped together, the rooting architectural category with a single primary adventitious root demonstrated the highest percentage of cuttings. The percentage of cuttings showed a descending pattern with an increase in the number of primary adventitious roots until a total of five roots. The category of many primary adventitious roots demonstrated a higher percentage of cuttings than the two categories with four and five primary adventitious roots.

Table 6.5 Rooting architecture showing number (%) of cuttings with primary adventitious roots with (+) or without (-) secondary and tertiary roots per treatment

Treatment	Rooting architecture												Total number of cuttings
	$R1^+$	$R1^-$	$R2^+$	$R2^-$	$R3^+$	$R3^-$	$R4^+$	$R4^-$	$R5^+$	$R5^-$	RM^+	RM^-	
T1	57	41	37	44	24	24	9	8	3	3	4	26	280
Total ($R^+ + R^-$)	98 (35.0)		81 (28.9)		48 (17.1)		17 (6.1)		6 (2.1)		30 (10.7)		
T2	59	57	33	38	10	24	6	10	3	4	1	18	263
Total ($R^+ + R^-$)	116 (44.1)		71 (27.0)		34 (12.9)		16 (6.1)		7 (2.7)		19 (7.2)		
T3	43	44	21	28	19	18	1	5	3	5	7	31	225
Total ($R^+ + R^-$)	87 (38.7)		49 (21.8)		37 (16.4)		6 (2.7)		8 (3.6)		38 (16.9)		
T4	54	50	37	27	15	21	7	11	3	6	2	28	261
Total ($R^+ + R^-$)	104 (40.0)		64 (24.5)		36 (13.8)		18 (6.9)		9 (3.4)		30 (11.5)		

R^+ = Primary adventitious roots with secondary and tertiary roots; R^- = Primary adventitious roots without secondary and tertiary roots.

A chi-square test of independence was performed to test if the different rooting architectural types were independent of the treatment. The test revealed that treatment did not affect rooting architecture significantly (χ^2 = 23.672, df = 15, P = 0.0709) (Table 6.6).

Table 6.6 Contingency table of the chi-square test of independence for rooting architectural types of Experimental Trial 1

Treatment	Experiment 1 root architecture vs. treatments					
	R1	R2	R3	R4	R5	RM
T1	*110.20* (1.35)	*72.11* (1.10)	*42.18* (0.80)	*15.51* (0.14)	*8.16* (0.57)	*31.84* (0.11)
T2	*103.51* (1.51)	*67.73* (0.16)	*39.62* (0.80)	*14.57* (0.14)	*7.67* (0.06)	*29.90* (3.98)
T3	*88.56* (0.03)	*57.94* (1.38)	*33.89* (0.28)	*12.46* (3.35)	*6.56* (0.32)	*25.58* (6.03)
T4	*102.73* (0.02)	*67.22* (0.15)	*39.31* (0.28)	*14.46* (0.87)	*7.61* (0.25)	*29.68* (0.00)

Expected values in italics and the χ^2 values displayed in parentheses.

A graphical representation of the visual perspective of the different architectural rooting types showed a clear pattern. When the different architectural types (+ and -) were grouped together within each rooting type (R^+ and R^-), the treatments performed overall in a similar manner. All treatments demonstrated a decreasing number of cuttings from R1 to R5 types of primary adventitious roots, with or without secondary and tertiary roots. However, the number of cuttings of the RM architectural rooting type was greater than the number of cuttings of the R4 and R5 architectural rooting types (Figure 6.9).

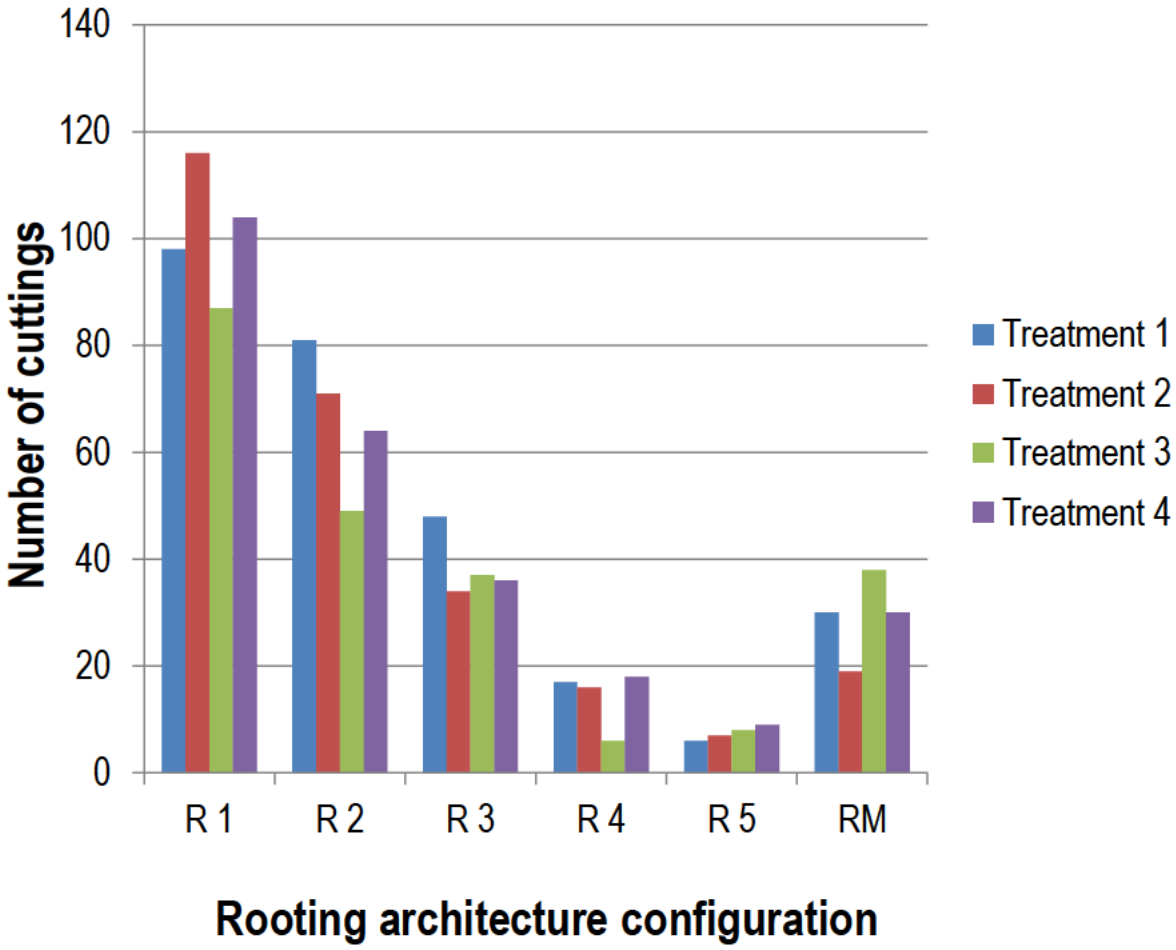


Figure 6.9 **Number of cuttings in each treatment with different rooting architectural configuration types**

6.6.3 Growth response of cuttings

Three growth measurements were made on each rooted cutting. Mean total growth, which included the shoot and root length, showed that the control treatment (T1) outperformed all the other treatments, by producing the longest rooted cuttings. A similar pattern could be discerned for both mean root length and means shoot length, where the control treatment (T1) also produced the longest roots and longest shoots (Table 6.7).

Table 6.7 Summary statistics for the growth response parameters of Experiment Trial 1

	Total length (root + shoot length)				Root length				Shoot length			
Treatment	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
Mean	25.3	23.0	20.6	22.3	10.5	9.6	8.3	9.7	14.8	13.4	12.2	13.8
SD	10.69	10.37	10.13	9.95	6.24	6.27	6.36	6.28	5.99	5.43	5.21	5.04
SE	0.60	0.58	0.57	0.56	0.35	0.35	0.36	0.35	0.33	0.30	0.29	0.28
Variance	114.30	107.62	102.56	98.98	38.97	39.28	40.50	39.41	35.88	29.49	27.12	25.36
Range	73	60.5	50	65.5	48	39.5	30	47	30	25	26	26
Minimum	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	73.0	60.5	50.0	65.5	48.0	39.5	30.0	47.0	30.0	25.0	26.0	26.0

SD = standard deviation; SE = standard error.

A graphical representation provides a visual perspective of the different growth responses for the four different treatments. The graph clearly demonstrates that the control treatment (T1) outperformed the other treatments, although the differences were relatively small when compared to T2 and T4. Treatment 3 appeared to show the poorest growth responses (Figure 6.10).

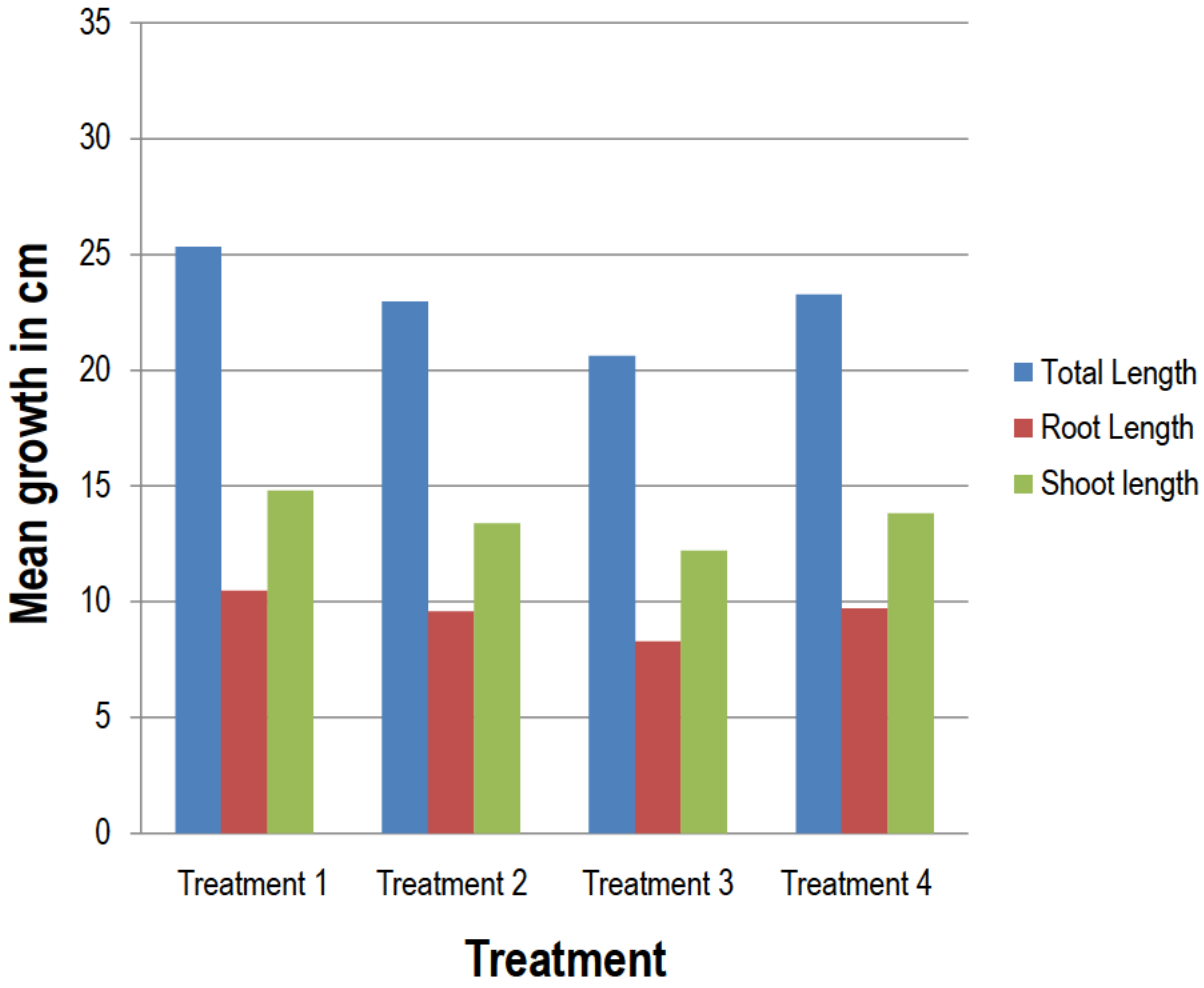


Figure 6.10 **Growth responses to different treatments showing mean total length, mean root length and mean shoot length of the cuttings**

Two factor ANOVA tests were performed on the respective growth responses to ascertain if significant difference existed between treatments, as well as between trays. All growth responses showed highly significant differences between the different treatments, as well as between trays. The tray versus treatment interaction also proved to be highly significantly different (Table 6.8).

Table 6.8 ANOVA tests for the growth responses, total length, root length and shoot length of cuttings for Experimental Trial 1

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
Total length	Tray	6164.97	9	684.99	7.02	**<0.0001**
	Treatment	3575.69	3	1191.89	12.21	**<0.0001**
	Tray/Treatment	7941.47	27	294.13	3.01	**<0.0001**
	Within	120973.45	1240	97.56		
Root length	Tray	1725.39	9	191.71	5.13	**<0.0001**
	Treatment	791.41	3	263.80	7.06	**<0.0001**
	Tray/Treatment	2411.43	27	89.31	2.39	**<0.0001**
	Within	46314.47	1240	37.35		
Shoot length	Tray	2598.03	9	288.67	10.88	**<0.0001**
	Treatment	1114.44	3	371.48	14.01	**<0.0001**
	Tray/Treatment	2105.93	27	77.99	2.94	**<0.0001**
	Within	32888.52	1240	26.52		

Tukey HSD tests were performed to ascertain which combination of treatments differed significantly. Most combinations of treatments differed significantly. Treatment 4, however, did not differ significantly from T1 and T2 for total length; from T3 for root length; and T1 and T2 for shoot length (Table 6.9).

Table 6.9 **Tukey HSD tests for Experimental Trial 1 (a) total length (TL), (b) root length (RL), and (c) shoot length (SL)**

a.

TL	T1	T2	T3	T4
T1				
T2	S			
T3	S	S		
T4	NS	NS	S	

b.

RL	T1	T2	T3	T4
T1				
T2	NS			
T3	S	S		
T4	S	S	NS	

c.

SL	T1	T2	T3	T4
T1				
T2	S			
T3	S	S		
T4	NS	NS	S	

S = significant at $\alpha = 0.05$, NS = not significant at $\alpha = 0.05$, TL = total length, RL = root length, SL = shoot length

6.7 Results of Experiment Trial 2: Hybrid GN 010

6.7.1 Survival and rooting of cuttings

Similar to Experiment Trial 1, more than 90% of the cuttings survived. For most trays the number of survived rooted cuttings exceeded 20, however tray T9.2 had less than 20 surviving rooted cuttings for all treatments, the total mean percentage of survived rooted cuttings was greater than 80% with the control treatment (T1) outperforming the other treatments (Table 6.10).

Table 6.10 **Survival and rooted number (%) of cuttings per tray per treatment**

Tray (T) and treatment (.x)	Number of cuttings that survived (%)	Number of cuttings that died (%)	Number of cuttings that survived and rooted (%)
T1.1	29 (90.6)	3 (9.4)	28 (96.6)
T1.2	29 (90.6)	3 (9.4)	26 (89.7)
T1.3	31 (96.9)	1 (3.1)	29 (93.5)
T1.4	32 (100.0)	0 (0.0)	28 (87.5)

Tray (T) and treatment (.x)	Number of cuttings that survived (%)	Number of cuttings that died (%)	Number of cuttings that survived and rooted (%)
T2.1	32 (100.0)	0 (0.0)	26 (81.3)
T2.2	30 (93.8)	2 (6.3)	26 (86.7)
T2.3	30 (93.8)	2 (6.3)	30 (100.0)
T2.4	32 (100.0)	0 (0.0)	30 (93.8)
T3.1	30 (93.8)	2 (6.3)	26 (86.7)
T3.2	32 (100.0)	0 (0.0)	28 (87.5)
T3.3	28 (87.5)	4 (12.5)	25 (89.3)
T3.4	32 (100.0)	0 (0.0)	25 (78.1)
T4.1	32 (100.0)	0 (0.0)	30 (93.8)
T4.2	30 (93.8)	2 (6.3)	25 (83.3)
T4.3	32 (100.0)	0 (0.0)	26 (81.3)
T4.4	32 (100.0)	0 (0.0)	28 (87.5)
T5.1	31 (96.9)	1 (0.0)	29 (93.5)
T5.2	32 (100.0)	0 (0.0)	25 (78.1)
T5.3	32 (100.0)	0 (0.0)	24 (75.0)
T5.4	25 (78.1)	7 (21.9)	25 (92.0)
T6.1	32 (100.0)	0 (0.0)	30 (93.8)
T6.2	30 (93.8)	2 (6.3)	23 (76.7)
T6.3	32 (100.0)	0 (0.0)	28 (87.5)
T6.4	31 (96.9)	1 (3.1)	26 (83.9)
T7.1	31 (96.9)	1 (3.1)	28 (90.3)
T7.2	30 (93.8)	2 (6.3)	26 (86.7)
T7.3	32 (100.0)	0 (0.0)	24 (75.0)
T7.4	31 (96.9)	1 (3.1)	28 (90.3)
T8.1	30 (93.8)	2 (6.3)	28 (93.3)
T8.2	31 (96.9)	1 (3.1)	27 (87.1)
T8.3	29 (90.6)	3 (9.4)	22 (75.9)
T8.4	31 (96.9)	1 (3.1)	28 (90.3)
T9.1	32 (100.0)	0 (0.0)	26 (81.3)
T9.2	32 (100.0)	0 (0.0)	18 (56.3)

Tray (T) and treatment (.x)	Number of cuttings that survived (%)	Number of cuttings that died (%)	Number of cuttings that survived and rooted (%)
T9.3	27 (84.4)	5 (15.6)	20 (74.1)
T9.4	29 (90.6)	3 (9.4)	23 (79.3)
T10.1	32 (100.0)	0 (0.0)	29 (90.6)
T10.2	32 (100.0)	0 (0.0)	30 (93.8)
T10.3	30 (93.8)	2 (6.3)	24 (80.0)
T10.4	31 (96.9)	1 (3.1)	29 (93.5)
T1 Total mean %	97.19	2.81	90.03
T2 Total mean %	96.25	3.75	82.47
T3 Total mean %	94.69	5.31	83.17
T4 Total mean %	95.63	4.38	87.58

Also, similar to Experiment Trial 1, a visual perspective of the total mean percentages of the cuttings that survived and total mean percentages of the surviving cuttings that rooted, shows clearly that more than 90% of the cuttings survived. However, T1 (control) performed marginally better than T2, T3 and T4. The mean percentage of cuttings that survived and had rooted in response to the four treatments were more than 80%. The control treatment (T1) showed the highest mean percentage of cuttings that rooted followed by T4; while T2 and T3 showed lower mean percentages of cuttings that survived and that had rooted (Figure 6.11).

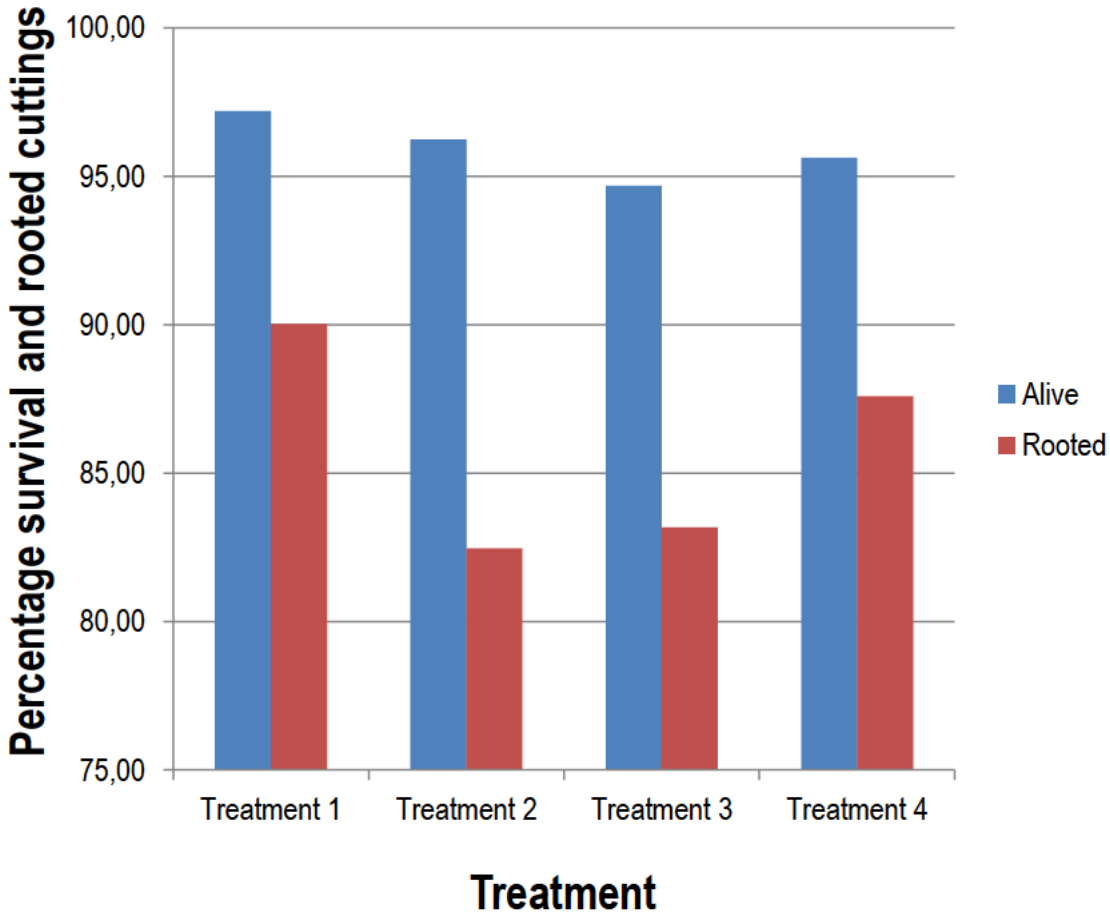


Figure 6.11 **Mean percentage of cuttings that survived and mean percentage of cuttings that rooted**

6.7.2 Rooting architecture of cuttings

Similar to Experiment Trial 1, the rooting architecture of the roots of the cuttings showed different presentations of the number of primary adventitious roots (R) with (R^+) or without (R^-) secondary and tertiary roots in response to the different treatments (Table 6.11). Also, similar to Experiment Trial 1, when the R^+ and R^- were grouped together, the rooting architectural category with a single primary adventitious root also demonstrated the highest percentage of cuttings. The percentage of cuttings also showed a descending pattern with an increase in the number of primary adventitious roots until a total of five roots. The category of many primary adventitious roots in Experiment Trial 2, also

showed a greater percentage of cuttings than the two categories with four and five primary adventitious roots.

Table 6.11 Rooting architecture showing number of cuttings (%) with primary adventitious roots with (+) or without (-) secondary and tertiary roots per treatment

Treatment	Rooting architecture												Total number of cuttings
	$R1^+$	$R1^-$	$R2^+$	$R2^-$	$R3^+$	$R3^-$	$R4^+$	$R4^-$	$R5^+$	$R5^-$	RM^+	RM^-	
T1	58	38	37	57	27	25	3	15	0	6	0	14	
Total ($R^+ + R^-$)	96 (34.2)		94 (33.5)		52 (18.5)		18 (6.4)		6 (2.1)		14 (5.0)		280
T2	49	47	29	40	12	34	1	19	0	4	6	13	
Total ($R^+ + R^-$)	96 (37.7)		69 (27.1)		46 (18.1)		20 (7.8)		4 (1.5)		19 (7.4)		254
T3	70	52	28	25	20	23	2	7	1	0	2	22	
Total ($R^+ + R^-$)	122 (48.2)		53 (20.9)		43 (16.9)		9 (3.5)		1 (0.4)		24 (9.4)		253
T4	63	44	30	43	15	26	8	13	1	5	1	19	
Total ($R^+ + R^-$)	107 (39.9)		73 (27.2)		41 (15.3)		21 (7.8)		6 (2.2)		20 (7.5)		268

R^+ = Primary adventitious roots with secondary and tertiary roots; R^- = Primary adventitious roots without secondary and tertiary roots

A chi-square test of independence was performed to test if the different rooting architectural types were independent of the treatment. The test revealed that treatment did not affect rooting architecture significantly ($\chi^2 = 22.996$, df = 15, P = 0.0842) (Table 6.12).

Table 6.12 Contingency table of the chi-square test of independence for rooting architectural types of Experiment Trial 2

Treatment	Experiment 2 root architecture vs. treatments					
	R1	R2	R3	R4	R5	RM
T1	*115.72* (2.14)	*80.77* (3.68)	*52.43* (0.24)	*21.71* (0.00)	*8.74* (0.18)	*24.63* (1.78)
T2	*105.06* (0.24)	*73.33* (0.00)	*47.60* (0.12)	*19.71* (0.27)	*7.93* (0.00)	*22.36* (0.02)
T3	*105.06* (4.17)	*73.33* (0.00)	*47.60* (0.01)	*19.71* (2.29)	*7.93* (1.08)	*22.36* (1.42)
T4	*111.15* (0.00)	*77.58* (0.00)	*50.36* (0.57)	*20.86* (0.82)	*8.39* (0.31)	*23.66* (0.01)

Expected values in italics and the χ^2 values displayed in parentheses.

A graphical representation of the visual perspective of the different architectural rooting types showed a similar pattern to the pattern in Experiment 1. The grouped architectural types, R^+ and R^-, demonstrated a decreasing number of cuttings from R1 to R5 types of primary adventitious roots, with or without secondary and tertiary roots, however the RM type has greater numbers than R4 and R5 (Figure 6.12).

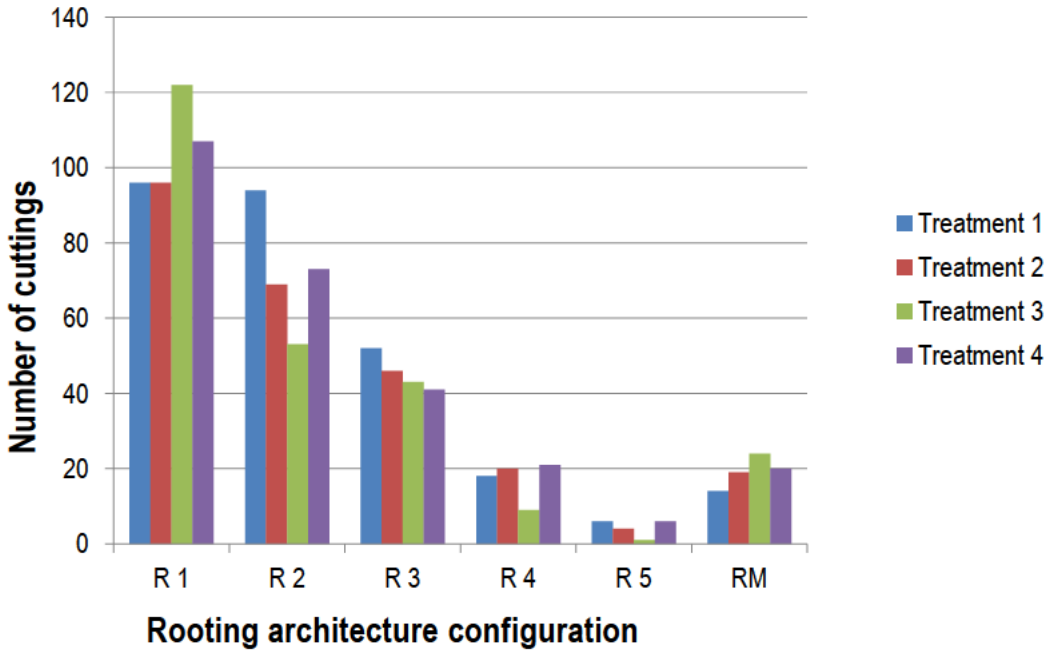


Figure 6.12 **Number of cuttings in each treatment with different rooting architectural configuration types**

6.7.3 Growth response of cuttings

Experimental Trial 2 presented with similar results as Experimental Trail 1 for the three growth responses of the rooted cuttings. The mean total length of the cuttings of the control treatment was longer than all the other treatments (Table 6.13). The mean total length of the cuttings of T3, however, was notably less than the other treatments. When considering the mean root length and the mean shoot length, T3 showed the lowest percentages. The other three treatments produced similar percentages.

Table 6.13 Summary statistics for the growth response parameters of Experiment Trial 2

	Total length (root + shoot length)				Root length				Shoot length			
Treatment	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
Mean	29.6	27.2	23.2	26.8	14.2	12.5	10.4	13.0	15.3	14.7	12.9	13.9
SD	12.67	12.73	11.59	11.45	8.41	8.12	7.47	7.58	5.82	6.0	5.46	5.31
SE	0.71	0.71	0.65	0.64	0.47	0.45	0.42	0.42	0.33	0.34	0.31	0.30
Variance	160.43	161.89	134.24	131.00	70.74	65.85	55.79	57.31	33.93	35.97	29.82	28.17
Range	71	73	53	57	49	48	34	38	31	30	30.6	28
Minimum	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	71	73	53	57	49	48	34	38	31	30	30.6	28

SD = standard deviation; SE = standard error.

A graphical representation shows that the different growth responses for the four different treatments were largely similar to Experimental Trial 1. In Experimental Trial 2, the mean total length of the control (T1) also outperformed the other treatments, although the mean root length and mean shoot length was rather similar for treatments 1, 2 and 4, with T3 marginally less than the other three treatments (Figure 6.13).

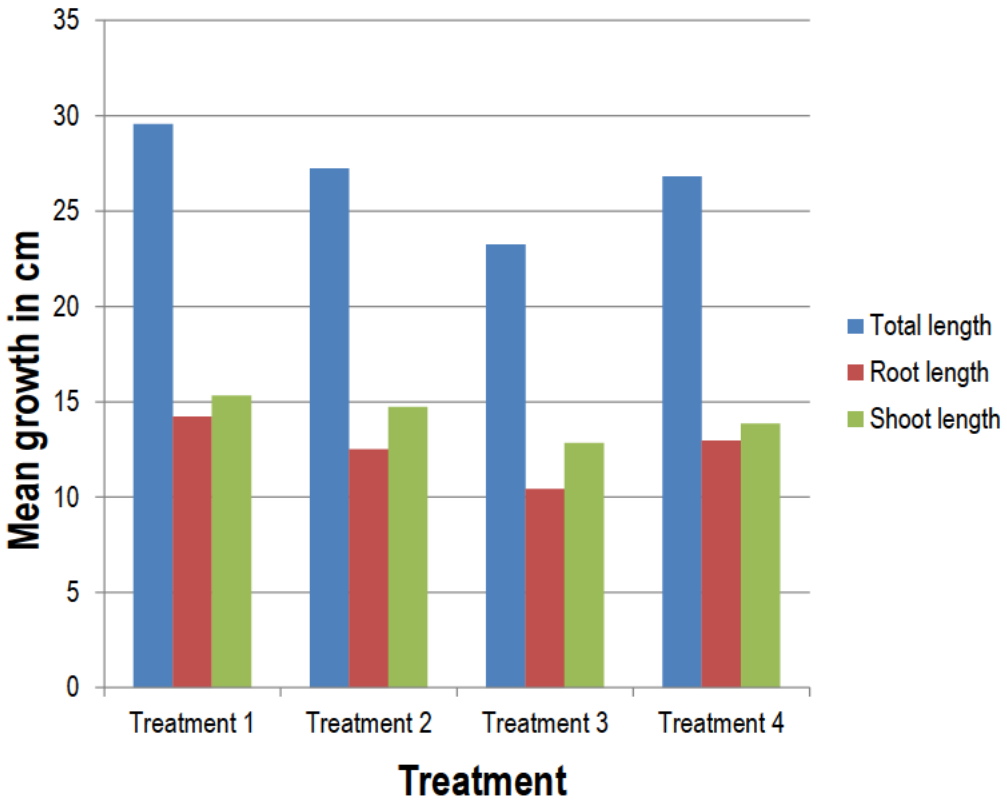


Figure 6.13 Growth responses to different treatments showing mean total length, mean root length and mean shoot length of the cuttings

Two factor ANOVA tests were performed on the respective growth responses to ascertain if significant difference existed between treatments, as well as between trays. Similar to Experimental Trial 1, to Experimental Trial 2 also produced highly significant differences between the different treatments, as well as between trays for all three growth measurements. Also, the tray versus treatment interaction also proved to be highly significantly different (Table 6.14).

Table 6.14 ANOVA tests for the growth responses, total length, root length and shoot length of cuttings for Experimental Trial 2

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
Total length	Tray	5504.79	9	611.64	4.32	**<0.0001**
	Treatment	6565.59	3	2188.53	15.45	**<0.0001**
	Tray/Treatment	6311.51	27	233.76	1.65	**<0.0001**
	Within	175614.11	1240	141.62		
Root length	Tray	2013.02	9	223.67	3.70	**<0.0001**
	Treatment	2408.20	3	802.73	13.28	**<0.0001**
	Tray/Treatment	2704.37	27	100.16	1.66	**<0.0001**
	Within	74933.35	1240	60.43		
Shoot length	Tray	1409.59	9	156.62	5.144	**<0.0001**
	Treatment	1127.53	3	375.84	12.34	**<0.0001**
	Tray/Treatment	1633.52	27	60.50	1.987	**<0.0001**
	Within	37754.15	1240	30.45		

Tukey HSD test were also performed to ascertain which combination of treatments differed significantly. As was the case in Experimental Trial 1, most combinations of treatments differed significantly (Table 6.15). However, T4 showed no significant differences with T2 for total length, T1 and T2 for root length and T2 and T3 for shoot length.

Table 6.15 Tukey HSD tests for Experimental Trial 2 (a) total length (TL), (b) root length (RL), and (c) shoot length (SL)

a.

TL	T1	T2	T3	T4
T1				
T2	NS			
T3	S	S		
T4	S	NS	S	

b.

RL	T1	T2	T3	T4
T1				
T2	S			
T3	S	S		
T4	NS	NS	S	

c.

SL	T1	T2	T3	T4
T1				
T2	NS			
T3	S	S		
T4	S	NS	NS	

S = significant at $\alpha = 0.05$, NS = not significant at $\alpha = 0.05$, TL = total length, RL = root length, SL = shoot length

6.8 Comparison of the hybrids of the two Experimental Trials

6.8.1 Survival and rooting of cuttings

A graphical representation of the number of cuttings that survived and the number of rooted cuttings that survived, was prepared. The graph shows that there were no clear differences between the different treatments of the two experiments for the number of surviving cuttings (Figure 6.14). Similarly, there were no clear differences between the numbers of rooted cuttings across treatments and experimental trials.

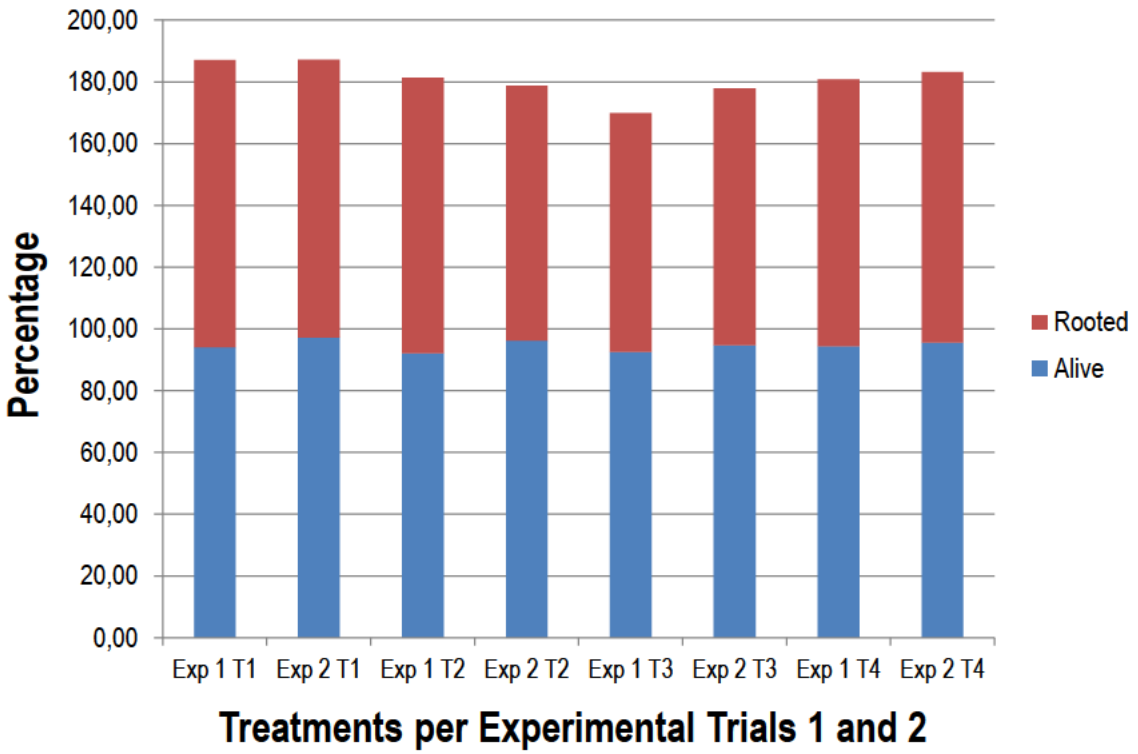


Figure 6.14 **Comparison of number of cuttings that survived and rooted for the two Experimental Trials (Exp 1 = Experimental Trial 1; Exp 2 = Experimental Trial 2)**

6.8.2 Rooting architecture of cuttings

The percentages of cuttings that demonstrated different levels of fibrosity were calculated. When considering Level 3 (highest level of fibrosity), it appears as if T3 and T4 did promote fibrosity in Experimental Trail 1, while for Experimental Trail 2, all treatments promoted fibrosity when compared to the control (T1) (Table 6.16). Goodness of fit chi-square tests were conducted to test to what extent treatments T2, T3 and T4 promoted fibrosity in comparison to the control (T1). Most tests were not significant at an $\alpha = 0.05$. However, the chi-square test for T3 of Experiment 1, proved to be highly significant ($\chi^2 = 12.14$, df = 2, P = 0.0023). Furthermore, it was noted that the chi-square test for T2 of Experiment 1 was also closely significant ($\chi^2 = 5.92$, df = 2, P = 0.051).

Table 6.16 Percentages of cuttings demonstrating the different levels of fibrosity for Experimental Trails 1 and 2

Experiment	Treatment	Level 1	Level 2	Level 3
1	1	63.9	23.2	12.9
	2	71.1	19.0	9.9
	3	60.4	19.1	20.4
	4	64.4	20.7	14.9
2	1	67.9	25.0	7.1
	2	65.0	26.0	9.1
	3	69.4	20.6	9.9
	4	67.2	23.1	9.7

6.8.3 Growth response of cuttings

Two factor ANOVA tests were performed on the respective growth responses of the two hybrids (Experimental Trial 1 and Experimental Trial 2). For the three growth responses, total length, root length and shoot length, the performances of the two hybrids were significantly different at an α = 0.05 (Table 6.17). Similarly, the outcomes of the treatments were also significantly different at an α = 0.05. However, the interaction between hybrid genotype and treatment was not significant.

Table 6.17 Two-way ANOVA test performed to compare growth responses on the two hybrids

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
Total length	Hybrids	8574.28	1	8574.28	67.85	**<0.0001**
	Treatments	9851.25	3	3283.75	25.98	**<0.0001**
	Hybrids/Treatments	290.03	3	96.68	0.76	0.4612
	Within	322510.30	2252	126.38		
Root length	Hybrids	5849.45	1	5849.44	114.74	**<0.0001**
	Treatments	2976.84	3	992.28	19.47	**<0.0001**
	Hybrids/Treatments	222.78	3	74.26	1.46	0.1972
	Within	130102	2552	50.98		
Shoot length	Hybrids	261.76	1	261.76	8.52	**<0.0147**
	Treatments	2105.28	3	701.76	22.85	**<0.0001**
	Hybrids/Treatments	136.69	3	45.56	1.48	0.1588
	Within	78389.73	2552	30.72		

6.9 Discussion

When preparing rhizospheric bacterial treatments as biological rooting stimulants, an understanding of the growth properties of the bacterial species is necessary. The growth properties of a bacterial species provides information about the growth rate under laboratory culturing conditions. Under such controlled conditions, bacterial cell cultures grow until they reach the stationary phase when growth-limiting factors, such as the depletion of essential nutrients, prevent a further increase in bacterial cell numbers, because the number of new bacterial cells produced are in balance with the number of bacterial cells that die. Therefore, it is important to determine the growth rate and the maximum population density that each species can achieve under laboratory conditions. In this study, some

rhizospheric bacterial species were able to reach a maximum population density within seven to eight hours, while other species required up to 10 hours. All species achieved a population density that was in the order of $\approx 10^7$ cfu/ml, which was used as a guide to prepare the inoculums for the treatment of the *Eucalyptus* cuttings. Although Teixeira et al. (2007) and Diaz et al. (2009) suggested that $\approx 10^8$ cfu/ml was the preferred bacterial cell concentration for the treatment of *Eucalyptus* cuttings, Zaspel and Ewald (2001) stated that $\approx 10^7$ cfu/ml was also appropriate. Thus based on this information all treatments were prepared at a concentration of $\approx 10^7$ cfu/ml.

This study revealed that the rooting responses of the cuttings were distinctly different when the two *Eucalyptus* hybrids were compared. These genotypic differences were noted particularly for growth rate of the cuttings and adventitious root development. Both hybrid clones demonstrated significant ($P = 0.0001$) genotypic differences for all three growth responses, total length, root length and shoot length. These outcomes support the findings of Brondani et al. (2012), who identified genotypic differences in adventitious root development in tree clones of *Eucalyptus benthamii* × *Eucalyptus dunnii.*

Several notable differences and similarities amongst the different treatments could be established within each of the experimental trials. Cuttings of both hybrids demonstrated relatively high survival rates and rooting percentage for all treatments. Although several rooting architectural configurations were prevalent in this study, there were indications that some of the treatments, particularly in Experiment Trail 1, promoted adventitious root development and fibrosity. Fibrosity could be of value when rooted cuttings are planted out in plantations (Egamberdieva, 2011). When the cutting growth responses were compared, both experimental trials showed highly significant differences amongst the treatments in both trials ($P < 0.0001$). For the most, the control treatment, the nursery standard, outperformed the other treatments, although marginally so for some treatments.

Chapter 7

Discussion and conclusions

7.1 Introduction

The many benefits that *Eucalyptus* clonal forestry offers have brought about commercial nurseries to exploit this avenue of forest tree production. The cloning of trees allows for the preservation of superior genotypes (de Assis et al., 2004). At the heart of cloning operations lies vegetative reproduction, which allows the commercial forestry industry to mass produce such superior genotypes of species and hybrids. Clonal forests are largely uniform, yielding homogenous products, which allows for more accurate prediction of yield and profit (Ferreira et al., 2004).

Vegetative reproduction of *Eucalyptus* involves the rooting of cuttings. In South African forestry nurseries, cuttings were traditionally taken from outdoor hedges, however more recently cuttings are more often taken from indoor mini-hedges. For cuttings to develop successfully, adventitious root formation must occur. Adventitious roots increase nutrient absorbing surfaces and results in better assimilation of water and nutrients from the soil (Egamberdieva, 2011). Most commercial nursery enterprises apply plant growth promoting regulators to cuttings to stimulate the formation of adventitious root primordia (Brondani et al., 2012). However, some valued *Eucalyptus* genotypes, especially of the temperate species, and those deployed in low productivity areas demonstrate relatively poor adventitious rooting abilities (Martellet & Fett-Neto, 2005; Brondani et al., 2010b).

Eucalyptus genotypes that demonstrate low rooting percentages are problematic for commercial forestry nurseries. Besides losses from unrooted cuttings, nurseries suffer extensive financial losses.

In addition, poor rooting limits the area planted up to a superior genotype as poor rooting makes less plants available for planting. Therefore, research is ongoing searching for methods that will ensure high rooting percentages, particularly for superior genotypes that demonstrate low rooting percentages. Rhizospheric bacteria have been shown to stimulate adventitious root formation in *Eucalyptus* cuttings (Teixeira et al., 2007; Diaz et al., 2009). For example, the treatment of cuttings of *E. globulus* with plant growth promoting rhizospheric bacteria such as, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Comamonas*, *Paenibacillus*, *Pseudomonas* and *Streptomyces* induced adventitious root formation because of auxin production and the inhibition of ethylene synthesis by these bacterial strains (Tripp & Stomp, 1997; Esitken et al., 2003; Teixeira et al., 2007). However, the potential use of plant growth promoting rhizospheric bacteria to stimulate adventitious root formation in cuttings of forest tree species has not been studied extensively (Teixeira et al., 2007). This study was thus undertaken to test rhizospheric bacterial preparations for their ability to promote rooting in cuttings of two *Eucalyptus* hybrids that generally demonstrate relatively low rooting percentages.

7.2 Rhizospheric microbial diversity

The exploitation of rhizospheric microorganisms for their properties in biotechnological applications in clonal forestry, such as for the enhancement of the rooting of cuttings; requires some understanding of the diversity of rhizospheric microorganism communities, conditions of the rhizosphere, and change over time (Di Cello et al., 1997). The diversity of rhizospheric microbial communities and their rhizospheric conditions were investigated by characterising the microbial communities of two different ages of *Eucalyptus* rhizospheres. The microbial community of 3-month and 5-year old rhizospheres were characterised by acid methyl esters (FAMEs) profiling. This type of comprehensive characterisation, of particularly *Eucalyptus* clonal rhizospheres, was the first of its kind.

This study revealed that the rhizospheric microbial community diversity evolved over time. The younger rhizospheric microbial communities displayed significantly greater diversity when compared to the older rhizospheric microbial communities. Three diversity indexes; FAME richness, Shannon and Simpson's indexes supported the notion that the younger rhizospheres were more diverse than the older rhizospheres. The rhizospheric environment of young plants accommodates mainly first root colonisers, resulting in the more diverse rhizospheric microorganism communities (Di Cello et al., 1997). Studies have shown that the proportions of saturated and unsaturated fatty acids are indicative of aerobic or anaerobic conditions of a rhizospheric environment (Quezada et al., 2016). This study revealed that as the rhizospheric microbial community aged, so did the community composition, resulting in a reduction in the proportion of saturated fatty acids. This implied that younger rhizospheric microbial communities are associated with aerobic conditions, while with aging the community evolves to a community of microorganisms that requires more anaerobic conditions (Patrick, 2013; Quezada et al., 2016). The greater proportion of polyunsaturated fatty acids in the older rhizospheric soil samples, especially linoleic (C18:2ω6c), α-linolenic (C18:3ω3c), γ-linolenic (C18:3ω3c) fatty acids, as well as the unique γ-linolenic fatty acid, suggested the growing establishment of fungi as the rhizosphere ages.

The rhizospheric microbial community evolution that was demonstrated by the two *Eucalyptus* age groups of rhizospheres can be attributed to the changes in the composition of plant root exudates (Rasch et al., 2006; Micallef et al., 2009; Halder & Sengupta, 2015). Plant roots release organic compounds through exudates, which act as a selective medium for specific groups of rhizospheric bacteria (Marilley & Aragno, 1999; Garcia et al., 2001). As a result, as plants grow and mature, their root exudates change, which in turn act as selective medium for rhizospheric bacteria that are competitively fit to occupy compatible niches without causing pathological stress on the plant (Etesami et al., 2015). Thus, changes in the composition of plant root exudates have an impact on the microbial community succession in the associated rhizosphere (Rasch et al., 2006; Micallef et

al., 2009; Halder & Sengupta, 2015; Musyoki et al., 2016). As the plant grows and the root system develops, the more favourable microorganisms that make optimal use of the rhizospheric constituents establish themselves (Di Cello et al., 1997; Musyoki et al., 2016).

7.3 Rooting enhancement of rhizospheric bacteria

To exploit rhizospheric microorganisms for their potential to enhance the rooting of *Eucalyptus* hybrid cuttings, rhizospheric microorganisms were isolated from *Eucalyptus* rhizospheres and applied as plant growth promoting treatments to the cuttings of two *Eucalyptus grandis* × *Eucalyptus nitens* hybrid clones. This necessitated the isolation, characterisation and up-scaling of rhizospheric bacterial cultures in order to exploit their potentials as plant growth promoting treatments.

The preparation of bacterial inoculums involves the up-scaling of rhizospheric bacterial cultures. Prior to the preparation of the bacterial inoculums, a search of the literature was undertaken to ascertain at which concentration(s) the inoculums should be prepared. This search revealed no studies in which the selected species were used as rooting enhancing agents of *Eucalyptus* cuttings. However, Teixeira et al. (2007) and Diaz et al. (2009) had applied other bacterial strains as rooting enhancing agents on *Eucalyptus* cuttings at a concentration of 10^8 cfu/ml. Teixeira et al. (2007) and Diaz et al. (2009) suggested that 10^8 cfu/ml was the preferred concentration at which a bacterial inoculums should be applied to the rooting medium of *Eucalyptus* cuttings. It was thus necessary to construct growth curves for each of the seven bacterial species in order to ascertain when in the growth cycle the preferred concentration had been reached so that the bacteria could be harvested and used for the preparation of the inoculums for the nursery experiments. The growth curve investigation revealed that not all of the seven bacterial species could attain a growth concentration of $\approx 10^8$ cfu/ml, but all of the species attained a concentration of $\approx 10^7$ cfu/ml within 7 to 10 hours of incubation. For this reason, the bacterial species were harvested at $\approx 10^7$ cfu/ml and used to prepare

the inoculums. This concentration is also regarded as a suitable concentration according to Zaspel and Ewald (2001).

Of the 32 bacterial strains that were isolated, 12 were unique species belonging to eight different genera. Of the 12 species, seven demonstrated the ability to produce indole-3-acetic acid and to solubilise phosphates. Three of these species were from the genus *Pseudomonas* and one from the genus *Bacillus*, both genera known for improving plant growth, and also to stimulate adventitious root development in *Eucalyptus* cuttings (Teixeira et al., 2007; Diaz et al., 2009). The species of the *Bacillus* and *Pseudomonas* were combined into one rooting treatment, while the other isolated bacterial species were combined into another rooting treatment. These two rhizospheric rooting treatments were compared to the standard of general practice in the nursery, which was the application of indole-3-butyric acid (control), and to a commercial product containing live fungus of *Trichoderma harzianum*.

All treatments in this study demonstrated high survival rates and high rooting percentages. The nursery standard outperformed all treatments, although mostly marginally so. The rhizospheric rooting treatment comprising of the non-*Pseudomonas-Bacillus* bacteria, as well as fungus treatment, were closest in performance to the nursery standard, while the *Pseudomonas-Bacillus* treatment often showed lower values when compared to the other treatments. The *Pseudomonas-Bacillus* bacterial treatment appeared to improve fibrosity of the rooting architecture of cuttings of the GN 018B hybrid. This outcome could be of value when rooted cuttings are planted out in plantations and requires further investigation to ascertain what other rooting enhancement traits these bacterial species possess (Egamberdieva, 2011; Brondani et al. 2012). This is because increased rooting percentages of *Eucalyptus* cuttings will not only mitigate against the financial losses, which is currently experienced by *Eucalyptus* commercial nursery growers, but will also allow for the maximum use of the limited space in the nursery environment.

A noteworthy outcome of this study was the finding that extensive genotypic differences existed between the two *Eucalyptus grandis* × *Eucalyptus nitens* hybrid clones. Although this was an expected outcome (Brondani et al., 2012; Halder & Sengupta, 2015), it was not expected that two clones would exhibit significant differences in their responses to the different growth and rooting parameters, because the clones were derived through hybridisation between the same two species. These genotypic differences in adventitious root development supports the findings of Brondani et al. (2012) with the clones of *Eucalyptus benthamii* × *Eucalyptus dunnii.*

7.4 Conclusions

This study provided some insight and understanding of the diversity of rhizospheric microbial communities of *Eucalyptus* hybrids and their evolution over time. This knowledge could be of value when formulating treatments of potential rhizospheric bacterial preparations for the enhancement of rooting of *Eucalyptus* cuttings. This first attempt to isolate and apply rhizospheric bacteria as root stimulating treatment revealed some shortcomings that requires further investigation. In particular, knowledge of other root promoting traits of the different species of rhizospheric bacteria should be obtained to select more appropriate potential root stimulating rhizospheric bacterial species. Furthermore, rhizospheric bacterial species as root promoting treatment also requires experimentation of application protocols. In particular, single bacterial species applications should be compared to multiple species applications, together with assessing various practical application methods. These include the timing of the application of a treatment, as well as the number of application of treatment, single or double applications (Diaz et al., 2009; Brondani et al., 2012), the concentration of the bacterial treatment (Ludwig-Müller et al., 2005; Brondani et al., 2012; Spassin et al., 2016), as well as the method of application of treatment, directly or through the watering system, requires study. Also, the impact of these treatments on tree growth and timber yields is an important

aspect that should be investigated by future researchers. In conclusion the importance of genotypic differences should be noted when developing new biotechnological applications.

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Appendix

FAME results

SGS

Test Report

SGS Agri Food Laboratory
2nd Floor Panther Park
11 Berkley Road
Maitland, Cape Town

Phone: +27 21 506 4931
Fax: +27 866885597
Web: www.sgs.com
Email: chricilia.jordaan@sgs.com

Customer name	**CENTRAL UNIVERSITY OF TECHNOLOGY**
Customer address:	Central University
	ZQ President Brand Street
	Bloemfontein
Contact person:	Melanie Patrick
Issue date:	31 October 2013
Quote no:	Q1309454
Order no:	E163802
Date received:	16 October 2013
Date started:	25 October 2013
Date completed:	31 October 2013
Sample type:	**SOIL**
No. of samples:	12
Condition of sample:	Room temperature. No deviation from standard
Sample Identification:	As per page 2 to 4

SAMPLE IDENTIFICATION					
Samples:					
01	5.1 +- 10g	**05**	5.5 +-10g	**09**	3m.3 +-10g
02	5.2 +- 10g	**06**	5.6 +- 10g	**10**	3m.4 +- 10g
03	5.3 +- 10g	**07**	3m.1 +- 10g	**11**	3m.5 +- 10g
04	5.4 +- 10g	**08**	3m.2 +- 10g	**12**	3m.6 +- 10g

Laboratory Manager
C Jordaan

Lab ID: 13100081

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SGS

Test Report

13100081	AOCS Ce 2-66 Fatty Acid Profile		01	02	03	04
C4:0	Butyric Acid	%m/m	nd	nd	nd	nd
C6:0	Caproic Acid	%m/m	nd	nd	nd	0,4
Unknown		%m/m	nd	3,5	3,0	0,8
C8:0	Caprylic Acid	%m/m	nd	nd	nd	0,5
Unknown		%m/m	nd	5,3	1,3	nd
Unknown		%m/m	nd	1,9	1,9	nd
C10:0	Capric Acid	%m/m	0,7	nd	nd	0,6
C11:0	Undecanoic Acid	%m/m	nd	nd	nd	nd
C12:0	Lauric Acid	%m/m	1,2	nd	nd	0,7
C13:0	Tridecanoic Acid	%m/m	nd	1,6	2,0	nd
C14:0	Myristic Acid	%m/m	4,0	1,8	1,6	2,3
Unknown		%m/m	nd	nd	1,2	0,4
Unknown		%m/m	nd	1,5	1,9	0,5
Unknown		%m/m	1,7	3,0	3,1	1,3
C14:1	Myristoleic Acid	%m/m	0,6	nd	nd	nd
C15:0	Pentadecanoic Acid	%m/m	nd	1,2	1,4	0,8
C15:1	Pentadecenoic Acid	%m/m	nd	nd	nd	0,4
C16:0	Palmitic Acid	%m/m	27,0	22,4	24,9	27,4
Unknown		%m/m	1,3	nd	nd	0,4
C16:1	Palmitoleic Acid	%m/m	1,0	3,0	2,8	1,1
C17:0	Heptadecanoic Acid	%m/m	nd	nd	nd	nd
Unknown		%m/m	1,6	3,1	2,8	1,2
C17:1	Heptadecenoic Acid	%m/m	nd	nd	nd	nd
C18:0	Stearic Acid	%m/m	22,0	10,2	12,9	14,8
C18:1n-9t	Elaidic Acid	%m/m	2,5	5,7	5,5	2,4
C18:1n-9c	Oleic Acid	%m/m	14,6	8,6	12,5	20,6
C18:2n-6t	Linolelaidic Acid	%m/m	nd	nd	nd	nd
C18:2n-6c	Linoleic Acid	%m/m	5,7	9,1	9,5	6,7
C18:3n-6	g - Linolenic Acid (GLA)	%m/m	nd	nd	3,5	nd
Unknown		%m/m	2,1	nd	nd	nd
C18:3n-3	a - Linolenic Acid (ALA)	%m/m	1,5	4,3	3,5	2,3
C18:4n-3	Stearidonic Acid	%m/m	nd	nd	nd	nd
C20:0	Arachidic Acid	%m/m	2,6	nd	nd	2,2
C20:1n-9	Eicosenoic Acid	%m/m	nd	nd	nd	nd
C20:2n-6	Eicosadienoic Acid	%m/m	nd	4,7	4,0	1,8
C20:3n-6	d-g-Linolenic Acid	%m/m	nd	nd	nd	nd
C20:3n-3	Eicosatrienoic Acid	%m/m	nd	nd	nd	nd
C20:4n-6	Arachidonic Acid	%m/m	nd	2,6	nd	nd
C20:5n-3	Eicosapentaenoic Acid (EPA)	%m/m	nd	nd	2,1	nd
C21:0	Heneicosanoic Acid	%m/m	nd	nd	nd	nd
Unknown		%m/m	2,5	nd	nd	nd
C22:0	Behenic Acid	%m/m	2,7	nd	nd	3,7
C22:1n-9	Erucic Acid	%m/m	nd	3,5	nd	nd
C22:2n-6	Docosadienoic Acid	%m/m	nd	nd	nd	2,0
C22:4n-6	Adrenic Acid	%m/m	nd	nd	nd	nd
C22:5n-3	Docosapentaenoic Acid (DPA)	%m/m	nd	nd	nd	0,7
C22:6n-3	Docosahexaenoic Acid (DHA)	%m/m	nd	nd	nd	nd
C23:0	Tricosanoic Acid	%m/m	nd	nd	nd	nd
C24:0	Lignoceric Acid	%m/m	4,2	nd	nd	2,7
C24:1n-9	Nervonic Acid	%m/m	nd	nd	nd	nd

nd: Not detected

Laboratory Manager
C Jordaan

Lab ID: 13100081

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SGS

Test Report

13100081	AOCS Ce 2-66 Fatty Acid Profile		05	06	07	08
C4:0	Butyric Acid	%m/m	nd	nd	nd	nd
C6:0	Caproic Acid	%m/m	nd	nd	nd	0,2
Unknown		%m/m	nd	nd	nd	0,3
C8:0	Caprylic Acid	%m/m	nd	nd	nd	0,4
Unknown		%m/m	nd	nd	nd	nd
Unknown		%m/m	nd	nd	nd	nd
C10:0	Capric Acid	%m/m	nd	nd	nd	0,4
C11:0	Undecanoic Acid	%m/m	nd	nd	nd	nd
C12:0	Lauric Acid	%m/m	nd	1,3	0,5	0,7
C13:0	Tridecanoic Acid	%m/m	nd	1,6	2,0	nd
C14:0	Myristic Acid	%m/m	1,3	3,1	2,0	2,4
Unknown		%m/m	nd	nd	nd	1,6
Unknown		%m/m	2,4	4,9	2,6	0,9
Unknown		%m/m	1,1	0,9	nd	0,2
C14:1	Myristoleic Acid	%m/m	nd	1,0	0,9	nd
C15:0	Pentadecanoic Acid	%m/m	nd	0,8	nd	0,8
C15:1	Pentadecenoic Acid	%m/m	nd	nd	nd	0,4
C16:0	Palmitic Acid	%m/m	30,0	19,9	17,1	12,5
Unknown		%m/m	nd	nd	2,0	1,6
C16:1	Palmitoleic Acid	%m/m	2,1	1,8	3,1	0,7
C17:0	Heptadecanoic Acid	%m/m	nd	nd	1,6	1,5
Unknown		%m/m	1,9	3,3	1,6	0,7
C17:1	Heptadecenoic Acid	%m/m	nd	nd	nd	nd
C18:0	Stearic Acid	%m/m	17,6	14,0	18,7	31,1
C18:1n-9t	Elaidic Acid	%m/m	4,0	3,3	2,9	2,3
C18:1n-9c	Oleic Acid	%m/m	16,8	14,7	11,4	11,0
C18:2n-6t	Linolelaidic Acid	%m/m	nd	nd	nd	0,4
C18:2n-6c	Linoleic Acid	%m/m	9,6	8,0	5,3	3,4
C18:3n-6	g - Linolenic Acid (GLA)	%m/m	nd	nd	nd	nd
Unknown		%m/m	2,1	2,8	3,1	2,7
C18:3n-3	a - Linolenic Acid (ALA)	%m/m	1,5	2,3	1,9	nd
C18:4n-3	Stearidonic Acid	%m/m	nd	nd	nd	nd
C20:0	Arachidic Acid	%m/m	2,8	3,9	1,9	3,0
C20:1n-9	Eicosenoic Acid	%m/m	nd	nd	nd	nd
C20:2n-6	Eicosadienoic Acid	%m/m	3,6	3,6	3,1	0,8
C20:3n-6	d-g-Linolenic Acid	%m/m	nd	nd	nd	nd
C20:3n-3	Eicosatrienoic Acid	%m/m	nd	nd	nd	nd
C20:4n-6	Arachidonic Acid	%m/m	nd	nd	nd	0,9
C20:5n-3	Eicosapentaenoic Acid (EPA)	%m/m	nd	nd	2,9	nd
C21:0	Heneicosanoic Acid	%m/m	nd	nd	nd	nd
Unknown		%m/m	nd	nd	nd	nd
C22:0	Behenic Acid	%m/m	nd	4,1	1,9	3,3
C22:1n-9	Erucic Acid	%m/m	nd	nd	2,1	nd
C22:2n-6	Docosadienoic Acid	%m/m	nd	nd	nd	2,7
C22:4n-6	Adrenic Acid	%m/m	nd	nd	nd	nd
C22:5n-3	Docosapentaenoic Acid (DPA)	%m/m	nd	nd	nd	0,7
C22:6n-3	Docosahexaenoic Acid (DHA)	%m/m	nd	nd	0,4	nd
C23:0	Tricosanoic Acid	%m/m	nd	nd	nd	nd
C24:0	Lignoceric Acid	%m/m	1,3	4,5	2,3	5,2
C24:1n-9	Nervonic Acid	%m/m	nd	nd	2,0	nd

nd: Not detected

Laboratory Manager
C Jordaan

SGS

Test Report

13100081	AOCS Ce 2-66 Fatty Acid Profile		09	10	11	12
C4:0	Butyric Acid	%m/m	nd	nd	nd	nd
C6:0	Caproic Acid	%m/m	nd	nd	nd	0,2
Unknown		%m/m	nd	nd	nd	0,3
C8:0	Caprylic Acid	%m/m	nd	0,2	nd	0,4
Unknown		%m/m	nd	0,9	nd	nd
Unknown		%m/m	nd	0,1	nd	nd
C10:0	Capric Acid	%m/m	nd	nd	nd	nd
C11:0	Undecanoic Acid	%m/m	nd	nd	nd	nd
C12:0	Lauric Acid	%m/m	1,4	0,3	0,3	0,6
C13:0	Tridecanoic Acid	%m/m	nd	0,1	nd	nd
C14:0	Myristic Acid	%m/m	6,9	3,5	1,5	1,6
Unknown		%m/m	0,6	0,4	nd	1,5
Unknown		%m/m	1,9	0,7	2,3	2,2
Unknown		%m/m	0,9	2,3	nd	1,0
C14:1	Myristoleic Acid	%m/m	0,4	0,7	1,0	0,9
C15:0	Pentadecanoic Acid	%m/m	0,5	0,7	0,8	nd
C15:1	Pentadecenoic Acid	%m/m	nd	nd	nd	nd
C16:0	Palmitic Acid	%m/m	13,9	17,5	12,0	14,3
Unknown		%m/m	2,2	1,6	1,7	2,5
C16:1	Palmitoleic Acid	%m/m	1,7	1,9	0,9	2,1
C17:0	Heptadecanoic Acid	%m/m	1,4	1,3	1,6	1,5
Unknown		%m/m	0,7	0,4	3,0	1,4
C17:1	Heptadecenoic Acid	%m/m	nd	nd	1,6	nd
C18:0	Stearic Acid	%m/m	23,9	24,3	27,8	20,8
C18:1n-9t	Elaidic Acid	%m/m	1,9	1,6	2,0	2,6
C18:1n-9c	Oleic Acid	%m/m	14,3	11,8	13,3	13,8
C18:2n-6t	Linolelaidic Acid	%m/m	nd	0,3	nd	0,4
C18:2n-6c	Linoleic Acid	%m/m	3,6	2,1	3,1	5,2
C18:3n-6	g - Linolenic Acid (GLA)	%m/m	nd	nd	nd	nd
Unknown		%m/m	3,2	2,6	2,4	2,8
C18:3n-3	a - Linolenic Acid (ALA)	%m/m	0,6	0,3	nd	0,8
C18:4n-3	Stearidonic Acid	%m/m	nd	nd	nd	nd
C20:0	Arachidic Acid	%m/m	2,6	2,8	5,3	2,6
C20:1n-9	Eicosenoic Acid	%m/m	nd	nd	nd	nd
C20:2n-6	Eicosadienoic Acid	%m/m	0,6	0,7	nd	1,4
C20:3n-6	d-g-Linolenic Acid	%m/m	nd	nd	nd	nd
C20:3n-3	Eicosatrienoic Acid	%m/m	nd	nd	nd	nd
C20:4n-6	Arachidonic Acid	%m/m	nd	0,4	nd	0,9
C20:5n-3	Eicosapentaenoic Acid (EPA)	%m/m	0,6	0,6	nd	nd
C21:0	Heneicosanoic Acid	%m/m	nd	nd	nd	nd
Unknown		%m/m	nd	nd	nd	nd
C22:0	Behenic Acid	%m/m	4,0	3,2	1,9	3,7
C22:1n-9	Erucic Acid	%m/m	nd	nd	nd	nd
C22:2n-6	Docosadienoic Acid	%m/m	3,0	nd	4,5	2,7
C22:4n-6	Adrenic Acid	%m/m	nd	nd	nd	nd
C22:5n-3	Docosapentaenoic Acid (DPA)	%m/m	nd	nd	nd	nd
C22:6n-3	Docosahexaenoic Acid (DHA)	%m/m	nd	2,1	0,4	nd
C23:0	Tricosanoic Acid	%m/m	nd	0,9	1,4	nd
C24:0	Lignoceric Acid	%m/m	5,0	4,3	8,5	4,9
C24:1n-9	Nervonic Acid	%m/m	nd	0,5	2,0	nd

nd: Not detected

Laboratory Manager
C Jordaan