

Identification of rhizospheric microorganisms associated with sorghum

By

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Abstract

Approximately 50% of sorghum (*Sorghum bicolor* (L.) Moench) produced globally is used as human food, with 95% of its total consumption occurring in Africa. Unfortunately, sorghum crops are prone to pathogenic attack, notably leading to a reduction in production yields. Generally, chemical agents are used as fertilizers and/or biocides to increase crop production. However, these chemicals can have a detrimental environmental impact including the eutrophication of fresh water and marine ecosystems. Thus, there is increased interest in plant growth promoting rhizobacteria (PGPR), as an alternative to chemicals, to facilitate eco-friendly biological control of soil-borne pathogens. PGPRs colonize the plant root system (i.e rhizosphere and rhizoplane) and promote growth and production yields essentially via the biological control of plant pathogens and their role in the nutrient cycles (e.g N fixation). The aim of this study is to characterize the microbial communities associated with sorghum in South Africa, and to identify common bacteria which could further be developed and applied to improve sorghum growth and yield. Sorghum rhizospheric environments (rhizoplane and rhizosphere) were collected from three sites characterized by different agricultural practices (Free State, Limpopo and North West). Denaturing gradient gel electrophoresis (DGGE) and Terminal-restriction fragment length polymorphism (T-RFLP) were used to identify microbial community molecular fingerprints. Sorghum-associated microbial communities were found to be different in all rhizospheric soil samples which could be explained by differences in soil chemistry, agricultural practices and geographical location. The analyses also clearly demonstrated that the sorghum bacterial community structures were similar in the rhizoplane, indicating the strong influence that the sorghum plant has in determining the rhizoplane colonizers. The archaeal community structure from rhizoplane and rhizosphere in each sampling site were dissimilar, which could be explained by differences in soil type and/or agricultural practices. Both the T-RFLP and DGGE analyses revealed that *Bacillus* sp. were consistently associated with South African Sorghum, *Arthrobacter* sp. were detected in the rhizoplane, while Uncultured archaea were detected in the rhizoplane of sorghum. These microorganisms represent valuable targets for engineering to promote growth and yield in sorghum.

Declaration

I declare that **Identification of rhizospheric microorganisms associated with sorghum** is my work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Mr Tshabuse Freedom

January 2012



Publications originating from this thesis

Poster/Presentation

1. **Tshabuse F., Bopda W., Ramond J-B., Cowan D.A., Tuffin I.M. (2011).** Characterisation of sorghum-associated microbial communities in and around South Africa. *SASM 2011*. Cape Town: South Africa. **Talk**
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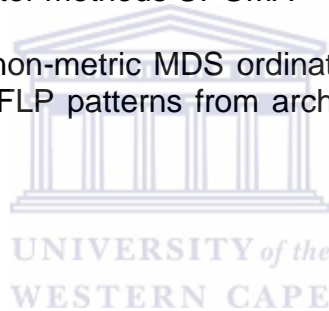
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Dedication

This thesis is dedicated to my Family, Thanks for believing in me.



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Chapter 1: Literature Review

1. Introduction

1.1 Sorghum overview

Sorghum is an annual grass belonging to the Poaceae family, the Andropogoneae tribe, subtribe Sorghinae, and the genus *Sorghum* Moench (Dillon *et al.*, 2007; Dicko *et al.*, 2006). The *Sorghum* genus consists of 25 species (USDA ARS, 2007), and is sub-separated into five taxonomic subgenera, namely: *Eu-sorghum*, *Chaetosorghum*, *Heterosorghum*, *Para-sorghum* and *Stiposorghum* (Dillon *et al.*, 2007). The sub-genera *Eu-sorghum* contains all cultivated sorghum races and varieties, as well as the wild and weed species, and is further classified into five botanical races, namely: *bicolor*, *candatum*, *durra*, *guinea* and *kafir* (Deu *et al.*, 2008; Dillon *et al.*, 2007). All *Sorghum bicolor* subsp. *bicolor* have $2n=2x=20$ chromosomes, and are described as having thick culms up to 6m in height, often branched with many tillers (Figure 1.1; Dillon *et al.*, 2007). Sorghum was firstly domesticated in Africa between 5000 and 7000 years ago (ICRISAT, 2005), and has spread via the movement of people and trade routes into India, China and eventually the United State of America (USA; Subdhi and Nguyen, 2000).



Figure 1.1: Picture of cultivated *Sorghum bicolor* (L) Moench. Sorghum varies between 0-6 m in height and has deep and spread roots with solid stem. The leaves are long and wide, with flat or wavy margins. The grain or cypse is rounded and bluntly pointed, from 4-8 mm in diameter and vary in size, shape and colour (Dicko et al., 2006).

Sorghum is the world's fourth most cultivated cereal crop after wheat, rice and maize (Smith and Frederiksen, 2000), with a global production of 60 million tons and a total cultivated area of 46 million hectares (FAO, 2005). Nigeria, the USA and India are the biggest sorghum producers in the world (Table 1.1; Dicko *et al.*, 2006). The African continent produces about 20 million tons of sorghum annually (FAO, 2005). Free State and Mpumalanga provinces account for 57% and 24% of sorghum produced, respectively (Figure 1.2). Of the sorghum produced in South African, 48% is used for human consumption, 2.2% for animal feed, 6.5% for industrial production such as beverages (Awika and Rooney, 2004), and the remainder (43%) is exported to other countries. A total production of 328 300 tons of sorghum is projected for the 2010/2011 marketing season in South Africa (FAO/WFP, 2010).

Table 1.1: World sorghum production 2007-2008

Country	Production (1000 tonnes)	Total production (%)
United State	12.827	20
Nigeria	10	16
India	7.78	12
Sudan	4.5	7
Ethiopia	3.23	5
Argentina	2.9	5
Australia	2.691	4
China	1.9	3
Burkina Faso	1.8	3
Brazil	1.7	3
Other Countries	6.88	12
Total	62.308	100

Source: ICRSAT, 2005

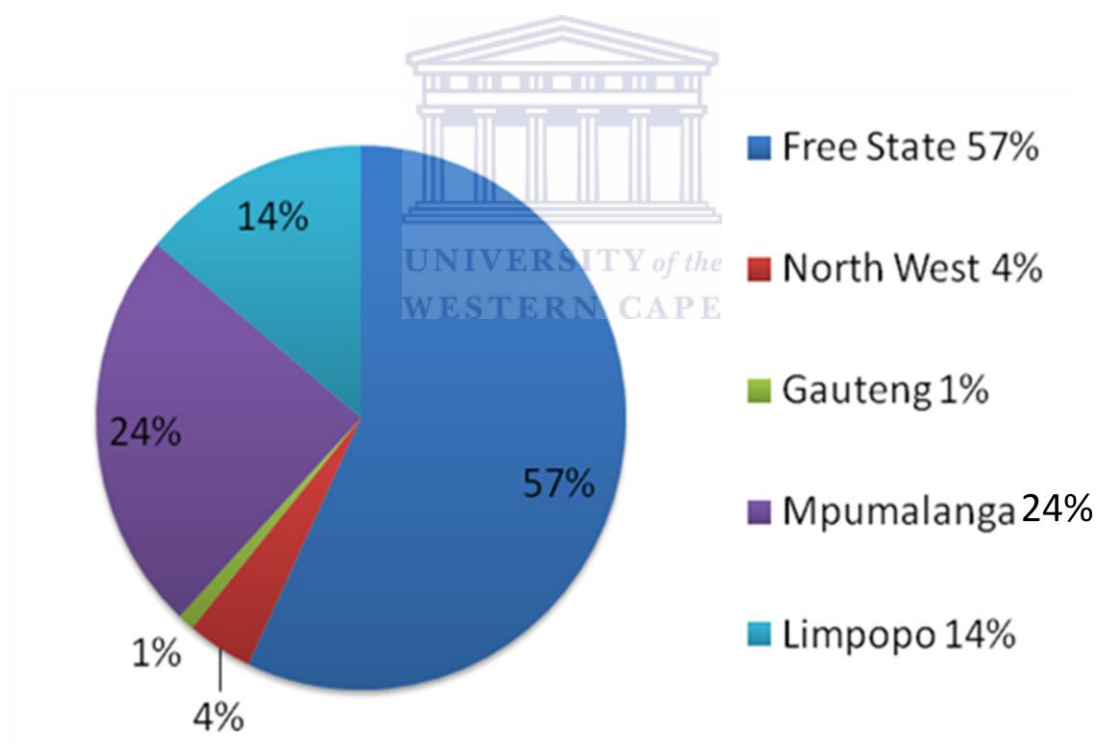


Figure 1.2: South African sorghum grain production by provinces 2007/2008 (Source: Grain sorghum, 2010/11)

According to the U.S National Sorghum Producers Association (2006), approximately 50% of the global produced sorghum is used as human food, with

95% of its total food use occurring in Africa and Asia (FAO, 1995). In Mexico, United States, Japan and Commonwealth of Independent States the use of sorghum as animal feed is successful because fodder processed from sorghum has high digestibility (Oliver *et al.*, 2004). Sorghum grain is a staple diet in Africa, the Middle East, Asia and Central America (Dicko *et al.*, 2006), and is consumed in forms such as porridge, steam-cooked, tortillas, baked goods and as beverages (CGIAR, 2009). In South Africa, sorghum is used to make the following products (Table 1.2): (i) Commercial malt: Sorghum is saturated and left to germinate. After germination, it is dried, packaged and sold for home brewing beer. Equal amounts of maize and commercial malt are then mixed with water and yeast to brew beer with an alcohol content of generally between 1-2%, (ii) Industrial malt: Germinated sorghum is soaked in water and then dried. The malt is used for production of sorghum beer with an alcohol content of about 3%, (iii) Sorghum flour, also known as *mabele*, is a very popular breakfast cereal. It is processed in the same way as maize during the dry milling process, and (iv) Sorghum as animal feed: Sorghum is a component of poultry, pet, pigeon and ostrich feeds.

Table 1.2: Total sorghum consumption in South Africa (tons)

Marketing year	Sorghum produced	Total food	Total feed	Industrial malt	Commercial malt	Total consumption
2001/02	302 100	189 900	16 200	28 700	84 300	206 100
2002/03	319 000	174 400	21 900	20 500	74 900	196 300
2003/04	288 000	168 900	10 100	21 100	73 700	179 000
2004/05	422 700	179 000	10 000	25 600	76 700	189 000
2005/06	437 400	190 900	12 000	24 600	87 900	202 900

Source: Grain sorghum, 2010/11

Agriculture remains the backbone of the Africa's economy, with about 70 % of Africans depending mainly on agriculture for their livelihood (FAO/WFP, 2010/11). Thus, the use of sorghum for human consumption is the driving force behind increasing its production (FAO, 1995). However, diseases caused by fungal pathogens and the unavailability of essential nutrients needed for plant growth are some of the major causes that affect growth and development, resulting in low sorghum yield (Davis and Bockus, 2001; Rodriguez and Fraga, 1999).

1.2 Soil ecosystems

Soils are structured media where the mineral and organic matter components are organized into aggregates that vary in size, porosity, continuity and composition (Hansel *et al.*, 2008). Soils are made up of four basic components: minerals (~45%), water (25%), air (25%) and organic matter (2%-5%). The mineral portion consists of three general groups of particles: sandy, silt, and clay (Sullivan, 2004): Sand and silt particles, which largely consist of quartz, contain no plant nutrients and thus are unnecessary for plant growth. Clay particles contain appreciable amounts of plant nutrients and are thus most productive (Sullivan, 2004). The relative proportions of these particles determine the soil texture, an important characteristic of soil (Table 1.3; Hansel *et al.*, 2008). For example, a loam soil contains sand, silt and clay in roughly equal proportions, a sandy loamy soil contains large amounts of sand with little clay, while a clay loam soil contains large amounts of clay and small amounts of sand (Table 1.3; Sullivan, 2004).

Table 1.3: Soil types and their characteristics

Soil type	Sand %	Silt %	Clay %
Sands	85+	–	0-10
Loamy sands	70-90	–	0-15
Sandy loams	43-85	–	0-20
Silt	–	80+	0-12
Silt loams	–	50-88	0-27
Loams	0-52	0-50	7-27
Sandy clay loams	45+	0-28	20-35
Clay loams	0-45	0-53	27-40
Silty clay loams	0-20	40+	27-40
Sandy clays	45+	0-20	35-55
Silty clays	–	40+	40+
Clays	0-45	0-40	40+

Source: Kinchloe, 2010

Soils are also characterized by their structure; i.e, the clumping together or aggregation of sand, silt and clay particles into secondary clusters such as aggregates (crumbs and granular units), blocks (large clods), platelets (resulting from sealing process), and prismatic structures (in sodic soils) (Blackwood *et al.*, 2006). Soil aggregates are formed by minerals, soil organic matter, fungal hyphae, roots and plant debris (Rillig and Mummey, 2006; Young and Ritz, 2000). Aggregates are the most beneficial secondary units of soil structure as they are determinant factors for the spatialization of soil microbiota (Blackwood *et al.*, 2006). The size of aggregates ranges from less than 50 μm to 4 mm, with their stability depending on their size and biological activities (Blackwood *et al.*, 2006). The most stable aggregates are found in the rhizospheric environment of graminaceous species, as plant exudates and microbial products provide agents for aggregation (Kandeler and Murer, 1993).

Because of the physical and chemical effects of rooting, the soil profile has been divided into the upper part, the solum, and the lower part, the parent material (Richter *et al.*, 2007). The solum consists of O, A, B horizons that are intensively affected by rooting and microbial activities. The parent material (C horizon) has very little biological activity (Figure 1.3; Richter *et al.*, 2007).

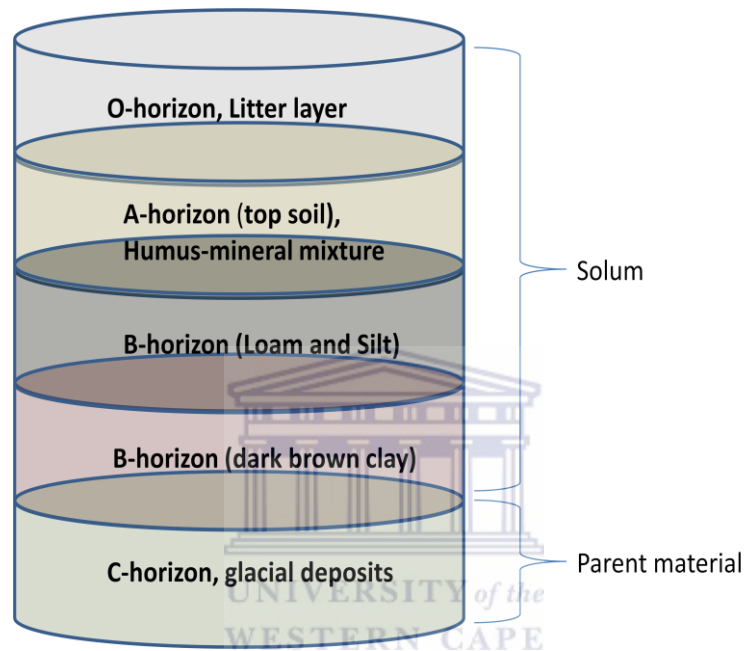


Figure 1.3: The vertical layered structure of soil, showing the four main horizons in a soil profile.

1.3 The root system

The root system is the “hidden half” of a plant. It is characterized by its important plasticity due to the continual propagation of new meristems, which are tissues consisting of undifferentiated cells found in zones where growth takes place (Bias *et al.*, 2006; Williamson *et al.*, 2001). In cereals such as sorghum, the root system is comprised of primary as well as adventitious roots, lateral roots and root hairs (Figure 1.4; Williamson *et al.*, 2001):

(i) Lateral roots develop from the pericycle layer deep within the parent root tissues. Their development is initiated in the differentiation zone of the root, where the pericycle is not actively dividing (Malamy and Benfey, 1997). The stimulated pericycle can either dedifferentiate (lose its immature characteristics) and proliferate to form a lateral root primordium (LRP) or redifferentiate to form a lateral root meristem, which establishes and perpetuates the organization of the lateral roots (Malamy and Benfey, 1997). This formation of lateral roots is a common characteristic of all root types and is responsible for much of a plants water and nutrient uptake (Hochholdinger *et al.*, 2004).



(ii) Root hairs are long tubular-shaped extensions from the lateral roots, ranging from about 70 μm to as small as 20 μm in diameter, and about 10 mm long (McCully, 1999). They can develop as second- or higher-order branches from other root hairs. The root hair has a very small stele (a central part of root), as narrow as 2.4 μm , surrounded by an epidermis (a cortex reduced to a hypodermis) and an endodermis (each with casparian strips) (Briggs and Ashford, 2001). The root hairs facilitate the uptake of immobilized nutrients by allowing the root to explore greater volumes of soil, thereby increasing the absorptive surface area of roots (Ma *et al.*, 2001). Phosphorus is the most acquired nutrient by root hairs because of the ^{32}P depletion zone around the root hairs (Ma *et al.*, 2001).

In addition to its roles of providing mechanical support and allowing water and nutrients uptake, plant roots also perform specialized roles such as the synthesis, accumulation and secretion of diverse compounds called root exudates (Bertin *et al.*, 2003; Walker *et al.*, 2003).

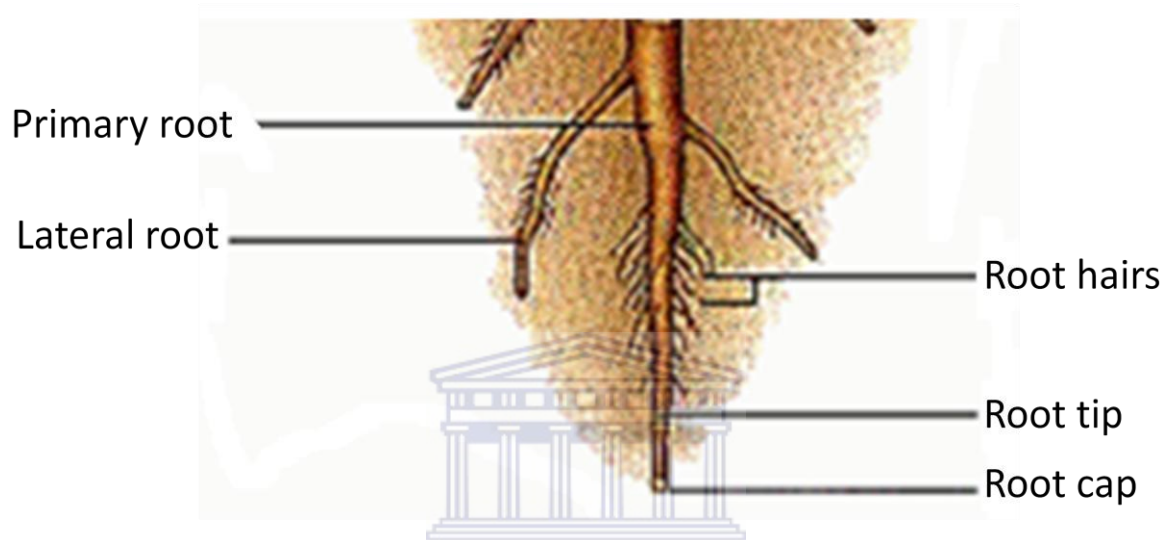


Figure 1.4: Representation of a plant root. The architectural patterns of a typical plant root showing the primary, lateral and root hairs.

Root exudates are composed of mucilage (gelatinous substance), root border cells, extracellular enzymes, simple and complex sugars, phenolics, amino acids, vitamins, organic acids, nitrogenous macromolecules (e.g, purines or nucleosides), and inorganic and/or gaseous molecules (HCO_3^- , OH^- , H^+ , CO_2 and H_2 ; Table 1.4; Nannipieri *et al.*, 2007; Walker *et al.*, 2003). The root exudates are released through the mechanism of rhizodeposition, which depends on plant genotype, age, and nutritional status as well as mycorrhizal fungi colonization (Wichern *et al.*, 2008). Rhizodeposition in many plants is achieved through diffusion process: low molecular weight root exudates (e.g, amino acids, sugars, carboxylic acids and phenolics) are released through this process. The diffusion of these compounds is dependent on

the physiological state of the root cell and the polarity of the compound to be exuded (Compant *et al.*, 2005). Although the functions of most root exudates are unknown, several compounds have been characterized and have known functions (Table 1.4): (i) to defend the rhizosphere against pathogenic microorganisms (e.g, root border cells), (ii) to maintain root-soil contact (e.g, mucilages), (iii) to chelate poorly soluble mineral nutrients (e.g, through the production of phytosiderophores), (iv) to catalyse nutrient release from organic molecules (e.g, enzymes) (Walker *et al.*, 2003; Neumann and Romheld, 2001; Hawes *et al.*, 2000).

Table 1.4: Functional role of root exudates

Component	Examples	Rhizosphere function
Phenolics	Flavanol, flavones, flavanones anthrocyanins, isoflavonoids and acetosyringone	Nutrients source, Chemoattractant signal to microbes Microbial growth promotion, <i>nod gene</i> inducer in rhizobia Resistance inducers against phytoalexins, Chelators of poorly soluble mineral nutrients Detoxifiers of aluminium Phytoalexins against soil pathogens
Organic acids	Acetic acid, Aconitic acid, Aldonic acid, Butyric acid, Erythronic acid and Fumaric acid	Nutrients source, Chemoattractant signal to microbes Chelators of poorly soluble mineral nutrients Acidifiers of soil Detoxifiers of aluminium and <i>node gene</i> inducers
Phytosiderophores	Bacteriocin	Chelators of poorly soluble mineral nutrients chemoattractant signals to microbes
Vitamins	Biotin, Niacin, Pantothenate, Riboflavin and Thiamie	Nutrient source
Nucleotides	Adenine, Cystidine, Gaunine and Uridine	Nutrient source
Enzymes	Amylase, Innvertase, Protease and Phosphatase	Catalysis for P release from organic molecules Biocatalysis for organic matter transformation in soil
Root border cells	root apical meristem	Produce signals that control mitosis and gene expression Stimulate microbial growth Release chemoattractants, mucilages and proteins Synthesize defence molecules for the rhizosphere Acts as decoys that keep root cap infectiuos free
Phytohormones	Auxins, Ethylene, Gibberellins and Cytokinins	Plant growth promotion

source: Faure *et al.*, 2008

The overall function of root exudates gives rise to a specialized biological niche called the rhizosphere. The term “rhizosphere” was firstly introduced in 1904, and can be defined as the volume of soil adjacent to and influenced by the living plant roots (Morgan *et al.*, 2005; Baudoin *et al.*, 2002). The rhizosphere environment is divided into three distinct ecological niches: (i) the ectorhizosphere, which is the soil influenced by the plant roots, (ii) the rhizoplane, which is the soil directly in contact with the plant root surface and, (iii) the root itself (endorhizosphere), which is the intercellular spaces or vascular tissues inside the plant root (Figure 1.5; Czaban, 2007; Morgan, 2005).

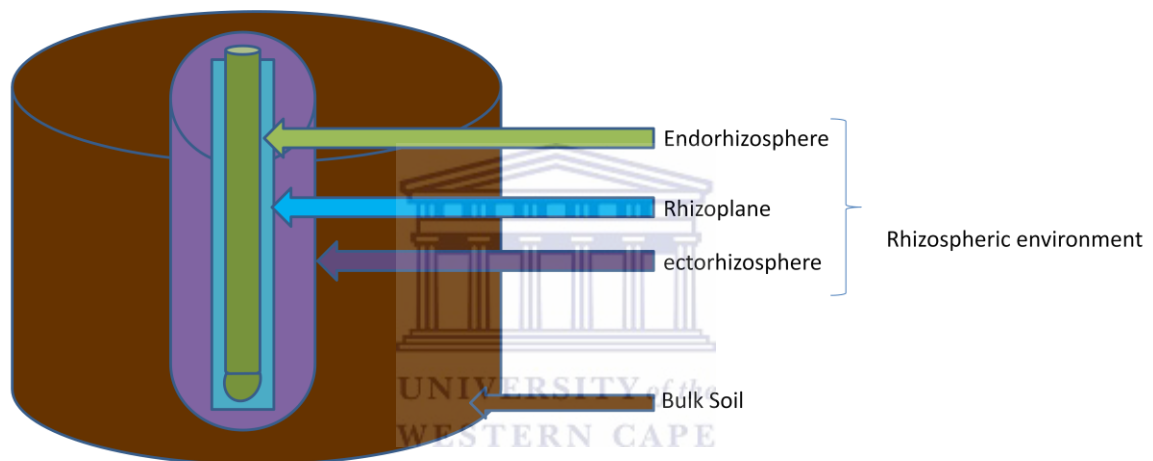
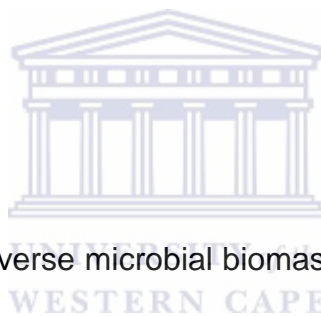


Figure 1.5: Diagrammatic representation of the rhizospheric environment. Distinct biological niches that can be habitable by microorganisms are shown.

Important and intensive interactions take place in the rhizosphere between the soil, the plant, the microorganisms and the soil fauna, which can all significantly influence plant growth and crop yields (Antoun and Prevost, 2005). These rhizospheric interactions transform the rhizospheric environment into a microbial “hot-spot” characterized by intense microbial activities (Morgan *et al.*, 2005). The microbial communities in the rhizospheric environment form a subset of the total microbial community present in the bulk soil and are fundamentally important in plant nutrition, growth promotion, and disease interactions (Buee *et al.*, 2009).

Plant diversity is the main parameter that can affect the rhizospheric colonization which is believed to be due to the composition of the root exudates released, which is plant-specific. The variation in chemical composition of the rhizodeposits can result in plant-species/specific rhizosphere microbial communities (Merbach *et al.*, 1999). This relies on the fact that chemical components of root exudates may inhibit one organism while attracting another, or two very different organisms may be attracted with differing consequences to the plant (Bais *et al.*, 2006). For example, the secretion of isoflavones by soybean roots can attract a mutualist (*Bradyrhizobium japonicum*) and/or a pathogen (*Phytophthora sojae*) (Morris *et al.*, 1998).



1.4 Rhizobacteria

The rhizosphere harbours a diverse microbial biomass, involved in various biological activities, including the decomposition of organic matter, the removal of toxins and the cycling of essential nutrients such as carbon, nitrogen and phosphorus (Walker *et al.*, 2003). Despite the severe technical limitations associated with traditional census-gathering methods of microscopy and bacteriology, 4×10^7 prokaryotic cells have been recorded in 1 g of forest soil (Richter and Markewitz, 1995), and 2×10^9 have been recorded in cultivated soil and grassland (Paul and Clark, 1989).

Rhizobacteria are rhizosphere competent bacteria that aggressively colonize all the ecological niches found on roots at all stages of plant growth (Antoun and Kloepper, 2001). Because of differences in the amount of nutrients exuded by plant roots, the microbial communities in the rhizosphere are not static, but are continually changing (Miller and Wood, 1996). Rhizobacteria are the most abundant microorganisms in

the rhizosphere and can have either neutral, detrimental or beneficial effects on plants (Compant *et al.*, 2005). Neutral rhizobacteria have no effects. The deleterious rhizobacteria naturally-occurring in the rhizosphere are toxigenic but not parasitic, and have the potential to suppress weed growth, to produce metabolites like phytotoxins and also to compete for nutrients (eg: *Pseudomonas* species) (Stur and Christie, 2003). Beneficial rhizobacteria are able to promote plant growth and development and are typically known as plant growth promoting rhizobacteria (PGPR; Lucy *et al.*, 2004).

1.4.1 Plant growth-promoting rhizobacteria (PGPR)

PGPRs are a wide variety of soil bacteria able to (i) colonize the root, (ii) to survive and multiply on root surfaces (in competition with other organisms) and, (iii) to promote plant growth (Joseph *et al.*, 2007; Barea *et al.*, 2005). They are classified into two major groups namely, “biocontrol plant growth promoting rhizobacteria” (Biocontrol PGPRs) and biofertilizer plant growth promoting rhizobacteria” (biofertilizer PGPR) (Vessey, 2003; Bashan and Holguin, 1998). Biocontrol PGPRs suppress plant pathogens by producing various types of inhibitory substances (e.g, *Bacillus* sp.), by increasing the natural resistance of the plant (*P. aeruginosa* 7NSK2), and by out-competing pathogens (e.g, *Pseudomonas* sp.; Chaiham *et al.*, 2008). Biofertilizer plant growth promoting bacteria (biofertilizer PGPR) promote plant growth via phytohormone production and by plant nutrition status improvement (Bia *et al.*, 2002). Based on their colonization site, PGPRs are divided into two subtypes (Table 1.5): extracellular Plant Growth Promoting Rhizobacteria (ePGPRs) and intracellular Plant Growth Promoting Rhizobacteria (iPGPRs). ePGPRs are

present in the ectorhizosphere, rhizoplane or in the spaces between root cortex cells. The iPGPRs are present in specialized nodular structure inside root cells and are called endophytes (Gray and Smith, 2005).

Table 1.5: Examples of PGPRs and their zone of colonization

PGPR	Examples of PGPR	Common plants colonized
iPGPR (endophytes)	<i>Bacillus</i> <i>Rhizobia</i> sp. <i>Frankia</i> sp.	Soybean, Alfalfa, Bean, Pea, Clover sp. Peanut, Acacia, Lentil, Red bud Corn, Wheat, Oat, Barley, Pea, Canola, Soybean, Potato, Tomato, Lettuce,
ePGPR (rhizoplane and rhizosphere)	<i>Actinobacter</i> sp., <i>Aeromonas caviae</i> , <i>Agrobacterium radiobacter</i> , <i>Alcaligenes</i> sp., <i>Bacillus</i> sp., <i>Serratia</i> sp., <i>Enterobacter</i> sp., <i>Erwinia herbicola</i> , <i>Flavobacterium</i> sp., <i>Pseudomonas</i> sp.	Radish Cucumber

Source: Gray and Smith, 2005

Some PGPRs can either be ePGPRs or iPGPRs. However *Burkholderia* sp. is an ePGPR present in the rhizoplane of *Sorghum bicolor* roots that has been observed endophytically in the root nodules of sugar cane (Boddey *et al.*, 2003; Chiarini *et al.*, 1998). Both iPGPR and ePGPR activities play important roles in improving growth and yield in crops (Brought *et al.*, 2003), and possess various mechanisms of action.

1.4.2 Archaea

Archaea, Bacteria and Eukarya comprise the three domains of life (Figure 1.6), with the Archaeal domain the least studied of the three groups (Bomberg and Timonen, 2007). The archaea consists of a small group of highly atypical microbial species, essentially known to occupy extreme environmental niches, such as thermophilic springs, hydrothermal vents, high-saline waters, and anoxygenic muds (Wuchter *et*

al., 2006). However, archaea are not restricted to extreme environments. They have also been isolated from mesophilic soils (DeLong, 1998). Archaea are similar to their bacteria counterpart in many respects, such as the size and organization of their chromosomes, the presence of polycistronic transcription units and the utilization of Shine-Dalgarno sequences for the initiation of translation (Gribaldo and Brochier-Armanet, 2006). However, they differ in their cell membrane: archaeal cell membranes have L-glycerol while the bacterial and eukaryotes are having D-glycerol, and the phospholipid side chains of bacteria and eukaryotes are fatty acids bound by ester linkages, while archaeal phospholipid side chains are built from isoprene bound using ether linkage (Bullock, 2000; Matheson, 1992). Within the archaeal domain there are two main phyla, crenarchaeota and euryarchaeota and two minor phyla, korarchaeota and nanoarchaeota (Bomberg and Timonen, 2007). The crenarchaeota contain most of the extreme thermophiles, while the euryarchaeota contain the halophiles, some extreme thermophiles, and the methanogens (Leigh, 2000).

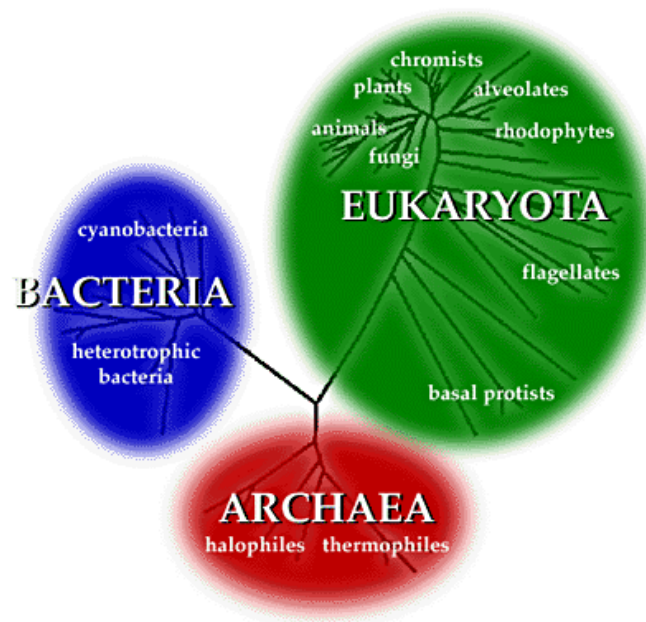


Figure 1.6: Phylogenetic representation of the three life domains (Wuchter *et al.*, 2006)

1.4.3 Plant growth promotion by archaea

Archaea can promote plant growth through their involvement in the nitrogen cycle. The nitrification process was previously thought to be confined to ammonia-oxidizing bacteria (AOB; Hu et al., 2010). Metagenomic studies have revealed a diverse set of putative archaeal ammonia monooxygenase encoding genes (*amoA*) necessary for ammonia oxidation to hydroxylamine before its conversion to nitrite (Wuchter *et al.*, 2006). Furthermore, Verhamme *et al.* (2011) revealed that ammonia-oxidising archaea (AOA) are generally more abundant than ammonia-oxidising bacteria (AOB) in soil. This finding provides strong evidence that Archaea play a major role in the nitrogen cycling process, which is needed for plant growth.



1.5 Mechanisms of action of PGPRs

PGPRs enhance and promote plant growth via direct and/or indirect mechanisms (Chaiham *et al.*, 2008). The direct growth promotion is mediated by active mechanisms of action including (i) the enhancement of iron uptake, (ii) phosphate solubilization, (iii) phytohormone production and (iv) ethylene level regulation (Gholani *et al.*, 2009). The indirect plant growth promotion consists of passive mechanisms of action which involve (i) antibiosis and (ii) induction of host plant resistance (Joseph *et al.*, 2007).

1.5.1 Direct mechanisms of plant growth promotion by PGPRs

1.5.1.1 Nitrogen fixation

Nitrogen is the most important nutrient for plants because of its influences all plant developmental processes such as the number of leaves and their rate of appearance, which subsequently affects the rate of photosynthesis (Hirel *et al.*, 2007). However, plants compete for nitrogen in soil with abiotic and biotic processes such as erosion, leaching and microbial consumption (Crawford, 1995). To be more competitive, plants assimilate inorganic forms of nitrogen such as ammonia, and organic forms such as urea which can be fixed by their symbiotic microorganisms. Some plant species are able to interact symbiotically with bacteria, forming root nodules. Within the nodules, bacteria reduce atmospheric nitrogen to ammonia, a usable nitrogen source (Albrecht *et al.*, 1999). Root nodule formation is initiated by the exchange of signal molecules (e.g. flavanoids compounds) between the plant and the bacteria, attracting the bacteria to the root surface (Tresvaskis *et al.*, 2002). Once the bacteria are bound to the root hair, the bacteria release *nod* factors (e.g. lipo-chitin oligosaccharides), which stimulate the reorientation of root hair cell walls, forming curled root hairs. Bacteria then enter the root either (i) through wounds, particularly where lateral roots protrude, (ii) through the root hairs, or (iii) between undamaged epidermal cells. In each case they induce the formation of an infection thread (Cocking, 2003). Within the infection thread, the bacteria proliferate and differentiate into bacteroids, eventually forming the root nodules. Bacteroids are then released into the cortex cell cytoplasm, and are surrounded by membrane-like structure called symbiosome membrane. Bacteroids enclosed within the

symbiosome membrane are the functional metabolic units of symbiotic nitrogen fixation (Albrecht *et al.*, 1999).

Symbiotic nitrogen fixation, reduction of atmospheric nitrogen to ammonia, is restricted to prokaryotic organisms (Cocking, 2003). Ammonia oxidation, which is the rate limiting step in nitrification, starts by the conversion of ammonia (NH₃) into hydroxylamine (NH₂OH) catalyzed by the enzyme ammonia monooxygenase (MNO). This is followed by the conversion of NH₂OH to nitrite (NO₂⁻) by a hydroxylamine oxidoreductase (HAO) enzyme (Hu *et al.*, 2010). Nitrite (NO₂⁻) is further converted to nitrate (NO₃), which is the absorbable form of nitrogen by nitrifying bacteria.

1.5.1.2 Enhancement of iron uptake

Iron is the fourth most abundant element of the earth's crust and is essential to both plants and microorganisms due to its involvement in the reduction of ribonucleotides and molecular nitrogen, and the energy-yielding electron transfer reactions of respiration and photosynthesis (Lemanceau *et al.*, 2009; Guerinot and Yi, 1994). Being a central element of the photosynthetic electron chain, iron plays an essential role in plant growth (Lemanceau *et al.*, 2009). Although iron is abundant on earth, it is majorly present as ferric iron (Fe³⁺) which is relatively insoluble in water and cannot be taken up by plants (Robinson *et al.*, 1999). Under limited iron supply, plant species acquire iron from the soil environment via two distinct mechanisms (Kovacs *et al.*, 2005). Strategy I involves the reduction of Fe(III) complexes to Fe (II) ions which can be absorbed by the plant. Strategy II involves the release of Fe(III)-binding phytosiderophores which solubilize Fe(III) ions, making it available for absorption (Lopez-Millan *et al.*, 2000). Strategy II is restricted to plants belonging to

the grass families such as Sorghum (Kovacs *et al.*, 2005). The enhancement of iron uptake in sorghum is necessary as sorghum releases phytosiderophores with low iron affinity (Von Wiren *et al.*, 2000). PGPRs enhance iron uptake from the rhizosphere by releasing hydroxamate siderophores (HS) with high affinity for iron-siderophore complexes. However, the absorption of the iron-siderophore complexes in the rhizosphere by plants such as sorghum is not well elucidated (Vessey, 2003).

1.5.1.3 Phosphate solubilization

Phosphorus is the second most important nutrient limiting agricultural production (Crespo *et al.*, 2011). Soils are often very rich in phosphorus, but 20-80% of the available soil phosphorus is in organic forms that cannot be directly utilized by plants (Richardson, 1994). For efficient phosphate absorption by plants, organic phosphate must first be hydrolyzed to inorganic phosphate, such as monobasic (HPO_4^{2-}) or dibasic (H_2PO_4^-) phosphate ions, through mineralization of phosphorus compounds (Fitriatin *et al.*, 2011). PGPRs are able to release enzymes such as phosphohydrolases, phytases, or phosphonatas, which solubilize organic phosphorus, making it available to plants (Table 1.6; Rodriguez *et al.*, 2006).

Phosphohydrolases are nonspecific phosphatases essentially involved in scavenging organic phosphoesters that cannot cross the cytoplasmic membrane. PGPRs such as *Francisella tularensis* are able to produce these enzymes (Rossolin *et al.*, 1998). Phytases constitute a specific group of phosphatases produced by *Pseudomonas* species which hydrolyse phytic acids (the major storage form of phosphorus in cereal, oil and legume) to myo-inositol and phosphoric acid, which can be taken up by the plant (Hasseinkhani *et al.*, 2009). Phosphonatas are enzymes cleaving the

C-P bonds of organophosphonates. Most of the phosphorus present in soils is released by these two enzymes (phosphonatases and phytases) because of their generally high substrate availability (Rodriguez *et al.*, 2006).



Table 1.6: PGPRs involved in phosphate-solubilization

PGPR	Produced enzymes	Host Plant	Reference
<i>Azotobacter</i> sp.	Phytases and Phosphonatasases	Wheat	Kumar and Narula, 1999
<i>Pseudomonas</i> sp.	phytases and Phosphatases	Soy bean	Cattelan <i>et al.</i> , 1999
<i>Bacillus</i> sp.; <i>Francisella tularensis</i>	Phytases, Phosphatases and Phosphonatasases	Wheat, Maize, Barley, Rice and Sorghum	Hayat <i>et al.</i> , 2011
<i>Enterobacter</i> sp.	Phytases	Tomato	Kim <i>et al.</i> , 1998
<i>Rhizobium</i> sp.	Phytases, Phosphatases and Phosphonatasases	Radish and Maize	Antoun <i>et al.</i> , 1998
<i>Azospirillum</i> sp.	Phytases, Phosphatases and Phosphonatasases	Sorghum, Maize and Wheat	Rodriguez and Fraga, 1999



1.5.1.4 Production of phytohormones

Phytohormones are chemical messengers, produced essentially by plants but also by microorganisms, necessary for the communication among cells, tissues and organs in plants, leading to growth promotion (Aloni, 2007). PGPRs are able to affect plant physiology by exuding important plant hormones such as auxins, gibberellins, and cytokinins (Chaiarn *et al.*, 2008).

(i) Auxins

The auxin hormone, or indole-3-acetic acid (IAA), is the most common natural occurring auxin-type hormone that plays a major role in vascular differentiation in plants (Aloni, 2001). Biosynthesis of auxins in plants is an extremely complex process, involving multiple pathways (Zhao, 2010). IAA can be synthesized from IAA conjugates by the hydrolytic cleavage of IAA-amino acids, IAA-sugars, and IAA-methyl esters (Zhao, 2010). The two most understood biological pathways of auxin production uses tryptophan as a precursor (Figure 1.7). Tryptophan is converted to indole-3-acetamide (IAM) by the enzyme tryptophan-2-monooxygenase, which is further hydrolyzed to indole-3-acetic acid (IAA) by hydrolases. Tryptophan can also be converted into IAA via the indole-3-pyruvic acid (iPyA) pathway, where it is converted to indole-3-acetaldehyde (IAAid) by the enzyme tryptophan deaminase. Indole-3-acetaldehyde is further decarboxylated to indole-3-acetic acid by an IAA decarboxylase enzyme (Zhao, 2010). The indole-pyruvic acid pathway is the most common pathway for IAA biosynthesis amongst the PGPRs. If auxins are directly taken up by plants in soil, plant growth promotion such as root growth occurs.

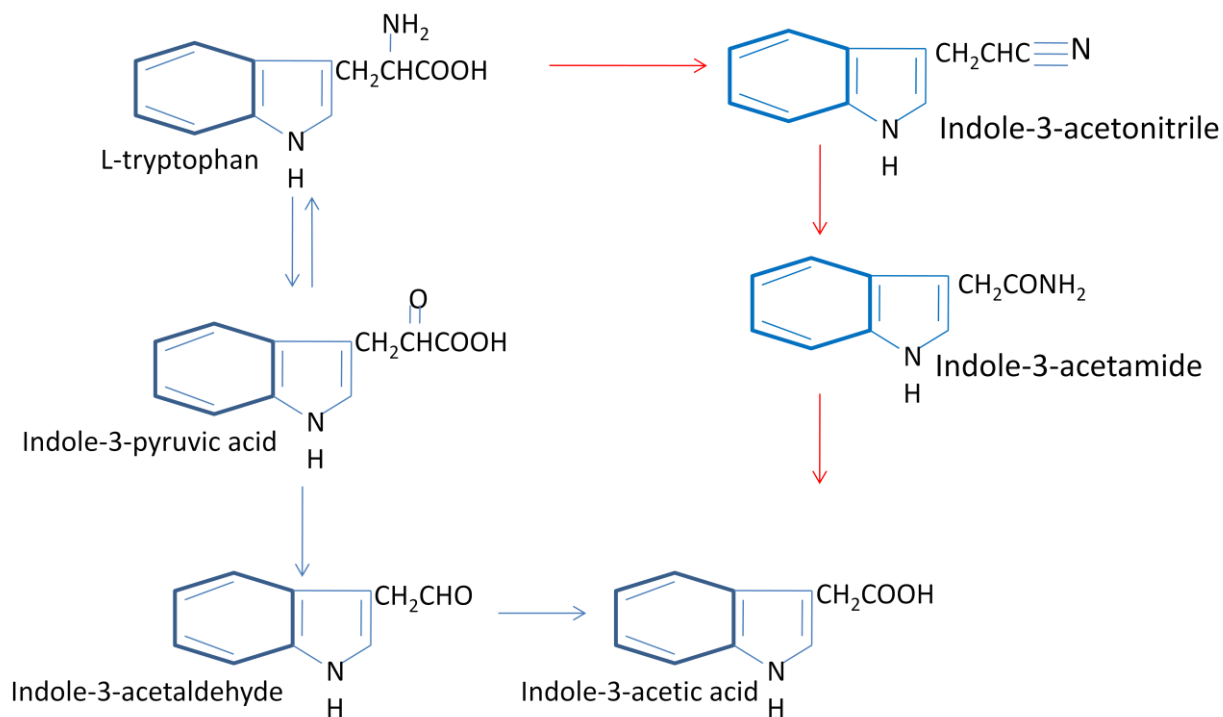
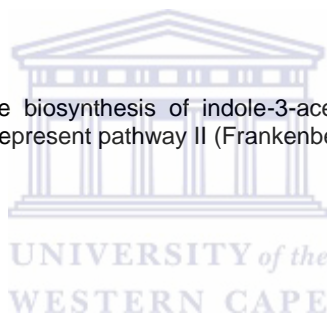


Figure 1.7: Proposed pathways for the biosynthesis of indole-3-acetic acid (IAA) in soil. The blue arrows represent pathway I and the red arrows represent pathway II (Frankenberger and Brunner, 1983).



(ii) Gibberellins

Gibberellins (GAs) correspond to a large family of more than 125 tetracyclic diterpenes that promote cell and stem elongation in plants (Taiz and Zeiger, 2006). GAs are widely known for controlling the induction of hydrolytic enzymes in cereal grains. During their germination, the aleurone layer (a layer of cells that surrounds the endosperm) secretes hydrolases (mainly α -amylases) into the endosperm. This releases nutrients that feed the growing seedlings. GAs regulate α -amylase gene transcription and thus the secretion of α -amylases from aleurone cells. PGPRs such as *Bacillus pumilis* and *Bacillus polymyxa* can promote plant growth by producing this hormone (Richardson *et al.*, 2001).

(iii) Cytokinins

Cytokinins (CKs) are adenine derivatives that are free or bound to molecules in both plants and microbial tissues (Arshad and Frankenberger, 1991). Cytokinins produced in the root tips are transported upward to shoots in the xylem, via vessels and tracheids (Aloni *et al.*, 2005). The upward movement of cytokinin is regulated by the transpiration stream. Cytokinins move to developing shoot organs with high transpiration rates and promote shoot development. Cytokinins also regulate (i) the stimulation of lateral bud growth, (ii) the leaf development and, (iii) the breaking of bud dormancy in deciduous trees during spring (Aloni, 2007). Their physiological role in plants is to promote seed germination, *de novo* bud formation, and leaf expansion and also delay senescence. *Bacillus megaterium* enhances growth promotion by producing this hormone (Arshad and Frankenberger, 1991).



(iv) Lowering of ethylene levels

Ethylene is a gaseous plant hormone produced by all plants and a large number of microorganisms. It controls seedling development, flowering, fruit ripening and senescence (Dakora and Philips, 2002). The ethylene production in plants is triggered by mechanical injury and pathogen attacks (Mworia *et al.*, 2010), and is dependent on the supply of mineral nutrients (P, S, Ca, Mg, Fe, and Cu). The limited supply of one or more of these mineral nutrients increases ethylene production in plants, thereby restraining their growth (Schmidt *et al.*, 1999). The ethylene levels in plants must be closely regulated, as ethylene concentrations as low as 10 ppb can induce plant response while concentrations of 25 ppb can inhibit plant growth (Arshad and Frankenberger, 1991).

Methionine is the sole ethylene biosynthetic precursor in plants. The first step in ethylene biosynthesis involves the conversion of S-adenosyl methionine to 1-aminocyclopropane-1-carboxylate (ACC), catalyzed by aminocyclopropane-1-carboxylate synthase (ACC synthase). ACC is then oxidized to ethylene by the enzyme ACC oxidase (Gray and Smith, 2005). When plants are unable to regulate their ethylene levels, PGPRs such as *Sclerotinia sclerotiorum* and *Enterobacter* release the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which sequesters the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) for hydrolysis. This induces a decrease of ethylene levels (Desbrosses *et al.*, 2009).

1.5.2 Indirect mechanisms of plant growth promotion by PGPRs

1.5.2.1 Antibiosis

Management of soil-borne diseases presents major challenges to plant pathologists, as generally the use of pesticides results in environmental pollution and the development of resistance among phytopathogens (Fernando *et al.*, 2005). Among various phytopathogens, fungi have been widely associated with plant diseases (Whitman *et al.*, 1998). For example, *Fusarium moniliforme* is the major causative agent of grain mold in sorghum, a serious disease which limits sorghum growth (Navi *et al.*, 2005). However, the primary mechanisms of biocontrol by PGPR involve the production of antibiotics along the plant root. Antibiotics produced by PGPRs have broad-spectrum action against several plant pathogens (Table 1.7; Lugtenberg and Kamilova, 2009).



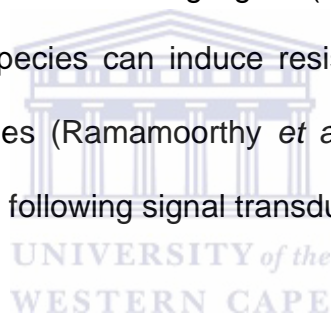
Table 1.7: Antibiotics: produced by PGPRs

PGPR	Antibiotics	Target pathogens	Disease
<i>Pseudomonas fluorescens</i> F113	2,4-diacetyl-phloroglucinol	<i>Pythium</i> sp.	Damping off
<i>Agrobacterium radiobacter</i>	Agrocin 84	<i>Agrobacterium tumefaciens</i>	Crown gall
<i>Bacillus subtilis</i> AU195	Bacillomycin D	<i>Aspergillus flavus</i>	Aflatoxin contamination, Wilt
<i>Bacillus amyloliquefaciens</i> FZB42	Bacillomycin, fengycin	<i>Fusarium oxysporum</i>	Damping off
<i>Lysobacter</i> sp. Strain SB-K88	Xanthobaccin A	<i>Aphanomyces cochlioides</i>	Root rots
<i>Trichoderma virens</i>	Gliotoxin	<i>Rhizoctonia solani</i>	Fire blight
<i>Pontea agglomerans</i> C9-1	Herbicolin	<i>Erwinia amylovora</i>	Damping off
<i>Bacillus subtilis</i> QST713	Iturin A	<i>Botrytis cinerea</i>	Damping off
<i>Bacillus subtilis</i> BBG100	Mycosubtilin	<i>Pythium aphanidermatum</i>	Take-all
<i>Pseudomonas fluorescens</i> 2-79 and 30-84	Phenazines	<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	Damping off
<i>Pseudomonas fluorescens</i> pf-5	Pyoluteorin, purrolnitrin	<i>Pythium ultimum</i> and <i>Rhizoctonia solani</i>	Damping off and rice blast
<i>Bacillus cepacia</i>	Pyrrrolnitrin, pseudane	<i>Rhizoctonia solani</i> , <i>Pyricularia oryzae</i>	
<i>Bacillus cereas</i> UW85	Zwittermicin A	<i>P.aphanidermatum</i> , <i>P. medicaginis</i>	Damping off

Source: Pal and McSpadden, 2008

1.5.2.2 Induction of host resistance

All plants possess active defense mechanisms against pathogens, called induced resistance (van Loon *et al.*, 1998). Induced resistance can be defined as an enhancement of the plant's defensive capacity against pathogens and pests, acquired after appropriate stimulation (Ramamoorthy *et al.*, 2001). These defense mechanisms can be triggered both by biotic (virulent pathogens, non-pathogens and elicitors of fungal cell wall metabolites) and abiotic agents (salicylic acid, ethylene, dichloro-isonicotinic acid and benzothiazole) (Table 1.8; Ramamoorthy *et al.*, 2001). Their quality and quantity depend on the genotype and physiological condition of the plant, as well as the nature of the inducing agent (Tuzun, 2001). PGPRs such as *Bacillus* and *Pseudomonas* species can induce resistance against fungi, bacteria, viruses, insects and nematodes (Ramamoorthy *et al.*, 2001). Induction of a plant defense response involves the following signal transduction cascades:



(1) Single gene disease resistance: Also known as the gene-for-gene resistance mechanism, it depends on the possession of a single resistance (R) gene by the plant, which interacts with a specific avirulence (*avr*) gene product of the pathogen. If a plant lacks the R gene compatible with one of the *avr* genes possessed by an invading pathogen, that plant will be unable to use its R genes to detect and stop the pathogen (Tuzun, 2001).

(2) Multigenic resistance: Also known as polygenic resistance, this mechanism refers to plant disease resistance generated via interactions between the products of

multiple plant genes. Multigenic resistance is considered to be non-specific as plants and pathogens do not require matching R and *avr* genes for a timely plant defense response to occur (Buell, 1999).

If induction of host plant resistance occurs throughout the plant tissues, the defense is called systemic acquired resistance (SAR), and is characterized by increasing the amounts of salicylic acid and pathogenesis-related proteins (van Loon *et al.*, 1998). The induced resistance is not always expressed systemically, but Localized Acquired Resistance (LAR) occurs when plant tissues are directly exposed to the primary invader (Tuzun, 2001). Both SAR and LAR are effective against various types of pathogens.



Table 1.8: PGPR-mediated induced systemic resistance in various plants

Plant species	PGPR	Challenging pathogen	Disease symptoms
Arabidopsis	<i>P. fluorescense</i> WCS417	<i>F.oxysporum f.sp raphani</i>	Vascular wilt
	<i>P. putida</i> WCS358	<i>P.syringae pv. Tomato</i>	Bacterial speck
Bean	<i>P. aeruginosa</i> 7NSK2	<i>P.parasitica</i>	Downy mildew
		<i>B.cinerea</i>	Gray mold and Anthracnose
Carnation	<i>P. fluorescense</i> WCS417	<i>C.lindemuthianum</i>	
Cucumber	<i>P. fluorescense</i> WCS417	<i>F.oxysporum f.sp dianthi</i>	Vascular wilt
	<i>P. aureofaciens</i>	<i>C.orbiculare</i>	Anthracnose
	<i>P. corrugate</i> 13	<i>P.aphanidermatum</i>	Crown rot
	<i>P. fluorescense</i> C15	<i>P.aphanidermatum</i>	Crown rot
	<i>P. fluorescense</i> G8-4	<i>C.orbiculare</i>	Anthracnose
	<i>S. plymuthica</i>	<i>C.orbiculare</i>	Anthracnose
	<i>P. putida</i> and <i>S. marcescens</i>	<i>A.vittatum</i>	Herbivory
		<i>C.orbiculare</i>	Anthracnose
Radish	<i>P. fluorescense</i> WCS417	<i>D.undecimpunctata</i>	Herbivory
		<i>E.tracheiphila</i>	Bacterial wilt
		<i>F.oxysporum f.sp lachrymans</i>	Angular leaf spot
		<i>F.oxysporum f.sp cucumerinum</i>	Vascular wilt
		<i>Cucumber mosaic virus</i>	Systemic mosaic
		<i>F.oxysporum f.sp raphani</i>	Vascular wilt
		<i>A.brassicicola</i>	Necrotic lesions
		<i>F.oxysporum</i>	Necrotic lesions
Tobacco	<i>P. fluorescense</i> WCS374	<i>F.oxysporum f.sp raphani</i>	Vascular wilt
	<i>P. fluorescense</i> WCS417	<i>A.brassicicola</i>	Necrotic lesions
	<i>S. marcescens</i>	<i>Tobacco necrosis virus</i>	Necrotic lesions
		<i>P.syringae pv. Tabaci</i>	Wildfire
Tomato	<i>P. fluorescense</i> CHAO	<i>Tobaccom mosaic virus</i>	Necrotic lesions
	<i>S. marcescens</i>	<i>T.basicola</i>	Black root rot
		<i>Tobacco necrosis virus</i>	Necrotic lesions
Tomato	<i>P. fluorescense</i> WCS417	<i>F.oxysporum f.sp lycopersici</i>	Vascular wilt
	<i>P. fluorescense</i> 89B-27	<i>Cucumber mosaic virus</i>	Systemic mosaic
	<i>S. marcescens</i>	<i>Cucumber mosaic virus</i>	Systemic mosaic

Source : van Loon *et al.*, 1998

1.6 Study rationale and Aim

Sorghum (*Sorghum bicolor* (L.) Moench) constitutes an important source of food and is cultivated all over the world. It is the fourth most important cereal crop behind wheat, rice and maize (Smith and Frederiksen, 2000). However, Sorghum crops are prone to diseases caused by pathogens (such as *Fusarium* sp.) (Navi *et al.*, 2005). To increase its production, various practices including fertilization, crop rotation, soil tillage and monoculture are used (Liu *et al.*, 2006). Fertilization is the most widely used practice in crop production for its influence on plant growth, but has been reported to negatively affect soil health (Ishaq *et al.*, 2002). The maintenance of soil health is essential for sustainable productivity of food, the decomposition of wastes, storage of heat, sequestration of carbon and the exchange of gases (Liu *et al.*, 2006). Despite the negative impact on the soil environment, the use of fertilizers (N in particular) in agriculture has positively impacted the food supply for both animal and human consumption (Hirel *et al.*, 2007). Nitrogen fertilizers are produced by the Harber-Bosch process which requires large quantities of energy, mostly derived from fossil fuels. Furthermore, the excessive use of nitrogen fertilizers has major detrimental impacts on the plant ecosystems, including the eutrophication of fresh water and marine ecosystems (Hirel *et al.*, 2007). The challenge for the future is to accommodate the needs for the rising world population by developing a highly productive agricultural practice whilst at the same time preserving the quality of the environment.

To counteract these issues, scientists are paying more attention to microorganisms that inhabit the plant rhizosphere, as an alternative to chemicals, to facilitate eco-friendly biological control of soil-borne pathogens. Bacteria that aggressively

colonize the plant root system and have a positive effect on the plant are called PGPRs. Plant growth promotion by PGPRs can occur via biological control of plant pathogens, suppression of diseases and nutrient cycling (Berea *et al.*, 2005). These rhizobacteria remain the most extensively studied domain in agricultural soils. However, there is a growing interest for the importance of archaea to the global carbon and nitrogen cycles in agricultural soils (Pratscher *et al.*, 2011). Some studies has revealed putative *amoA* (the A subunit of ammonia monooxygenase) gene sequences derived from uncultivated crenarchaeota in natural and agricultural soils, suggesting the genetic capacity for ammonia oxidation (Treusch *et al.*, 2005). This finding provides strong evidence of a major role played by archaea in nitrogen cycling, an important process that promotes plant growth.

The aim of this study is to identify naturally occurring Archaea and Bacteria which are associated with South African sorghum roots, such organisms could be subsequently exploited to improve sorghum growth and yield. The overall objective was to identify common rhizobacteria and/or archaea that might have the growth promoting capabilities in the rhizoplane and/or rhizosphere of sorghum and also to compare the archaeal diversity and abundance to the diversity and abundance of bacteria in the sorghum rhizosphere.

Chapter 2: Materials and Methods

2.1 Study area and sorghum sampling collection

Ten grams sorghum rhizospheric soils (rhizosphere and rhizoplane) were collected from three farming fields located in Free State (Commercial farm), North West (Academic farm), and Limpopo provinces (House hold farm) of South Africa, in April 2010 (Figure 2.1).



Figure 2.1: South African provinces where sorghum samples were collected.

Three sorghum plants were identified and tagged within each sampling site using a GPS (Table 2.1). Rhizosphere soils were collected by pulling out the plant and shaking it into a sterile plastic bag. Soils directly attached to the sorghum root (rhizoplane) were bagged together with the root. Open soils samples were collected

at every site to be used as experimental controls. All samples were kept on ice and transported to the Institute for Microbial Biotechnology and Metagenomics (IMBM) located in the University of the Western Cape (UWC) where they were stored at -80°C prior to analysis.

Table 2.1: Sorghum sampling sites and their GPS coordinates.

Sampling sites	Type of farms	Sampling points	GPS coordinates
Free State	Commercial	FSP 1	S27°02.975' E027°31.405'
		FSP 2	S27°03.665' E027°31.780'
		FSP 3	S27°03.660' E027°31.811'
North West	Academic	NSP 1	S26°43.741' E027°04.870'
		NSP 2	S26°44.063' E027°04.721'
		NSP 3	S26°34.063' E027°03.944'
Limpopo	House-hold	LSP 1	S24°38.620' E029°52.484'
		LSP 2	S24°39.375' E029°53.593'
		LSP 3	S24°40.822' E029°52.146'



2.2 Metagenomic DNA extraction

2.2.1 Modified Zhou et al (1996) extraction method

Metagenomic DNA from 0.5 g of soil was extracted using the Zhou *et al.* (1996) protocol. To avoid heat shock, soil samples stored at -80°C were transferred to -20°C for 24 h and then thawed on ice. Using a sterile spatula, 0.5 g was weighed aseptically into a 1.5 ml sterile eppendorf. One millilitre of freshly prepared lysis buffer (25mM Tris-HCL pH 8, 50mM glucose, 10mM EDTA, 25mg/ml lysozyme, 20mg/ml proteinase K) was added. The tubes were incubated at 37 °C overnight. Fifty microlitres of 1% Sodium Dodecyl Sulphate (SDS) was added to each tube, which were then incubated at 65 °C for 30 minutes. The tubes were centrifuged at 14

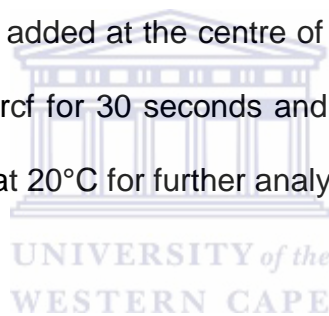
000 rcf for 2 minutes and the supernatants transferred to sterile eppendorfs. An equal volume of phenol (Sigma, pH 8) was added to each tube and mixed gently. The phenol extraction was repeated when the supernatant was deeply colored, which is indicative of significant concentrations of humic acids (known PCR inhibitors). The tubes were then centrifuged at 10 000 rcf for 1 minute, and the supernatants transferred to new sterile tubes. An equal volume of chloroform: iso-amyl alcohol (24:1, v/v) was added, and the tubes re-centrifuged at 10 000 rcf for 1 minute. The supernatants were transferred to sterile eppendorf tubes and 1 ml of ice cold isopropanol was added, followed by a centrifugation at 10 000 rcf for 1 minute. The supernatants were descanted and the tubes left to dry for 2 hours. The dried DNA pellets were resuspended in 50 μ l TE (10 mM Tris, 1 mM EDTA, pH 8).



2.2.2 PowerSoil® DNA Isolation Kit

Metagenomic DNA from sorghum rhizospheric and bulk soil was extracted using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc). 0.25 g was added into the PowerBead Tubes and vortexed gently to mix. Sixty microlitres of solution C1 was added into the tubes and the tubes were inverted several times. The PowerBead Tubes were vortexed horizontally at maximum speed for 10 minutes on the flat-bed vortex pad. Two-hundred microlitres of solution C2 was then added and the tubes were incubated for 5 minutes at 4°C. The tubes were then spun at 10 000 rcf for 1 minute, and 600 μ l of the supernatant was transferred into a clean 2 ml collection tube. Two-hundred microlitres of solution C3 was added into the tubes, centrifuged and the tubes were then incubated for 5 minutes at 4°C. The tubes were centrifuged for 1 minute at 10 000 rcf, and 750 μ l of supernatant was transferred in a clean 2 ml

collection tube. 1.2 ml of solution C4 was added to the supernatant and vortexed for 5 minutes. 650 μ l of supernatant mixture was transferred onto a Spin Filter and centrifuged at 10 000 rcf for 1 minute at room temperature. The flow-through was discarded and an additional 675 μ l of the supernatant was added to the Spin Filter and centrifuged at 10 000 rcf for 1 minute at room temperature. The flow-through was discarded and the remaining supernatant was loaded onto the Spin Filter and centrifuged at 10 000 rcf for 1 minute at room temperature. Five hundred microlitres of solution C5 was added and the tubes centrifuged at 10 000 rcf for 30 seconds. The flow-through was discarded and the tubes were centrifuged again at 10 000 rcf for 1 minute. The Spin Filter was carefully placed into a clean 2 ml collection tube, and 100 μ l of solution C6 was added at the centre of the white filter membrane. The tube was spun at 10 000 rcf for 30 seconds and the Spin Filter was discarded. The isolated DNA was stored at 20°C for further analyses.



2.3 DNA purification

2.3.1 Purification using Polyvinylpyrrolidone (PVPP) minicolumns

Metagenomic DNA was purified using the Polyvinylpyrrolidone (PVPP) spin columns constructed using P20 tips (end of tip cut off) placed inside 0.6 ml PCR tubes (with end and lid cut off) enclosed in a 1.5 ml tube (lid removed). Hundred and fifty microlitres of 50% PVPP in TE was loaded inside the filter tip. Columns were centrifuged at 200 rcf for 2 minutes and the flow-through discarded. The previous step was repeated. The columns were washed twice with 125 μ l TE and centrifuged as above. The columns were then centrifuged at 600 rcf for 10 minutes to dry, and placed in sterile eppendorfs. Twenty microlitres DNA was added to the columns and

incubated for 1 minute at room temperature, and was eluted by centrifugation at 600 rcf for 5 minutes and 17 000 rcf for 10 minutes.

2.3.2 Purification using a commercial kit

The Illustra™ GFX™ PCR DNA and gel band purification kit (GE Healthcare Limited, Buckinghamshire, UK) was used to purify PCR products from solution or agarose gels according to the manufacturers' specifications.

2.4 Agarose gel electrophoresis

DNA analysis was performed using agarose gel electrophoresis. Horizontal 0.8% (w/v) TAE (40mM TRIS base (w/v), 0.2 mM Glacial acetic acid (w/v), 10mM EDTA (w/v), pH 8.0) agarose slab gels containing ethidium bromide (0.05µg/ml) were cast and electrophoresed at 100 V for 30 min in 1 X TAE buffer. The DNA fragments were visualised and gel images captured under UV illumination using the Alphaimager 3400 Imaging System (AlphaInnotech Corporation™ San Leandro, CA). DNA fragments of known concentrations and sizes were used as reference markers.

2.5 DNA quantification

The Concentration (calculated as $OD_{260} \times 50 \text{ ng}/\mu\text{l}$) and purity (ratio OD_{260}/OD_{280}) of the processed DNA were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA).

2.6 Polymerase chain reaction (PCR) of phylogenetic marker genes

High molecular weight metagenomic DNA extracts from the rhizospheric and bulk soils were used as the template for the PCR amplifications. The PCR were performed in 0.2ml thin walled tubes using Applied Biosystem (AB 2720, Singapore) and Hybaid (Thermo Hybaid, Ashford, GB) thermocyclers. The basic PCR reaction mix for 20 μ l reactions consisted of: ~ 50 ng template DNA, 0.5 μ M of each primer, 1X Dream Taq buffer (100mM Tris-HCl pH 8.3, 15mM MgCl₂, 0.01 % [w/v] gelatin), 200 μ M dNTPs, 1.5U Taq DNA polymerase and distilled water adjusted to a final volume of 20 μ l. Negative controls containing all reagents and no DNA template were always included. The PCR products for all reactions were visualized by electrophoresis on 1.5 % (w/v) agarose gels. The primers used as well as the cycling conditions applied are given in Table 2.2

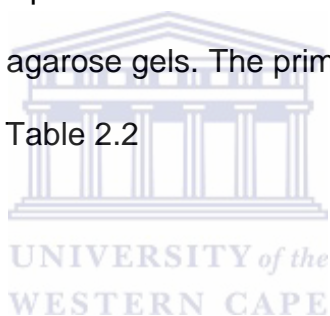


Table 2.2: Primer combinations and PCR parameters used in this study

Primer Name	Sequence (5' to 3')	Specificity	PCR cycling Conditions	Reference
E9F U1510r	GAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Eubacterial 16S rRNA gene/ T-RFLP-PCR of Eubacterial 16S rRNA gene	94°C for 4 min 30 cycles: 94°C for 30s, 55°C for 30s, 72°C for 145s 72°C for 10 min	Hansen <i>et al.</i> , 1998; Reysenbach and Pace, 1995
341F-GC* 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	DGGE-PCR of Eubacterial 16S rRNA gene	94°C for 4 min 20 cycles: 94°C for 45s, 65°C for 45s, 72°C for 1 min 20 cycles: 94°C for 30s, 55°C for 30s, 72°C for 1 min 72°C for 10 min	Muyzer <i>et al.</i> , 1993
Act235F Act562R	CGCGGCCTATCAGCTTGTTG ACGTATTACCGCGGCTGCTGGC	Actinobacterial 16S rRNA gene	94°C for 4 min 20 cycles: 94°C for 45s, 62°C for 45s, 72°C for 1 min 20 cycles: 94°C for 30s, 55°C for 30s, 72°C for 1 min 72°C for 15 min	Stach <i>et al.</i> , 2003b; Muyzer <i>et al.</i> , 1993
341F-GC* Act562R	CCTACGGGAGGCAGCAG ACGTATTACCGCGGCTGCTGGC	DGGE-PCR of Actinobacterial 16S rRNA gene	94°C for 4 min 20 cycles: 94°C for 45s, 62°C for 45s, 72°C for 1 min 20 cycles: 94°C for 30s, 55°C for 30s, 72°C for 1 min 72°C for 15 min	Stach <i>et al.</i> , 2003b; Muyzer <i>et al.</i> , 1993
A3Fa AB927R	TCCGTTGATCCYGCCGG CCCGCCGAATTCCTTTAAGTTTC	Archaeal 16S rRNA gene (except for Nanoarchaea)	94°C for 4 min 20 cycles: 94°C for 45s, 55°C for 30s, 72°C for 75s 10 cycles: 94°C for 30s, 65°C for 45s, 72°C for 75s 72°C for 10 min	Ovreas <i>et al.</i> , 1997
A430F-GC* A533R	GCCCTACGGGGYGCASCAG TTACCGCGGCKGCTG	DGGE-PCR of Archaeal 16S rRNA gene	94°C for 4 min 30 cycles: 94°C for 30s, 53.5°C for 30s, 72°C for 1 min 72°C for 10 min	Ovreas <i>et al.</i> , 1997
8fa 1492R	TCYSGTTGATCCTGCS GCTTACCTTGTTACGACTT	T-RFLP-PCR of archaeal 16S RNA genes	94°C for 4 min 30 cycles: 94°C for 30s, 55°C for 30s, 72°C for 1 min 72°C for 10 min	Costello and Schmidt, 2006

^a K=T/U; S=G/C; Y=C/T.* GC-clamp: 5'-CGCCCCCGCGCGCGCGGGCGGGGCGGGGGCACGGGGG-3'

2.7 Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out as described by Muyzer et al., (1993) with some modifications. PCR products amplified with primers containing a GC-clamp were separated on 9% (w/v) polyacrylamide gels containing a chemical denaturing gradient. Denaturing gradient gels were poured using a gradient mixer (Bio-Rad) containing 'high' (maximum amount of denaturants required) and 'low' (minimum amount of denaturant required) gels solutions. 'High' and 'low' solutions were prepared by mixing '0%' and '100%' denaturant gel stock solutions to give the required denaturant concentrations. The '0%' solution contained 40% acrylamide: N,N' bis-acrylamide (37.5:1) and 1 X TAE (40mM Tris-HCL, 10Mm glacial acetic acid, 1 mM EDTA, pH 8.0). The '100%' solution included the addition of 7 M urea and 40% (v/v) deionised formamide as the denaturants. The denaturing of each phylogenetic marker genes used was determined by first using the broad 30% to 70% denaturing gradient, then by narrowing the range depending on the level of separation achieved. For the Eubacterial primer set (341F-GC and 543R) 40%/60% gels, for the Actinobacterial primer set (341F-GC and ACT562R), both 35%/75% and 40%/60% gels, and for the Archeal primer set (A341F-GC and A533R) 40%/60% gels were used (Table 2.3).

Table 2.3: Denaturing gradient table showing volumes in millilitres (ml) of “low” solution (denaturing stock solution A: 8% acrylamide in 0.5X TAE (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8) and “high” solution (denaturing stock solution B: 8% acrylamide, 7M urea, 40% formamide in 0.5X TAE buffer) mixed to form a gradient within the gel.

Denaturing percentage (%)	High solution (ml)	Low solution (ml)
30	5.4	12.6
35	6.3	11.7
40	7.2	10.8
45	8.1	9.9
50	9	9
55	7.2	8.1
60	10.8	7.2
65	11.7	6.3
70	12.6	5.4
75	13.5	4.5

Electrophoresis was performed using the Bio-Rad DCode™ DGGE system. The PCR products were separated using a constant 100 V for 16 h (in 1 X TAE) at a constant temperature of 60°C. After electrophoresis, gels were stained in 1 X TAE containing ethidium bromide (10mg/ml) for 40-50 min, then destained in 1 X TAE for 10 min. Gels were viewed and the images captured under UV using the Alphamager (AlphaInnotech) imaging system.

2.7.1 Statistical analysis of DGGE gels

Image analysis of DGGE profiles and banding patterns was performed using the software GelCompar® II, version 5.0 (Applied Maths). The same DGGE marker was electrophoresed with the samples. This allowed the normalisation of the gels; i.e, the standardisation of the band migration in all gels, for comparison of all community profiles. Bands on the DGGE gels were considered as present or absent and

recorded as binary matrix. This matrix was used to generate distance matrices which were transformed into dendograms and multi-dimensional scaling (MDS) plots.

2.7.2 Isolation and sequencing of DGGE bands

Prominent common bands were excised from the UV-illuminated acrylamide gels using sterile surgical blades, and the DNA was eluted from the excised gel by incubation in 50 µl filter-sterilised water at 4°C overnight. Elutes was used for PCR reactions, as described above, and the products were analyzed by DGGE. PCR products with single bands on the third DGGE were purified for analysis using the GFX kit with a final elution volume of 20 µl. Purified PCR products from DGGE bands were directly sequenced with the same primer sets at the University of Stellenbosch. Chromas was used for editing sequences. Edited sequences were compared with those in GenBank using BLAST on the NCBI's homepage. According to the similarities in the BLAST hits and alignments from all the sequences obtained, the aligned sequences were used to phylogenetic identification.

2.8 Terminal restriction length polymorphism (TRFLP)

Terminal restriction length polymorphism (TRFLP) of the 16S rRNA PCR product amplified using the E9F/U1510R primers for bacteria and 8Fa/1492 primers for archaea was digested in a 25µl reaction containing: 2U of HAE III, 10X buffer X, and distilled water adjusted to a final volume of 25 µl. Both forward primers were fluorescently labelled. The digestion of the PCR products was confirmed on 2.5% agarose gel before TRFLP analysis. The lengths of fluorescently labelled terminal

restriction fragments (T-RFs) were determined by comparison with internal standard size markers (ROX 1.1; Applied Biosystems, INC) and were analyzed using a range of computational and statistical approaches. T-RFLP electropherograms were processed using the PeakScanner™ software V1.0 (PE Applied Biosystems) as well as the T-RFLP Analysis Expedited (T-REX) software (<http://trex.biohpc.org>). Analysis was performed using a size cut-off for peaks, where all peaks shorter than 35 bp (minimum length) and longer than 1200 bp (maximum length) were excluded from analysis. To visualize relative similarities between T-RFLP profiles non-metric multi dimensional scaling (NMDS) and dendrograms were generated using PRIMER 6 software package (PRIMER-E Ltd., UK). This resemblance matrix is represented in low-dimensional space (usually 2d) in ordination plots. The relative distance between sites in these ordination plots indicates the relative similarity of community structure. NMDS was performed with 25 random restarts and results were plotted in 2-dimensions. Peak abundance in samples was transformed using the presence/absence model prior to generating a Bray-Curtis matrix.

2.9 Isolation of *Bacillus* sp. from the sorghum rhizoplane samples

2.9.1 Culturing of *Bacillus* sp.

Zero point one gram of sorghum rhizoplane samples were aseptically added to the test tubes containing 10 ml of saline solution (0.9% sodium chloride). The tubes were mixed by vigorous vortexing for 60s to dislodge bacteria from soil particles and allowed to settle for a minute. The tubes were then heated at 85°C for 20 minutes to kill all non-spore forming bacteria and 1 ml of suspension used for serial dilution up to 10⁻³ times. All diluted soil suspensions were plated onto LB, R2A and Nutrient

agar plates at pH 8. The plates were then incubated both at room temperature and 37°C for 3 days. The isolated bacteria were identified.

2.9.2 Purification of cultures

Colonies of different morphologies were preferentially selected based on their size, appearance and pigmentation and purified by streaking onto new agar plates of the same medium they were isolated. Pure isolates were kept on agar plates at 4°C for further analysis.

2.9.3 Amplified ribosomal DNA restriction analysis (ARDRA)

The selected pure cultures were amplified using the universal bacterial primer set E9E and U1510R (Table 2.2), with the initial boiling step incorporated in the PCR parameter to ensure cell lysis. ARDRA of PCR amplicons was done in 20 µl reaction mixtures containing ~100 ng of DNA, 1U of *Hae*III (Fermentas) and the 1X buffer R (Fermentas). The reactions were incubated overnight at 37°C, and the digests separated using agarose gel electrophoresis (2.5% gels).

Chapter 3: Identification of rhizobacteria associated with sorghum

3.1 Introduction

The rhizospheric environment (i.e. rhizosphere and rhizoplane) is the volume of soil adjacent to and influenced by the living plant roots (Baudoin *et al.*, 2002; Morgan *et al.*, 2005). It is characterized by high microbial activities, arising from intense interactions between the soil, the plant, the microorganisms and the soil fauna (Antoun and Prevost, 2005; Morgan *et al.*, 2005). Culture-dependent methods, such as plate-count methods, have been used to study microorganisms from environmental samples for decades (Staley and Konopka, 1985). However, the inability of the majority (less than 99%) of microbial cells to grow on common nutrient agar is recognized as a severe draw back, limiting the use of this technique to study the diversity of environmental bacterial communities (Amann *et al.*, 1995). Thus, to unlock the 'black box' of microbial life in soil, culture-independent techniques, that also allow the processing of many samples simultaneously, such as denaturing/temperature gradient gel electrophoresis (D/TGGE) or terminal restriction fragment length polymorphism (T-RFLP) analysis, are necessary to study soil microbial diversity (Kowalchuk *et al.*, 2007). For this study, DGGE and T-RFLP were used to assess microbial communities associated with the sorghum rhizospheric environment. Both molecular techniques have previously been used to assess the microbial community composition in soils (Enwell and Hallin, 2009), and their principles are shown in Figure 3.1.

T-RFLP is a rapid profiling method that combines restriction fragment analysis of a PCR-amplified gene with automated sequencing gel technology (Liu *et al.*, 1997). The technique requires that one or both of the primers used to amplify the target gene is labelled with a fluorescent dye (Figure 3.1.A). The resulting amplicon is digested with either one or two selected restriction endonucleases, providing fragments which are then separated by high-resolution gel electrophoresis on automated DNA sequencers. Each peak on a chromatogram is considered to be a terminal restriction fragment (T-RF) that theoretically corresponds to one bacterial population in the original community. Its height (or area) can be used as a proxy of the relative abundance of that population in the total community. Microbial diversity can thus be estimated by analyzing the number of peaks and peak heights (or area) of terminal restriction fragments (T-RFs) in complete T-RFLP patterns. Because of its high level of sensitivity and accuracy, T-RFLP has been used to study microbial communities from various environments including activated sludge, bioreactors, enriched cultures, marine sediments, lake sediment, soils, plant roots as well as deep gold mines of South Africa (Liesack and Dunfield, 2004).

DGGE (Figure 3.1.B), is also a fingerprinting technique that separates PCR-amplified genes on the basis of heterogeneities in their GC-content and sequence, leading to differences in their melting properties as they migrate through a gel matrix containing a gradient of denaturing chemicals. Complete separation of the sequence is prevented by using PCR primers with a GC-clamp. Each band is considered as an operational taxonomic unit (OTU; Muyzer *et al.*, 1993), and theoretically corresponds to one population in the original community. The band intensity can be used as a proxy of abundance of that population in a total community.

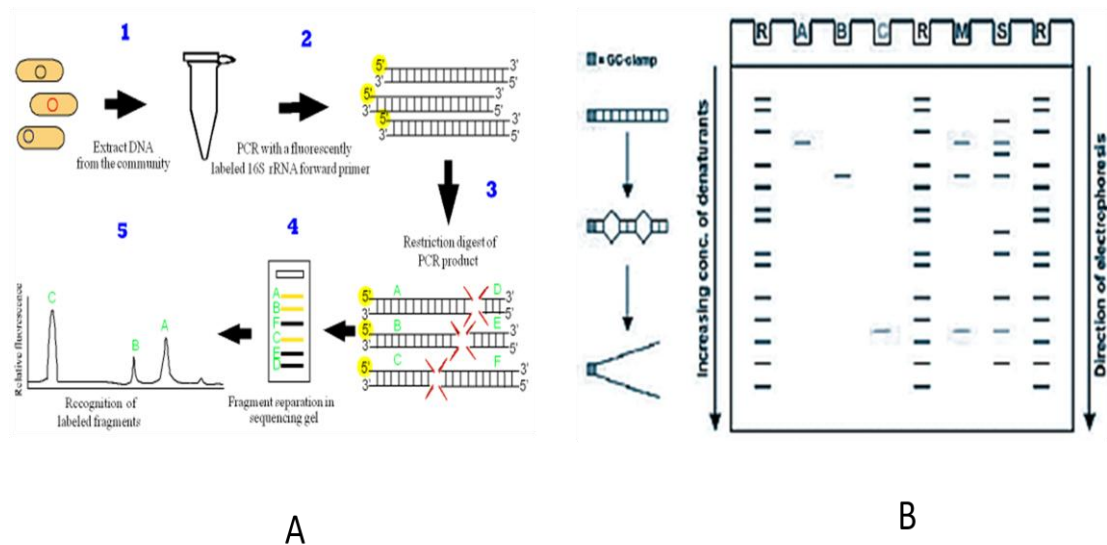


Figure 3.1: A diagrammatic sketch showing steps involved in terminal-restriction fragment length polymorphism (T-RFLP; A) and denaturing gradient gel electrophoresis (DGGE; B).

3.2 Study area and sample collection

Rhizosphere and rhizoplane samples from sorghum were collected from three farms located in the three South African provinces (Free State, Limpopo and North West) in April 2010 (Table 3.1). Within each sampling site, three mature and healthy sorghum plants at an interval of 100 m were collected and one bulk soil was also collected to be used as the experimental control. In addition to the differences in soil types (Table 3.2), these farms were also characterized by their use of different agricultural practices to cultivate sorghum: both the Free State (commercial farm) and North West (academic farm) farms were characterized by “modern” agricultural practices (such as N fertilization), while the Limpopo farm (small household farm) was characterized by traditional farming methods such as the use of cow dung as a fertilizer.

The three different sampling sites allowed the study of the impact of agricultural practices and geographical localization on sorghum rhizospheric microbial communities. Differences in agricultural practices have been shown to have an effect on the physical and the chemical compositions of the soil as well as on the structure of the microbial community (Girvan et al., 2003).



Table 3.1: Sorghum samples used in this study

Sampling site	Sample name	GPS coordinates	Description
Free State	FSRP-1	S27°02.975' E027°31.405'	Rhizoplane from sorghum plant 1
	FSRS-1	S27°02.975' E027°31.405'	Rhizosphere from sorghum plant 1
	FSB		Bulk soil
	FSRP-2	S27°03.665' E027°31.780'	Rhizoplane from sorghum plant 2
	FSRS-2	S27°03.665' E027°31.780'	Rhizosphere from sorghum plant 2
	FSRP-3	S27°03.660' E027°31.811'	Rhizoplane from sorghum plant 3
	FSRS-3	S27°03.660' E027°31.811'	Rhizosphere from sorghum plant 3
Limpopo	LPRP-1	S24°38.620' E029°52.484'	Rhizoplane from sorghum plant 1
	LPRS-1	S24°38.620' E029°52.484'	Rhizosphere from sorghum plant 1
	LPB		Bulk soil
	LPRP-2	S24°39.375' E029°53.593'	Rhizoplane from sorghum plant 2
	LPRS-2	S24°39.375' E029°53.593'	Rhizosphere from sorghum plant 2
	LPRP-3	S24°40.822' E029°52.146'	Rhizoplane from sorghum plant 3
North West	LPRS-3	S24°40.822' E029°52.146'	Rhizosphere from sorghum plant 3
	NWRP-1	S26°43.741' E027°04.870'	Rhizoplane from sorghum plant 1
	NWRS-1	S26°43.741' E027°04.870'	Rhizosphere from sorghum plant 1
	NWB		Bulk soil
	NWRP-2	S26°44.063' E027°04.721'	Rhizoplane from sorghum plant 2
	NWRS-2	S26°44.063' E027°04.721'	Rhizosphere from sorghum plant 2
	NWRP-3	S26°34.063' E027°03.944'	Rhizoplane from sorghum plant 3
NWRS-3	S26°34.063' E027°03.944'	Rhizosphere from sorghum plant 3	

LPRP-3 and LPRS-3= breed from the North West province

3.3 Bulk soil and rhizospheric soil chemical properties

Soil organic matter (SOM) includes a mixture of bio-organic components in variable proportions and evolutionary stages (Arias *et al.*, 2005). Intensive agricultural practices can change the soil organic matter content, which will directly affect the soil microbial biodiversity (Girvan *et al.*, 2003). In this study, different SOM contents were expected in three selected sampling sites, because all sampling sites have different agricultural practices which should affect the chemical and physical composition of these soils. Three rhizospheric soil samples from each sampling point in each province were mixed and chemically analysed. Bulk soil samples were also analyzed. The rhizospheric soil pH values varied between 4.2 and 6.3 (Table 3.2). No significant differences in soil total N was found between Free State and Limpopo sites. Soil $\text{NH}_4^+\text{-N}$ content ranged from 8.68 to 9.6 mg/kg. The highest $\text{NH}_4^+\text{-N}$ content was in the North West rhizosphere. Significant differences in soil $\text{NO}_3^-\text{-N}$ contents were detected, with the North West bulk soil having the highest content, and the Free State rhizosphere having the lowest. This could be due to the differences in the types of fertilizers and agricultural practices used, which can affect the soil nutrient content.

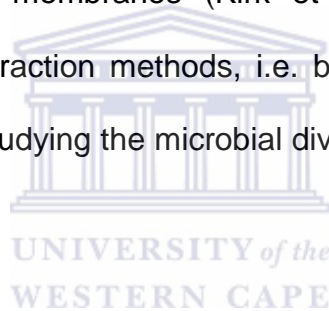
Table 3.2: Bulk soil and rhizospheric soil characteristics of the three sampling sites (Free State, Limpopo and North West).

Province	Sample name	pH (KCL)	$\text{NH}_4\text{-N}$ (mg/kg)	$\text{NO}_3\text{-N}$ (mg/kg)	C (%)	N (%)
Free State	Bulk soil	4.7	7.88	1.44	0.58	0.11
	Rhizosphere	4.2	8.68	0.52	0.4	0.11
Limpopo	Bulk soil	5.4	9.6	3.72	0.19	0.09
	Rhizosphere	6.3	8.36	4.72	0.36	0.1
North West	Bulk soil	6.2	8.44	11.88	0.96	0.14
	Rhizosphere	6	9.6	5.8	0.94	0.14

C=Organic carbon content (%), N=Organic nitrogen content (%), $\text{NO}_3\text{-N}$ = Nitrate containing compounds, $\text{NH}_4\text{-N}$ = Ammonium containing compounds.

3.4 Metagenomic DNA extraction

To obtain an accurate environmental microbial community diversity when using molecular tools such as T-RFLP and DGGE, DNA extraction methods that result in high quality DNA yield, with minimum biases should be used (Miller *et al.*, 1999), as DNA extraction methods strongly influence the interpretation of the environmental microbial community structure derived from the data acquired (Inceoglu *et al.*, 2010). This is essentially due to variations in microbial cell wall structures, Gram-negative bacteria being susceptible to gentle extraction methods because of their thin cell membrane, while Gram-positive bacteria need harsh extraction methods since they are composed of thick cell membranes (Kirk *et al.*, 2004). As a result, the effectiveness of two DNA extraction methods, i.e. bead-beating and chemical cell lysis were assessed prior to studying the microbial diversity associated with sorghum rhizospheric environment.



High molecular weight DNA (~11500bp) was obtained either using the Zhou *et al.* (1999) DNA extraction method (chemical method; Figure 3.2 A) or the Power Soil DNA Isolation Kit (Bead beating; Figure 3.2 B). However, the DNA extracted using the chemical method needed an extra purification step, using PVPP minicolumns, to eliminate humic contaminants, which are known to inhibit DNA polymerase during PCR amplification (Muyzer *et al.*, 1993). Indeed, without this purification step, no amplicons were observed (data not shown), validating this purification step prior to any downstream application.

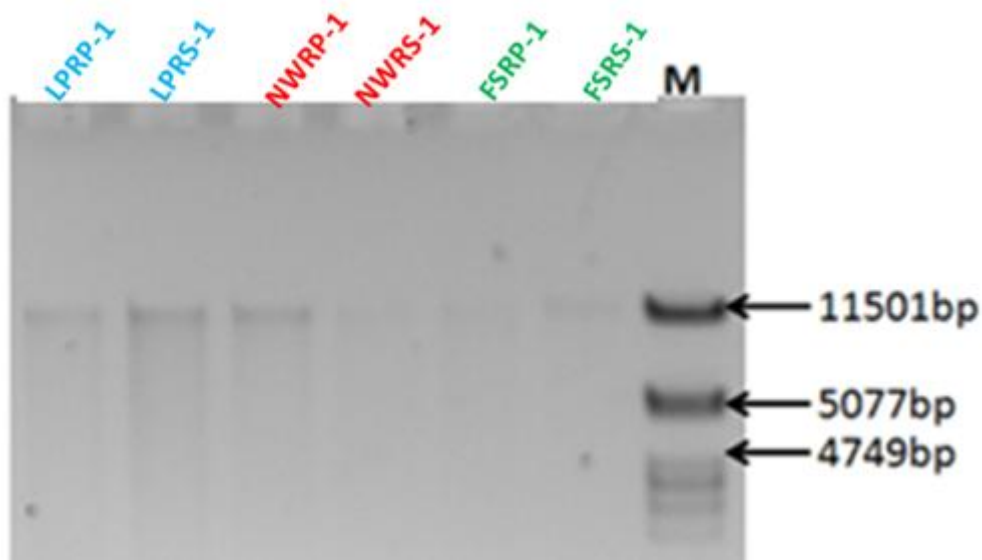


Figure 3.2 A: Agarose gel electrophoresis of PVPP minicolumn- purified DNA extracted using a modified Zhou *et al* (1999) DNA extraction method. Sample names refer to Table 3.1. M= *Pst* I marker. The DNA was resolved on a 0.8% agarose gel.

Contrastingly, use of the Power Soil DNA Isolation kit resulted in high quality DNA yields (Figure 3.3) that was directly suitable for downstream applications.

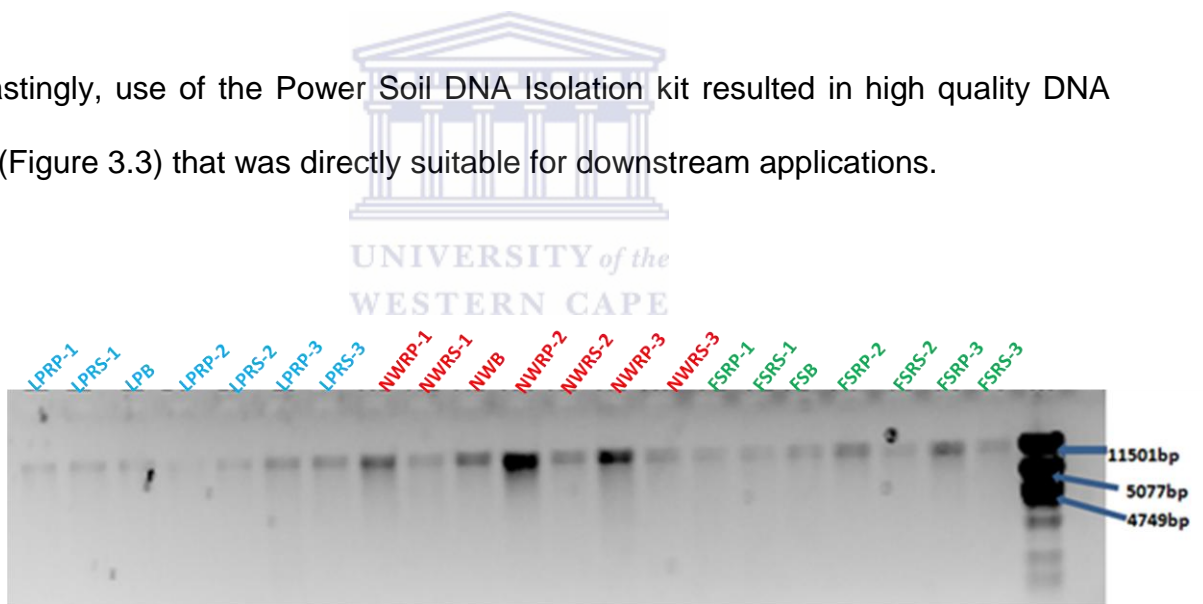


Figure 3.2 B: Agarose gel electrophoresis of total DNA extracted using the Power Soil DNA Isolation Kit from sorghum rhizospheric soil. Sample names refer to Table 3.1. Lane M= *Pst* I marker. Soil extracted DNA loaded onto the gel represent the total amount derived from 0.5 g of soil. The DNA was resolved on a 0.8% agarose gel.

To conclude, metagenomic DNA was obtained with both extraction methods. However, only the chemical lysis method was coupled with the presence of humic contaminants in the extracted DNA. Purification of the DNA did eliminate these humic contaminants but resulted in significant loss of DNA (data not shown),

potentially biasing the molecular diversity analysis (Kirk *et al.*, 2004). The use of the Power Soil DNA Isolation kit resulted in high quality metagenomic DNA ($A_{260}/A_{280} \approx 1.6-2$) that did not require purification for downstream analyses. Therefore, the Power Soil DNA isolation kit showed to be a more suitable method for DNA extraction from sorghum rhizosphere and bulk soil samples in order to study their diversity and was the preferred method used in this study.

3.5 Diversity of rhizobacteria associated with South African sorghum

3.5.1 Bacterial 16S rRNA PCR-DGGE analysis

In this study, the 16S rRNA gene (a component of the 30S subunit of prokaryotic ribosomes) was used as the phylogenetic marker to study the bacterial diversity in the sorghum rhizospheric environment. The use of 16S rRNA genes to study the microbial phylogeny and taxonomy has been adopted since the early 1990s (Giovannoni *et al.*, 1990), and still remains the most commonly used phylogenetic marker gene to date (Kitts, 2001). PCR, together with extraction of nucleic acid from environmental samples played a central role in the development of culture-independent techniques to study environmental microbiology. It enables their analysis within environmental samples by selectively amplifying the target gene sequence specific to the primer used.

In spite of inherent limitations associated with PCR-based approaches, such as the differential amplification of target genes which also bias molecular diversity analysis

(Kirk *et al.*, 2004), PCR has proved to be a more trustworthy approach to effectively elucidate microbial community composition, particularly in comparison to the conventional culturing approach. In this study, the 16S rRNA gene was amplified from metagenomic DNA the using the universal bacterial primer set, E9F and U1510R (Figure 3.3).

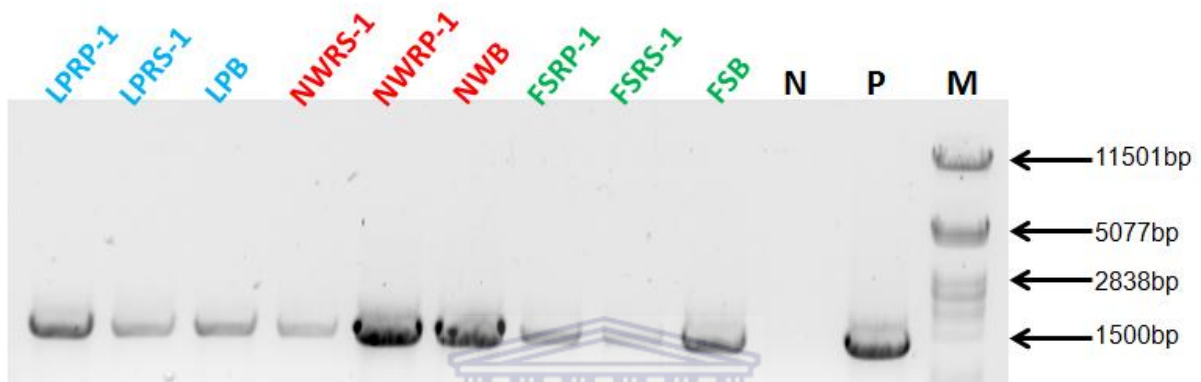


Figure 3.3: Agarose gel electrophoresis showing PCR products of ca. 1500bp from bacterial 16S rRNA first round PCR amplification using E9F and U1510R primers (Table 2.2). Sample names refer to Table 3.1. P = positive control (*E. coli* strain), N = negative control and M = *Pst* I marker

Foe DGGE analysis, the 1500pb fragment obtained was then used as a template in a nested PCR reaction using the bacterial-specific 16S rRNA gene DGGE primers 341F-GC and 543R (Muyzer *et al.*, 1993). DGGE-PCR amplicons yielded 202bp fragments (Figure 3.4). Distilled water was used as the negative control, and did not amplify in all the PCR reactions.

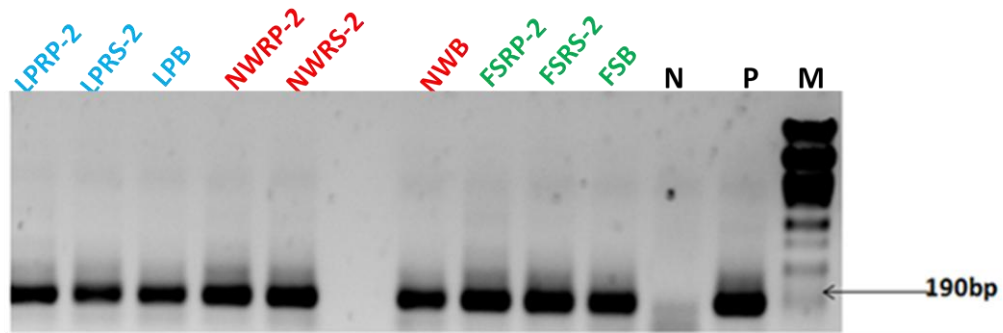
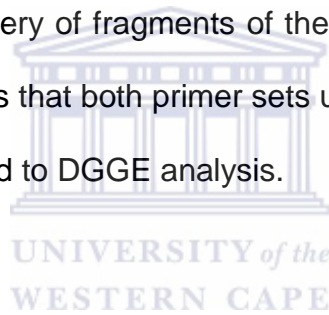


Figure 3.4: Agarose gel electrophoresis showing PCR product of ca. 190bp amplified using the first round PCR amplicons as templates with eubacterial PCR-DGGE primer set 341F-GC and 534R (Table 2.2). Sample names refer to Table 3.1, P = positive control (*E. coli* strain), N = negative control and M = Lambda DNA marker.

The successful PCR amplification of 16S rRNA gene fragments from the extracted DNA validates the effectiveness of the chosen DNA extraction method (Kirk et al., 2004). Furthermore, the recovery of fragments of the expected sizes in both rounds of PCR amplifications indicates that both primer sets used were specific. The nested-PCR amplicons were subjected to DGGE analysis.



3.5.2 Homogeneity of bacterial diversity in South African sorghum rhizosphere and rhizoplane.

DGGE separations were performed to assess the homogeneity between samples collected from the Free State, Limpopo and North West provinces of South Africa. Three samples, i.e. rhizosphere, rhizoplane and bulk soil from each sampling site were studied (Figure 3.5 A). Visual examination of the gel revealed different banding patterns between the Free State, Limpopo and North West samples, indicating different microbial diversity in South African sorghum rhizospheric environments and in bulk soils. Also, each sample consisted of unique fingerprint patterns, indicating unique microbial community structures in each soil studied.

Cluster analysis was performed to differentiate the banding patterns and the results were shown as a dendrogram (Figure 3.5 B). However, recognition of significant clusters within a dendrogram is largely a subjective process (Beckstead, 2002).

The dendrogram (Figure 3.5 B) is divided into two distinct clusters (clusters 1 and 2) sharing 3% similarity with each other. Cluster 1 consists of representatives from the Free State, Limpopo and North West samples which shared 42% similarity with each other and 7% to the NWRS-1 and NWB samples which form an out-group. Cluster 2 consists of representatives from the Free State and Limpopo rhizosphere samples which shared 73% similarity, with NWRS-1 and NWRS-3 forming an out-group. The clear distinction between the rhizosphere and rhizoplane bacterial community as shown by the dendrogram indicates that the sampling method used was accurate, enabling the separation of the two rhizospheric biological niches of interest. The fact that some bands appeared to be rhizospheric specific (Figure 3.5 A; bands a,b,c), or rhizoplane-specific (d,e,f) confirm this. Furthermore, clustering of the Free State and

Limpopo rhizospheric samples indicates that the sorghum plant has a strong influence on bacterial populations in the vicinity of its roots, which is in agreement with studies that showed that plants from different soils can have similar rhizospheric microbial community structures (Marschner *et al.*, 2004). However, scattering of the North West rhizosphere samples on the dendrogram shows that the sorghum rhizospheric community structures in the North West are different. Since the nutrients in the soil may not be evenly distributed, differences in the microbial community structures in the same soil can be observed (Wang *et al.*, 2009).

It can be concluded that sorghum plants in South Africa recruit similar bacterial populations in the rhizoplane, regardless of the differences in soil type and/or agricultural practices. In both the Free State and Limpopo provinces, the sorghum plant strongly influenced the rhizospheric bacterial composition. However, in the North West province, both the soil type and/or agricultural practices seem to influence the rhizosphere bacterial community structure, and this is in agreement with the findings of Marschner *et al.* (2004), which showed that some soils can override the plant effects on rhizospheric microorganisms.

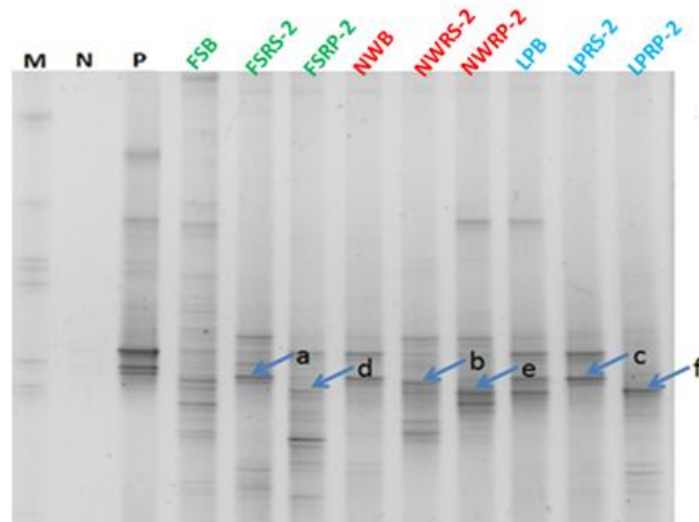


Figure 3.5 A: DGGE analysis of bacterial 16S rRNA genes amplified by PCR from sorghum rhizosphere and rhizoplane samples collected from three South African provinces. Sample names: FSB= Free State Bulk Soil, FSRS-2 = Free State Rhizosphere from sorghum plant 2, FSRP-2= Free State Rhizoplane from sorghum plant 2. LPB= Limpopo Bulk Soil, LPRS-2 = Limpopo Rhizosphere from sorghum plant 2, LPRP-2= Limpopo Rhizoplane from sorghum plant 2. NWB= North West Bulk Soil, NWRS-2 = North West Rhizosphere from sorghum plant 2, NWRP= North West rhizoplane from sorghum plant 2. M = marker, P= Positive control, and N= Negative control.

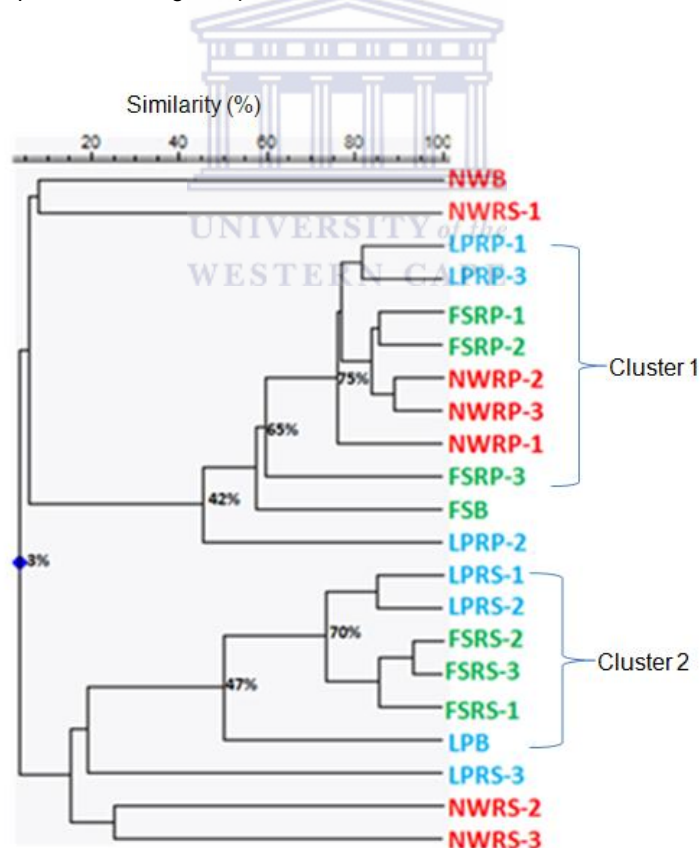


Figure 3.5 B: Cluster analysis of community bacterial 16S rRNA gene fingerprints from sorghum plant using the Pearson correlation and UPGMA algorithm. Sample names: FSB= Free State Bulk Soil, FSRS/RP-(1-3) = Free State Rhizosphere/Rhizoplane from plant 1 to 3, LPB= Limpopo Bulk Soil, LPRS/RP-(1-3) = Limpopo Rhizosphere/Rhizoplane from plant 1 to 3. NWB= North West Bulk Soil, NWRS/RP-(1-3) = North West Rhizosphere/Rhizoplane from plant 1-3.

3.5.3 Comparative DGGE analysis of bacterial diversity in sorghum rhizosphere

Figure 3.6, shows the DGGE fingerprints of the bacterial communities of the bulk soils and rhizosphere samples of the three sorghum plants collected from each of the three provinces. The rhizosphere banding patterns of the 3 plants from the Free State showed little variation, but differ to the bulk soil patterns. This indicates that the bacterial community structure in the rhizospheres of the 3 plants from the Free State sampling sites is similar, but different to the community in the bulk soil. This is in agreement with Marschner and Timonen (2006), who reported that the species composition of microbial communities in the rhizosphere differs from the bulk soil. This is a clear indication that the sorghum plants growing in the Free State have a strong influence on the sorghum rhizosphere samples; In the North West samples the rhizosphere banding patterns are different for each plant and for the bulk soil. This indicates that each sorghum plant sampled in the North West province recruited different bacterial populations. Carelli et al. (2000) showed that the plant-developmental stage of *Sinorhizobium meliloti* had strong effects on the microbial community structure in the rhizosphere. Within the Limpopo sampling site, samples LPRS-1 and LPRS-2 had similar banding patterns that were also similar to that of the bulk soil, but significantly different to that of the sample LPRS-3. This suggests that the LPRS-1 and LPRS-2 samples consist of similar bacterial community structures, but are different to that in sample LPRS-3. Sample LPRS-3, although grown in the Limpopo province, is a North West province breed. It has been documented that the plant genotype can modify the root exudate compositions, which in turn influence the rhizospheric microorganisms, because microbial species differ in their ability to metabolise and compete for different carbon sources (Smalla et al., 2001).

Overall analysis of the DGGE gel revealed that the banding patterns of the bacterial populations growing in the sorghum rhizosphere from the three South African provinces are different, suggesting that sorghum plants recruit different bacterial populations to their rhizosphere in the three provinces. This may be due to differences in the soil factors such as nutrient availability and/or pH (Table 3.2). This is in agreement with studies done by Latour et al. (1999), which suggested that soil was the most important factor for determining the rhizospheric community structure.

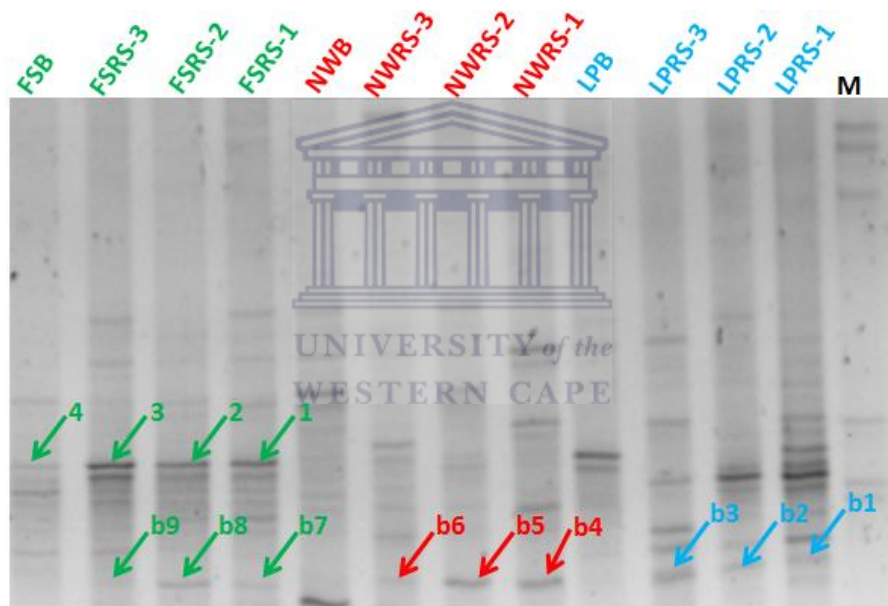


Figure 3.6: DGGE analysis (60%/40%) of bacterial 16S rRNA genes amplified by PCR from sorghum rhizosphere samples collected from three South African provinces. Sample names: FSB= Free State Bulk Soil, FSRS-(1-3) = Free State Rhizosphere from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRS-(1-3) = Limpopo Rhizosphere from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRS-(1-3) = North West Rhizosphere from sorghum plant 1-3. M is the marker.

Cluster analysis (Figure 3.7) revealed no homogeneity in sorghum rhizosphere bacterial diversity but one cluster is observed consisting of all Free State rhizosphere samples and two Limpopo rhizosphere samples (LPRS-1, LPRS-2) sharing 73% similarity. The Free State share 85% similarity with each other and the two Limpopo

samples (LPRS-1 and LPRS-2) also share 85% similarity. The high similarity between the Free State and Limpopo samples indicates that the sorghum rhizospheres in both communities comprise a similar structure, with the exception of LPRS-3 that forms an out-group. Very low similarity (25%) is observed within the North West samples, indicating that the bacterial community structure in the sorghum rhizosphere differ. This could be due to differing developmental stages of the sorghum plant during sampling, which also influences bacterial community composition (Wieland *et al.*, 2001).

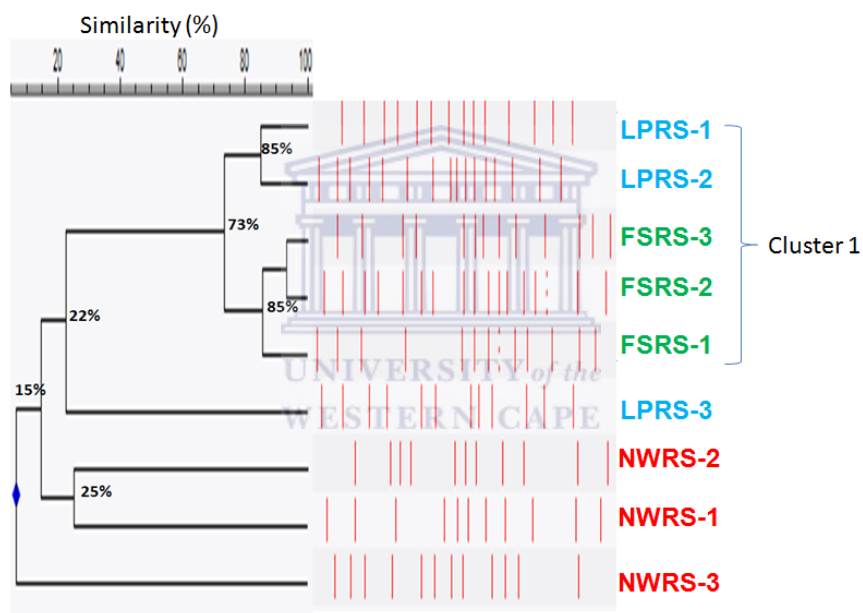


Figure 3.7: Cluster analysis of community bacterial 16S rRNA gene fingerprints using the Pearson correlation and UPGMA algorithm. In the dendrogram, sample names refer to Figure 3.7.

OTUs (marked b1-b9) in Figure 3.6 that migrated at the same position on the gel were observed across all rhizosphere banding patterns. Bands were excised and sequenced to verify if they represented a potential common bacterial species present in the rhizospheres of South African sorghum plants.

3.5.4 Sequence analysis of common DGGE bands from the sorghum rhizosphere

To verify if common DGGE bands identified represented the same bacterial species through all the rhizospheric samples (band b1-b8; Figure 3.6) sequencing was performed (please note: difficulties in amplification of b9 were experienced and therefore was eliminated from the study). The obtained sequences were compared with public databases using BLAST analysis. Three bacterial phyla were identified (Table 3.3). The similarities of the blasted sequences to the closest GenBank match ranged from 88% to 100%. The average length of the sequenced bands was 118 bp, which provided not only adequate coverage, but also excellent recovery for classification at the genus level (Liu *et al.*, 2008). The results confirmed that the co-migrated of bands in a DGGE gel were from different bacterial species. This is one of the disadvantages of the DGGE techniques. Therefore, common rhizospheric bacteria were not identified. Although the sequences identified were not the same, common phylogenetic clades were observed: clades b1, b2, b6 and b7 belonged to the bacteriodetes, clades b3-b5 belonged to the actinobacteria and clade b8 to the proteobacteria. Actinobacteria are a group of Gram-positive bacteria known to produce many important metabolites including enzymes and antibiotics, and are known to promote plant growth (Qin *et al.*, 2011). Proteobacteria have been commonly found in the rhizosphere of wheat (Lu *et al.*, 2011). The presence of *Escherichia coli* was observed in the rhizosphere of sorghum plants growing in the Limpopo and Free State farms. The farm in Limpopo is an organic farm where bovine manure was used as a fertilizer. Bovine manure is a well known source of pathogenic bacteria such as *E. coli* (Zschock *et al.*, 2000), and using cow faeces

without pre-treatment to destroy pathogenic bacteria can result in the contamination of the agricultural soil (Ingham *et al.*, 2004). In the North West farm, *E. coli* may have been introduced by irrigating with contaminated water. Orlikowski *et al.* (2009) reported that contaminated irrigation water is a primary source for the introduction of pathogens into agricultural soils.

Erwinia sp. which were identified in the Limpopo farm soils are the causative agent of fire blight in apple and pear plants (Sebahai *et al.*, 2010) and soft rot in potato, maize and other crops (Avrova *et al.*, 2002). However, some *Erwinia* sp are able to solubilise insoluble phosphate making it available to the plant, thereby acting as plant growth promoters (Rodriguez and Fraga, 1999).



Table 3.3: Blast analysis of bacterial 16S rRNA gene fragments recovered from the DGGE gel of the sorghum rhizosphere

Province	Band ID	GenBank accession no.	Closest GenBank match ^a	% Identity (no. bp)	Source	Phylogenetic clade
Limpopo	b1	EU490596.1	<i>Erwinia psidii</i> str 8423	94 (122)	Environmental samples	Bacteriodetes
	b2	CP002212.1	<i>Escherichia coli</i> clone Di 14	100 (118)	Environmental samples	Bacteriodetes
	b3	GU271756.1	<i>Unc. Erythrobacter</i> Clone 4_304	100 (112)	Soil	Actinobacteria
North West	b4	GU271756.1	<i>Unc. Erythrobacter</i> Clone 4_304	100 (110)	Soil	Actinobacteria
	b5	EU300221.1	Unc. Actinobacterium clone GASP-KC3S2_B09	98 (117)	Soil	Actinobacteria
	b6	CP002212.1	<i>Escherichia coli</i> clone Di 14	100 (119)	Environmental samples	Bacteriodetes
Free Stare	b7	AB597547.1	Unc. <i>Vibrio</i> sp clone YDB21	88 (129)	Environmental samples	Bacteriodetes
	b8	AB597547.1	Unc. <i>Acidobacteria</i> bacterium clone KBS T1	91 (119)	Environmental samples	Proteobacteria

^aonly one of best matches with database entries is listed per band sequence, unc: unculturable.



3.5.5 Comparative DGGE analysis of bacterial diversity in the sorghum rhizoplane

Figure 3.8 presents the fingerprints of both sorghum rhizoplane and bulk soil microbial communities. Compared to the rhizosphere banding patterns, the sorghum rhizoplane consisted of fewer but more intense OTUs. Furthermore, the banding pattern is very similar between all the sampling sites, indicating that sorghum plants have similar bacterial community structures in the rhizoplane, regardless of differences in the soil type and/or agricultural practices and geographical location. This is in line with the findings of Miethling *et al.* (2000), who reported that plants may have similar microbial community structures in different soils. It can therefore be concluded that sorghum plants growing in South Africa recruit similar bacterial community structures to their rhizoplanes, which indicates that the plant has a major influence in determining the rhizoplane community structure. This was reported by Gomez *et al.* (2010) who demonstrated the mangrove has a strong influence in determining the rhizospheric microbial community structure.

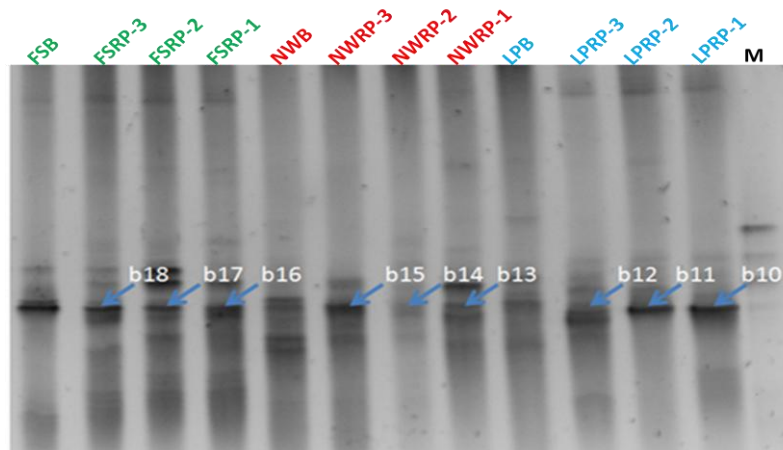


Figure 3.8: DGGE analysis (60%/40%) of bacterial 16S rRNA genes amplified by PCR from sorghum rhizoplane samples collected from three South African provinces. Sample names: FSB= Free State Bulk Soil, FSRP-(1-3) = Free State Rhizosphere from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRP-(1-3) = Limpopo Rhizosphere from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRP-(1-3) = North West Rhizosphere from sorghum plant 1-3. M is the marker.

With the exception of LPRP-2, cluster analysis of rhizoplane DGGE banding patterns revealed homogeneity in sorghum rhizoplane bacterial diversity (60% similarity) (Figure 3.9). In addition, representatives from the Free State (FSRP1 and FSRP-2), Limpopo (LPRP-1 and LPRP-3) and North West (NWRP-1, NWRP-2 and NWRP-3) samples shared a 76% similarity. It can thus be concluded that sorghum plants in South Africa recruit similar bacterial populations to their rhizoplane regardless of the differences in the soil type, geography and/or agricultural practices.

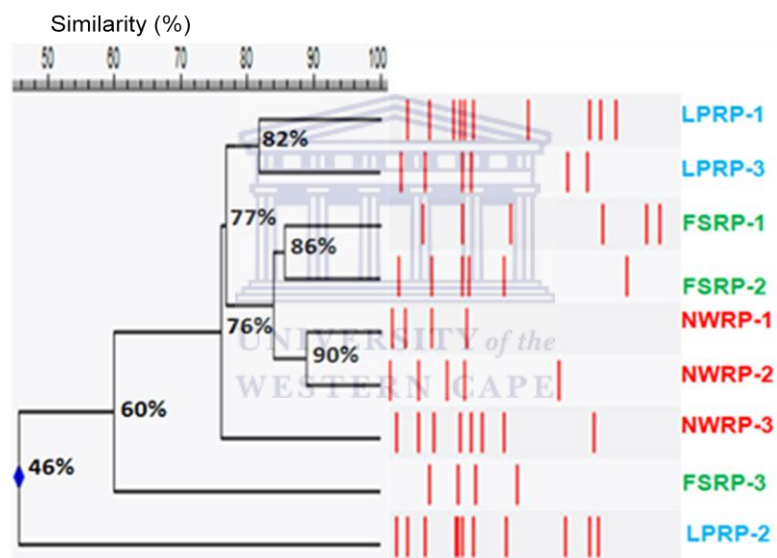


Figure 3.9: Cluster analysis of community bacterial 16S rRNA gene fingerprints using the Pearson correlation and UPGMA algorithm. In the dendrogram, sample names refer to Figure 3.8.

Co-migration of bands was also observed, which could indicate the presence of common bacteria associated with South African sorghum rhizoplanes (b10-b18).

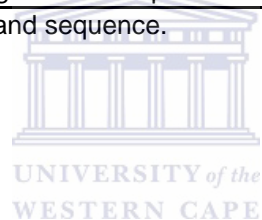
3.5.6: Bacterial sequence analysis of sorghum rhizoplane samples

Nine bands that migrated at the same distance on the DGGE gel (b10-b18; Figure 3.8) were excised and sequenced (Table 3.4). All the blasted sequences revealed high similarities to *Bacillus megaterium* and *Bacillus aryabhatai*, indicating that these microorganisms are common in the rhizoplane of South African sorghum. *Bacillus* species are a group of Gram-positive bacteria able to produce phytohormones, which enhance growth of plant roots, thereby increasing the ability of a plant to take-up nutrients and water (Saharan and Nehra, 2011). It can be concluded that the occurrence of *Bacillus* species in the rhizoplane indicates that although the rhizosphere and the rhizoplane are closely related in physical distance, the availability of nutrients, which is important in determining the bacterial populations in these environments, is different. The presence of these microorganisms in the sorghum rhizoplane raises a suspicion that they may be involved in promoting the growth of sorghum.

Table 3.4: Blast analysis of bacterial 16S rRNA gene fragments recovered from the DGGE gel of the sorghum rhizoplane

Province	Band ID	GenBank accession no.	Closest GenBank match ^a	% Identity (no. of bp)	Source	Phylogenetic clade
Limpopo	b10	JN656233.1	<i>Bacillus megaterium</i> str p705	100 (116)	soil	Bacteriodetes
	b11	JN642548.1	<i>Bacillus megaterium</i> EN2	100 (119)	soil	Bacteriodetes
	b12	JN656233.1	<i>Bacillus megaterium</i> str p705	100 (104)	soil	Bacteriodetes
North West	b13	JN656233.1	<i>Bacillus megaterium</i> str p705	99 (108)	soil	Bacteriodetes
	b14	JN656233.1	<i>Bacillus megaterium</i> str p705	99 (118)	soil	Bacteriodetes
	b15	JN656233.1	<i>Bacillus megaterium</i> str p705	99 (121)	soil	Bacteriodetes
Free State	b16	JN656233.1	<i>Bacillus megaterium</i> str p705	97 (119)	soil	Bacteriodetes
	b17	JN656233.1	<i>Bacillus megaterium</i> str p705	100 (104)	soil	Bacteriodetes
	b18	JN656233.1	<i>Bacillus megaterium</i> str p705	95 (111)	soil	Bacteriodetes

^aonly one of best matches with database entries is listed per band sequence.



3.5.7 T-RFLP analysis of bacterial diversity in the sorghum rhizospheric environment

The T-RFLP fingerprinting method was used to study the sorghum rhizospheric-associated bacterial communities and to validate the results obtained using DGGE. The non-metric multidimensional scaling plot (NMDS; Figure 3.10) is a data analysis technique that displays the structure of distance-like data as a low dimensional space (usually in 2D) in ordination plots. In these plots, a complex microbial community is represented by a point, and the closer the scattered points are, the more similar the communities are to each other and vice versa (Chiffman *et al.*, 1981).



Figure 3.10 revealed that there is only a 20% similarity in the bacterial community structure in the sorghum rhizospheric environments sampled in South Africa. Thus, T-RFLP fingerprinting showed that the bacterial community structure in the sorghum rhizospheric environment is different in the three provinces. This could be due to differences in the soil types and agricultural practices which may have a selective influence on rhizospheric microorganisms (Maschner *et al.*, 2004).

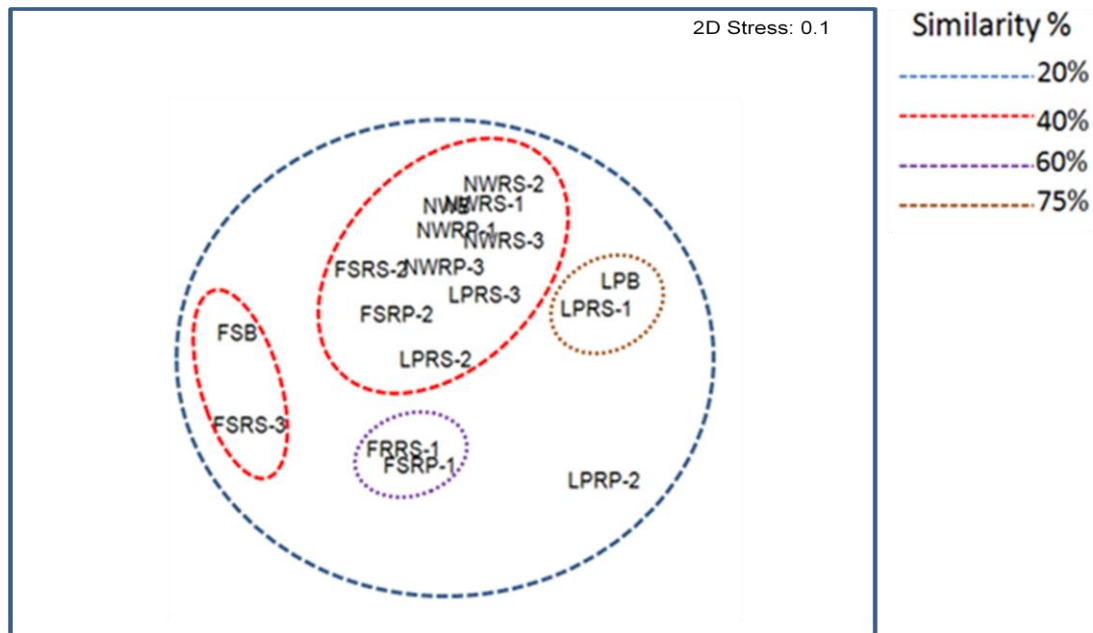


Figure 3.10: 2D-dimensional, non-metric MDS ordination (stress = 0.1) based on S17 Bray Curtis similarities of T-RFLP patterns from bacterial 16S rRNA gene fragments digested with *Hae*-III. Sample names: FSB= Free State Bulk Soil, FSRP/RP-(1-3) = Free State Rhizosphere/Rhizoplane from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRS/RP-(1-3) = Limpopo Rhizosphere/Rhizoplane from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRS/RP-(1-3) = North West Rhizosphere/Rhizoplane from sorghum plant 1-3.



T-RFLP measurements detected higher bacterial diversity in the Limpopo samples yielding a total of 160 TRFs and an average of 27 TRFs in each sample, followed by the North West with 152 TRFs and an average of 25 TRFs in each sample. The Free State had the lowest bacterial diversity with 148 TRFs, and an average of 25 TRFs in each sample. Table 3.5 summarises the common TRFs in sorghum rhizosphere and rhizoplane samples based on T-RFLP fragments.

Table3.5: common TRFs in the rhizosphere and rhizoplane samples from Free State, Limpopo and North West.

Rhizospheric Environment	Sample Location	Common TRFs (bp)
Rhizoplane	Free State	61, 211, 214
	Limpopo	
	North West	
Rhizosphere	Free State	72, 192, 211,238
	Limpopo	
	North West	

Four T-RFs were found to be common in all the sorghum rhizosphere samples, and three T-RFs in the sorghum rhizoplane (Table 3.5). A common T-RF (211bp) was present in the rhizosphere and rhizoplane of all samples.

The 16S rRNA genes corresponding to the common T-RFs were further analyzed to identify the possible bacterial species corresponding to the T-RFs. The 16S rRNA genes amplified with bacterial primers (E9F and U1510R) were subjected to *in silico* in order to identify the possible species associated with the samples. Predictive identification with the MiCA3 program (Microbial Community Analysis III) was achieved but the results did not pinpoint a single species. The OTU for the 61bp T-RF was assigned to *Isosphaera pallida*, a member of planctomycetes, that were originally isolated from aquatic habitats and are involved in ammonium oxidation (Chouari *et al.*, 2003; Fuerst and Sagulenko, 2011). The 192bp T-RF was assigned to several by *Rhizobium* species, known for symbiotic nitrogen fixation activities when growing in association with leguminous plants (Zahran *et al.*, 1999). Furthermore, Coelho *et al.* (2008) has identified *Rhizobium* sp in the sorghum rhizosphere, providing evidence for their potential growth promotion by fixing nitrogen. The 238bp T-RF was assigned to *Paenibacillus* species which which exhibit hyperparasitic activity, by producing extracellular chitinase, suppressing

Fusarium oxysporum f.sp. *Cucumerium* (Acuna *et al.*, 2011). The OTU of the 211bp T-RF matched to many bacterial species of which *Bacillus*, *Burkholderia*, and *Streptomyces* were dominant (Table 3.6). The OTU of the 72bp T-RF couldn't be assigned to any bacterial species. These bacterial species have been found to be associated with plant roots acting as growth promoters (Compant *et al.*, 2005). The OUT of 214bp corresponds to *Geobacter metallireducens*, a bacterium with iron-reducing capabilities (Aklujkar *et al.*, 2009).

Table 3.6: *In silico* digestion of bacterial T-RF with *HaeIII* restriction enzymes.

T-RF	Species Match
61bp	<i>Isosphaera pallida</i> str. IS 1B ATCC 43644 (T). (X64372)
192bp	<i>Rhizobium</i> CCBAU71462 CCBAU 71462. (AF195031) <i>Rhizobium etli</i> str. SEMIA 0430 USDA 2667. (U47303) <i>Rhizobium gallicum</i> str. R602sp. (U86343) <i>Rhizobium hainanense</i> str. I66 CCBAU 57015 (T). (U71078) <i>Rhizobium leguminosarum</i> IAM 12609 (T). (D14513)
211bp	<i>Bacillus coagulans</i> JCM 2257. (D78313) <i>Brevibacillus agri</i> NRRL NRS-1219 (T). (D78454) <i>Brevibacillus borstelensis</i> NRRL NRS-818 (T). (D78456) <i>Brevibacillus brevis</i> JCM 2503 (T). (D78457) <i>Brevibacillus brevis</i> NCIMB 9372 (T). (X60612) <i>Brevibacillus centrosporus</i> NRRL NRS-664 (T). (D78458) <i>Brevibacillus choshinensis</i> str. HPD52. (D78459) <i>Brevibacillus formosus</i> NRRL NRS-863 (T). (D78460) <i>Brevibacillus parabrevis</i> IFO 12334 (T). (D78463) <i>Brevibacillus reuszeri</i> NRRL NRS-1206 (T). (D78464) <i>Burkholderia 'SAP II'</i> str. SAP II. (AF052387) <i>Burkholderia</i> sp. (U37344) <i>Streptomyces espinosus</i> NRRL 5729. (X80826) <i>Streptomyces</i> sp. NRRL 3890. (X81574)
214bp	<i>Geobacter metallireducens</i> str. GS-15 ATCC 53774 (T). (L07834)
238bp	<i>Paenibacillus macquariensis</i> str. 673 NCTC 10419 (T.) (X60625) <i>Paenibacillus</i> sp. Str. KK19 (AJ011322)

3.5.8 T-RFLP analysis of the bacterial community structure in the sorghum rhizosphere

As in the previous section, T-RFLP analysis was used for comparative analysis of the sorghum rhizospheric bacterial community structure. The NMDS ordination plot (Figure 3.11) showed a very low similarity in bacterial community structure across all sampling sites, suggesting that the sorghum plants have different bacterial communities in the various rhizospheres. It is divided into two clusters, cluster 1 consisted of Free State (FSRS-2) and Limpopo (LPRS-2) samples sharing only 40% similarity and cluster 2 consisted of all North West rhizosphere samples also sharing 40% similarity. This low similarity observed between Free State and Limpopo indicates that the sorghum plant in the two provinces is influenced by soil type and/or agricultural practices in determining the bacteria rhizosphere colonizers. The clustering together of the North West samples indicates that the sorghum plants recruit similar bacterial community structures.

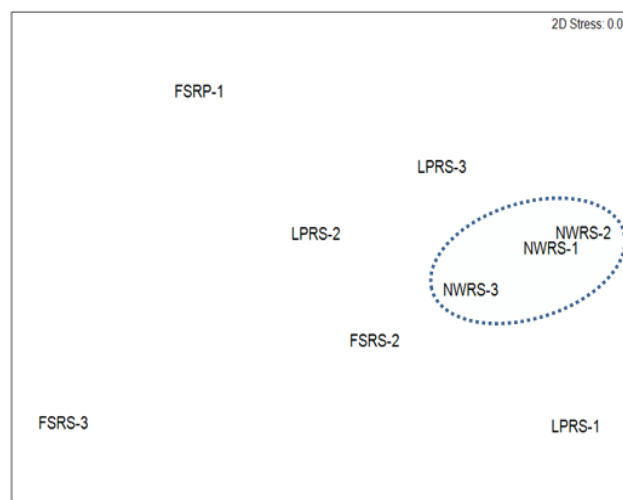


Figure 3.11: NMDS ordination plot showing the relatedness of T-RFLP profiles of *HaellI*-digested 16S rRNA amplicons from sorghum rhizosphere. Sample names refer to Figure 3.10.

3.5.9 T-RFLP analysis of bacterial community structure in sorghum rhizoplane

Very low similarity (<3%) was observed for bacterial community structure of sorghum rhizoplane between the three sampling sites (Figure 3.11). Free State (FSRP-1 and FSRP-2) and North West (NWRP-1 and NWRP-3) shared 50% similarity to each other, while the Limpopo samples scatter throughout the dendrogram. This indicates that the bacteria community in the Free State and North West rhizoplane is similar, but differ from the Limpopo rhizoplane.

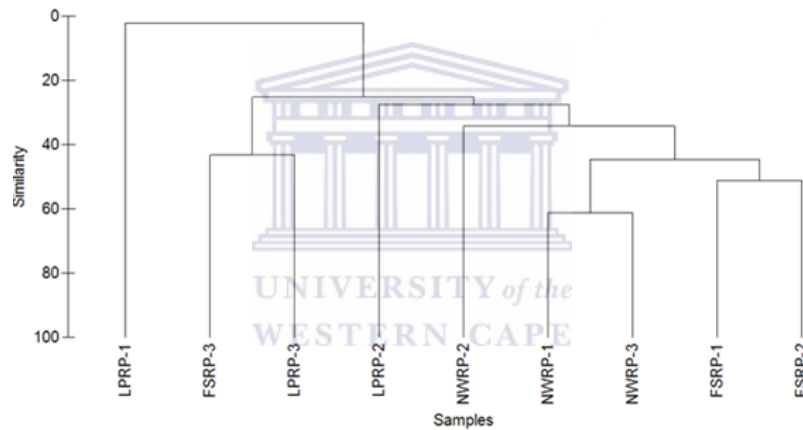


Figure 3.12: Dendrogram showing the relatedness of T-RFLP profiles of *HaellI*-digested 16S rRNA from sorghum rhizoplane.

3.5.10 Isolation of *Bacillus* sp. from the sorghum rhizoplane

Using DGGE and T-RFLP analysis *Bacillus* sp. was identified as a common bacterium in the sorghum rhizospheric environment of the three sampling sites. Three colonies from all three provinces were analysed, based on differences in the phenotypic appearance; i.e. on shape, size and colour. The colonies were subjected to ARDRA analysis (Figure 3.13). ARDRA is a molecular tool used to differentiate strains of various bacterial species. The results are read as profile patterns on an agarose gel (Lucchini and Altwegg, 1992). Bacteria producing similar profiles following digestion with the same restriction enzyme are considered to be the same OTU, often the same strain. This is further confirmed by sequencing of the 16S rRNA. ARDRA has been successfully used to study soil microbial structure during cropping period cotton (Rai *et al.*, 2010).

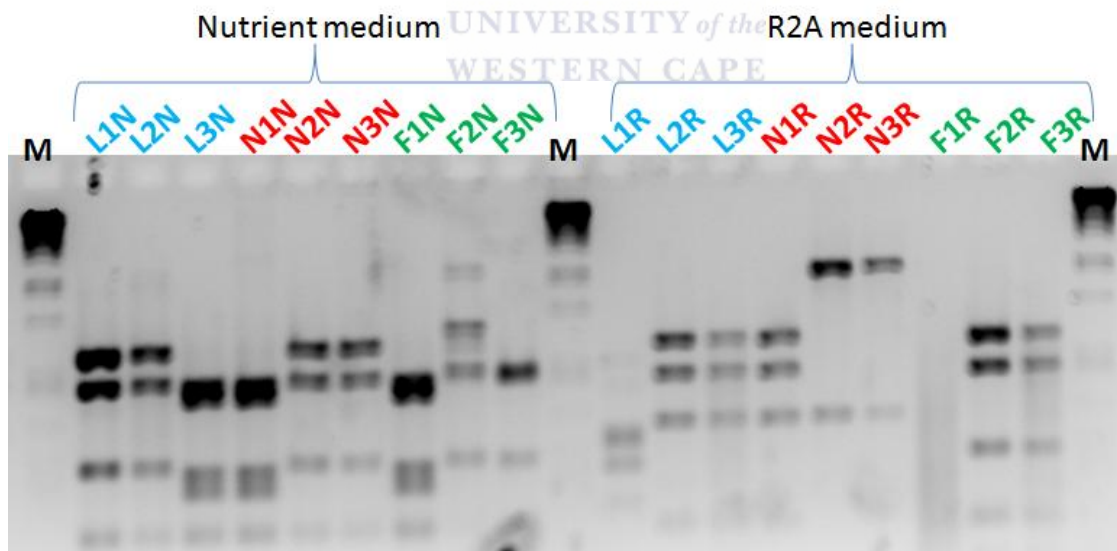


Figure 3.13: Genotyping of bacterial 16S rRNA amplified from the bacterial colonies using the bacterial primer set, E9F and U1510R. The PCR-amplicons were digested with *Hae*III enzyme. Lane 1-3 are Limpopo isolates, Lanes 4-6 are North West isolates, Lanes 6-9 are Free State isolates. N is nutrient agar medium, R is R2A medium and M is the molecular weight marker.

Of the 7 patterns observed, two common patterns were identified in ARDRA analysis of the putative *Bacillus* species from the three provinces (Figure 3.13): pattern 1 (F1N, L3N and N1N) and pattern 2 (F2R, F3R, L1N, L2N, N2N and N3N). PCR amplification was performed on the samples corresponding to the common ARDRA patterns using the bacterial-specific primers (E9F and U1510R). The amplicons were sequenced to identify the isolated bacteria (Table 3.9). Pattern 1 corresponded to *Brevibacillus* species and the second ARDRA pattern identified as *B. megaterium*. It can therefore be concluded that the *Bacillus* species are the common bacteria colonizing the sorghum rhizoplane of plants cultivated in South Africa.



Table 3.7: Blast analysis of bacterial 16S rRNA gene fragments recovered from bacteria showing common ARDRA profiles (*HaeIII* restriction enzyme)

Common ARDRA patterns	Band ID	GenBank accession no.	Closest GenBank match ^a	% Identity (no. of bp)	Source	Phylogenetic clade
Pattern 1	N1N	GQ284340.1	<i>Brevibacillus</i> sp. PCWC 539	98% (1106)	Soil	Bacteriodetes
	L3N	DQ192211.1	<i>Brevibacillus</i> sp. L67	95% (1158)	Soil	Bacteriodetes
	F1N	DQ192211.1	<i>Brevibacillus</i> sp. L67	97% (1225)	Soil	Bacteriodetes
Pattern 2	L1N	HQ336310.1	<i>Lysinibacillus sphaericus</i> str Bi3	95% (1117)	Soil	Bacteriodetes
	L2N	AY822760.1	<i>Bacillus</i> sp. KR076	96% (1164)	Soil	Bacteriodetes
	N2N	FN433022.1	<i>Bacillus megaterium</i> sp. PCWS 39	97% (1071)	Soil	Bacteriodetes
	N3N	GQ284340.1	<i>Brevibacillus</i> sp. PCWS 39	98% (1106)	Soil	Bacteriodetes
	F2R	GU048867.1	<i>Bacillus megaterium</i> str TOBCMDU-1	96% (1172)	Soil	Bacteriodetes
	F3R	JN215486.1	<i>Bacillus megaterium</i> str MB1	97% (893)	Soil	Bacteriodetes

^aonly one of best matches with database entries is listed per band sequence

3.6 Actinobacterial community diversity analysis

3.6.1 Actinobacterial 16S rRNA PCR-DGGE analysis

Actinobacteria were detected in the sorghum rhizosphere using the universal bacterial primers (Table 3.3), so we went further using actinobacterial specific primers which allow for the detection of more actinobacterial genera. Actinobacteria are a group of Gram-positive bacteria known for their production of many important bioactive compounds such as antibiotics and enzymes (Lam, 2006; Nonoh *et al.*, 2011). Forty five percent of the 22000 known biologically active compounds are produced by actinobacteria, especially from the *Streptomyces* genus (Qin *et al.*, 2011). Several studies have shown that actinobacteria can colonize plant organs (roots, stems, leaves, fruits and seeds) and promote plant growth (Qin *et al.*, 2011). In this study, PCR was used to amplify the actinobacterial 16S rRNA gene in the sorghum rhizospheric environmental samples and bulk soils as described in chapter 2. The first round PCR amplicons yielded ca. 320 bp fragments as expected (Figure 3.14).

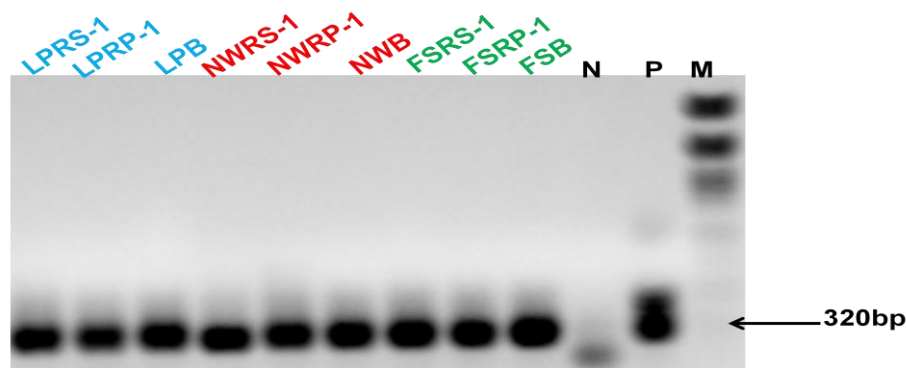


Figure 3.14: Agarose gel electrophoresis showing PCR products of ca. 320bp from actinobacterial 16S rRNA first round PCR amplification using the act235r and act562r primer set (Table 2.2). Sample names: FSB= Free State Bulk Soil, FSRP/RS-1 = Free State Rhizosphere/Rhizoplane from sorghum plant 1, LPB= Limpopo Bulk Soil, LPRP/RS-1= Limpopo Rhizosphere/Rhizoplane from sorghum plant 1. NWB= North West Bulk Soil, NWRP/RS-1 = North West Rhizosphere/Rhizoplane from sorghum plant 1. P= Positive control, N= Negative control, M= is the marker.

A nested PCR using the actinobacterial-specific primers 341f-GC and act562r (Table 2.2) was then performed for DGGE analysis. DGGE amplicons yielded a *ca.* 220bp fragment (Figure 3.15) was then performed for the DGGE analysis. Distilled water was used as the negative control, and didn't amplify in all reactions, indicating that there was no contamination.

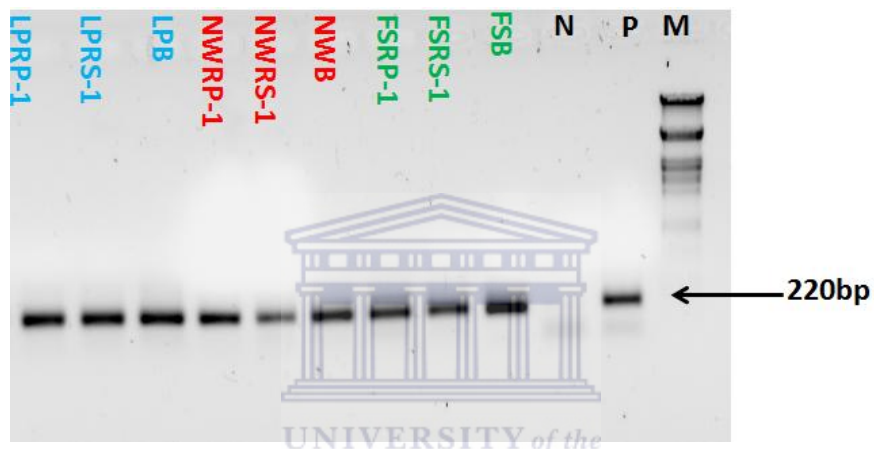


Figure 3.15: Agarose gel electrophoresis showing PCR product of *ca.* 220bp from actinobacterial 16S rRNA first round PCR amplified using 341f-GC and act562r primer set (Table 2.2). sample names refer to Figure 3.14.

3.6.2 Comparative DGGE analysis of actinobacterial diversity in sorghum rhizosphere

Figure 3.16 shows the DGGE fingerprints of the actinobacterial community structure present in the sorghum rhizosphere. Visual analysis of the DGGE banding patterns revealed diverse actinobacterial communities. The DGGE banding pattern within the North West province samples are similar but different from those in the other provinces. Overall, analysis of the DGGE gel revealed that sorghum plants recruit different actinobacterial populations in their rhizosphere, which could be due to the strong impact of geographical, agricultural practices, plant age and many other environmental factors (Smalla *et al.*, 2001). Common bands were not observed in the rhizosphere of sorghum.

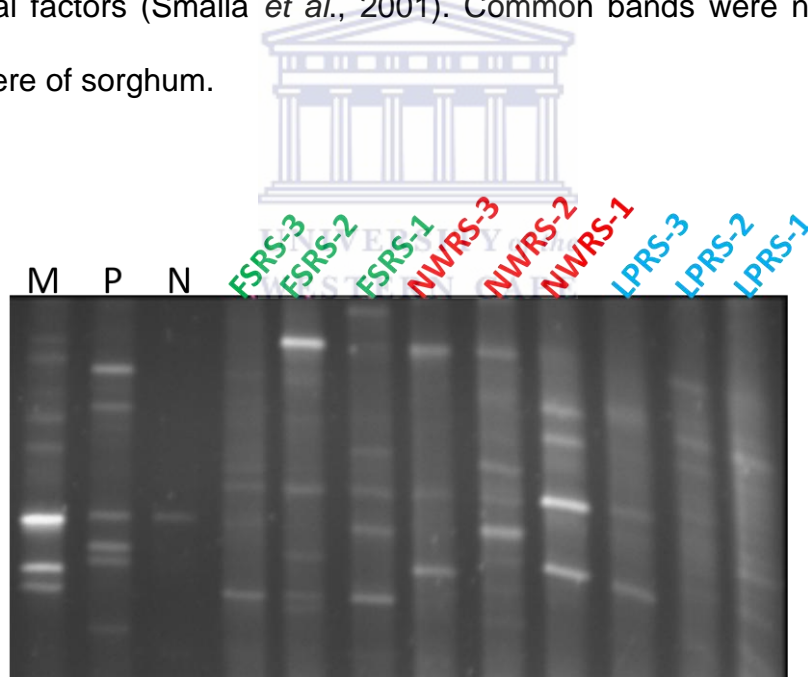


Figure 3.16 DGGE analysis of actinobacterial 16S rRNA genes amplified by PCR from sorghum rhizosphere samples collected from three South African provinces. Sample names: FSB= Free State Bulk Soil, FSRs-(1-3) = Free State Rhizosphere from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRS-(1-3)= Limpopo Rhizosphere from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRS-(1-3) = North West Rhizosphere from sorghum plant 1 to 3. P= Positive control, N= Negative control, M= is the marker.

The rhizosphere DGGE gel was further subjected to MDS analysis. The MDS (Figure 3.17) revealed that the North West samples form a cluster, indicating similar actinobacterial community between them. The Free State and Limpopo samples scatter throughout the plot, showing that their actinobacterial community structure consist of different populations. In conclusion, the sorghum plants recruit similar actinobacterial populations in North West, but different populations in Free State and Limpopo.

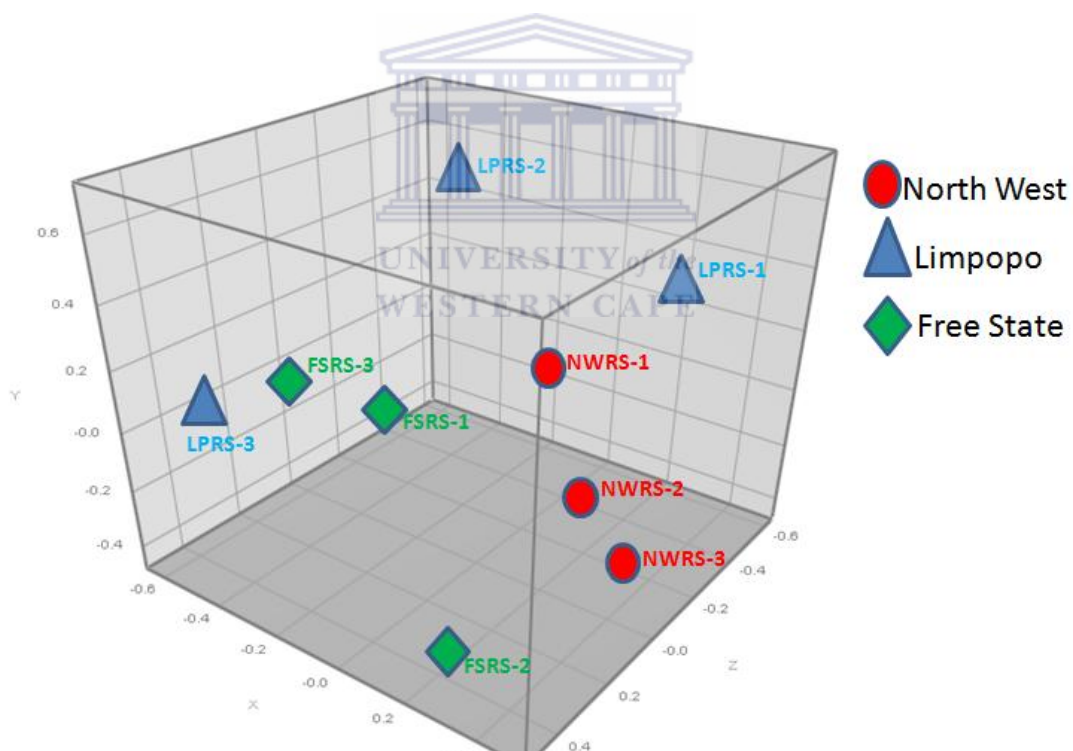


Figure 3.17: Multi-dimensional scaling (MDS) analysis of rhizoplane actinobacterial 16S rRNA community fingerprints highlighting the positions occupied by representatives of the three sampling sites (Free State, Limpopo, and North West).

3.6.3 Comparative DGGE analysis of actinobacterial diversity in the sorghum rhizoplane

Figure 3.8 A, shows the fingerprints of the actinobacterial community both in the bulk soil and rhizoplane samples. Visual investigation of the fingerprints showed that both the Limpopo and the North West rhizoplane banding patterns have more OTUs than their bulk soils; this could be due to the root exudates released which stimulates growth of microorganisms around the plant roots. The rhizoplane and bulk soil banding patterns in Free State are very similar, indicating the presence of site-specific actinobacterial populations.

An MDS (Figure 3.18 B) was generated from the DGGE gel. Limpopo sorghum rhizoplane communities formed a cluster together and separately from their bulk soil. This shows that the actinobacterial community in the sorghum rhizoplane in Limpopo is similar, and differed from their bulk soil. Contrastingly, the Free State and North West samples scattered on the NMDS plot, indicating that the sorghum rhizoplane actinobacterial community structure in the two provinces consist of different populations. In conclusion, the sorghum plants in Limpopo province recruit similar actinobacterial populations to their rhizoplanes, but recruit different populations in both in the Free State and North West.

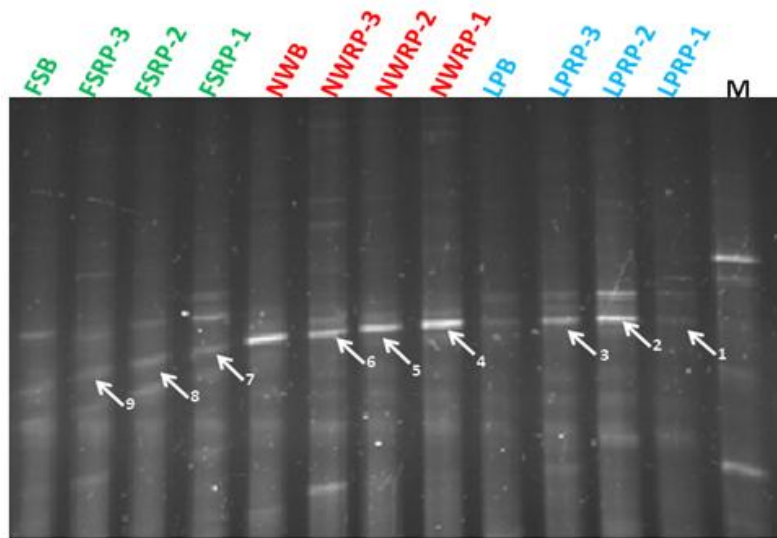


Figure 3.18 A: DGGE analysis of actinobacterial 16S rRNA genes amplified by PCR from sorghum rhizoplane samples collected from three South African provinces. Sample names: FSB= Free State Bulk Soil, FSRP-(1-3) = Free State Rhizoplane from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRP-1= Limpopo Rhizoplane from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRP-1 = North West Rhizoplane from sorghum plant 1 to 3. P= Positive control, N= Negative control, M= is the marker. This is however not the best DGGE image, but was used as the representatives of archaea in the rhizoplane.

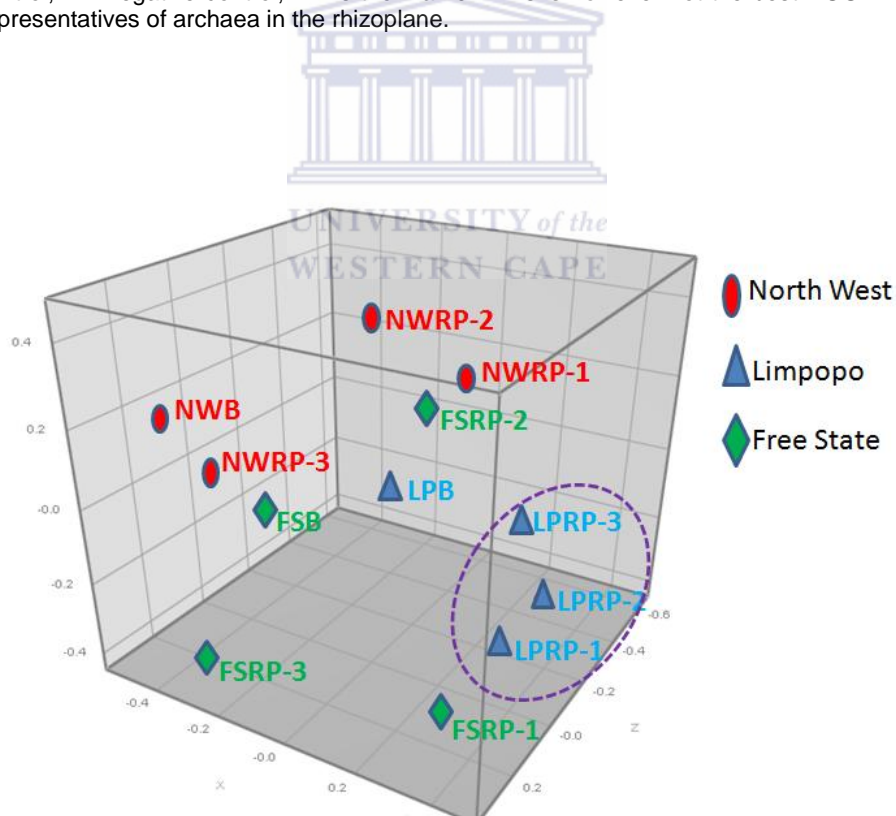


Figure 3.18 B: Multi-dimensional scaling (MDS) analysis of the rhizoplane actinobacterial 16S rRNA community fingerprints highlighting the positions occupied by representatives of the three sampling sites (Free State, Limpopo, and North West).

3.6.4 Actinobacterial sequence analysis from sorghum rhizoplane

A common band was identified from each sample (Fig 3.18 A) and was excised and sequenced from each sample (Table 3.10). All the blasted sequences revealed high similarity to *Arthrobacter* species which are known rhizosphere-colonizing bacteria (Tokala *et al.*, 2002). *Arthrobacter* has been routinely isolated from rhizospheric soil of various plants such as tomato, rice, wheat and soybean (Banerjee *et al.*, 2010). In the rhizosphere, *Arthrobacter* improve solubilization of fixed soil phosphorus, resulting in higher crop yields. Our study showed the presence of *Arthrobacter* sp. in rhizoplane of sorghum cultivated in the three different provinces. The phosphate solubilising activity of *Arthrobacter* sp. is a desirable trait of competent PGPRs.



Table 3.8: Blast analysis of actinobacterial 16S rRNA gene fragments recovered from the DGGE gel of the sorghum rhizoplane

Province	Band ID	GenBank accession no.	GenBank closest match	% Identity (no. of bp)	Source	Phylogenetic clade
Limpopo	1	JN676108.1	<i>Arthrobacter</i> sp. PAO32	96% (158)	Soil	Actinobacteria
	2	JN676108.1	<i>Arthrobacter</i> sp. PAO32	96% (160)	Soil	Actinobacteria
	3	JN676108.1	<i>Arthrobacter</i> sp. PAO32	96% (139)	Soil	Actinobacteria
North West	4	JN676108.1	<i>Arthrobacter</i> sp. PAO32	96% (159)	Soil	Actinobacteria
	5	AY833101.1	<i>Actinobacterium</i> EC5	98% (154)	Soil	Actinobacteria
	6	JN676108.1	<i>Arthrobacter</i> sp. PAO32	96% (154)	Soil	Actinobacteria
Free State	7	JN676108.1	<i>Arthrobacter</i> sp. PAO32	94% (121)	Soil	Actinobacteria
	8	JN676108.1	<i>Arthrobacter</i> sp. PAO32	92% (157)	Soil	Actinobacteria
	9	JN676108.1	<i>Arthrobacter</i> sp. PAO32	91% (138)	Soil	Actinobacteria

^aonly one of the best matches with the database entries is listed per band sequence.

Chapter 4: Identification of archaea associated with sorghum

4.1 Introduction

The nitrification process was previously thought to be confined to ammonia-oxidizing bacteria (Hu *et al.*, 2010). Metagenomic studies have revealed a diverse set of putative archaeal ammonia monooxygenase encoding genes (*amoA*) necessary for the oxidation of ammonia to hydroxylamine before its conversion to nitrite (Wuchter *et al.*, 2006). Furthermore, Verhamme *et al.* (2011) revealed that ammonia-oxidising archaea (AOA) are generally more abundant than ammonia-oxidising bacteria in soil. This finding provides strong evidence that Archaea play a major role in the nitrogen cycling processes, a process which is important for plant growth.



This study is aimed at identifying naturally occurring Archaea which are always associated with the roots of South African sorghum plants (rhizosphere and/or rhizoplane) regardless of differences in the biotic and abiotic factors as these may be exploited to improve sorghum growth and yield.

4.2 Results

4.2.1 Archaeal 16S rRNA PCR-DGGE analysis

The diversity and composition of the archaeal community structure in the sorghum rhizospheric environment of sorghum plants has not been previously studied, but there is evidence of archaeal signals being present in agricultural soil (Treusch *et al.*, 2005). In this study, a PCR was used to amplify the archaeal 16S rRNA gene in the sorghum rhizosphere and bulk soils as described in chapter 2. The first round PCR amplicons yielded a *ca.* 930 bp fragment (Figure 4.1).

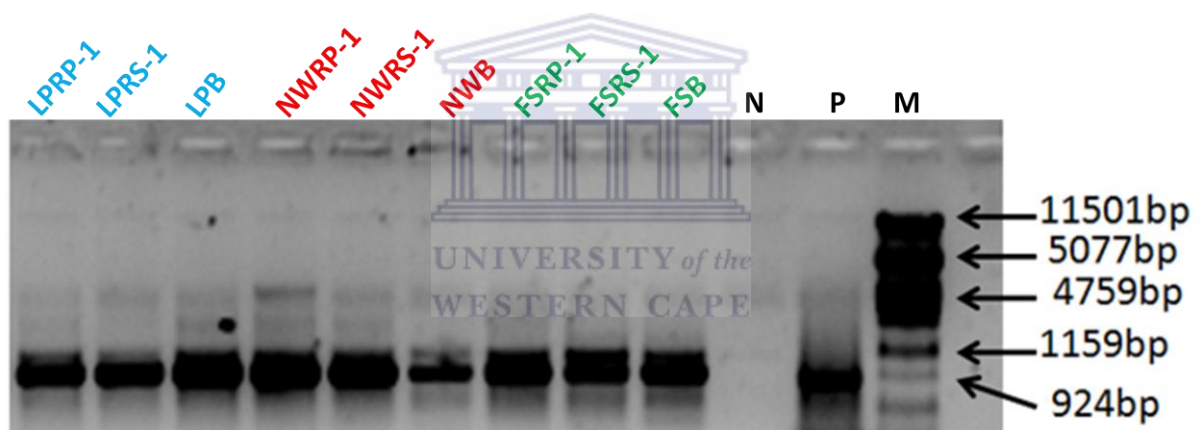


Figure 4.1: Agarose gel electrophoresis showing PCR products of *ca.* 930bp from archaeal 16S rDNA first round PCR amplified using A3Fa and AB927R primers (Table 2.2). Lanes LPRP-1, NWRP-1, FSRP-1 are rhizoplane samples, lanes LPRS-1, NWRS-1, FSRS-1 are rhizosphere samples, and LPB, NW and FB are bulk soils. P = positive control, N = negative control and M = molecular marker ladder.

For DGGE analysis, this 930 bp fragment was then used as template in a nested PCR (using the archaeal-specific 16S rRNA gene DGGE primers A340F-GC and 533R). DGGE-PCR amplicons yielded a *ca.* 170 bp fragment (Figure 4.2). Distilled water was used as the negative control, and didn't amplify in all the PCR reactions, indicating that there was no contamination.

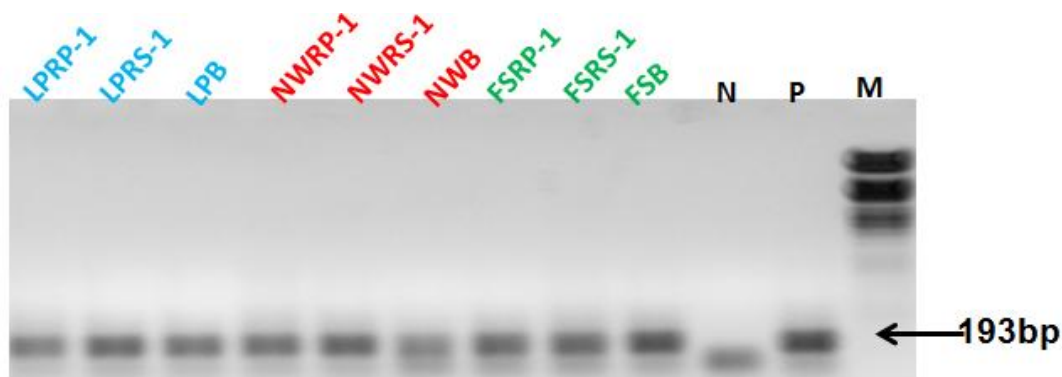


Figure 4.2: Agarose gel electrophoresis showing PCR products of ca. 170bp amplified using the first round PCR amplicons as templates with archaeal PCR-DGGE primer set A340f-GC and A533r (Table 2.2).on the agarose, sample names refer to Figure 4.1. P = positive control, N = negative control and M = molecular marker ladder.

The recovery of expected fragments in both rounds of archaeal PCR amplifications indicated that both primer sets used were specific. The nested-PCR amplicons were subjected to DGGE analysis.



4.2.2 Archaeal community diversity analysis

4.2.2.1 Comparative DGGE analysis of archaeal diversity in sorghum rhizosphere

The archaeal diversity in the sorghum rhizosphere was studied as previously described (section 3.5.2). Visual examination of the DGGE banding patterns (Figure 4.3) revealed that the archaeal community structure in the Free State rhizosphere is very similar, and differs from the surrounding bulk soil, by the presence of some bands (f1-f3). The rhizosphere banding patterns within the North West samples are very similar to each other and to their bulk soil. This indicates the presence of site specific archaeal communities, but not sorghum species association. Furthermore, the Limpopo

rhizosphere banding patterns are also similar to each other, but differ from the bulk soil. This again indicates the impact of “rhizosphere effect” in determining the rhizosphere colonizers of sorghum.

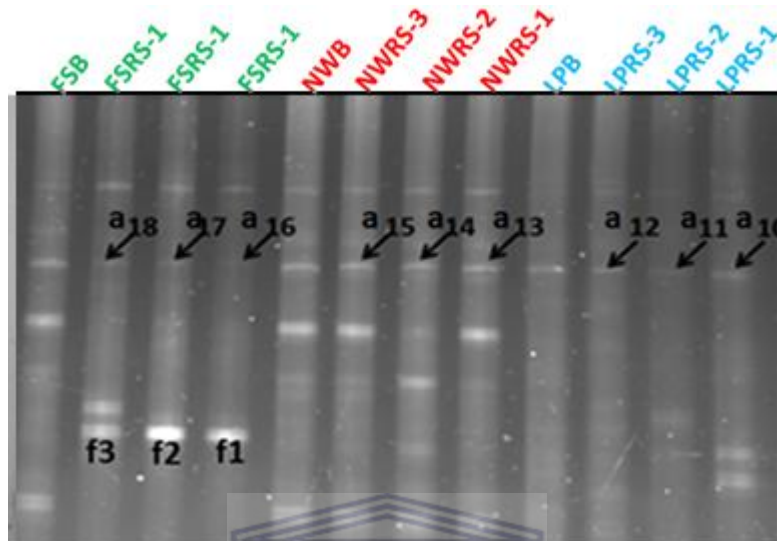


Figure 4.3: DGGE analysis of archaeal 16S rRNA genes amplified by PCR from the rhizosphere of sorghum plants samples collected in three South African provinces. Sample names: FSB= Free State Bulk Soil, FSRS-(1-3) = Free State rhizosphere from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRS-(1-3) = Limpopo rhizosphere from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRS-(1-3) = North West rhizosphere from sorghum plant 1-3, M= marker.

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Cluster analysis (Figure 4.4) of the DGGE banding patterns of archaeal communities collected from the sorghum rhizosphere revealed two Clusters sharing 34% similarity. Cluster 1 consisted of all the Free State rhizosphere samples which shared a 71% similarity. This high similarity indicates that the archaeal community structure in the rhizosphere of sorghum plants growing in the Free State province are conserved. This may be due to the soil type and/or agricultural practices. Cluster 2 is further subdivided into two sub-clusters (I and II) which share a 76% similarity and a 50% similarity to LPRS-1. Sub-cluster I consisted of two of the rhizosphere samples from Limpopo and sub-cluster II consisted of all the rhizosphere samples from the North West province, sharing 86% and 90% similarity respectively. This high similarity indicates that the archaeal

community structures in the two provinces are similar. This may be due to similar rhizosphere pH values (6.3 for Limpopo and 6 for North West) in both provinces. The dissimilarity between the LPRS-1 and other rhizosphere samples (LPRS-2 and LPRS-3) from the Limpopo province may be due to many environmental factors such as rhizodeposition in different root zones and differences in plant age. The clustering of the archaeal community from the same sampling sites can be indicative of the high influence of soil type over that of sorghum plant in determining the archaeal rhizospheric structure.

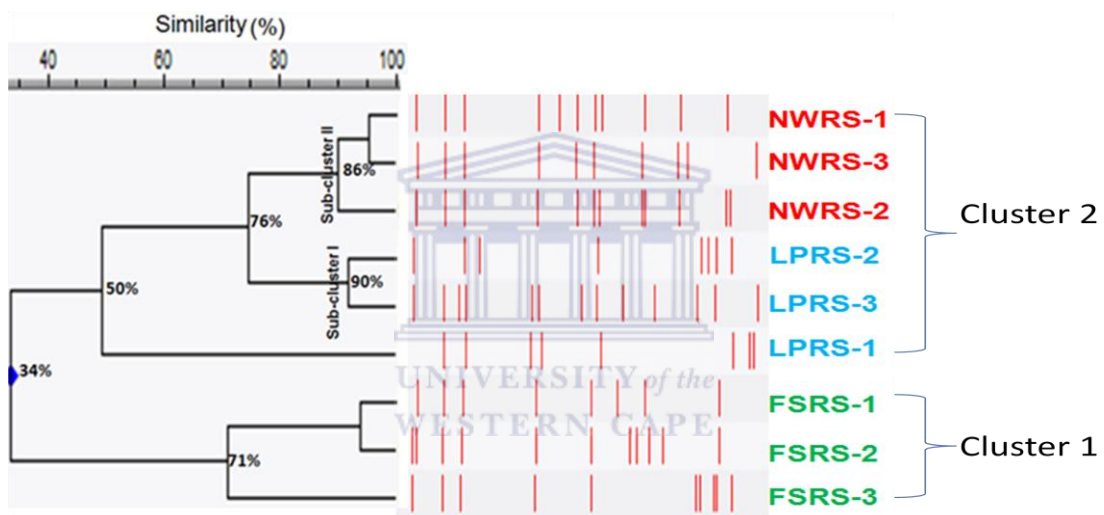


Figure 4.4: Dendrogram was calculated using the similarity matrix based on the Pearson correlation coefficient and cluster methods UPGMA. In the dendrogram, Sample names refer to Figure 4.3.

A common OTU (a10-18) was observed across all samples both in the rhizosphere and bulk soils, This may indicate the presence of the same archaeal species in the soils.

4.2.2.2 Archaeal sequence analysis of sorghum rhizosphere

The common DGGE bands (a10-a18; Figure 4.3) were sequenced. Online homology searches using NCBI and BLAST algorithm were conducted and all sequences obtained showed a 80-88% level of similarity to sequences of uncultured Archaeon (Table 4.1). The results confirmed that bands that had migrated to the same position in the same DGGE gel do not necessarily represent the same phylotype (Jackson *et al.*, 2000). Interestingly, only uncultured archaeal sequences were recovered, indicating that the majority of the archaea in the soil and/or rhizosphere are unknown. Thus, more research is needed in order to elucidate their role in the rhizosphere of sorghum plants.



Table 4.1: Identification of bacterial 16S rRNA sequences of the DGGE bands obtained from sorghum rhizosphere

Province	Band ID	GenBank accession no.	GenBank closest match	% Identity (no. of bp)	Source	Phylogenetic clade
Limpopo	a10	AB561482.1	Unc. <i>Archaeon</i>	83% (144)	soil	Archeaa
	a11	GQ871387.1	unc. <i>Archaeon</i> W5P2-B08	88% (149)	soil	Archeaa
	a12	AB561482.1	Unc. <i>Archaeon</i>	81% (143)	soil	Archeaa
North West	a13	AB561482.1	Unc. <i>Archaeon</i>	81% (152)	soil	Archeaa
	a14	GU126498.1	Unc. <i>Archaeon</i> clone FR2-C15a	83% (143)	soil	Archeaa
	a15	EU558261.1	Unc. <i>Archaeon</i> clone Ttate Houine-AP35	87% (147)	soil	Archeaa
Free State	a16	AB561482.1	Unc. <i>Archaeon</i>	84% (145)	soil	Archeaa
	a17	AB561482.1	Unc. <i>Archaeon</i>	86% (147)	soil	Archeaa
	a18	AB561482.1	Unc. <i>Archaeon</i>	89% (146)	soil	Archeaa

^aonly one of best matches with database entries is listed per band sequence. Unc: uncultured.

4.2.2.3 Comparative DGGE analysis of archaeal diversity in the sorghum rhizoplane

The DGGE banding patterns (Figure 4.5) showed high archaeal diversity in sorghum rhizoplane and bulk soils of all the provinces under investigation, indicating that archaea are abundant in the soil environment (Slikwinski and Goodman, 2004). The North West rhizoplane banding patterns are very similar and also similar to the bulk soil. Furthermore, the profiles showed common bands, i.e. n1-n4 of the same intensity that are unique in the North West fingerprints. Each lane within the Free State rhizosphere banding pattern was different from each other and also to the bulk soil. The same trend was observed within the Limpopo banding patterns.

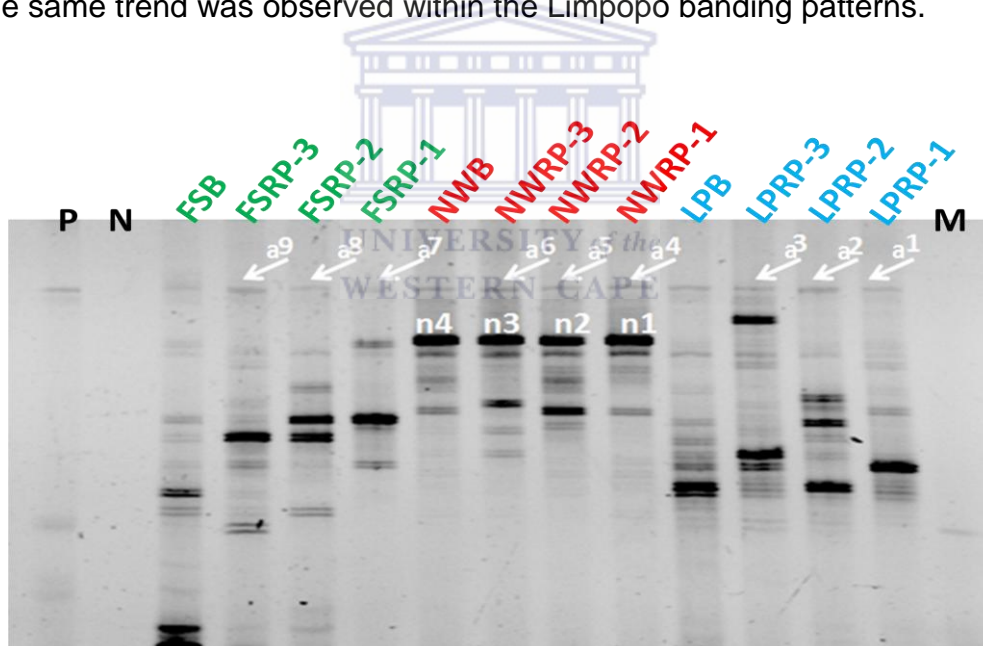


Figure 4.5: DGGE analysis of archaeal 16S rRNA genes amplified by PCR from sorghum rhizoplane samples collected from three South African provinces. Sample names: FSB= Free State Bulk Soil, FSRP-(1-3) = Free State rhizoplane from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRP-(1-3) = Limpopo rhizoplane from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRP-(1-3) = North West rhizoplane from sorghum plant 1-3. M is the marker. P= positive control, N= negative control and M= marker.

Cluster analysis (Figure 4.6) generated from the DGGE banding patterns of rhizoplane archaeal community structure revealed 2 clusters sharing 5% similarity. This indicates that the archaeal community in sorghum rhizoplane in the three

provinces under study is very different. Cluster 1 consisted of 2 representatives from Free State (FSRP-1 and FSRP-3) sharing 71% similarity, and 33% similarity to the sample FSRP-2. Cluster 2 consisted of all North West rhizoplane samples sharing 83% similarity. This indicates that the archaeal community structures within both provinces are very similar. Furthermore, the archaeal community structure in the Limpopo is very different; this might be due to differences in the developmental stages of the three plants sampled. It can therefore be concluded that the colonization of archaeal communities in sorghum rhizoplane is determined by the agricultural and/or soil type, thus they are highly similar within each province.

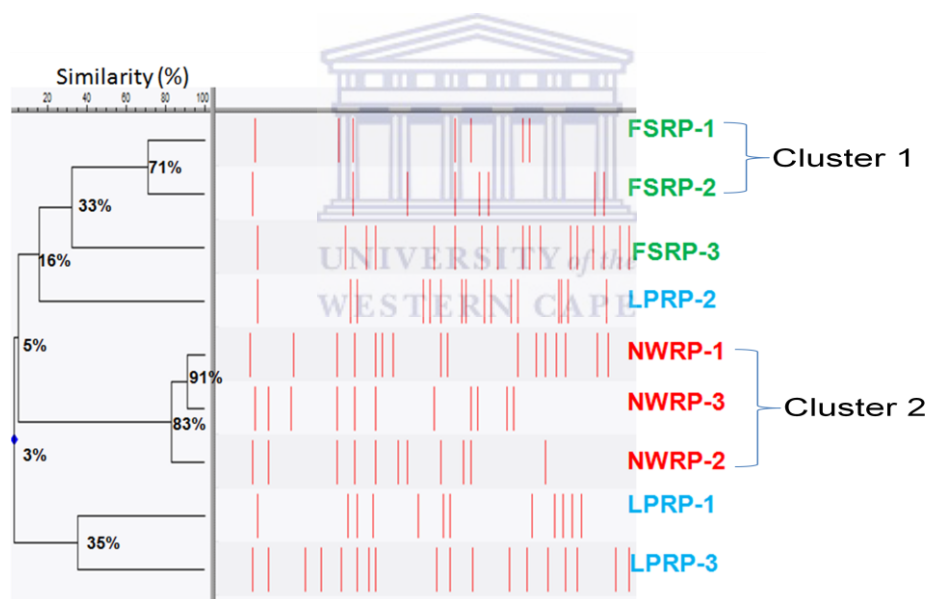


Figure 4.6: Dendrogram was calculated using the similarity matrix based on Pearson correlation coefficient and cluster methods UPGMA. Sample names refer to figure 4.5.

Despite the differences in the banding patterns between the three provinces, common bands that migrated at the same position, which could be indicative of the same archaeal population were also observed.

4.2.2.4 Archaeal sequence analysis of sorghum rhizoplane

The common band within all the samples showed an 80-89% level of similarity to sequences of uncultured *archaeon* (Table 4.2). The archaea domain has also been found globally distributed in many non-thermophilic habitats (Verhamme *et al.*, 2011). Because of a limited number of sequences analyzed, our study suggests the presence of previously unsuspected and diverse archaea in sorghum rhizospheric environment and supports the view that these microorganisms are more cosmopolitan than previously thought. Furthermore, detection of archaeal 16S rRNA sequences in the sorghum rhizospheric environment in the present study confirms the suspicion that they might be a factor in promotion of plant growth. Other articles on the possible role of archaea in growth promotion have recently been published (Verhamme *et al.*, 2011).

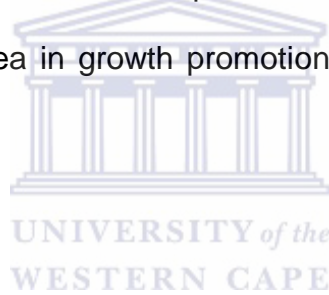


Table 4.2: Identification of the archaeal 16S rRNA sequences of the DGGE bands obtained from the sorghum rhizoplane

Province	Band ID	GenBank accession no.	GenBank closest match	% Identity (no. of bp)	Source	Phylogenetic Clade
Limpopo	a2	AB561482.1	Unc. <i>Archaeon</i>	88% (144)	soil	Archaea
	a3	GQ127624.1	Unc. <i>Archaeon</i> ARCu-75	89% (144)	soil	Archaea
North West	a4	AB561482.1	Unc. <i>Archaeon</i>	81% (152)	soil	Archaea
	a5	EU365315.1	Unc. Soil <i>archaeon</i> clone SLG-567	81% (141)	soil	Archaea
	a6	AF058730.1	Unc. <i>Crenarchaeota</i> KBSHat20	83% (141)	soil	Archaea
Free State	a7	AB561482.1	Unc. <i>Archaeon</i>	85% (145)	soil	Archaea
	a8	AB561482.1	Unc. <i>Archaeon</i>	83% (141)	soil	Archaea
	a9	AB561482.1	Unc. <i>Archaeon</i>	87% (148)	soil	Archaea

^aonly best matches with database entries in listed per band sequence. Unc: uncultured.

4.2.3 T-RFLP analysis of archaeal diversity in sorghum rhizospheric environments

The T-RFLP fingerprinting method was also used to study sorghum rhizospheric-associated archaeal community and to validate the results obtained using DGGE. NMDS (Figure 4.7) revealed that the archaeal community structure in the sorghum rhizospheric environment is very dissimilar, and is shown by the scattering of samples throughout the NMDS plot. All the Free State samples cluster together, sharing a minimum of 40% similarity with each other. Within the Free State samples, FSRS-1 and FSRP-1 (collected from the sorghum plant 1) and FSRS-2 and FSRP-2 (collected from the sorghum plant 2) share 50% similarity to each other. Four of the Limpopo (LPRS-1, LRRP-1, LPRP-3, and LPB) samples and three of the North West (NWB, NWRP-2, NWRS-1) form a cluster sharing 60% similarity. Samples collected from Limpopo plant two (LPRS-2 and LPRP-2) cluster together sharing 70% similarity to each other, and the North West rhizoplane (NWRP-1 and NWRP3) samples also cluster together sharing 40% similarity. It can therefore be concluded that the archaeal community structure in the rhizospheric environment of sorghum plants in the Free State is site-specific. Free State rhizospheric soil is acidic with a pH 4.2 while the soils in the Limpopo and North West provinces are slightly acidic with pH values of 6.3 and 6 respectively. The clustering of the samples from the Limpopo and North West provinces could be due to the fact that both sampling sites share a similar soil pH.

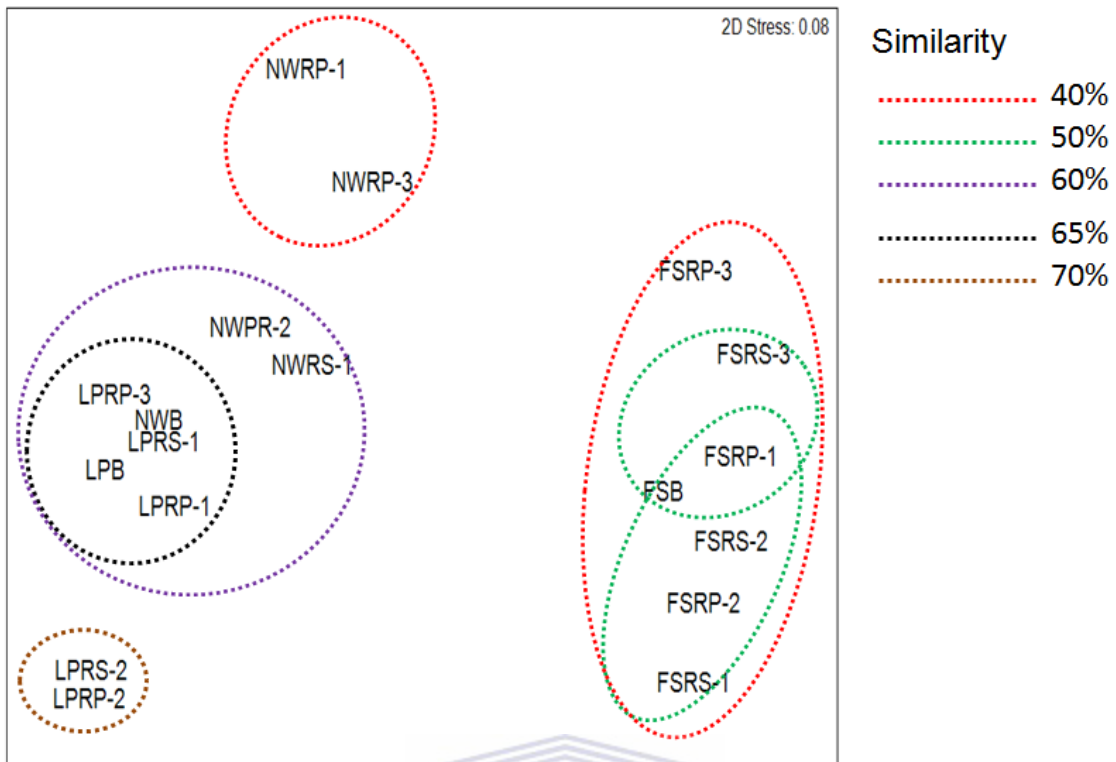
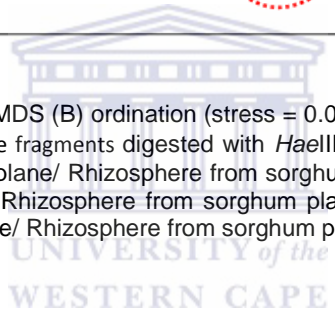


Figure 4.7: 2D-dimensional, non-metric MDS (B) ordination (stress = 0.08) based on S17 Bray Curtis similarities of T-RFLP patterns from archaeal 16S rDNA gene fragments digested with *Hae*III. Sample names: FSB= Free State Bulk Soil, FSRP/RS-(1-3) = Free State Rhizoplane/ Rhizosphere from sorghum plant 1 to 3, LPB= Limpopo Bulk Soil, LPRP/RS-(1-3) = Limpopo Rhizoplane/ Rhizosphere from sorghum plant 1 to 3. NWB= North West Bulk Soil, NWRP/RS-(1-3) = North West Rhizoplane/ Rhizosphere from sorghum plant 1 to 3.



T-RFLP analysis detected higher archaeal diversity in the Free State samples than in the North West and the Limpopo sampling sites. Table 4.3 shows the common TRFs in sorghum rhizosphere and rhizoplane samples.

Table 4.3: Summary of the common T-RFs in samples from Free State, Limpopo and North West.

Rhizospheric Environment	Sample Location	Common TRFs
Rhizoplane	Free State	26, 490
	Limpopo	
	North West	
Rhizosphere	Free State	26, 75, 160, 200,
	Limpopo	246, 299, 340,
	North West	350,389, 490

Two TRFs were found to be common in the sorghum rhizoplane of all the samples, and three TRFs in the sorghum rhizosphere. Common TRFs, 409bp were found to be present in the rhizosphere and rhizoplane of all samples and were further analyzed to identify the possible archaeal species it represents using the MiCA3 software (available at <http://mica.ibest.uidaho.edu/pat.php>). The OTU of 75bp to uncultured *Crenarchaeote* and uncultured *archaeon*, 160bp to uncultured *archaeon*, and 200bp to *Methanobacteriaceae archaeon*. It was impossible to assign archaeal species to T-RFs 26bp, 246bp, 299bp, 340bp, 350bp, 389bp, and 490bp, because archaea are not well studied in the agricultural soil environment. Theis (2007) reported that poorly studied organisms are poorly represented in the T-RFLP database.

Table 4.4: *In silico* digestion of archaeal T-RFs digested with *HaeIII* restriction enzymes

T-RF	Species Match
75bp	uncultured Crenarchaeote WMS64 uncultured archaeon OUTa 18
160bp	uncultured archaeote P3C Euryarchaeote DGGE gel band
200bp	Methanobacteriaceae archaeon 77aZ

Chapter 5: General discussion and conclusion

Africa is a semi-arid continent threatened by drought and desertification, seriously impacting its food security since the continent relies heavily on rain-fed agriculture (Camberlin *et al.*, 2009). The continent's agriculture is dominated by maize and sorghum production. Sorghum is considered as the "future crop", due to the fact that it is well adapted to the continent's climatic conditions, being the most drought resistant cereal crop (Taylor, 2003).

Plant roots are important in the survival of the plant as they are involved in root-root, root-microbe and root-insect communications (Walker *et al.*, 2003). For this study we investigated the root-microbe interactions occurring in the rhizospheric environment (rhizoplane and rhizosphere); i.e., the narrow zone of soil immediately surrounding the plant roots (Morgan *et al.*, 2005). Many studies have demonstrated that soil microorganisms in the rhizosphere interact with plant roots and promote their growth and yield (Barea *et al.*, 2005). They are known as Plant growth promoting rhizobacteria (PGPR), and are able to suppress pathogens, and solubilise nutrients (Gholami *et al.*, 2009). This work was aimed at identifying naturally occurring microorganisms always associated with the South African sorghum rhizospheric environment, which could be exploited to improve growth and yield.

As previously shown, the composition of these microorganisms in the rhizosphere can be influenced by its organic content (Girvan *et al.*, 2003). Furthermore, Das and Dkhar (2011) have shown that microbial biomass in soil treated with organic fertilizers such as plant compost and integrated plant compost was higher than that treated with inorganic fertilizers such as NPK (nitrogen, phosphorus and potassium). It was also reported that agricultural management has effects on the physical and

chemical composition of the soil, which in turn influence specific microbial community structure (Girvan *et al.*, 2003). In the three provinces studied, the organic matter content was assumed to be different, as different agricultural practices were used (Das and Dkhar, 2011). Therefore, since the three provinces sampled have different soil types, and chemistry, differences in the microbial community structure were expected between the samples from different provinces.

5.1 Bacterial 16S rRNA genes analysis

To counteract the limitation imposed by culture-dependent techniques (Smalla *et al.*, 2001), DGGE and T-RFLP fingerprinting techniques were used to study the bacterial community structure in the sorghum rhizospheric environment (rhizosphere and rhizoplane) and bulk soil (root-free soil). DGGE analysis (Figure 3.5 A) showed higher bacterial diversity in the rhizosphere environment than in bulk soils. This was also reported by Sliwinski *et al.* (2004), who proposed that this is due to exudates released by the plant root which turn the rhizosphere into an oasis in the soil. Statistical analysis of the DGGE banding patterns (Figure 3.5 B), showed differences between the bacterial communities of the rhizoplane and rhizosphere of sorghum plants, as previously reported (Wieland *et al.*, 2001). This validated the sampling procedure of keeping the two biological niches (rhizoplane and rhizosphere) for each sorghum plant separate.

The bacterial communities of the rhizospheres of the three sampling sites were separately analysed (Figure 3.6). Higher bacterial diversity was observed in the

rhizosphere of sorghum in all provinces compared to the bulk soils. This was also reported by Shi et al. (2011), who found that the plant rhizosphere has a higher microbial biomass in comparison to the surrounding bulk soil as a result of the presence of root exudates. Cluster analysis (Figure 3.7) showed a similar bacterial community structure in the Free State and Limpopo sampling sites, which differed from that present in the North West site. This could be due to differences in the nutrient contents of the soils. Both the Free State and Limpopo rhizosphere soils have lower organic nitrogen and carbon contents than the North West rhizospheric soil. Nutrient concentration can have a selective effect on bacterial diversity (Aagot *et al.*, 2001).

Compared to the rhizosphere, the bacterial diversity was lower in the sorghum rhizoplane of all sampling sites (Figure 3.8). This could be due to the fact that root exudates stimulate specific groups of microorganisms. This was seen in leguminous plants where exudates (isoflavonoids and flavonoids) stimulated the *Rhizobium* rhizosphere colonization (Bais *et al.*, 2004). Furthermore, cluster analysis (Figure 3.9) showed that the bacterial community structure in the rhizoplane of all the sampling sites was similar, highlighting the strong effect that the sorghum plant has in selecting its rhizoplane colonizers which could be important to the plant as they out-compete pathogen colonization, thereby acting as biocontrol (Lugtenberg and Kamila, 2009).

Based on DGGE analysis, the overall results showed both soil factors and agricultural practices to be important in determining the composition of rhizosphere microbial communities in sorghum. Similar results were observed by Latour et al. (1996), who identified soil type as the most important factor affecting *Pseudomonas* populations in the rhizosphere of flax and tomato. However, the high similarity in the

sorghum rhizoplane bacterial community structure of all sampling sites indicated that the soil influence decreased from the rhizosphere to rhizoplane, as previously reported (Wieland *et al.*, 2001).

In contrast to the DGGE analysis, T-RFLP (Figure 3.10) analysis showed no apparent similarity in the microbial community structures between the three sampling sites. This could be due to the higher sensitivity of the T-RFLP technique in determining the relationship of microbial communities in complex environments (Moeseneder *et al.*, 1999). However, sorghum plants in the Free State sampling site have similar bacterial community composition, and are different from that of Limpopo and North West sites. This could be due to differences in pH between the sampling sites, with Free State having lower pH value (4.2) and Limpopo and North West having similar pH values of 6.3 and 6 respectively. As previously reported by Arifuzzaman *et al.* (2010), soil pH can strongly influence microbial diversity in agricultural soils.

Using both DGGE and T-RFLP analysis *Bacillus* species were always present within the sorghum rhizospheric environment, in the three provinces. Several studies have found *Bacillus* species with plant growth promoting activities inhabiting the rhizosphere of maize, potato, sugar cane and soy bean crops (Ikeh *et al.*, 2001; Calvo *et al.*, 2010). They promote growth in many plant species including sorghum by a number of mechanisms including phytohormone production and phosphate solubilization (Acuna *et al.*, 2011; Idris *et al.*, 2009). Finding *Bacillus* species in the rhizospheric environment of sorghum across the South African provinces provided strong evidence that members of this species could be involved in sorghum growth promotion.

However, attempt to isolate *Bacillus megaterium* identified using the culture-independent techniques was unsuccessful, so future work will involve the use of *Bacillus megaterium* specific media or other molecular techniques such as Fluorescence *in situ* Hybridization (FISH).

5.2 Actinobacterial 16S rRNA genes analysis

There is growing interest in studying the actinobacterial component of PGPRs as these organisms have the potential for crop growth promotion. This is due to the production of a large number of bioactive compounds against pathogens (Franco *et al.*, 2007). The presence of actinobacteria in the sorghum rhizospheric environment can be seen as an advantage as they provide direct evidence for potential growth promotion. Several studies have reported actinobacteria in the rhizospheric environment of crops such as wheat, maize and cabbage (Janso and Carter, 2010).

In the rhizosphere, the DGGE (Figure 3.16) showed that the three provinces have different actinobacterial community structure. This indicated that the actinobacterial community in the sorghum rhizosphere is influenced by soil type and/or agricultural practices. Several studies have reported that the actinobacterial populations in soil was influenced by soil type, pH, organic matter content, cultivation, aeration and moisture content; soil pH being reported to be the most important driver of changes in actinobacterial community in agricultural soils (Arifuzzaman *et al.*, 2010; O'Donnell *et al.*, 2001). Cluster analysis of the rhizosphere DGGE banding patterns (Figure 3.17) showed that the sorghum plant recruits similar actinobacteria in the North West, while in the Free State and Limpopo provinces the communities in the

rhizosphere differs. This suggests that nutrient concentration can have a selective effect on bacterial diversity; as reported by Aagot et al. (2001).

In the rhizoplane (Figure 3.18 A), the DGGE banding pattern showed that there are several dominant groups of actinobacteria that are always present in the sorghum rhizoplane and bulk soil in all the sampling sites, representing general soil inhabitants. Furthermore, higher actinobacterial diversity was observed in the rhizoplane as compared to the rhizosphere. This could be due to the exudates gradient that is high near the root (rhizoplane), and decreasing as it moves far away from the root (Gao *et al.*, 2011). MDS Analysis of the rhizoplane DGGE banding patterns (Figure 3.18 B) showed that the Limpopo samples form a cluster, indicating similar actinobacterial populations in their rhizoplane, and no cluster was formed by both the Free State and North West. This could suggest that the sorghum plant in both Free State and North West sampling sites override the soil type and/or agricultural practices in determining the microorganisms that colonize its root, while in Limpopo the environmental factors are the main determinant.

Athrobacter species were found to always be associated with sorghum rhizoplane environment by DGGE. *Arthrobacter* species have been isolated from the rhizosphere of many crops such as wheat and barley, inhibiting growth of pathogens by producing antibiotics (Gorlach-Lira and Stefaniak, 2009). Therefore, identification of *Arthrobacter* species in the sorghum rhizoplane is a strong indication that they could be promoting growth naturally in South African sorghum.

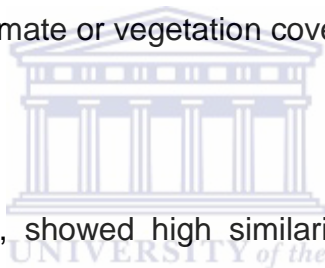
5.3 Archaeal 16S rRNA genes analysis

The archaeal domain is known to occupy extreme environmental niches, such as thermophilic springs, hydrothermal vents, high-saline waters, and oxygenic muds (Wuchter *et al.*, 2006). However, Chaban *et al.* (2006) reported that the members of the archaeal domain are more widely distributed in the soil than previously thought. Furthermore, several studies have revealed archaea association with plant rhizospheres (Sliwinski and Goodman, 2004). This was also the case in the present study, where high archaeal diversity was found in the sorghum rhizosphere (Figure 4.3) and rhizoplane (Figure 4.5) as revealed by DGGE.

The DGGE patterns of the sorghum rhizosphere (Figure 4.3) showed higher archaeal abundance in the North West sampling sites than the Free State and Limpopo. This could be due to the fact that the soil chemistry in the two provinces has similar $\text{NH}_4\text{-N}$, Carbon and Nitrogen concentrations which are different to the North West. Archaeal community composition may indeed be influenced by soil organic matter (SOM) as suggested by Nelson *et al.* (2010). Cluster analysis (Figure 4.4) of the DGGE revealed that the archaeal communities in the Limpopo and North West sampling sites are very similar, but different to that in the Free State. This indicates that the archaeal composition diversity in the three sampling sites is strongly influenced by pH, since the Limpopo and North West rhizospheric soil have similar pH but different to that in the Free State rhizospheric soil. This was reported by Nicol *et al.* (2008), who suggested that the archaeal community structure can be influenced by soil pH.

Analysis of the DGGE analysis of the sorghum rhizoplane (Figure 4.5) showed that the three sites samples in the Free State and Limpopo soils have diverse archaeal

communities, while samples from the North West have a similar community structure. This was also observed in the bacterial community structure of the rhizosphere, and may be due to differences in the nutrient concentrations between the sampling sites which may have a selective influence on microbial diversity (Aagot *et al.*, 2001). Cluster analysis (Figure 4.6) showed that the archaeal community structure in the sorghum rhizosphere was similar within each sampling site, but differed between the three sampling sites in the three provinces. This suggests that the soil type and/or agricultural practices strongly influence the archaeal community structure in the sorghum rhizosphere. This is in agreement with the studies done by Bates *et al.* (2010) who reported that the archaeal community structure in soil can be influenced by pH, elevation, climate or vegetation cover.



T-RFLP analysis (Figure 4.7), showed high similarity in the archaeal community structure from Free State. The Limpopo and North West samples cluster together, this might be due to the fact that they have similar rhizospheric pH. Soil pH is important in the solubility of nutrients and activity of soil microorganisms (Sandaa *et al.*, 1999). Furthermore, higher archaeal diversity was observed in both Free State and North West, compared to the Limpopo sampling site, clearly indicating that the inorganic fertilizers have a beneficial effect on archaeal community in the sorghum rhizosphere. This finding is in contrast with the finding by Das and Dkhar (2011) who reported that the application of organic fertilizers can result in increased microbial population compared to inorganic treatment. Due to lack of knowledge on archaea in the plant rhizospheric environment, our understanding of the different degrees and dynamics of archaeal community variation as induced by soil type, plant type or plant development is still at its initial stage.

Common T-RFs (Table 4.3) and the excised DGGE bands (Table 4.1 and 4.2) corresponded to uncultured *archaea*. Further research into the metabolic activities of these archaea is required.

5.4 Conclusion

In conclusion, this study showed that sorghum-associated rhizospheric microbial community composition in South Africa is strongly influenced by the soil type and/or agricultural practices. Contrastingly, the rhizoplane microbial community composition was more influenced by the sorghum plant itself, as they are highly similar across all sampling sites. Despite the differences in the soil type and agricultural practices, *Bacillus* species were found to be always associated with sorghum roots, and *Arthrobacter* species was always associated with the sorghum rhizoplane. These findings show that the sorghum plant in South Africa has common bacterial populations that can be engineered to enhance growth. Furthermore, this study provided some knowledge on the archaea present in sorghum-associated ecosystems. Studies of the uncultured archaea associated with the sorghum roots and their role as PGPRs would be valuable, since identification of the archaea specific to sorghum roots in South Africa could only be identified to have similarity to uncultured archaea.

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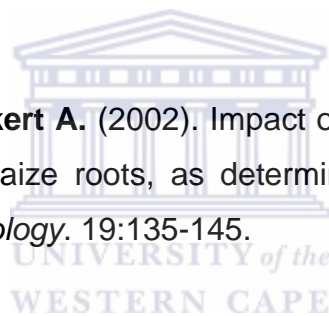
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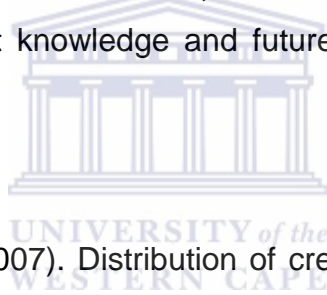
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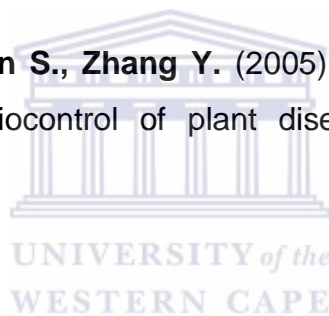
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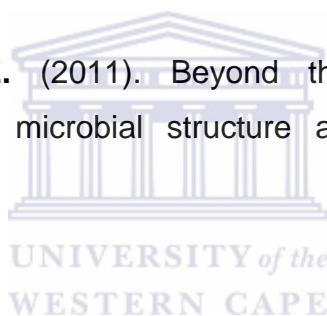
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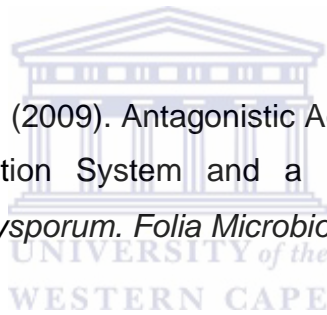
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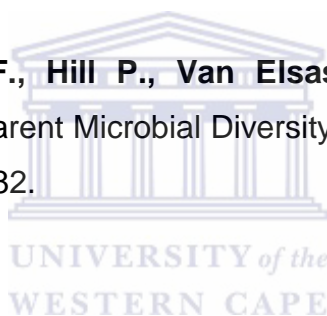
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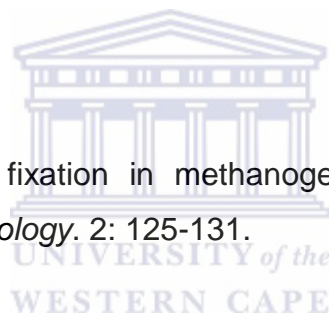
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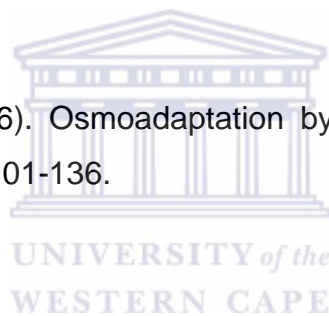
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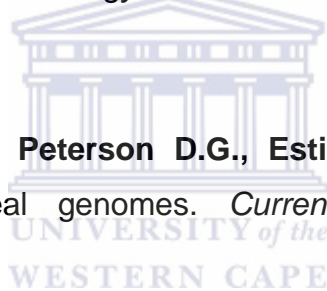
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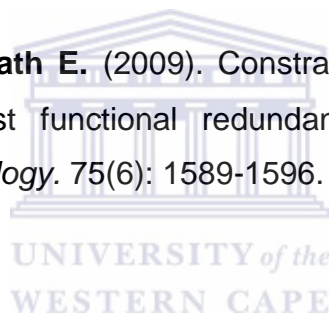
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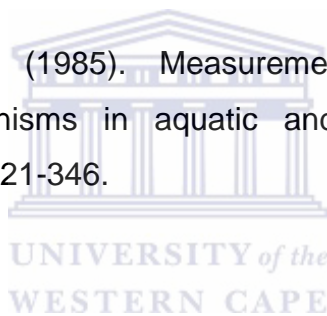
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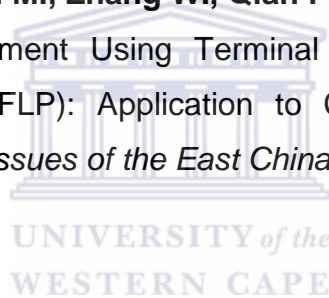
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