IMBM Institute for Microbial Biotechnology and Metagenomics

UNIVERSITY of the WESTERN CAPE

Characterisation of the endophytic bacterial communities associated with South African sorghum plants: looking for potential plant growth-promoting endophytes

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DECLARATION

Hereby I, Cyprien William Bopda Finyom, declare that *Characterisation of the endophytic bacterial communities associated with South African sorghum plants: looking for potential plant growth-promoting endophytes* is my own 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.

Date: January 2012

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ABSTRACT

The term endophyte is used to define all microorganisms that, during a part of their life cycle, colonize the internal tissues of a plant host. Many endophytes have been found to promote plant growth by acting either as biocontrol agents, biofertilizers or phytohormone producers. This study aimed to characterise the endophytic microbial community diversity associated with sorghum farmed in South Africa. Members of any common endophytic bacterial species identified during the study might in future studies be developed to improve sorghum production. Sorghum tissues (roots, shoots, stems) were sampled in three South African provinces (Free State, Limpopo and North West), each site being characterised by the use of different agricultural practices. Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analyses were used to characterise the endophytic bacterial communities. The analysis clearly demonstrated that the endophytic bacterial community structure in the three sorghum tissue types differed, suggesting that endophyte colonization is tissue-specific. The endophytic bacterial community structure is quite similar in each tissue when comparing the populations present in the sampling sites. In the sorghum endophytic microbial communities, common bacterial species were identified using molecular tools: The cyanobacterium *Synechococcus* and *Staphylococcus saprophyticus* were identified in the root samples. *Pantoea* sp., *Erwinia* sp., *Enterobacter* sp. and *Klebsiella* sp. were found in all shoot samples. *Nocardia fluminea*, *Bacillus cereus* and *Microbacterium* sp. were isolated as common shoot endophytic bacteria. This study defines, for the first time, the endophytic bacterial species associated with South African sorghum plants. These common endophytic bacterial species can be used to enhance the yield of sorghum crops.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair
$CaCl_2$	calcium chloride
CTAB	cetyl-trimethyl-ammonium bromide
dNTPs	deoxyribonucleic-5'-triphosphates
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
°C	degrees celsius
EtOH	ethanol
EDTA	ethylene diamine tetraacetic acid
g	gram
GYT	glycerol yeast extract plus tryptone
H_2SO_4	sulfuric acid
H	hour
IPTG	isopropyl β-D-thiogalactosidase
Kb	kilo basepairs
KCl	potassium chloride
λ	lambda
l	liter
LB	Luria-Bertani
µl	microliter
M	molar
ml	milliliter
Min	minute
$MgCL_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
mM	millimolar
$MnCL_2$	manganese chloride
NaCl	sodium chloride
NaOH	sodium hydroxide

PCR	polymerase chain reaction
rpm	revolutions per minute
rDNA	ribosomal deoxyribonucleic acid
s	second
SDS	sodium dodecyl sulphate
TA	tris acetic acid
TAE	tris acetic acid EDTA
TBE	tris boric acid EDTA
TCA	trichloroacetic acid
U	unit
X-gal	5-bromo-4-chloro-3-indoly-β-D-galactoside

CHAPTER ONE

Crop-associated microorganisms

TABLE OF CONTENTS

1.1 Plant-linked ecosystems

Ecosystems are systems with active interactions between living organisms, such as plants, animals and microorganisms and their physical location. All components work together as an efficient unit. Amongst all the potential interactions occurring in ecosystems, in this study the interactions between the plants and microorganisms inhabiting the portion of soil surrounding plant roots (the rhizosphere) (Morgan *et al*., 2005) and the interactions between plants and microorganisms found inside the plant tissues (the endosphere) (Saito *et al*., 2007) were of interest.

1.1.1 The rhizosphere ecosystem

The rhizosphere was first defined by Hiltner in 1904 as the zone of stimulated bacterial growth around living plant roots that is influenced by root activities. This description has since been made more explicit by describing the rhizosphere as the volume of soil distant of seven mm from living plant roots, which is influenced by root activities and shared with soil bacteria (Raynaud, 2010). The rhizosphere is the largest terrestrial ecosystem on earth, and it has been estimated that plants release between 20 to 50 % of their photosynthates through their roots (Buchenauer, 1998; Bottner *et al*., 1999).

1.1.1.1 Formation of the rhizosphere

The rhizosphere comprises three micro-environments (Figure 1.1): the rhizoplane, the endorhizosphere and the ectorhizosphere (Morgan *et al*., 2005). The rhizoplane is defined as the soil in contact with the root surfaces which consists of the epidermis of the root and the mucilaginous polysaccharide layer surrounding the epidermis. The endorhizosphere is the root tissue including the endodermis and cortical layers. The ectorhizosphere is the soil directly adjacent to the root and influenced by the root (Morgan *et al*., 2005).

The principal inducer of rhizosphere formation is the expansion of water and solute gradients around plant roots (Raynaud, 2010). These gradients can modify the physical, chemical and biological properties of the soil and originate from the absorption and/or the expulsion of water and solutes by the plant roots at their surface. The gradient can be represented as depletion profiles, accumulation profiles or more complex profiles. In the case of a depletion profile, the lowest solute concentrations are situated at the root surface which leads to the absorption of the solutes by the plant as mineral nutrients. In the case of accumulation profiles, the highest solute concentrations are at the plant root surface leading to the release of solutes by the plant into the rhizosphere. In complex profiles, the solutes are stable due to interactions amongst solutes, soil properties and soil organisms (Raynaud, 2010).

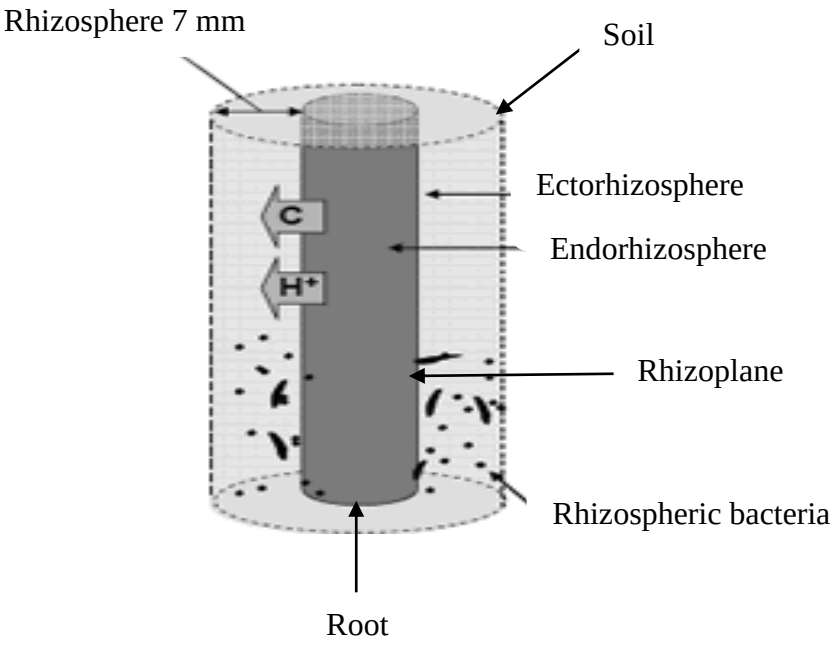

Figure 1.1 The overall cross section of the rhizosphere with its three micro-environments (Morgan *et al*., 2005; Walter, 2007).

1.1.1.2 The rhizosphere: a hot-spot for microbial growth

Plants release up to 50 % of freshly assimilated carbon into the root environment (Degenhardt *et al*., 2003). The rhizosphere is therefore an environment containing high concentrations of substances such as carbohydrates (sugars and oligo-saccharides), organic acids, vitamins, nucleotides, flavonoids, enzymes, hormones and volatile compounds (Table1.1). These compounds are transferred from the plant roots to the rhizosphere as exudates (Kumar *et al*., 2006) where they perform different functions as described in Table 1.2. Microbes in the rhizosphere consume between 64 % and 86 % of the carbon released by the roots (Hutsch *et al*., 2002).

Table 1.1 List of carbon compounds released by plant roots into the rhizosphere (Kumar *et al*., 2006).

Amino acids	Organic acids	Sugars	Vitamins	Purines/nucleosides	Enzymes	Inorganic ions/gaseous molecules
α-Alanine	Citric	Glucose	Biotin	Adenine	Acid/alkaline	HCO^{-3}
Asparagine	Oxalic	Fructose	Thiamin	Guanine	phosphatase	OH^{-}
Aspartate	Malic	Galactose	Niacin	Cytidine	Invertase	H
Cystine	Fumaric	Maltose	Pantothenate	Uridine	Amylase	CO^{2}
Acetic Xylose	Acetic	Xylose				
Glutamate	Butyric	Rhamnose				
Glycine	Valeric	Arabinose				
Leucine	Piscidic	Deoxyribose				
Lysine	Formic	Oligosaccharides				
Methionine	Aconitic					
Serine	Lactic					
Threonine	Pyruvic					
Proline	Glutaric					
Valine	Malonic					
Tryptophan	Aldonic					
Ornithine	Erythronic					
Histidine	Tetronic					
Arginine						
Homoserine						
Phenylalanine						
Aminobutyric acid						
Aminoadipidic acid						

Table 1.2 Possible functional roles of plant root exudates in the rhizosphere (Kumar *et al*., 2006).

Component (from root exudates)	**Function**
Phenolics	Nutrient source
	Chemo-attractant signals to microbes
	Microbial growth promoters
	Nod gene inducers in *Rhizobia*
	Nod gene inhibiters in *Rhizobia*
	Resistance inducers against phytoalexins
	Act as chelaters
	Phytoalexin against soil pathogens
Organic acids	Nutrient source
	Chemo-attractant signals to microbes
	Chelators of poorly soluble mineral nutrients
	Acidifiers of soils
	Detoxifiers of aluminium
	Nod gene inducers
Amino acids and phytosiderophores	Nutrient source
	Chemo-attractant signals to microbes
	Chelaters of poorly soluble mineral nutrients
Vitamins	Promoters of plant and microbial growth
	Nutrient source
Purines	Nutrient source
Enzymes	Catalysts for phosphorus release from organic molecules
	Biocatalyst for organic matter transformation in soil
Sugars	Nutrient source
	Promoters of microbial growth

As root exudates serve as nutrient sources and microbial growth promoters (Table 1.2), microorganisms in the rhizosphere are 19 to 32 times more abundant than in bulk soils, i.e. in soils not impacted by roots (Bodelier *et al*., 1997). A recent comparative study by Walter (2007) on the microbial communities present in the rhizosphere of wheat (*Triticum aestivum* L.) and its related bulk soil has shown that bacteria were 23 times more abundant in the rhizosphere than in the bulk soil while actinomycetes were seven times more abundant (Table 1.3).

Table 1.3 The number of microorganisms in the rhizosphere of wheat (*Triticum aestivum* L.) and in the associated bulk soil and the R/S ratio (bacteria in rhizosphere/ bacteria in bulk soil) (Walter, 2007).

Microbial group	Rhizosphere (CFU g^{-1} soil)	Bulk soil CFU g^{-1} soil)	R/S ratio
Bacteria	1.2×10^{9}	5.3×10^{7}	23
Actinomycetes	4.6×10^{7}	7.0×10^{6}	7

1.1.2 The endosphere ecosystem

The endosphere is defined as the microbial environment localized in plant organs (Saito *et al*., 2007). In this study the focus was on the endophytic environments of the roots, shoots and stems of sorghum (*Sorghum bicolour* L).

1.1.2.1 The root

The root is the plant organ located in the soil. It provides support for plant growth and plants absorb water and minerals through this organ.

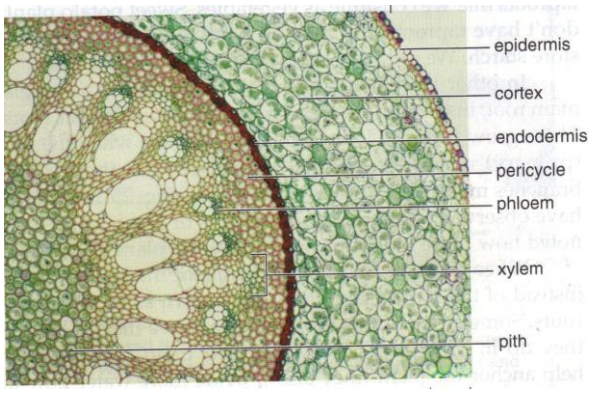

Figure 1.2 The overall cross section of the internal part of a monocotyledenous root (Madder, 2004).

The specialized tissues within a monocotyledonous plant root are shown in Figure 1.2. The epidermis is the outer layer of the root. It consists of a single layer of cells whose function is to protect the root from the external environment. The cortex is situated close to the epidermis. It consists of multiple layers of large, thin walled parenchyma cells. Their shape allows water and minerals to move across them without entering them. The endodermis consists of a single layer of rectangular cells that separates the cortex and the inner vascular cylinder. The inner vascular cylinder contains the xylem and phloem. The xylem is responsible for water and nutrient uptake from the roots to the leaves. The phloem is responsible for the transport of sucrose and other organic compounds including hormones from the leaves to the roots. The pericycle is the first layer of cells inside the vascular cylinder and is responsible for lateral root initiation and is involved in root growth. The pith is the ground tissue in the center of the root. It is surrounded by vascular rings formed by alternating xylem and phloem bundles (Madder, 2004).

1.1.2.2 The shoot

The shoot of a plant consists of the stem, the branches and the leaves (Madder, 2004). Plant growth starts post-embryonically from the action of shoot apical meristems which are undifferentiated cells that produce progeny cells that can differentiate into leaves, stems and flowers (Fletcher and Meyerowitz, 2000).

1.1.2.3 The stem

The stem is used by plants to support the leaves, to conduct water and minerals from the roots to the leaves via the xylem and to transport organic compounds from the leaves to the roots via the phloem (Madder, 2004).

The specialized tissues within the stem of a monocotyledonous plant are shown in Figure 1.3. The epidermis is the outer layer covered by a waxy cuticle and is used by the plant to avoid water loss. The vascular bundle is formed of the xylem and the phloem. In each bundle, the xylem is located towards the inside of the stem while the phloem is located towards the outside. The ground tissue is responsible for the storage of carbohydrates produced by the plants during photosynthesis (Madder, 2004).

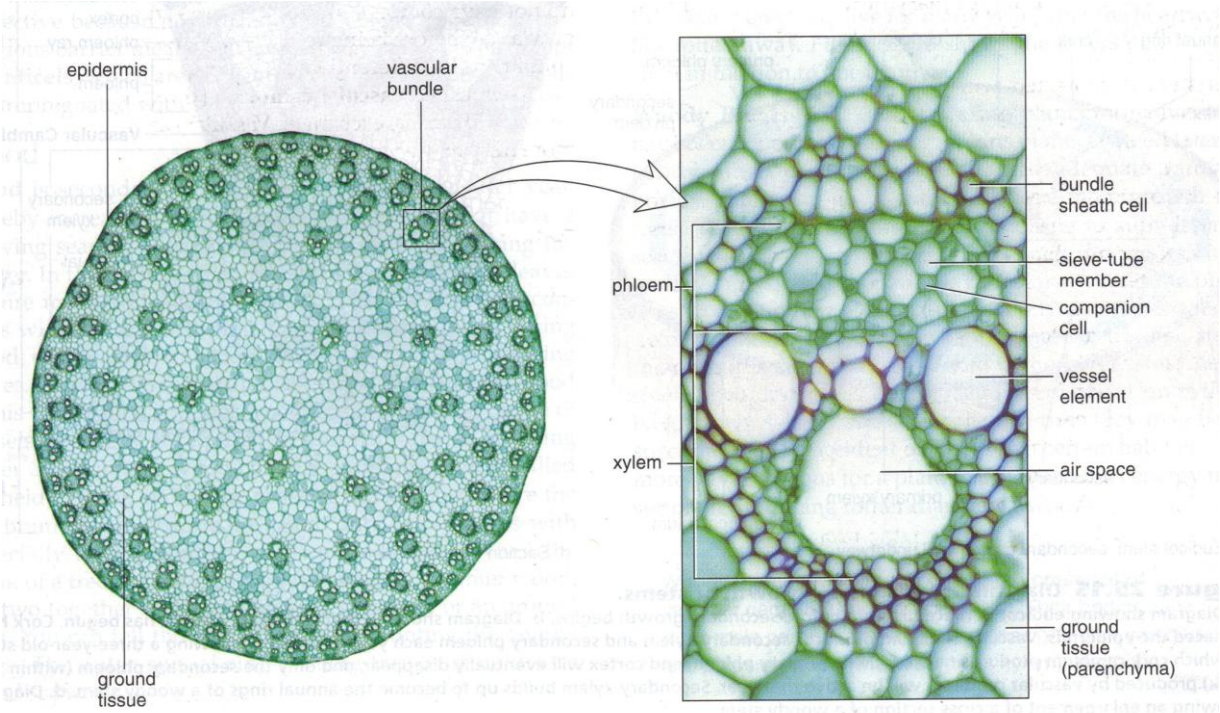

Figure 1.3 The overall cross section of the internal part of a monocotyledenous stem (Madder, 2004).

1.2 Plant-associated bacterial communities

Bacterial communities distributed in the rhizosphere and the endosphere may develop mutualistic relationships with plants. The bacteria present in the rhizosphere form rhizospheric bacterial communities. Those present in the endosphere form endophytic bacterial communities (Andreote *et al*., 2009).

1.2.1 The rhizobacterial communities

During germination and seedling development growing plants interact with a range of microorganisms which are present in the surrounding soil. As seeds germinate and roots develop the release of exudates in the rhizosphere transforms this environment into a significant carbon tank. These carbon tanks are nutrient-rich niches which attract a diverse population of microorganisms (Compant *et al*., 2005; Nihorimbere *et al*., 2011).

Bacteria living in the rhizosphere form the rhizospheric bacterial communities as they (1) depend on root exudates to get their carbon sources and (2) have efficient systems for the uptake and catabolism of organic compounds found in the root exudates (Tilak *et al*., 2005). The rhizospheric microbial communities, depending on their relationship with plants, can be beneficial (i.e. with a symbiotic relationship), neutral or harmful (i.e. with pathogenic action).

Beneficial interactions have been observed between plants and nitrogen-fixing bacteria, mycorrhizal fungi, some actinomycetes as well as free-living bacterial saprophytes and endophytes (Sturz and Christie, 2003). Harmful interactions were observed between plants and some bacteria such as *Pseudomonas cyanogenesis, Arabidopsis thaliana* (Rudroppa and Bais, 2008) and *Erwinia* sp. (mostly *E. carotova* and *E. chrysanthem).* The latter two organisms cause soft-rot in *Arabidopsis thaliana* (L.) (Norman *et al*., 1999). Other rhizobacteria associated with plants are listed in Table 1.4.

Table 1.4 Examples of rhizobacteria associated with plants (Vessey, 2006; Martínez-Viveros *et al*., 2010).

Rhizospheric bacteria	Host crops
Azospirillum sp.	Maize
Azospirillum sp.	Rice
Azospirillum sp.	Wheat
Azotobacter sp.	Maize
Azotobacter sp.	Wheat
Bacillus polymyxa	Wheat
Cyanobacteria∗	Rice
Cyanobacteria∗	Wheat
Bacillus M3	Apple
Bacillus OSU142	Apple
Microbacterium FSO1	Apple
Bacillus subtilis FZB24	Cotton
Azotobacter	Maize
Pseudomonas fluorescens MPp4	Maize
Burkholderia sp. (MBp1,MBf21)	Maize

Azospirillum sp. (Ch06, Ch08)	Oats
Pseudomonas sp. Ch09	Oats
Bacillus cereus (KBE7-8)	Sorghum
Bacillus cereus (NAS4-3)	Sorghum
Azospirillum brasilense CW903	Tomato

∗Numerous species; predominantly of the genera *Anabaena* and *Nostoc*

1.2.2 The endophytic bacterial communities

Plants and animals generally form relationships with many varieties of microorganisms. In the gut of mammals, bacteria have many functions including facilitating digestion and inducing immunity and allergic symptoms (Wohlgemuth *et al*., 2010). In the same way plant-associated bacteria, or endophytic bacteria, induce plant defences against phytopathogens and stimulate plant-growth through the production of secondary metabolites such as phytohormones (Rosenblueth and Martinez-Romero, 2006).

The term endophyte is used to define all microorganisms that, during a variable period of their life cycle, colonize the internal tissues of their plant host without causing harm and which are able to establish a mutualistic relationship with their host (Rajkumar *et al*., 2009). Figure 1.4 shows two species of endophytic bacteria present in the xylem and root cortex of pea plants (Geramine *et al*., 2006)

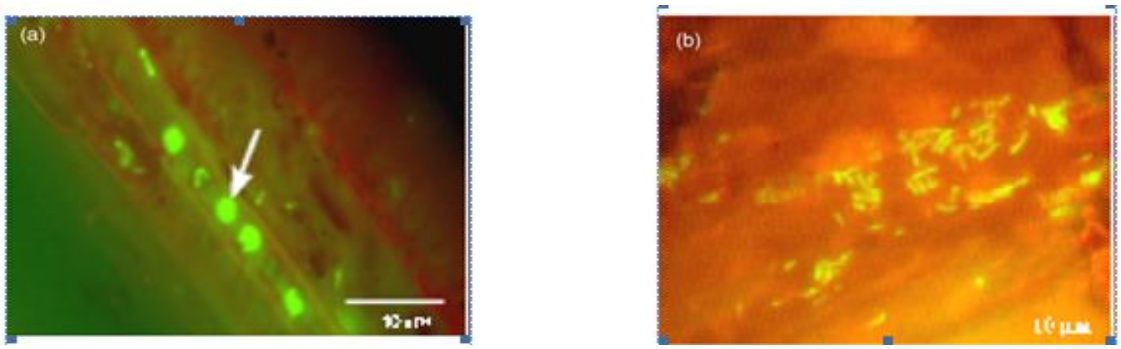

Figure 1.4 Endophytic bacteria in a pea plant. (a) The green spots show the presence of *Pseudomonas putida* VM 1453 cells (×1000) inside the xylem tracheid pits of pea plants. Scale bar = 10 µM. (b) The yellow spots show the presence of *Pseudomonas putida* VM 1450 colonies inside the root cortex of pea plants (×1000) (Germaine *et al*., 2006).

The evidence of an association between plants and microorganisms in fossilised tissues of stems and leaves indicates that an association between endophytes and host plants may have originate from the time of appearance of higher plants on the Earth (Zhang *et al*., 2006).

1.2.2.1 Classification of endophytic bacteria

Depending on their mode of development, endophytic microorganisms are classified as obligate or facultative endophytes. Obligate endophytes must live inside their host in order to develop and survive. Examples include some species of *Herbaspirillum* and *Burkholderia*. Facultative endophytes have to spend time outside their host plant in order to fulfil their life cycle. Some strains of *Azospirillum* are facultative endophytes (Baldani *et al*., 1997; Rajkumar *et al*., 2009).

1.2.2.2 The colonization of the endopshere

Figure 1.5 depicts the possible zones of penetration and colonization of roots by bacteria. The soil of the rhizosphere and the root surface or rhizoplane, including the dead cells of the

outside cell layers of the roots, are colonized by rhizobacteria termed epiphytic microorganisms.

In general, endophytic microorganisms penetrate the plant tissues through two routes: the epidermis and the root hairs (Figure 1.5). These organs consist mainly of cells whose cell walls containing pectin. Most endophytic microorganisms produce pectin degrading enzymes which allow them to penetrate the plant organ without harming it. They are able to live within cells, in the intercellular spaces or in the phloem and xylem vascular tissues (Reinhold-Hurek and Hurek, 1998).

The endodermis is the internal layer of cells neighbouring the central stele which is composed of xylem vessels and phloem vessels. This zone is colonized by diazotrophic endophytes. In Figure 1.5 these are represented by red ovals (not to scale) and their zone of penetration is represented by red arrows (Reinhold-Hurek and Hurek, 1998).

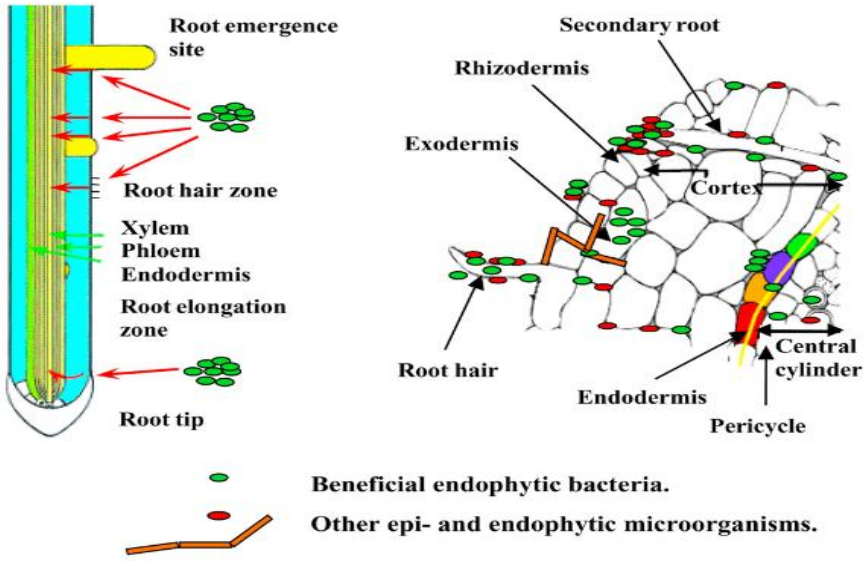

Figure 1.5 Sites of plant colonization by endophytic bacteria (Comptant *et al.*, 2010).

Endophytic bacteria in the root zone can establish negative or positive colonisations. Negative colonisation results in the inhibition of the growth of some endophytic bacteria by other microorganisms due to nutrient competition (Sturz and Nowak, 2000). Positive colonisation results in a variety of relationships between invading microorganisms, such as commensalism, mutualism and synergism. These relationships promote the growth of the colonisers within the environment (Sturz and Nowak, 2000).

Using different strategies, endophytic bacteria may spread inside the plant and colonize other plant organs such as stems and leaves (Hardoim *et al*., 2008). The main strategy is to use the xylem lumen vessels to spread throughout the plant via the perforated plates (Figure 1.6). The movement of the bacteria is facilitated by the use of flagella and/or the plant transpiration stream to colonize other plant organs (Compant *et al*., 2010).

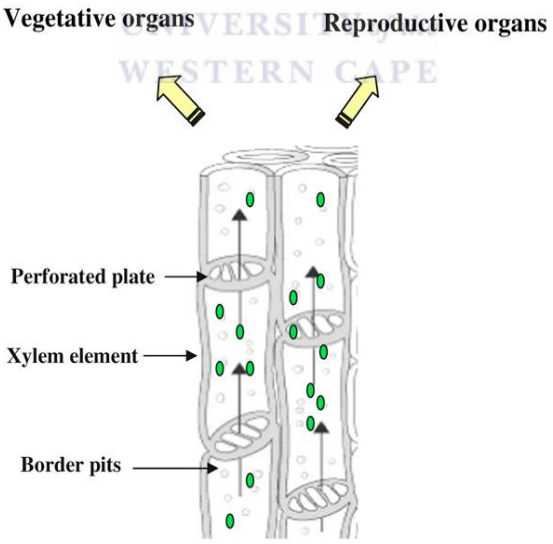

Figure 1.6 Bacterial spread inside xylem vessels in aerial plant parts. Arrows show the movement process (Compant *et al*., 2010).

1.2.2.3 The diversity of endophytic bacteria

The endophytic microbial communities in the root are composed of bacteria and fungi. They can be obligate symbionts or saprophytic mutualists which promote plant growth and in return receive protection against biotic and abiotic stresses (Backman and Sikora, 2008). Endophytic bacterial populations that have been identified in various plants are listed in Table 1.5.

Table 1.5 Endophytic bacterial species found in various plants (adapted from Rosenblueth and Martinez-Romero, 2006; Vessey, 2006).

Endophytes	Plant species
α Proteobacteria	
Azorhizobium caulinodans	Rice
Bradyrhizobium japonicum	Rice
Methylobacteruim mesophililuim	Citrus plants
Methylobacteruim extorquens	Citrus plants
Rhizobium radiobacter	Carrots, rice
Sinorhizobium meliloti	Sweet potato
Sphingomonas paucimobilis	Rice
Gluconacetobacter sp.	Sorghum
Diazotrophicus sp.	Sugarcane
β Proteobacteria	
Burkholderia cepacia	Citrus plants
Chromobacterium violaceum	Rice
Pantoea sp.	Sugarcane
Azoarcus sp.	Sorghum
Burkholderia sp.	Rice
Herbaspirillum sp.	Rice
γ Proteobacteria	
Citrobacter sp.	Banana
Enterobacter sakazakii	Soybean
Enterobacter asburiae	Sweet potato
Escherichia coli	Lettuce
Pseudomonas fluorescens	Carrot
Klebsiella sp.	Sugarcane
Erwinia sp.	Sand dune plants

Firmicutes	
Bacillus sp.	Citrus plants
Actinobacteria	
Microbacterium testaceum	Maize

1.3 Plant growth-promoting bacteria

Plant growth-promoting bacteria (PGPB) are associated with many plant species and are present in many environments such as in the rhizosphere and the endopshere (Comptant *et al*., 2005). PGPBs are divided into two groups: (i) plant growth-promoting endophytic bacteria which are bacteria present in the endosphere and (ii) plant growth-promoting rhizobacteria which are bacteria present in the rhizosphere and colonize the roots surfaces (Comptant *et al*., 2005).

1.3.1 Plant growth-promoting endophytic bacteria

Plant growth-promoting endophytic bacteria living inside plant tissues have been observed in almost all plant studies to date (Schulz *et al*., 1993). They participate in the physiological activities of the host plants by promoting the growth of the plant. These bacteria can enhance plant growth by participating in the process of nitrogen fixation in the host plant (biofertilizers). The fixed nitrogen acts as a phytostimulator. Alternatively they can promote plants by expressing plant hormones or by inhibiting the growth of plant pathogenic bacteria (biocontrol) (Comptant *et al*., 2005).

1.3.1.1 Biofertilizers

Plant growth and metabolism are affected by the amount of nutrients present in the soil. For example, in soil with a higher content of nitrogen than phosphorus and potassium, foliage growth is more developed than the growth of the reproductive organs. Thus crops such as lettuce, which are farmed for leaves, need soils high in nitrogen whereas crops farmed for

their seeds, such as corn or sorghum do not. In order to increase the nitrogen content of the soil the use of biofertilizers is an ecologically-friendly solution when compared to the use of synthetic fertilizers. Synthetic fertilizers may also affect the soil structure (Barassi *et al*., 2007).

The biofertilization process usually starts with a symbiosis between an endophytic bacterium and a plant host during the development of the root or stem nodules (Brewin, 1991). The molecular mechanism of symbiosis appears to be based on at least two stages of molecular signalling. Figure 1.7 shows the signalling mechanism in *Rhizobium leguminosarum* and its host. First the plant roots secrete flavonoids that induce the transcription of the bacterial *nod* genes and induce nodulation. *R. leguminosarum* replies with the production and secretion of an acylated lipooligosaccharide signal molecule (Spaink *et al*., 1993). These signal molecules induce the formation of new plant organs called nodules. Once nodules have been established on the root or the stem the bacteria penetrate the cortex, multiply and differentiate into bacteroids. Bacteroids are able to produce a nitrogenase enzyme complex inside the plant organ. The plant responds by generating a low concentration of oxygen which allows bacterial nitrogenase to convert atmospheric nitrogen into ammonia (Bloemberg and Lugtenberg, 2001).

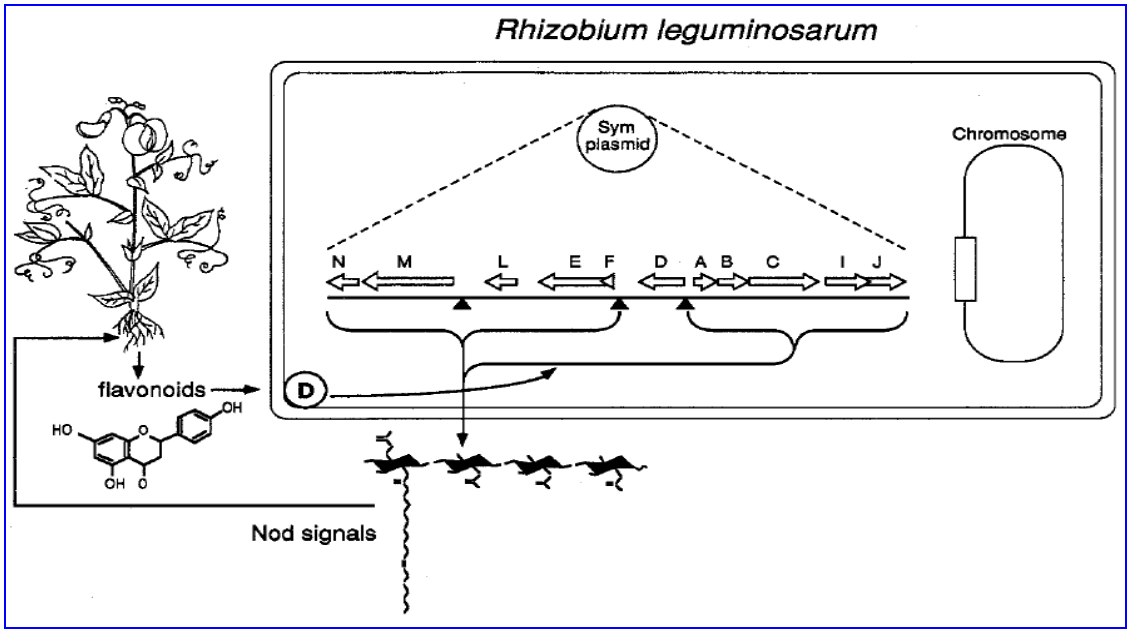

Figure 1.7 Signal interaction between *R. leguminosarum* and its host. The flavonoid inducers are secondary metabolites secreted by the roots of leguminous plants. These are absorbed by the endophytic bacteria. Inside the *Rhizobium* cell membrane the flavonoid inducers interact with the *Rhizobium* regulator *NodD* protein to activate the expression of the *R. leguminosarum* nodulation genes. The *nodABCD* genes are specific for the synthesis of the lipooligosaccharide signal molecule (Spaink *et al*., 1993).

1.3.1.2 Phytostimulators

There are five groups of phytohormones: cytokinins, auxins, gibberellins, abscisic acid and ethylene (Opik and Rolfe, 2005). Cytokinins are vital for plants as even at low concentration they promote cell division. Auxins (indole 3-acetic acid) regulate the development of lateral roots, the formation of vascular systems and the development of fruits. Gibberellins stimulate growth and flowering in long day plants and delay leaf senescence and dormancy breaking in seeds. Abscisic acid is useful for plants under stress conditions: in cases of water scarcity, it is released by plants through the roots or chloroplasts, causing stomata to close. Ethylene is a stress hormone for fruit ripening, and is also involved in plant responses to wounds and phytopathogens (Opik and Rolfe, 2005).

Many rhizobacteria produce phytohormones. Endophytic *Pseudomonas* and *Bacillus* species have been reported to produce phytohormones including indole-3-acetic acid, gibberellins and cytokinin-like substances (Lugtenberg *et al*., 1991; Panchal and Ingle, 2011). As previously noted, one action of phytohormones is to promote the development of lateral roots. This action was demonstrated by the association formed between a genetically modified *Azospirillum* with wheat plants. The genetically modified *Azospirillum* was able to produce auxins as secondary metabolites. An increase in the elongation of the roots was observed to result from that association. The study confirmed the idea that elongation of roots can be induced by auxin synthesis (Dobbelaere *et al*., 2008).

1.3.1.3 Biocontrol compounds

The term biocontrol refers to the inhibition of the growth of phytopathogenic organisms by other microorganisms. A commonly known mechanism of biocontrol is the production of siderophores by the biocontrol agents. This substance is produced by members of bacterial species including *Pseudomonas* and *Enterobacter cloacae* (Compant *et al*., 2005).

Iron is a vital element for the growth of all living organisms. Low iron availability leads to competition for the element. In circumstances of iron scarcity bacteria secrete siderophores, molecules with a high affinity for ferric iron in the soil. This action inhibits phytopathogenic fungi such as *Fusarium* sp. which cannot absorb ferric iron in the soil as efficiently as the siderophore secreting microorganisms (Bais *et al*., 2004).

Microorganisms can also possess a hyperparasitic activity which inhibits pathogens through the production of hydrolases that degrade the bacterial cell wall (Comptant *et al*., 2005). *Serratia plymuthica* produces a chitinase that inhibits spore growth and germ-tube elongation

in the pathogen *Botrytis cinerea* (Comptant *et al*., 2005). The expression of chitinase has been implicated in the ability of *Serratia marcescens* to inhibit the growth of *Sclerotium rolfsie* and for *Paenibacillu*s sp. strain 300 and *Streptomyces* sp. strain 385 to inhibit the growth of *Fusarium oxysporum* (Comptant *et al*., 2005).

Further biocontrol action includes the detoxification of pathogen virulence factors such as the albicidin toxin produced by *Xanthomnas albilineans*. Detoxification of albicidin by *Klebsiella* is due to the expression of the *AlbA* gene, which produces the AlbA protein that is able to reversibly bind the toxin (Zhang *et al*., 1998). Irreversible binding of albicidin was observed to be due to the expression of an esterase by *Pantoea dispersa* (Comptant *et al*., 2005).

1.3.2 Plant growth-promoting rhizobacteria

Two to five percent of the bacteria found in the rhizosphere have a beneficial effect on plant growth (Comptant *et al*., 2005). These bacteria are termed plant growth promoting rhizobacteria (PGPRs). Competition for the nutrients in the rhizosphere is the principal mechanism by which PGPRs protect plants from plant pathogenic bacteria. The movement of the PGPRs to the root surface is facilitated by flagella and is conducted by chemotactic responses (Compant *et al*., 2005). External PGPRs are a subset of beneficial bacteria that are able to colonize the root environment (Barea *et al*., 2005). In the rhizosphere they are found primarily in the rhizoplane due to the presence of high concentrations of root exudates in that location. In this environment PGPRs may act as biofertilizers, biocontrollers and produce phytohormones. Some species including *Burkholderia* have shown the ability to transform atmospheric nitrogen to ammonia suitable for use by the plants (Caballero-Mellade *et al*., 2007).

In addition these groups of PGPRs, termed diazotrophic PGPRs (Section 1.3.2.1) have the ability to inhibit the growth of plant pathogenic bacteria. This is due to the production of iron-chelating siderophores, antibiotics or lytic enzymes. An example is *Pseudomonas putida* strain WCS358 (Compant *et al*., 2005). PGPRs including *Bacillus* sp. may produce plant hormones such as auxin and cytokinin (Lugtenberg *et al*., 1991).

1.3.2.1 Diazotrophic PGPRs

Dioazotrophs are microorganisms that fix atmospheric nitrogen to ammonia, a form of nitrogen which is suitable for plant uptake and growth. Bacteria from the genera *Rhizobium, Sinorhizobium, Bradyrhizobium, Mesorhizobium* and *Azorhizobium* are diazotrophic PGPRs. These bacteria, generally named rhizobia, are the microbial symbiotic partners of leguminous plants. They induce the formation of the atmospheric nitrogen fixing nodules. Other nitrogen fixing PGPRs are the actinomycetes from the genus *Frankia* which form root nodules in contact with actinorhizal plant species (Vessey *et al*., 2004).

1.3.2.2 *Bacillus* species

Some *Bacillus* species such as *Bacillus subtilis* have been isolated from the rhizosphere of a range of plant species at concentrations higher than 10^7 CFU.g^{-1} (Wipat and Harwood, 1999). They have been reported to be phytostimulators, expressing plant hormones such as auxins, gibberellins and cytokinin (Lugtenberg *et al*., 1991). Many species have been reported to demonstrate plant growth promoting activities. These include *Bacillus polymyxa* BcP26 which stimulates nitrogen, phosphorous and potassium uptake in maize, particularly in soil with low nutrient levels (Egamberdiyeva, 2007). Some *Bacillus* species can inhibit the growth of soil-borne pathogens of the chickpea plant (*Cicer arietinum* L.) by secreting siderophores (Joseph *et al*., 2007).

1.3.2.3 Pseudomonads

Pseudomonads have been widely isolated from crop roots and hare characteristics associated with PGPRs such as biocontrol and phytohormone production (Gravel *et al*., 2007). Among the most efficient root colonizing pseudomonads is *P. putida* RCO6 which induces the growth of sugar beet by fixing atmospheric nitrogen to ammonia (ÇakmakÇı *et al*., 2007). Biocontrol activity was observed in the secretion of the secondary metabolites chitinase and laminarinase by *Pseudomonas stutzeri* growing in low-glucose PA medium. These metabolites digested and lysed mycelia of the plant pathogenic fungus *Fusarium solani* (Compant *et al*., 2005).

1.4 Biotechnologies using endophytic bacteria

The soil environment exerts many unintended environmental pressures on plants including water scarcity, salinity, deforestation and the pressure of pathogenic organisms. These issues directly influence agricultural and potential production. The use of PGPBs in crop production is one of the strategies which may be useful in avoiding the risk of low agricultural yields and food insecurity issues (Kohler *et al*., 2006; Barassi *et al*., 2007). The use of PGPBs is a safe practise in food production and may have benefits over the use of synthetic fertilizers. Organic farming practises prohibit the use of synthetic fertilizers and in this arena PGPBs are of immense importance.

Plant growth promoting rhizobacteria do not build up in the food chain and are self sustaining due to the ability of the microbes to replicate themselves. Plant pathogenic microorganisms rarely develop a resistance against PGPRs and PGPRs acting as biocontrol agents have not been shown to be dangerous to ecological processes and the environment (Gould, 1990).

1.4.1 The application of endophytic bacteria in agriculture

The beneficial action of PGPRs has lead to the development of products which are used in agricultural production, mostly to inhibit the growth of phytopathogenic organisms. These products produced by endophytic microbial species are described in Table 1.6

Table 1.6 Natural products of endophytic microorganisms used in agriculture (Guantilaka *et al*., 2006).

Microbial strain	Plant host (s) family Plant part or tissue	Culture conditions	Natural Products	Biological Activity
Acremonuim zeae NRRLB 540	*Zea maydis*. L (Poaceae); kernel	Whole maize kernel in d H20 25°C 30 days, PDA 25°C 7 days	pyrrocidine A pyrrocidine B	Antibacterial Antifungal
Fusaruim sp. IFB-121	*Quercus Variabilis.* L (Fagaceae); bark	PDB; 28 °C; 6 days	cerebroside	Antibacterial; xanthine oxidase inhibitor
Periconia sp. OBW-15	*Taxus cuspidate* Siebold inner bark	S-7 (liquid) medium 25°C; 21 days	periconicin A	Antimycotic root growth accelerator (at low conc)
Streptomyces aureofaciens CMUAc130	*Zingiber officinale* Roscoe; Root	ISP-2 broth; 30°C; 5 days	5,7-dimethoxy- 4-phenylcoumarin	Antifungal
Streptomyces sp. NRRL 30562	*Kennedia nigricans* Lindley; Stems	PDB still culture; 23°C; 21 days	munumbacins A-D (peptides)	Antibiotic

1.4.2 The application of endophytic bacteria in bioremediation

Metallurgic industries discharge toxic waste streams into the environment severely impacting the affected ecosystems. In order to treat the waste streams and to prevent the toxic wastes from polluting neighbouring environments, chemical (precipitation and oxidation/reduction), physical (filtration) and biological methods have been used. In most instances the bioremediating agents are microorganisms which can be exploited to eliminate hazardous chemicals found in the environment (Guo *et al*., 2010).

Pentachloronitrobenzene (PCNB) is a polychlorinated aromatic compound that was used as a fungicide in Japan until 1997 and was also used against soil borne diseases including clubroot disease of *Brassica* plants caused by *Plasmodiophora brassicae* (Motoyama *et al*., 2001). Polychlorinated aromatic compounds are the most toxic chemical pollutants in the environment as they are very stable. They can remain for long periods (between four and ten months) without being degraded (Motoyama *et al*., 2001). PCNB degrades into molecules such as PCA (pentachloroaniline), PCTA (pentachlorothioanisole) and PCP (pentachlorophenol). However these molecules are resistant to mineralization by many microorganisms. The use of the symbiotic relationship between *Sphingomonas* sp PGPBs and plants (where plants provide exudates to the bacteria and bacteria degrade PCA) can be exploited in bioremediation. The PCP molecule can be degraded by an enzyme encoded by the PCP-inducible *pcpB* gene which is present in *Sphingomonas chlorophenolica* (Crawford *et al*., 2007). The gene expresses PCP-4-monooxygenase which hydroxylates PCP to tetrachlorohydroquinone (Orser *et al*., 1993). PCP-4-monooxygenase is active against a range of substrates and is reported to catalyse the hydroxylation of the para position of a diverse range of polyhalogenated phenols with an ortho substitute (Xun *et al*., 1992).

1.5 Research project: the aim of the research was to characterise the endophytic bacterial communities associated with South African sorghum plant, looking particularly for potential plant growth-promoting endophytes. To better understand the interactions between endophytic bacteria and plants, it is important to identify and characterise the endophytic bacterial communities associated with plants.

1.5.1 Sorghum: origin, culture and production in South Africa

Figure 1.8 Sorghum plants (Photo courtesy of Sorghum plants).

Sorghum is a member of the grass family, the Gramineae (Almodares *et al*., 2009). Sorghum is native to parts of Africa, Asia and Latin America (Dicko *et al*., 2006). *Sorghum bicolour* (L.) is the common sorghum farmed in Africa (Figure 1.8). It is a tropical grass and is mainly cultivated in dry areas, particularly on shallow and heavy soils. Sorghum is cultivated with difficulty on sandy soils except where a heavy textured sub-soil is present. It is suitable for growth on soils with a pH between 5.5 to 8.5. Sorghum is a warm weather crop and its germination is optimal in an environment with temperatures between 7°C and 15°C. After the germination stage, optimum growth and development is achieved in ambient temperatures

between 27°C and 30°C. Sorghum production in South Africa is between 100 000 tonnes to 180 000 tonnes per year. Mpumalanga and Free State provinces are the largest areas where sorghum is cultivated (Murdy *et al*., 1994). Sorghum plants, as other crops, are affected by the excessive use of fertilizers. The excessive use of fertilizers causes a nitrogen deficit which decreases the rate of photosynthesis and directly affects plant growth (Boussadia *et al*., 2010).

1.5.2 The importance of improving sorghum production

Sorghum is the fifth most cultivated cereal in the world. In Africa and Asia it is farmed mostly for food consumption. Sorghum grain contains proteins, fat, non-starch polysaccharides, starch and resistant starch. This resistant starch reduces its digestibility, particularly for babies, but it can be used to reduce human obesity and nourish diabetic individuals (Dicko *et al*., 2006). In Africa, sorghum is consumed by 500 million people in more than 30 countries including South Africa. In most West African countries, 50 % of the total cereal crop land surface is occupied by sorghum (Taylor, 2003).

An increase in the sorghum growth yield and productivity in Africa, a continent often subjected to food scarcity, can thus be an important step forward in reducing famine, and a driver of the African economy.

1.5.3 Aims and objectives

The interactions between endophytic bacteria and plants are generally symbiotic relationships, where the endophytic bacteria promote plant growth either as biocontrol agents, biofertilizers or phytohormone producers. The plants in return protect endophytic bacteria from biotic and abiotic stresses (Rosenblueth and Martinez-Romero, 2006).

The aim of this study was to characterise the endophytic bacterial communities associated with South African sorghum plants, looking particularly for potential endophytic plant growth-promoting bacteria that are always associated with South African sorghum plants.

The procedure to achieve this aim is outlined in Figure 1.9. Roots, shoots and stems of sorghum plants were the source of sorghum metagenomic DNA. Total culturable microbial community studies were used to identify the common culturable endophytic bacterial species associated with sorghum plants. Metagenomic analysis was used to examine the unculturable endophytic populations.

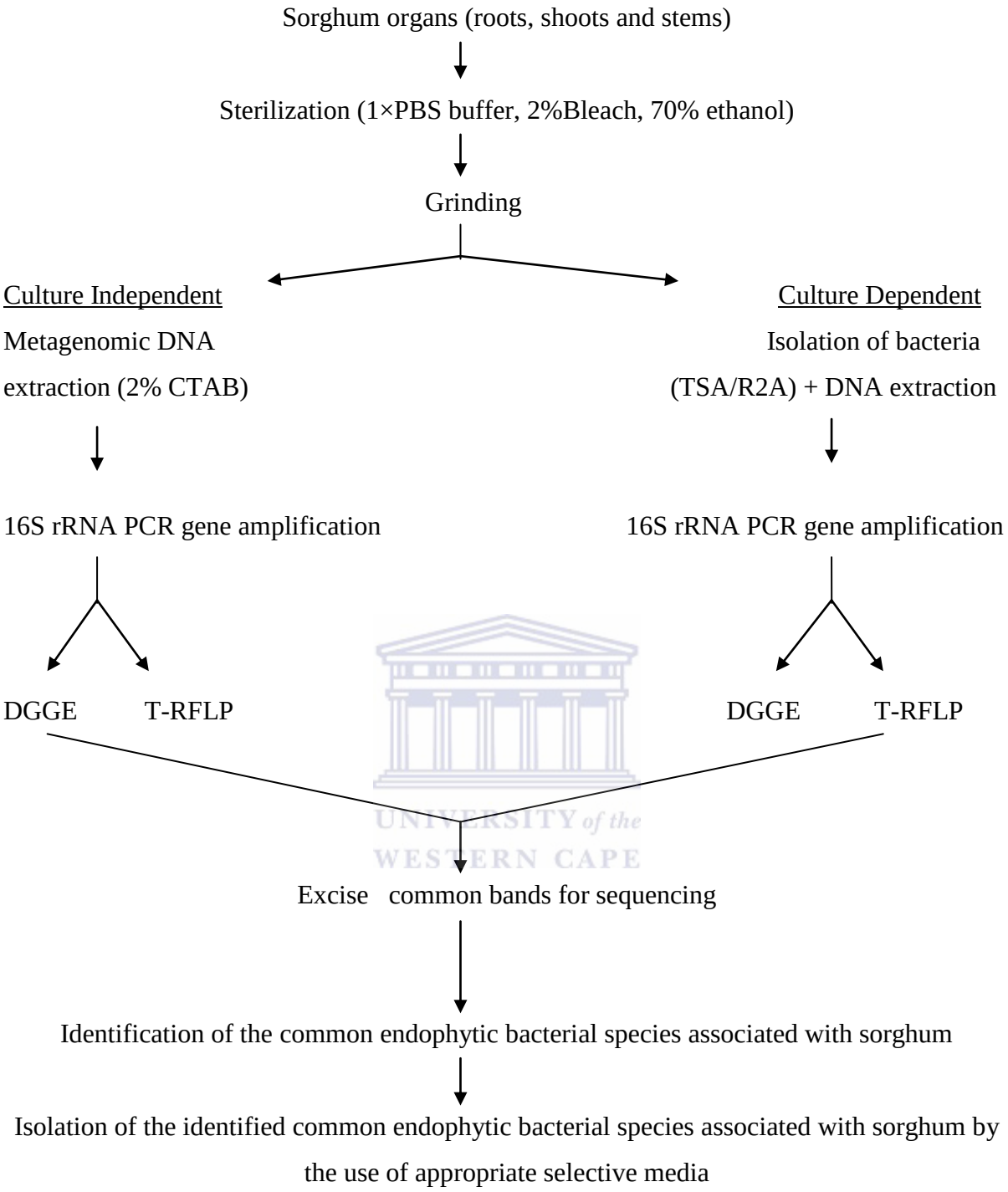

Figure 1.9 Methods used in this study to identify and isolate common endophytic bacterial species associated with sorghum. DGGE: Denaturing Gradient Gel Electrophoresis T-RFLP: Terminal Restriction Fragment Length Polymorphism TSA: Trypticase Soy Agar R2A: R2A agar.

CHAPTER TWO

Materials and methods

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2.1 Media

The suppliers of the media and general laboratory chemicals are listed in appendix 1.

LB agar Medium (Luria-Bertani Medium) (Sambrook and Russell, 2001)

Constituent	**L^{-1}**
Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Agar	15.0 g

The pH was adjusted to pH 7.0 with 5 N NaOH.

This medium was used for the growth of *Escherichia coli* and the isolation of *Microbacterium* sp., *Bacillus cereus* and *Pseudomonas* sp.

SOB Medium (**Sambrook and Russell, 2001**)

Constituent	**L^{-1}**
Tryptone	20.0 g
Yeast extract	5.0 g
NaCl	0.5 g
250 mM KCl	10.0 ml

The pH was adjusted to pH 7.0 before autoclaving. The medium was cooled to ~50°C and 5.0 ml of a filter sterilized 2 M $MgCl_2$ solution was added aseptically.

This medium was used for the growth of recombinant strains of *E. coli.*

SOC Medium (Sambrook and Russell, 2001)

Constituent	**L^{-1}**
Tryptone	20.0 g
Yeast extract	5.0 g
NaCl	0.50 g
250 mM KCl	10.0 ml

The pH was adjusted to pH 7.0 before autoclaving. The medium was cooled to ~50^{o}C and the following filter sterilized solutions were added aseptically; 5 ml of 2 M of $MgCl_2$ and 20 ml of 1 M glucose. This medium was used for the growth of recombinant strains of *E. coli.*

2x YT Medium (Sambrook and Russell, 2001)

Constituent	**L^{-1}**
Tryptone	16.0 g
Yeast extract	10.0 g
NaCl	5.0 g

The pH was adjusted to pH 7.0 before autoclaving.

This medium was used for the growth of recombinant strains of *E. coli.*

TSA (Trypticase Soy Agar) Medium (Mendes *et al*., 2007)

Constituent	**L^{-1}**
Trypticase Soy Broth	24.0 g
Yeast extract	2.4 g
Agar	12.0 g

The pH was adjusted to pH 7.4 before autoclaving.

This medium was used to cultivate fast growing endophytic bacteria.

R2A Medium (Dong-Sung *et al*., 2007)

Constituent	**L^{-1}**
Peptone	0.5 g
Yeast extract	0.5 g
Casein	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Potassium phosphate	0.3 g
Magnesium sulphate	0.05 g
Agar	12.0 g

This medium was autoclaved and used to cultivate slow growing endophytic bacteria.

LGI-P Medium (Loiret *et al*., 2004)

Constituent	**L^{-1}**
Peptone	5.0 g
Yeast extract	1.0 g
NaCl	5.0 g
Potato dextrose broth	39.0 g
Agar	15.0 g

The pH was adjusted to pH 7.4 before autoclaving.

This medium was used to isolate *Pantoea* strains.

BD Trypticase Soy Agar Medium (Funke *et al*., 1995)

Constituent	L^{-1}
Pancreatic digest of casein	15.0 g
Papaic digest of casein	5.0 g
NaCl	5.0 g
Agar	15.0 g

The pH was adjusted to pH 7.4 before autoclaving.

This medium was used to isolate *Microbacterium* strains.

Pseudomonas Selective Agar Medium (Krueger and Sheikh, 1987)

Constituent	L^{-1}
Pseudomonas selective agar	48.4 mg
Glycerol	10.0 ml

The medium was autoclaved and cooled to ~50°C and the following supplements were added

Cetrimide	5.0 mg
Fucidin	5.0 mg
Cephaloridine	25.0 mg

This medium was used to isolate *Pseudomonas* strains.

Nitrogen Free Medium (BG-11) (Porta *et al*., 2003)

Constituent	L^{-1}
Sodium nitrate	10 ml (30.0 g/200 ml)
Dipotassium phosphite	10 ml (0.8 g/200 ml)
Magnesium sulphate	10 ml (0.15 g/200 ml)
Calcium chloride	10 ml (0.72 g/200 ml)
Citric acid	10 ml (0.12 g/200 ml)
Ammonium ferric citrate	10 ml (0.12 g/200 ml)
Sodium edetate dihydrate	10 ml (0.02 g/200 ml)
Sodium carbonate	10 ml (0.4 g/ 200 ml)
BG-11 Trace Metals	1 ml

BG-11 Trace Metal solution

Constituent	L^{-1}
Boric acid	2.86 g
Manganese chloride	1.81 g
Zinc sulphate	0.22 g
Sodium molybdate	0.39 g
Copper II sulphate	0.079 g
Cobaltic nitrate	49.4 mg

The pH was adjusted to pH 7.4 before autoclaving.

This medium was used to isolate *Synechococcus* strains and endophytic cyanobacteria.

Nitrogen Deficient Medium Salt Agar (NDMSA) (Almadini *et al*., 2011)

Constituent	L^{-1}
d-Mannitol	10.0 g
Potassium phosphate	0.5 g
Magesium sulphate	0.1 g
Sodium chloride	0.2 g
Ferric chloride hexahydrate	0.02 g
Ammonium molybate	0.0025 g
Calcium carbonate	10.0 g
Agar	12.0 g

The pH was adjusted to pH 7.4 before autoclaving.

This medium was used to isolate nitrogen fixing endophytic bacteria.

Medium for the growth of *Klebsiella* strains (Gilmore *et al*., 1982)

Constituent	L^{-1}
Tryptone	10.0 g
Yeast extract	1.0 g
Agar	12.0 g

The pH was adjusted to pH 7 before autoclaving.

This medium was used to isolate *Klebsiella* strains.

NG Medium (Lee and Yu, 2006)

Constituent	L^{-1}
Potato detrose broth	24.0 g
Yeast extract	1.5 g
Glycerol	10.0 ml
Calcium chloride	290.0 mg
Magnesium chloride	190.0 mg
Manganese chloride	395.0 mg
Agar	15.0 g

The pH was adjusted to pH 6.5 before autoclaving.

This medium was used to isolate *Erwinia* strains.

2.2 Buffers

Table 2.1 Buffers used in this study (Sambrook and Russell, 2001).

Buffer	Components	pH
6X Agarose loading buffer	30 % (v/v) Glycerol 0.25 % (w/v) Bromophenol blue 15 % (w/v) Glycerol	
10X Orange G loading buffer	60 % Glycerol 0.25 % (w/v) Orange G	
10X PCR	50 mM Tris-HCl 100 mM NaCl 25 mM $MgCl_2$ 1 % Triton X-100	8
50X TAE	2 M Tris base 10 mM Glacial acetic acid 0.5 M EDTA	8
1X TE	10 mM Tris-HCl (pH 8.0) 1 mM EDTA (pH 8.0)	8
Lysis buffer	50 mM Tris-HCl (pH 7.6) 50 mM NaCl 5 % SDS (pH 8.0)	
PBS buffer	140 mM NaCl	

	2.5 mM KCl	
	10 mM $Na_2HPO_4.2H_20$	
	1.5 mM KH_2PO_4	
2X CTAB	1 M CTAB	
	1 M Tris-HCl (pH 8.0)	8
	5 M NaCl	
	1 M PVP 40	
1X PCR buffer	5 mM Tris-HCl	
	10 mM NaCl	
	2.5 mM $MgCl_2$	
	0.1 % Triton X-100	

2.3 Strains and vectors

Table 2.2 Strains and vectors used in this study.

Strains/plasmid	Source
E. coli (Gene Hog)	Invitrogen USA
pGEMTeasy	Promega, Madison, Wis. USA

2.4 Sorghum sample collection

Sorghum samples (Table 2.3) were collected on the 6th and 7th of April 2011 from farms located in three South African provinces (Free State, Limpopo and North West) shown in Figure 1. Different agricultural practices are used on the three farms to cultivate sorghum.

Figure 2.1 Map of South Africa indicating key farming sites used for the research (Google map).

The commercial farm outside of Parys in the Free State uses synthetic materials including nitrogen, potassium and phosphorus fertilizers and manure to grow sorghum. The Limpopo farm is a family farm that uses cow feces as an organic fertilizer. The research farm of the Agriculture Research Council in the North West province also uses synthetic materials to grow sorghum. Only sorghum plants in good condition were harvested. Individually, the roots, shoots and stems were aseptically cut and collected. The samples were individually packed and transported in a cooler box containing ice to the Institute for Microbial Biotechnology and Metagenomics at the University of the Western Cape where they were stored at -80°C.

Table 2.3 Roots, shoots and stems from three sample plants from each of the three locations used for the study.

Province	Samples	GPS location
Free State	SP1	S27°02975´ E027°31405´
	SP2	S27°03665´ E027°31780´
	SP3	S27°03660´ E027°31780´
Limpopo	SP1	S24°38620´ E029°52484´
	SP2	S24°39375´ E029°53593´
	SP3	S24°40822´ E029°52146´
North West	SP1	S26°43741´ E027°04870´
	SP2	S26°44063´ E027°04721´
	SP3	S26°43063´ E027°03944´

2.5 Soil analysis

Bulk soil and soil from the rhizosphere (soil within 7 mm of the sorghum plant roots) were collected for analysis. The pH, the total carbon, the total nitrogen, the ammonium and the nitrate content were determined (Bemlab, Somerset West).

2.6 Plant organ sterilization process

The plant organs were sterilized using a modified protocol described by Mendes *et al*. (2007). For each sorghum plant, the roots, shoots and stems were washed five times with sterile distilled water to remove the remaining soil particles. Each plant organ was placed in flask containing 400 ml of 1X PBS buffer and incubated for 2 hrs at room temperature with shaking (Figure 2.2). Samples were sequentially washed by shaking in (i) a 70 % ethanol solution for 10 mins, (ii) a 2 % (v/v) sodium perchlorate solution for 10 mins, (iii) a 70 % ethanol solution for 5mins and (iv) finally rinsed three times with autoclaved distilled water for 1 min. A 100 µl aliquot of the last rinse was plated on TSA and R2A agar plates supplemented with actidione (100 mg/ml) to evaluate the efficacy of the sterilization procedure. The plates were incubated at 28°C for 4 days. If no growth was observed on the plates, the sterilization of the samples (roots, stems and shoots) was considered successful. If growth was observed, the complete sterilization process was repeated until successful. Once sterilized, the plant organs (roots, stems and shoots) were stored at 4°C for subsequent analysis.

Roots **Shoot** **Stem**

Figure 2.2 Sterilized root, shoot and stem samples.

2.7 Isolation of the total culturable sorghum-associated endophytic bacterial community

Sterilized plant organs were ground into a fine powder using sterilized mortars and pestles containing liquid nitrogen. The powdered sample material retrieved from each plant organ was placed into a sterilized microcentrifuge tube and kept on ice. Samples were serially diluted with 0.85 % NaCl. 100 µl aliquots from the 10^{-1} and 10^{-2} dilutions were plated on TSA and R2A medium containing 100 mg/ml of the fungicide actidione and incubated at 28°C for 4 days. Selective media were similarly inoculated to isolate members of specific endophytic bacterial genera. Colonies present on the plates were examined and those showing similar growth characteristics from the three sampling sites were identified and streaked onto the appropriate selective medium to obtain pure cultures. Colonies from the pure isolates were placed into 50 µl of sterile water prior to 16S rRNA PCR gene amplification and ARDRA analysis (see section 2.8.8.1).

2.8 Molecular biology

2.8.1 Genomic DNA extraction from total culturable endophytic bacteria communities and isolated endophytic bacteria

Metagenomic DNA extractions from culturable endophytic bacteria were performed by a modified version of the method described by Miller *et al*. (1999). Total culturable communities on TSA and R2A media and cells of the individual bacterial isolates isolated on selective media were harvested and mixed with 500 µl of sterile distilled water in microcentrifuge tubes. The bacterial suspensions were centrifuged for 5 mins at 4500 rpm to pellet the bacterial cells. Pellets were re-suspended in 1 ml of lysis buffer and incubated overnight at 37°C. 1 ml of the mixture was transferred to a 2 ml tube containing 500 µl of a

phenol, chloroform and isoamyl alcohol solution (25:24:1v/v). The mixture was vortexed for 20 secs. After centrifugation at 14000 rpm for 3mins the upper aqueous phase was transferred to a new 2ml tube with 500 µl of a chloroform/isoamyl alcohol solution (25:24:1, v/v). The DNA was precipitated with 700 µl of ice-cold isopropanol, followed by a centrifugation step at 14000 rpm (30 mins, 10°C). The supernatant was discarded. The pellet was washed with 70 % ethanol and centrifuged at 14000 rpm for 5 mins. After removing the supernatant, the pellet was air dried at room temperature for 30 mins and resuspended in 100 µl of 1X TE buffer (Table 2.1) containing 100 $\mu g.ml^{-1}$ RNAseA. The solution was incubated at 37°C for 30 mins, and re-extracted with an equal volume of phenol:chloroform (1:1). The tube was gently inverted a few times and centrifuged at 14000 rpm for 5 mins. After centrifugation the upper aqueous phase was transferred to a 2 ml tube and an equal volume of ice-cold isopropanol was added. The tube was gently mixed by inverting and centrifuged at 14000 rpm for 5 mins. After removing the supernatant the pellet was resuspended in 50 µl of 1 X TE buffer (Table 2.1) and stored at 4°C

2.8.2 Metagenomic DNA extraction from sorghum organs

Total metagenomic DNA extraction was performed using a modified version of the method described by Murray and Thompson (1980). Sterilized plant organs were ground into a fine powder using sterilized mortars and pestles containing liquid nitrogen. Sterilized microcentrifuge tubes were used to collect the powder from the plant organs and placed on ice. A pre-heated solution of 700 µl of 2 X CTAB (Table 2.1) and 1 µl of β- mercaptoethanol was added to the powder. Tubes were vortexed for 20secs and incubated at 65°C for 60 mins. 600 µl of a chloroform/isoamyl alcohol (24:1 v/v) solution was added to each tube. Tubes were mixed by inverting for 5 mins and centrifuged at 12000 rpm for 5 mins. The upper liquid layer (500 to 550 µl) was collected and transferred into microcentrifuge tubes. An

equal amount of ice-cold isopropanol and RNase A (10 $mg.ml^{-1}$ final concentration) was added to the supernatant and mixed briefly by inversion. The tubes were incubated at room temperature for 20 mins and centrifuged at 12000 rpm for 5mins to recover the metagenomic DNA. The isopropanol was discarded. Once dried, the DNA pellets were washed twice with 250 µl of 70 % ethanol and centrifuged at 12000 rpm for 5 mins prior to drying a laminar flow cabinet. The DNA was resuspended in 50 µl of 1 X TE buffer (Table 2.1) and stored at 4°C.

2.8.3 DNA quantification

DNA was quantifed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Delaware-USA) at 260 nm, where one OD unit corresponded to a dsDNA concentration of 50 $\mu g.ml^{-1}$. Genomic DNA was considered pure when the $A_{260nm/280nm}$ ratio was between 1.8 and 2.0 (Murmur, 1963).

2.8.4 Agarose gel electrophoresis

Total extracted DNA and PCR products were separated in 0.7 % and 1.5 % (w/v) agarose gels containing 2.5 µl of ethidium bromide (50 $\mu g.ml^{-1}$) respectively. Samples were mixed with a 6 X agarose loading buffer (Table 2.1) before loading onto the agarose gels. Electrophoresis was performed in 1X TAE buffer (Table 2.1) at 100 V for 30 mins. The size of DNA bands was determined by comparing the migration of the bands to the migration of bands in DNA molecular weight markers (e.g. λ*Pst*I). Gels were visualized under ultraviolet light and photographed using a digital imaging system (Alphaimager 2000, Alpha Inotech, San Leandro, CA).

2.8.5 Polymerase chain reaction

PCR amplifications were done in 0.2 ml thin-walled tubes using an Applied Biosystems thermocycler. The amplification conditions and primer sets used are described in Table 2.4. A standard 50 µl reaction was set up with 1X PCR buffer (Table 2.1) 0.2 mM of each dNTP, 0.5 M of each primer, 0.3 µl of Taq DNA polymerase and 25 ng of DNA as template. For colony PCR, small amounts of freshly grown colonies were transferred to the reaction tubes using a sterile tooth-pick.

Table 2.4 Primer combinations, cycling conditions and targeted genes of the primer sets used in this study.

Primer	Sequence (5'-3')	Amplification Cycle	Target	References
E9F U1510R	GAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	94°C/4 min30×(94°C/30s-52°c/30s-72°C/105s), 72°c/10mins	Bacteria (16S rRNA gene)	Farrelly *et al*., 1995
341F-GC 534R	CCTACGGGAGGCAGC ATTACCGCGGCTGCTGG	94°C/4 min20×[(94°C/45s-65°C/45s-72°C/60s) (94°c/30s-55°c/30s72°c-60s)],72°c/10mins	Most bacteria	Muyzer *et al*., 1993
M13F M13R	GTAAAACGACGGCCAGT CAGGAAACAGCTATGAC	94°C/9 min35×(94°C/30s-52°c/30s-72°C/105s,72°c-10mins	Cloning vector pGEMTEasy	Yanisch-Perron *et al*., 1985
PolF PolR	TGCGAYCCSAARGCBGAC ATSGCCATCATYTCRCCGGA	94°C/4min35×(94°C/60s-55°C/30s-72°C/105s, 72°c/10mins	*nif*H gene	Poly *et al*., 2000

2.8.6 Denaturing gradient gel electrophoresis (DGGE)

The diversity of the endophytic bacterial communities was studied using DGGE. Ribosomal sequences from metagenomic DNA and genomic DNA were amplified with the E9F and U1510R primers (Table 2.4). 2 µl of the PCR products were further amplified with the primer set 341F-GC and 534R (Table 2.4). DGGE was performed as follows: DGGE plates were cleaned thoroughly with methanol and twice with ethanol to remove all traces of grease. Urea-formamide gel denaturing gradients were formed using the Bio-Rad Gradient-former

(Bio-Rad, Hercules, USA). 0.5 % (w/v) APS and 0.02 % (v/v) TEMED were added to acrylamide: bisacrylamide (37.5:1(w/w)) solutions as catalysts for gel polymerization prior to gradient development. 30-70 % urea-formamide gradients (a 100 % urea-formamide solution contains 7 M urea and 40 % (v/v) formamide) were used to separate PCR-DGGE products from the primer set 341F-GC, 534R. DGGE was performed using the Bio-Rad electrophoresis apparatus on 16.5 mm x 16.5 mm x 1 mm thick 9 % (w/v) polyacrylamide gels, at 100 V and 60 °C for 16 hrs in 1X TAE buffer (Table 2.1). After electrophoresis, the gels were stained using ethidium bromide (0.5 μg/ml final concentration) in 1X TAE buffer (Table 2.1) for 15 mins and destained in 1X TAE buffer (Table 2.1) for 30 mins before visualizing and capturing the image using the Alphaimager 3400 Imaging System UV transilluminator (Alphainotech Corporation™, San Leandro, CA). Prominent common bands were excised from the gels and reamplified. The new PCR products were purified using the GFX™ purification kit (GE Healthcare UK) to remove the unused deoxynucleoside triphosphates, and the purified PCR products were cloned into a pGEM-T Easy vector (Promega, Madison, Wis. USA) in accordance with the manufacturer's instructions (Section 2.7.7). The analysis of the DGGE gel fingerprint data was performed using the clustering method of the GelCompar II R software (Applied Maths NV, Belgium). Bands on the DGGE gels were considered as present or absent and recorded as a matrix. This matrix was used to generate distance matrices which were interpreted into dendrograms and multi-dimensional scaling plots to assess the similarity of community profiles using cosine and ward algorithm.

2.8.7 Cloning into pGEM-T™ Easy

Ligation reactions were set up at 3:1 insert (PCR product)/vector molar ratios in a total volume of 30 μL according to the manufacturer's instructions (Promega, Madisson, Wis. USA). Reactions were performed in rapid ligation buffer (Promega, Madson, Wis. USA)

containing T4 DNA ligase. The ligation reactions were set up on ice as described in Table 2.5.

Table 2.5 Ligation reactions used in this study.

	Standard reaction	Background control
2X Rapid ligation bufferT4DNA ligase	15 µl	15 µl
pGEM-T-Easy vector(25 ng)	0.5 µl	0.5 µl
PCR product	6 µl	-
T4 DNA ligase (3 U/µl)	1 µl	1 µL
Deionized water	7.5 µl	13.5 µl
Total reaction set up	30 µl	30 µl

The reactions were mixed by vortexing for a few seconds and then incubated overnight at 37°C to maximise ligation.

2.8.7.1 Preparation of electrocompetent *E. coli* cells

Glycerol stocks of *E. coli* (Gene Hog) strains (Invitrogen. USA) were streaked onto LB agar plates. The plates were incubated for 24 hrs at 37°C. Pre-culturing was performed by transferring a single colony into 10 ml LB medium and incubating overnight at 37°C in a shaking incubator. A volume of 10 ml the overnight culture was inoculated into a 5 L flask containing 1 l 2X YT medium. The flask was incubated with shaking at 37 °C for 3.5-4 hrs to an optical density at 600 nm of 0.6-0.9. The flask was placed on ice and divided into 4 equal volumes in ice-cold centrifugation bottles. The cultures were centrifuged at 4°C for 25 mins at 4000 rpm. The supernatant was discarded and the pellet was resuspended in 200 ml sterile ice cold distilled water and centrifuged at 4°C for 25 mins at 4000 rpm. The previous step was repeated but the volume of ice cold distilled water was reduced to 100 ml. The supernatant was discarded and the pellets resuspended in a solution containing 20 ml ice cold 15 % (v/v) glycerol and 2 % (w/v) sorbitol, and centrifuged at 4°C at 4000 rpm for 10 mins. The centrifuge tubes were placed on ice, the supernatant discarded and the pellet resuspended

in a solution containing 1ml ice cold 15 % v/v glycerol and 2 % w/v sorbitol. Aliquots of cell suspensions were transferred into microcentrifuge tubes, snap frozen in liquid nitrogen or ice-cold EtOH/dry ice and stored at -80°C (Sambrook and Russell, 2001).

2.8.7.2 Transformation of electrocompetent *E. coli* cells

The electrocompetent *E. coli* (Gene Hog) cells were transformed with purified DNA. A microcentrifuge tube containing 50 µl of electro-competent cells was removed from -80°C storage and allowed to thaw on ice. A 2 µl aliquot of a ligation mixture (Section 2.7.7) was added to the thawed cells and gently mixed. The mixture was pipetted into a prechilled 0.1 cm sterile electroporation cuvette (Bio-Rad). Electroporation was performed under the following conditions: 1.8 kV, 25 µF, 200 Ω on the BioRad Gene Pulser (Biorad). Following electroporation, 1 ml SOB broth medium (Section 2.1) was added to the cuvette. The cells were transferred to a 15 ml tube and incubated at 37°C for 1 hr with agitation. 100µl of cells were plated onto LB-agar plates supplemented with ampicillin (100 $mg.ml^{-1}$), IPTG (20 $\mu g.ml^{-1}$) and X-Gal (30 $\mu g.ml^{-1}$).

2.8.7.3 Colony PCR

Recombinant transformants were selected by blue/white colour selection based on insertional inactivation of the *lac*Z gene. White colonies were picked from overnight culture plates using sterile toothpicks and resuspended in 50 µl 1 X TE buffer (Table 2.1). 2 µl of the suspension was used as a template for PCR. PCR amplifications were achieved with the cycling parameters specific for each primer combination as detailed in Table 2.5. The PCR products were separated by agarose gel electrophoresis (section 2.7.4).

2.8.7.4 Plasmid purification

Plasmid extraction was performed using the Zippy™ Plasmid Miniprep Kit (Qiagen, Hilden, Germany) with minor modifications to the manufacturer's instruction. 600 µL of bacterial culture was added to 1.5 ml microcentrifuge tubes. 100 µl of 7 X Lysis Buffer (Zyppy™) was added and mixed by inverting the tube 5 times. 350 µl of cold Neutralization Buffer (Zyppy™) was added and mixed thoroughly for 2 mins. A centrifugation step at 13000 rpm for 4 mins was performed before transferring the supernatant to the Zymo-Spin™ II column. The column was placed in a collection tube and centrifuged for 20 secs. 200 µl of Endo-Wash Buffer (Zyppy™) was added to the column and the column was centrifuged for 20 secs. 400 ul of Zyppy™ Wash Buffer was (Zyppy™) added to the column and the column was centrifuged for 30 secs. The column was transferred to a clean 1.5 ml microcentrifuge tube with 25 µl of Zyppy™ Elution Buffer being added directly to the column matrix. A final centrifugation for 20 secs at 11000 rpm followed to elute plasmid DNA.

2.8.8 Microbial community fingerprinting

2.8.8.1 Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA analysis of PCR amplicons was done in 30 µl reaction mixtures containing 10 µl of PCR product, 2 U of restriction endonuclease (*Hae*III, *Alu*I), 2 µl of the appropriate 10 X buffer and 18 µl of autoclaved distilled water. Reactions were incubated for 16 hrs at 37°C before the restriction patterns were analysed. The restriction digests were separated using 2.5 % agarose gels.

2.8.8.2 Terminal restriction fragment length polymorphism (T-RFLP)

Partial 16S rRNA gene sequences were amplified using a fluorescently labeled 16S rRNA forward E9F primer and U1510R reverse primer (Table 2.4) (Farrelly *et al*., 1995). The forward primer E9F was labeled at the 5' with the fluorescent dye FAM. For each sample two individual PCR reactions were run in a 50 µl volume (Section 2.7.5) and visualized on a 1 % agarose gel to minimize stochastic PCR biasis. Duplicate PCR reactions were pooled and purified using the GFX^{TM} kit (GE Healthcare, UK). Purified T-RFLP PCR products quantified using the 16S rDNA amplicons were purified using the GFX^{TM} kit (GE Healthcare, UK), according to the manufacturer′s instruction with minor modifications. The purified 16S rDNA amplicons were quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Delaware USA) (Section 2.7.3). All samples were quantified in duplicate. 150 ng of the purified amplicons were digested at 37°C for 16 hrs in 20 µl reactions containing 2 U of restriction endonuclease (Fermentas, Vilnius, Lithuania), 2 µl of the appropriate 10 X buffer and 15 µl of UltraPure^{TM} distilled water (Invitrogen Ltd). The digested T-RFLP purified amplicons were re-purified using the GFX^{TM} kit (GE Healthcare, UK) and sent to the Central Analytical Facility of the University of Stellenbosch for terminal resctriction fragment length polymorphism analysis on an automated Applied Biosystems genetic analyzer. Data obtained from genotyping was analysed and interpreted using a range of computational and statistical approaches. T-RFLP electrophoregrams were processed using the Peak Scanner^{TM} V1.0 (PE Appllied Biosystems). The analysis was performed using a size cut-off for peaks, where all peaks shorter than 35 bp and longer than 1200 bp were excluded from analysis. Peaks between these two intensity cut-offs were analysed using the online T-REX software (http://trex.biohpc.org/). Only TRFs between 35 bp and 1200 bp present in two of the replicate samples were considered for further analysis using the Primer 6 software (Primer E, Plymouth, UK). Similarities were calculated between every pair of

samples as the Bray-Curtis similarity coefficient (Bray and Curtis, 1957), using the standardized T-RFLP profiles and the similarity functions in Primer 6. The similarity matrices were used for multidimensional scaling (MDS) plots. Non-metric MDS were created by the rank order of Bray-Curtis similarity matrices. MDS constructs a configuration of the samples, in a specific number of dimensions, which attempts to satisfy all the conditions imposed by the Bray-Curtis similarity matrix (Clarke and Warwick, 2001) i.e. the MDS algorithm tries to construct a sample map whose inter-point distances have the same rank order as the corresponding Bray-Curtis matrix. The term operational taxonomic unit (OTU) is used to refer to individual restriction fragments in T-RFLP patterns (based on variation in the 16S rRNA gene), with recognition that each OTU may comprise more than one distinct bacterial ribotype (Blackwood *et al*., 2007). Putative identification of selected OTUs was performed by in silico restriction using the MiCA3 program (available at htt://mica.ibest.uidaho.edu/pat.php) and the Ribosomal Data Project dataset (RDP release 9.51).

2.8.9 Sequencing

DNA sequencing was performed at the Central Analytical Facility of the University of Stellenbosch using the Hitachi 3730xl DNA Analyzer (Applied Biosystems). The Big Dye Terminator v3.1 system based on the Sanger method was used.

2.8.10 Sequence analysis

DNA sequences were edited using the software Chromas (version 2.01) and aligned using the software DNAMAN (version 4.15). Analysis of DNA sequences and homology searches were carried out using the database of the National Centre for Biotechnology Information. The Basic Local Alignment Search Tool (BLASTn and BLASTp) programmes (Altschul *et*

al., 1997) were used to determine sequence similarity and identity to known species and genes in the GenBank database (www.ncbi.nlm.nih.gov).

CHAPTER THREE

The endophytic bacterial diversity associated with South African sorghum

TABLE OF CONTENTS

3.1 Introduction

In South Africa food shortages are a daily problem and long-term projections suggest that regional food production per capita is likely to decline in the future (Menghestab, 2005). Sorghum (*Sorghum bicolor* L.) is the second most cultivated cereal grain in Africa after maize (Taylor, 2003) and improving its yields could circumvent potential food shortages in Africa.

Production in South Africa is estimated to be between 100 000 to 180 000 tonnes per year with sorghum being farmed mainly in the Free State and Mpumalanga provinces of South Africa. Sorghum is the major staple food consumed by many rural people (Murdy *et al*., 1994).

The productivity and consistency of sorghum production, like other crops, is affected by both biotic (such as the action of fungal and bacterial diseases) and abiotic (such as the nitrogen deficit caused by the excessive use of fertilizers) factors (Boussadia *et al*., 2010). Current control methods make use of herbicides, organic fertilizers, pesticides and/or fungicides which have limited efficiencies. There is an increasing demand for new strategies to inhibit and control diseases of sorghum. One of the proposed eco-friendly strategies is to use the properties of endophytic bacteria as they have been shown to promote growth and to increase yields of various plants (Rajkumar *et al*., 2009).

The use of the culture-dependent methodologies in the studies of the microbial communities has provided useful information for evaluating microbial diversity in different environments including the endosphere (Saito *et al*., 2007). However, it is generally accepted that approximately 0.1 to 1 % of microorganisms from environmental samples can be cultured by

standard laboratory techniques (Torsvik *et al*., 1998). As culture-independent techniques give information on the unculturable microorganisms in the environment, both culture-dependent and culture-independent techniques were used in this study in order to gain a full understanding of the endophtytic microbial communities present in the sorghum endosphere environment.

To evaluate the population of endophytic bacteria in sorghum plants, only healthy sorghum plants were harvested. Following surface sterilization, three different sorghum plant organs (roots, shoots and stems) from plants from the three provinces were pulverized and used to isolate bacteria and for metagenomic DNA extraction.

As fast growing bacterial species rapidly use the nutrients in the medium, they inhibit the growth of slow growing bacterial species. Thus two media were used to cultivate the endophytic bacteria of sorghum. TSA was used to isolate fast growing bacteria and R2A to isolate slow growing bacteria. Molecular tools used to evaluate the unculturable endophytic bacterial population included PCR amplification and analysis of the bacterial 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE) analysis and terminal restriction fragment length polymorphism (T-RFLP) analysis.

3.2 Results

3.2.1 Soil properties

Sorghum is cultivated in areas of South Africa with moderately high temperatures (27°C-30°C). The crop can tolerate a broad range of soil compositions (Almodares and Hadi, 2009).

Rhizospheric soil is the volume of soil seven mm from living plant roots (Walter, 2007). Plants release 20 to 50 % of their photosynthates through their roots (Bottner *et al*., 1999) and these modify the physical, chemical and biological properties of the soils in the rhizosphere. Compounds transferred from the plant roots to the rhizosphere include carbohydrates and organic acids (see Table 1.1). Root exudates serve as nutrient sources for rhizospheric microorganisms (Bodelier *et al*., 1997) and influence the soil chemistry.

Analysis of the rhizosphere and bulk (non-rhizospheric) soils from the sampling sites in the three South African provinces under investigation (Free State, Limpopo and North West) was undertaken to determine the pH, ammonium (NH_4-N), nitrate (NO_3-N) (being the readily assimilable forms of nitrogen), total carbon and total nitrogen content of the soil (Table 3.1).

Table 3.1 Rhizosphere soil and bulk soil analyses from sorghum fields in the Free State, Limpopo and North West provinces.

Environment	Parameter	Free State [a]	North West [a]	Limpopo [b]
Bulk soils	pH	4.7	6.2	5.4
	C %	0.58	0.96	0.19
	N %	0.11	0.14	0.09
	NH_4-N(mg/kg)	7.88	8.44	9.6
	NO_3-N (mg/kg)	1.44	11.88	3.72
Rhizosphere soils	pH	4.2	6	6.3
	C %	0.4	0.94	0.36
	N %	0.11	0.14	0.1
	NH_4-N(mg/kg)	8.68	9.6	8.36
	NO_3-N (mg/kg)	0.52	5.8	4.72

C: carbon. N: nitrogen. NH_4-N: ammonium. NO_3-N: nitrate. a: synthetic fertilizers. b: organic feritlizers.

The pH of the soils in the three sampling sites was acidic and ranged from pH 4.7 (Free State) to pH 6.2 (North West) in the bulk soils. Soil pH's from the rhizosphere of sorghum plants ranged from 4.2 (Free State) to 6.3 (Limpopo) (Table 3.1). A decrease in the pH of the rhizospheric soils in the Free State (pH 4.7 in the bulk soil to pH 4.2 in the rhizosphere) and

North West (pH 6.2 to pH 6) provinces was recorded while the soil of rhizosphere in the Limpopo sampling site was less acidic (pH 6.3) than that of the bulk soil (pH 5.4) (Table 3.1). The sorghum fields in Limpopo province were amended with organic fertilizers (cow feces) and contained significantly less carbon (0.19 %) in the bulk soil than soils from the Free State and North West sampling sites (0.58 and 0.96 % respectively) where the soils were amended with synthetic fertilizers. The carbon content of the soil in the rhizosphere samples from the Limpopo farm increased significantly over that in the bulk soil (from 0.19 to 0.36 %). This was not observed in soils from the other sites. No clear pattern of ammonium or nitrate forms of nitrogen utilization emerged (Table 3.1).

Two of the sampling sites (Free State and North West) were situated on farms which were farmed according to scientific principals and made use of synthetic fertilizers whereas the family farm in Limpopo used traditional farming methods which included the use of cow feces as manure. This clearly influenced the chemistry of the bulk soil. In all cases, altered chemistry of the soils in the rhizosphere was noted.

3.2.2 DNA extraction from sorghum -associated endophytic bacterial communities

The total endophytic bacteria from sorghum plant organs of the three South African provinces studied (Free State, Limpopo and North West) grown on both TSA and R2A media were harvested and their total genomic DNA was extracted. Using a modified version of the method of Miller *et al.* (1999), high DNA yields ranging from 3.5×10^3 to 5.2×10^3 ng/µl were obtained from the root, shoot and stem samples. This method produced good quality ($A_{260}/_{280}$ ~ 1.8-1.9) high molecular weight DNA and little RNA was observed (Figure 3.1). The DNA samples required no further purification for downstream processing operations.

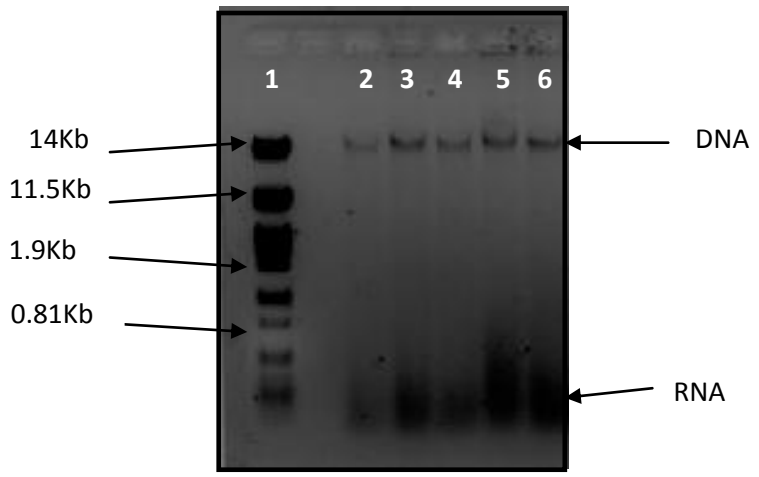


Figure 3.1 Total genomic DNA extracted from total culturable sorghum endophytic bacteria samples. Lane 1: (λ*Pst*I) DNA molecular size marker; Lane 2: Root isolates cultured on TSA; Lane 3: Root isolates cultured on R2A; Lane 4: Shoot isolates cultured on TSA; Lane 5: Shoot isolates cultured on R2A; Lane 6: Stem isolates cultured on TSA.

3.2.3 Metagenomic DNA extraction from sorghum organs

Metagenomic DNA extraction from sorghum plant organs was initially attempted using a 3 % CTAB extraction protocol (Murray and Thompson, 1980). This method produced little high molecular weight DNA and an average DNA yield of 21.1(± 7.27) ng/µl, ranging from 13.4 to 36.4 ng/µl (Figure 3.2) (Table 3.2). The 2 % CTAB extraction protocol produced high molecular weight DNA with average DNA yield of 329.1(± 275.5) ng/µl ranging from 89.5 to 999.3 ng/µl (Figure 3.2) (Table 3.2) which was significantly higher than the 3 % CTAB extraction (T-test, p=0.003). Less RNA was coextracted using the latter protocol and a high degree of DNA purity (A_{280}/A_{260} 1.70 to 2.02) was obtained. The 2 % CTAB DNA extraction protocol was used for further studies.

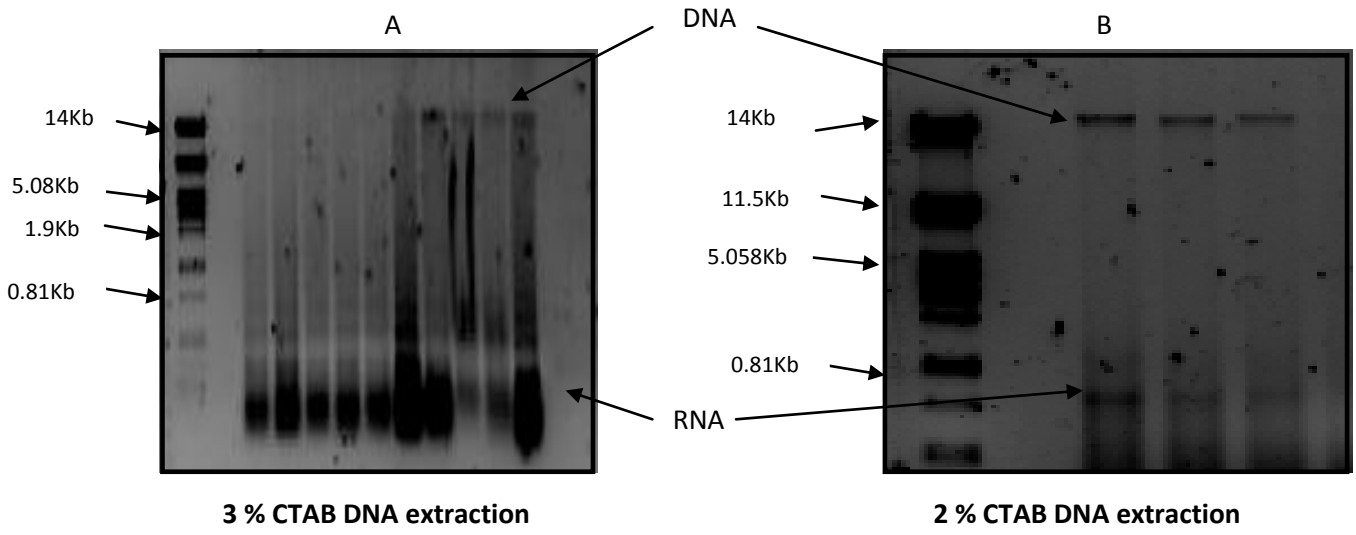


Figure 3.2 Agarose gels showing metagenomic DNA extracted from sorghum plant organs from plants growing in the Free State province using the 3 % (A) and 2 % (B) CTAB DNA extraction procedures. Similar results were obtained for DNA extractions from plant organs growing in the North West and Limpopo provinces (data not shown).

Table 3.2 Sorghum metagenomic DNA extraction yields using the 2 % and 3 % CTAB DNA extraction protocols. Root, shoot and stem samples from the three sampling sites were used.

		3 % CTAB DNA extraction			2 % CTAB DNA extraction			
Province	**Sample**	**DNA yield(ng/µl)**	**A_{280}/A_{260}**	**$A_{260}/_{230}$**	**Sample**	**DNA yield(ng/µl)**	**A_{280}/A_{260}**	**$A_{260}/_{230}$**
Free State	Roots	13.4	1.97	0.99	Roots	89.5	2.01	1.24
	Shoots	25.5	1.98	0.83	Shoots	112.8	1.96	1.01
	Stems	18.7	2.67	0.43	Stems	310.2	2.29	0.78
Limpopo	Roots	36.4	2.15	0.72	Roots	105.8	2.04	1.4
	Shoots	25.5	2.27	1.01	Shoots	999.3	1.83	0.92
	Stems	14.6	2.53	0.38	Stems	502.8	1.79	1.02
North West	Roots	15.5	2.06	1.53	Roots	112.8	1.94	1.19
	Shoots	25.8	2.27	1.01	Shoots	361	1.85	0.74
	Stems	14.6	2.53	0.38	Stems	367.3	2.08	1.3

3.2.4 Microbial community fingerprinting using DGGE

Denaturing gradient gel electrophoresis is a well known molecular technique used to separate amplified 16S rRNA gene fragments of the same length but of different base pair composition (Malik *et al*., 2008). Using this technique a DNA fragment is amplified using a primer modified with a 35-40 bp GC-clamp attached to its 5' end. The GC-clamp maintains the amplified DNA fragment in a double stranded configuration while migrating through a denaturing gradient gel. This allows DNA fragments to migrate differentially on the gel and to be separated based on differences in base pair composition. Bands that migrate in the acrylamide gel at the same level (and at the same temperature) are considered to have the same melting temperature (Muyzer *et al*., 1993). DGGE was used to obtain an overview of the endophytic bacterial community structure and diversity in the sorghum plant organs in plants grown in the three South African provinces, and to show whether or not common endophytic bacteria were observed in the sorghum plants from the three sampling sites.

In this study a 1500 bp DNA fragment from the 16S rRNA genes of the endophytic bacteria was successfully amplified from both total genomic (from the cultured organisms) and total metagenomic DNA using the universal bacterial 16S rRNA gene PCR primers (Section 2.7.6) from the various sorghum plant organs (Figure 3.3: the root PCR amplification gels are shown as a representative). Shoot and stem amplifications are not shown, but generated the same results. Using this amplicon as a template, a small DNA fragment (193 bp) from the variable V3 region of the 16S rRNA gene was amplified using the 341FGC/534R primer set (Figure 3.4). PCR products with the GC-clamp were separated using 9 % (w/v) polyacrylamide gels with a denaturing gradient of 30-70 % (Figure 3.6a, 3.7a, 3.8a).

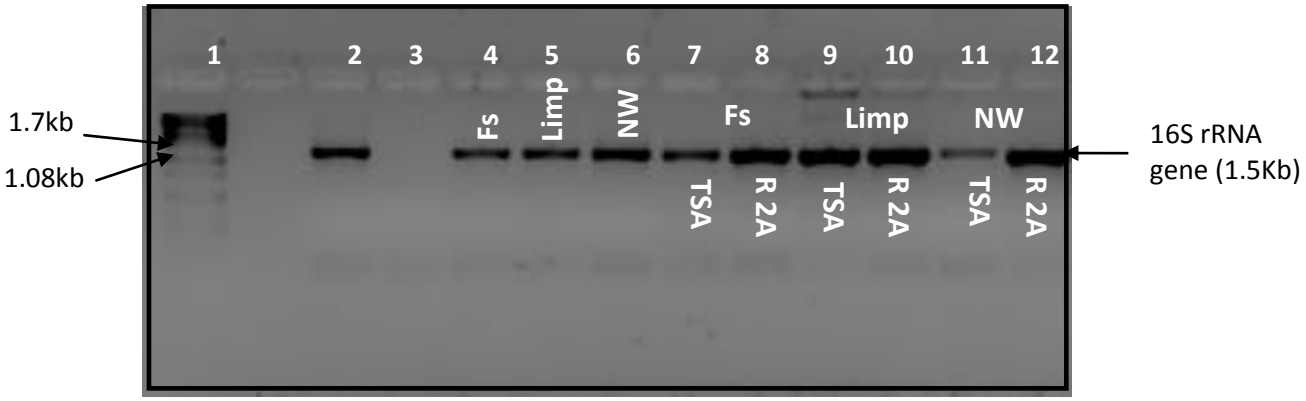


Figure 3.3 PCR amplification of the bacterial 16S rRNA gene using the primer set E9F/ U1510R. Lane 1: (λ*Pst*I) DNA molecular weight marker; Lane 2: positive control (*Pseudomonas* sp); Lane 3: negative control (no DNA); Lane 4-6: sorghum metagenomic DNA of root samples as template; Lane 7-12: total genomic DNA of the total culturable microbial communities of the root. FS: Free State. Limp: Limpopo. NW: North West. TSA: Tryptic soy agar R2A: R2A agar.

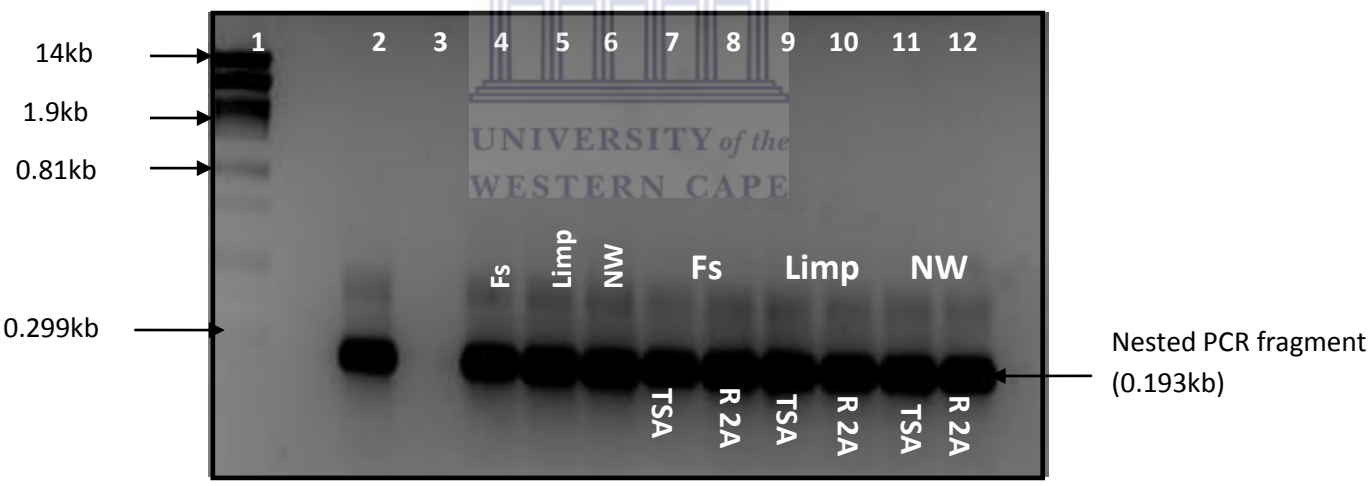


Figure 3.4 Nested PCR amplification of the bacterial 16S rRNA gene using primers 341F-GC and 534R. Lane 1: (λ*Pst*I) DNA molecular weight marker; Lane 2: positive control (*Pseudomonas* sp); Lane 3: negative control (no DNA); Lane 4-6: sorghum metagenomic DNA; Lane 7-12: total genomic DNA of the total culturable microbial communities of the root. FS: Free State. Limp: Limpopo. NW: North West. TSA: Tryptic soy agar. R2A: R2A agar.

3.2.4.1 Comparative analysis of the root, shoot and stem endophytic bacterial communities

DGGE banding patterns of the root, shoot and stem samples from the three sampling locations were compiled and analysed in order to study the homogeneity of the endophytic microbial communities of sorghum plants. A dendrogram was constructed which showed that the sorghum plant endophytic bacterial communities, independent of the DNA extraction procedure used and the sampling site, shared a minimum of 38 % similarity (Figure 3.5). The endophytic bacterial communities of the roots, shoots and stems of sorghum differ. However, the endophytic bacterial communities of the sorghum plant organs were grouped into two clusters. The endophytic bacterial communities of all shoot samples formed cluster one. These shared a minimum of 76.30 % similarity. Included in cluster one were the isolates from the total culturable microbial communities from the roots of plants grown in the North West province. Cluster two was formed by the endophytic bacterial communities of all stem samples, and the majority of the endophytic bacterial communities of the root samples (excluding the three samples which were included in cluster one). These shared a minimum of 80.26 % similarity. Thus the endophytic bacterial communities from the shoot and stem samples are dissimilar but both shared some endophytic bacterial species with the root samples.

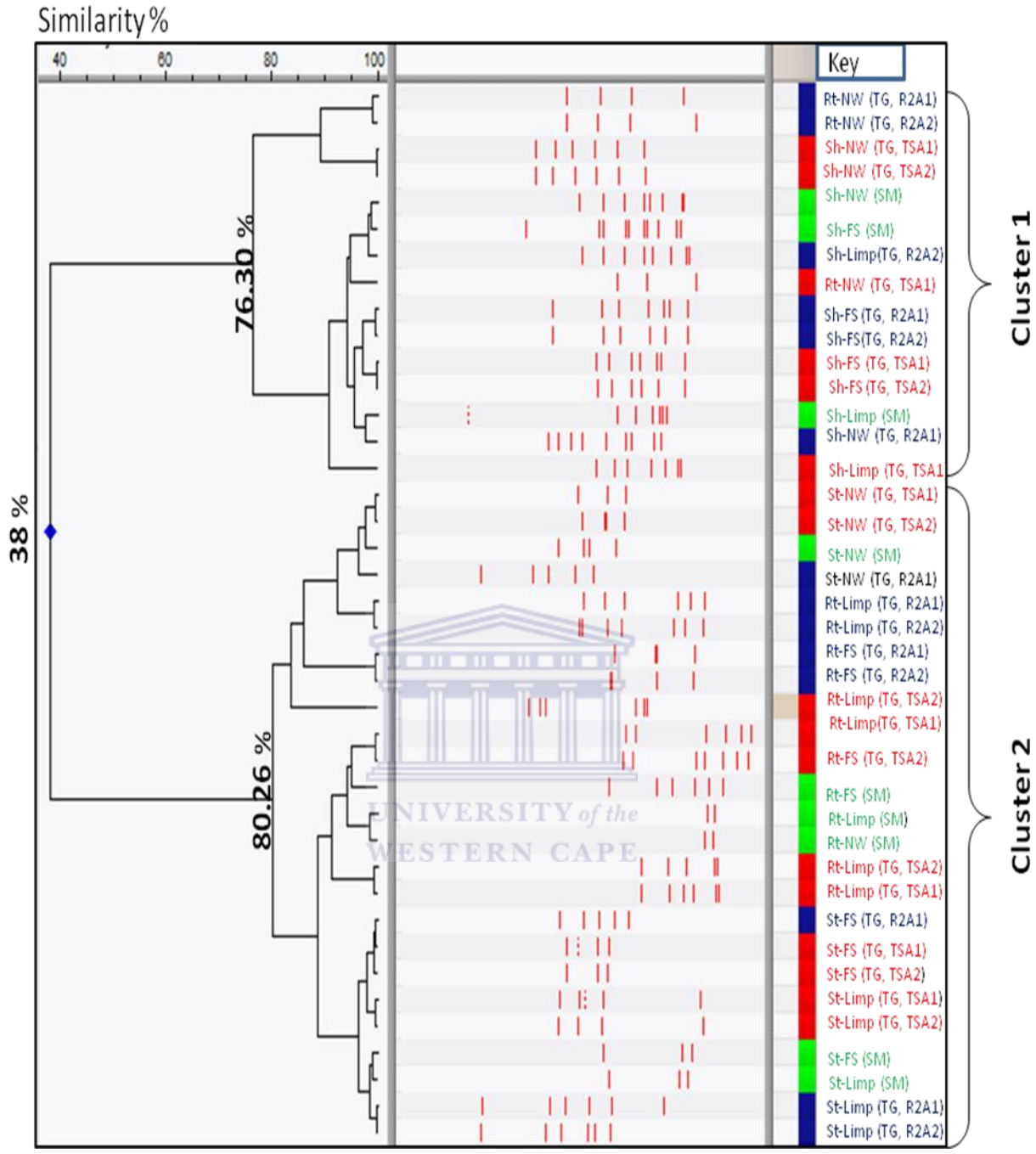


Figure 3.5 Cluster analysis of the DGGE fingerprints of the root, shoot and stem associated endophytic bacterial communities of the three sampling sites. FS: Free State. Limp: Limpopo. NW: North West. Rt: root. Sh: shoot. St: stem. SM: Sorghum metagenomic DNA (green). TG: Total genomic DNA of the total culturable microbial communities on TSA (red). TG: Total genomic DNA of the total culturable microbial communities on R2A (blue). 1, 2: replicates. Uncertain band.

3.2.4.2 Root microbial communities

Three (meta)genomic approaches to analyzing community diversity within root tissue (sorghum metagenomic DNA, genomic DNA isolated from fast growing (TSA) and slow growing (R2A) species) were used to analyze the endophytic bacterial communities of the sorghum roots using DGGE analysis (Figure 3.6a). DGGE patterns obtained from the various DNA samples differed, indicating that the endophytic microbial communities isolated using the three isolation procedures were different. This result validated the decision to use multiple approaches to analysing community diversity within sorghum root tissues. Duplicate samples from the sites showed the same banding patterns, showing that DGGE is a reproducible molecular tool. (Figure 3.6a). Using sorghum metagenomic DNA samples as a template, 6 bands were observed in the Free State sample, 2 in the Limpopo sample and 2 in the North West sample (Figure 3.6a, lanes 2-4 and Figure 3.6b). From the total fast growing (TSA) communities, 7 bands were observed in the Free State sample, 6 in the Limpopo sample and 3 in the North West sample (Figure 3.6a, lanes 5-9 and Figure 3.6b). From the total slow growing (R2A) communities, 3 bands were observed in the Free State sample, 6 in the Limpopo sample and 4 in the North West sample (Figure 3.6a, lanes 10-15 and Figure 3.6b).

A lower diversity was recorded from the sorghum metagenomic DNA samples (Figure 3.6a, lanes 2-4) when compared to that from the total fast growing (TSA) and slow growing (R2A) communities (Figure 3.6a, lanes 5-9 and lanes 10-15). These results were unexpected as in general only up to 1 % of the microorganisms in an environmental sample are assumed to be culturable (Torsvik *et al*., 1998). These results might arise from the fact that during the metagenomic DNA extraction of the sorghum plant organs, both plant DNA and endophytic microbial DNA were co-extracted and the latter DNA was consequently present in a low concentration. Overall, different DGGE patterns were observed in the samples from the three

provinces indicating that the root endophytic bacterial communities differed according to the origins of the sorghum plants.

A dendrogram was obtained which showed two clusters and a high degree of similarity within the sorghum root endophytic bacterial communities. The similarity was independent of the DNA extraction procedure used and sampling site (Figure 3.6b). The clusters shared a minimum of 80.7 % similarity (Figure 3.6b).

Cluster one was formed by two sub-clusters (sub-cluster 1a and sub-cluster 1b). Sub-cluster 1a was formed by the metagenomic DNA and the total fast growing (TSA) communities of the roots of plants grown in the Free State and Limpopo. These shared a minimum of 91.4 % similarity. Sub-cluster 1b was formed by the total slow growing (R2A) endophytic bacterial communities of the roots of plants grown in the Free State and from the total fast growing (TSA) endophytic bacterial communities of the roots of plants grown in the North West. These shared a minimum of 93.4 % similarity. These results indicate that the sorghum root recruits similar endophytic communities in the three geographical locations studied and that the communities are independent of the agricultural practices used for the cultivation of the sorghum plants. Duplicates shared 90 % similarity, confirming that DGGE is a reproducible molecular tool.

Cluster two was a small cluster comprising members of the slow growing (R2A) endophytic inhabitants of roots of sorghum plants growing in the North West sampling site. The endophytic bacterial communities isolated from the remaining samples were represented in cluster one.

A common band observed in the DGGE fingerprint of the metagenomic DNA from the Free State, Limpopo and North West samples was identified and sequenced (Bands a1, a2, a3, Figure 3.6a). Table 3.3 presents the nearest match which in all cases was to a cyanobacterium (*Synechococcus* sp.). Endophytic cyanobacteria (*Anabaena* sp.) have been reported in the stems of sweet potato plants where they were characterized as plant growth-promoting endophytes as they assisted the plant host in nitrogen fixation (Terakado-Tonooka *et al*., 2008).

To conclude, despite the different agricultural practices used to cultivate the sorghum plants, the endophytic bacterial communities associated with sorghum roots shared significant similarities and habour a common endophytic bacterium of the genus *Synechococcus* with potential plant growth promotion capacities.

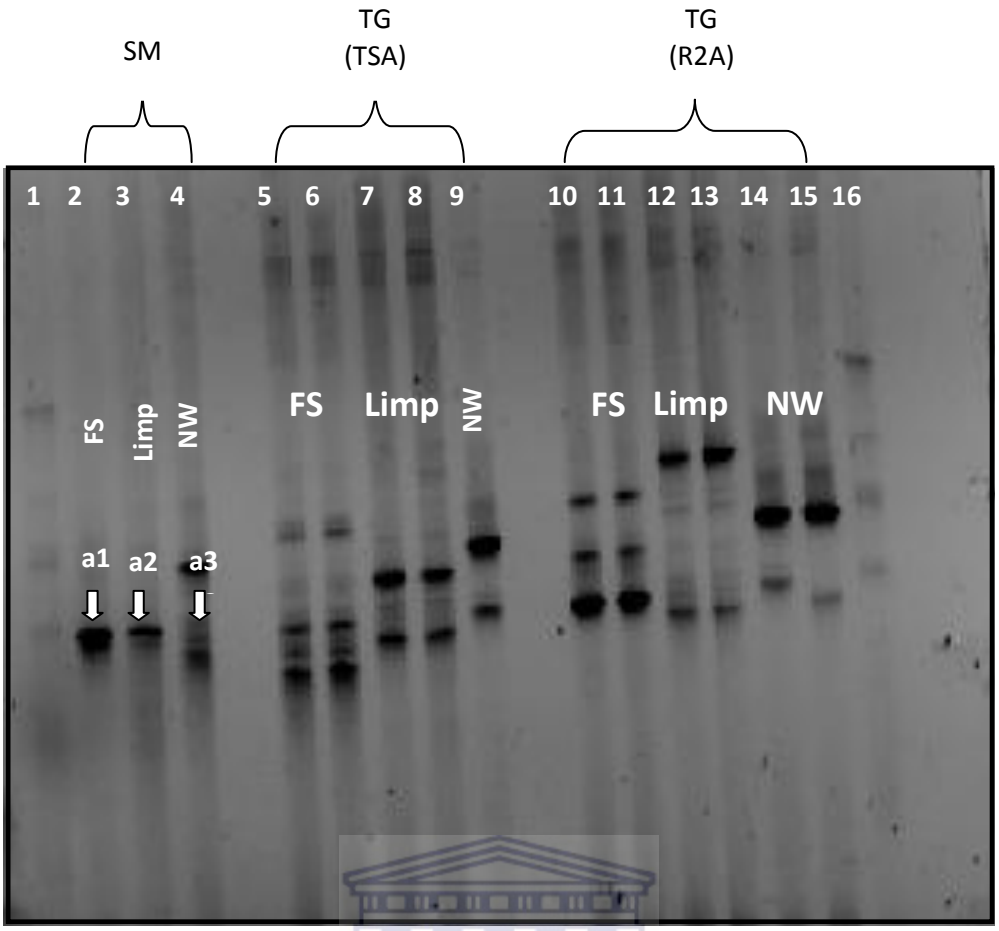


Figure 3.6a DGGE fingerprints of sorghum root endophytic microbial communities from three South African provinces. Lane 1: DGGE marker; Lane 2-4: sorghum metagenomic DNA; Lane 5-9 (TG, TSA); Lane 10-15 (TG, R2A); Lane 16: DGGE marker. SM: sorghum metagenomic DNA. TG: total genomic DNA of the total fast growing (TSA) or slow growing (R2A) culturable microbial communities. FS: Free State. Limp: Limpopo. NW: North West.

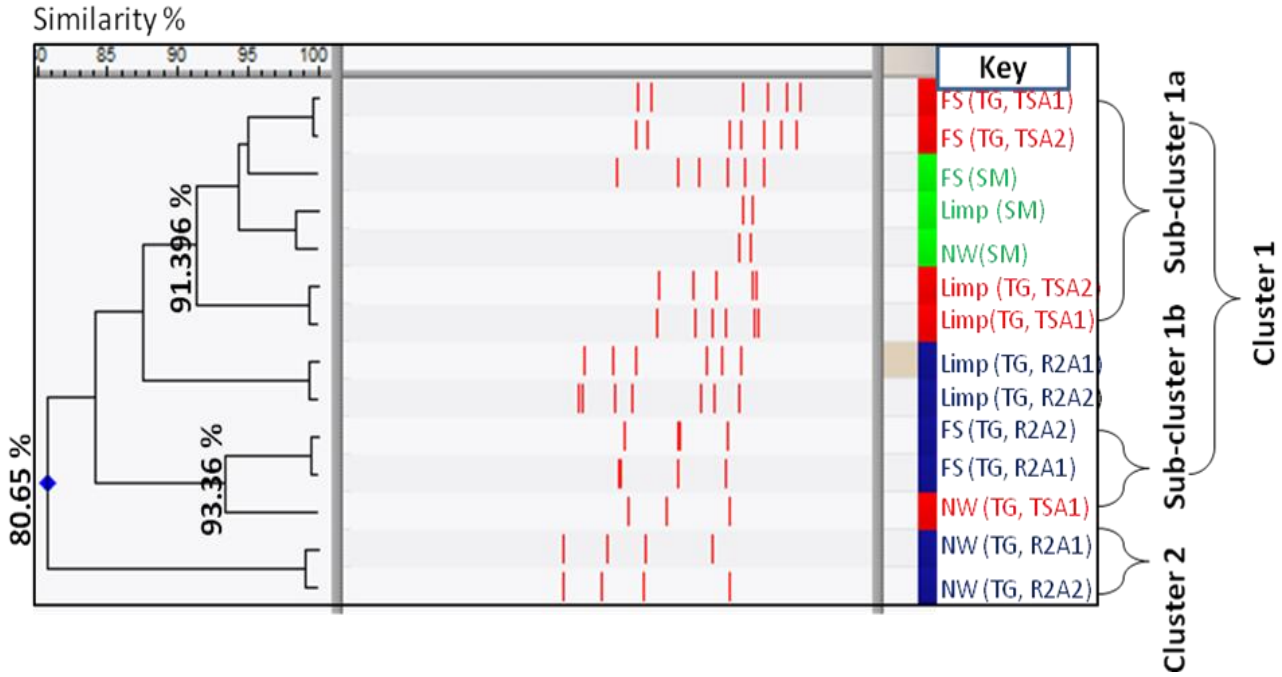


Figure 3.6b Cluster analysis of the DGGE fingerprints of the root associated endophytic bacterial communities of the three sampling sites. FS: Free State. Limp: Limpopo. NW: North West. SM: sorghum metagenomic DNA (green). TG: total genomic DNA of the total fast growing (TSA) culturable microbial communities (red). TG: total genomic DNA of the total slow growing (R2A) culturable microbial communities (blue). 1, 2: replicates.

Table 3.3 Sequences obtained from the common small variable (V3) region of the 16S rRNA gene found in the root samples.

Province	Band	Accession	Description	Isolation source	E-value	Max-identical (no. of bp)
Free State	a1	HQ018568	Uncultured *Synechococcus* sp. clone R4CP3R1F09	Rhizosphere soil treated with nitrogen fertilizer (sugarcane)	2e-78	100 % (170 bp)
Limpopo	a2	HQ018568	Uncultured *Synechococcus* sp. clone R4CP3R1F09	Rhizosphere soil treated with nitrogen fertilizer (sugarcane)	2e-80	99 % (193 bp)
North West	a3	HQ018568	Uncultured *Synechococcus* sp. clone R4CP3R1F09	Rhizosphere soil treated with nitrogen fertilizer (sugarcane)	9e-83	100 % (193 bp)

3.2.4.3 Shoot microbial communities

As with the root endophytic communities (Section 3.2.4.2) the bacterial diversity identified by analyzing the three (meta)genomic approaches to analyzing community diversity within shoot tissue (sorghum metagenomic DNA and genomic DNA isolated for fast growing (TSA) and slow growing (R2A) species) differed, validating the use of multiple approaches to analysisng community diversity within shoot tissues. From the total slow growing (R2A) communities, 7 bands were observed in the Free State sample, 8 bands in the Limpopo sample and 9 bands in the North West sample (Figure 3.7a, lanes 1-5 and Figure 3.7b). From the total fast growing (TSA) communities, 7 bands were observed in the Free State sample, 8 bands in the Limpopo sample and 12 bands in the North West sample (Figure 3.7a, lanes 6-10 and Figure 3.7b). Using sorghum metagenomic DNA as a template, 10 bands were observed in the Free State sample, 6 bands in the Limpopo sample and 6 bands in the North West sample (Figure 3.7a, lanes 11-13 and Figure 3.7b). The DGGE banding patterns of the shoot microbial communities shared a minimum of 76.30 % similarity (Figure 3.7b), indicating that they were highly conserved independent of the sample location and agricultural practices used for their cultivation. This suggests that the endophytic bacterial communities present are dependent on the sorghum plants and that the host plants recruit many specific endophytic bacterial species.

Common bands were identified in the DGGE analysis of the shoot DNA samples from the three sampling sites (Figure 3.7a, b1-3; c1-3; d1-3). The nearest sequence matches are presented in Table 3.4. Matches to three potential endophytic bacteria were found: *Erwinia* sp. and *Pantoea* sp. were identified in the three provinces while band b3 had a nearest sequence match to a *Klebsiella* sp. Endophytic *Klebsiella* have been isolated from maize root tissues (Chelius and Triplett, 2000) where they have plant growth enhancement potential as

they are able to produce nitrogenase under appropriate plant cultivation conditions. Many *Pantoea* sp. are endophytic bacteria found in sugarcane where they are described as potential nitrogen fixing microorganisms (Loiret *et al*., 2004). Endophytic *Erwinia* sp. have also been previously reported in soybean plants where they are potential plant growth promoters due to their capacity to produce the auxin indole acetic acid (Kuklinsky-Sobral *et al*., 2004).

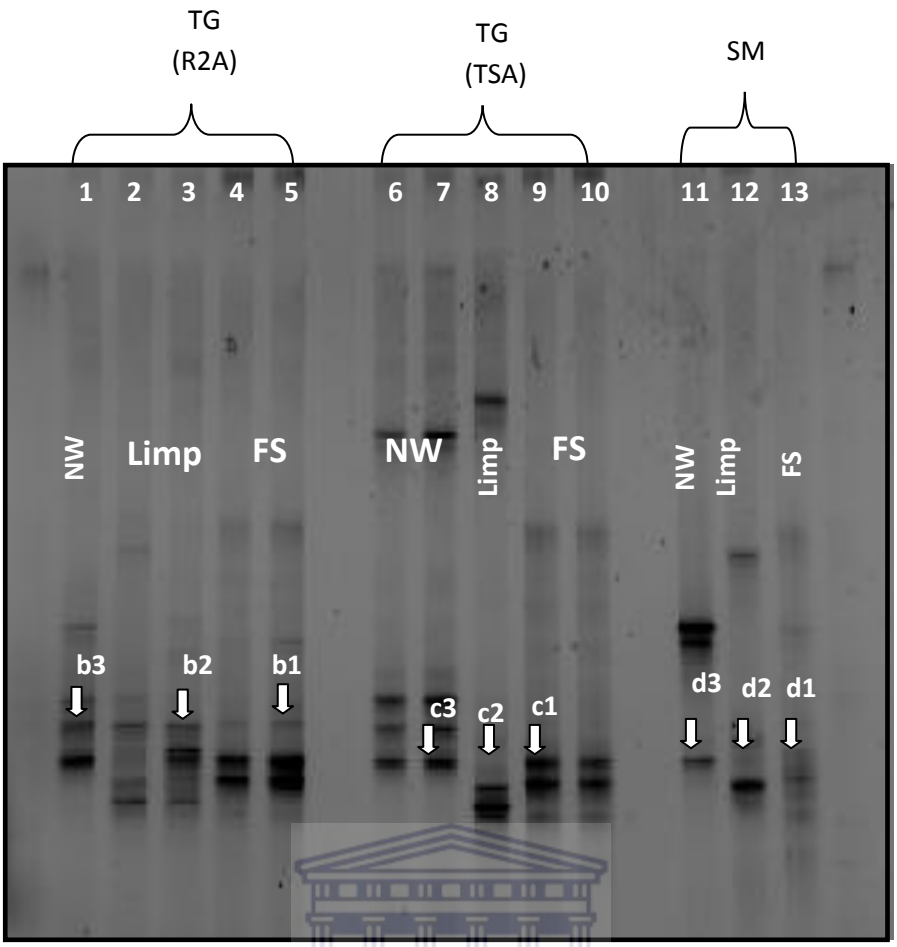


Figure 3.7a DGGE fingerprints of the sorghum shoot endophytic microbial communities from three South African provinces. Lane 1-5 (TG, R2A); Lane 6-10 (TG, TSA); Lane 11-13: sorghum metagenomic DNA. SM: sorghum metagenomic DNA TG: total genomic DNA of the total fast growing (TSA) or slow growing (R2A) culturable microbial communities. FS: Free State. Limp: Limpopo. NW: North West.

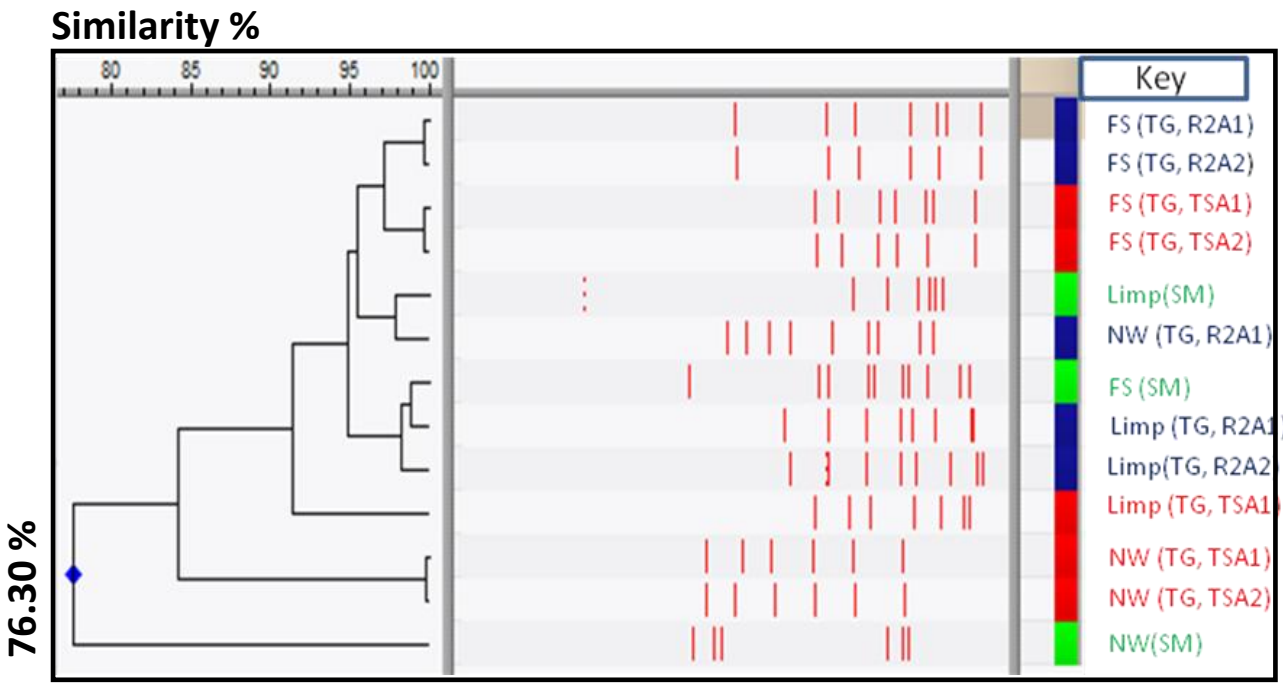


Figure 3.7b Cluster analysis of the DGGE fingerprints of the shoot associated endophytic bacterial communities of the three sampling sites. FS: Free State. Limp: Limpopo. NW: North West. SM: sorghum metagenomic DNA (green). TG: total genomic DNA of the total fast growing (TSA) culturable microbial communities (red). TG: total genomic DNA of the total slow growing (R2A) culturable microbial communities (blue). 1, 2: replicates. :Uncertain band.

Table 3.4 Sequences obtained from the common small variable (V3) region of the 16S rRNA gene found in the shoot samples.

Province	Band	Accession	Description	Isolation source	E-value	Max-ident (no.of bp)
Free state	b1	DQ413253	*Pantoea* sp. HPC1071 Clone G3-3	Activated biomass of a treatment plant	1e-35	99 % (180 bp)
Limpopo	b2	HQ443235	*Pantoea dispersa* strain TBRh9	Rhizosphere	6e-95	99 % (180 bp)
North west	b3	EU196756	*Klebsiella* sp. XJ15	Rhizosphere of litchi plant	8e-33	83 % (180 bp)
Free State	c1	HQ706112	*Erwinia* sp. AB294	Surface of disinfected plant tissue	2e-59	98 % (180 bp)
Limpopo	c2	HQ706112	*Erwinia* sp. AB294	Surface of disinfected plant tissue	1e-80	97% (180 bp)
North West	c3	HQ706112	*Erwinia* sp. AB294	Surface of disinfected plant tissue	1e-81	97% (180 bp)
Free state	d1	JF262564	*Pantoea* sp. UYSO13	Surface of disinfected plant tissue	6e-95	99 % (180 bp)
Limpopo	d2	JF262564	*Pantoea* sp. UYSO13	Surface of disinfected plant tissue	2e-95	100 % (180 bp)
North West	d3	GQ853415	*Pantoea* sp. N3	*Tartarian* (buckwheat) plant	4e-95	99 % (180 bp)

3.2.4.4 Stem microbial communities

The endophytic microbial community structure of the sorghum stem was analysed as described for the root and shoot communities. Fewer DGGE bands (a total of 42 bands were amplified from all sources and provinces) were observed indicating that the sorghum stem microbial communities are less diverse than those of the root and shoot. Using sorghum metagenomic DNA as a template, 4 bands were observed in the Free State sample, 6 bands in the Limpopo sample and 3 bands in the North West samples (Figure 3.8a, lanes 2-4). From the total fast growing (TSA) communities, 3 bands were observed in the Free State sample, 4 bands in the Limpopo sample and 4 bands in the North West sample (Figure 3.8a, lane 5-10). From the total slow growing (R2A) communities, 6 bands were observed in the Free State sample, 6 bands in the Limpopo sample and 5 bands in the North West sample (Figure 3.8a, lanes 11-14).

Analysis of the DGGE banding patterns revealed two clusters (Figure 3.8b). The clusters shared a minimum of 89.5 % similarity indicating that the endophytic microbial communities in the stems of the sorghum plants used in the study are conserved. The Free State and Limpopo communities clustered together, sharing a minimum of 96 % similarity. Cluster 2 represented the North West province stem endophytic bacterial communities which share a minimum of 96.2 % similarity. To conclude, stems displayed highly similar endophytic bacterial communities, independent of the DNA extraction procedures, sample location and agricultural practices used to cultivate the crop. However, those from Free State and Limpopo provinces are more closely related than those from the North West province.

No common endophytic bacterium was observed in the DGGE fingerprints of the metagenomic DNA of the sorghum stem samples.

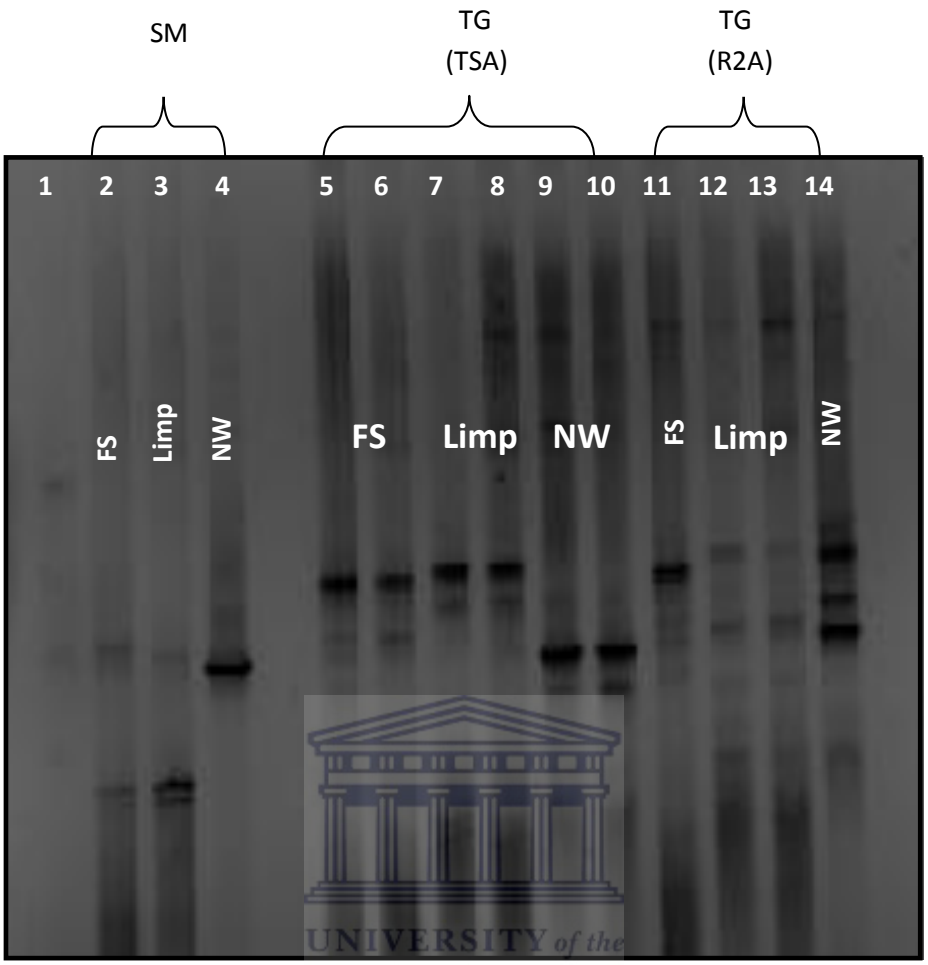


Figure 3.8a DGGE fingerprints of the sorghum stem endophytic microbial communities from three South African provinces. Lane 1: DGGE marker; Lane 2-4 (SM): FS, Limp, NW; Lane 5-10 (TG, TSA): FS, Limp, NW; Lane 11-14 (TG, R2A): FS, Limp, NW. SM: sorghum metagenomic DNA. TG: total genomic DNA of the total fast growing (TSA) or slow growing (R2A) culturable microbial communities. FS: Free State. Limp: Limpopo. NW: North West.

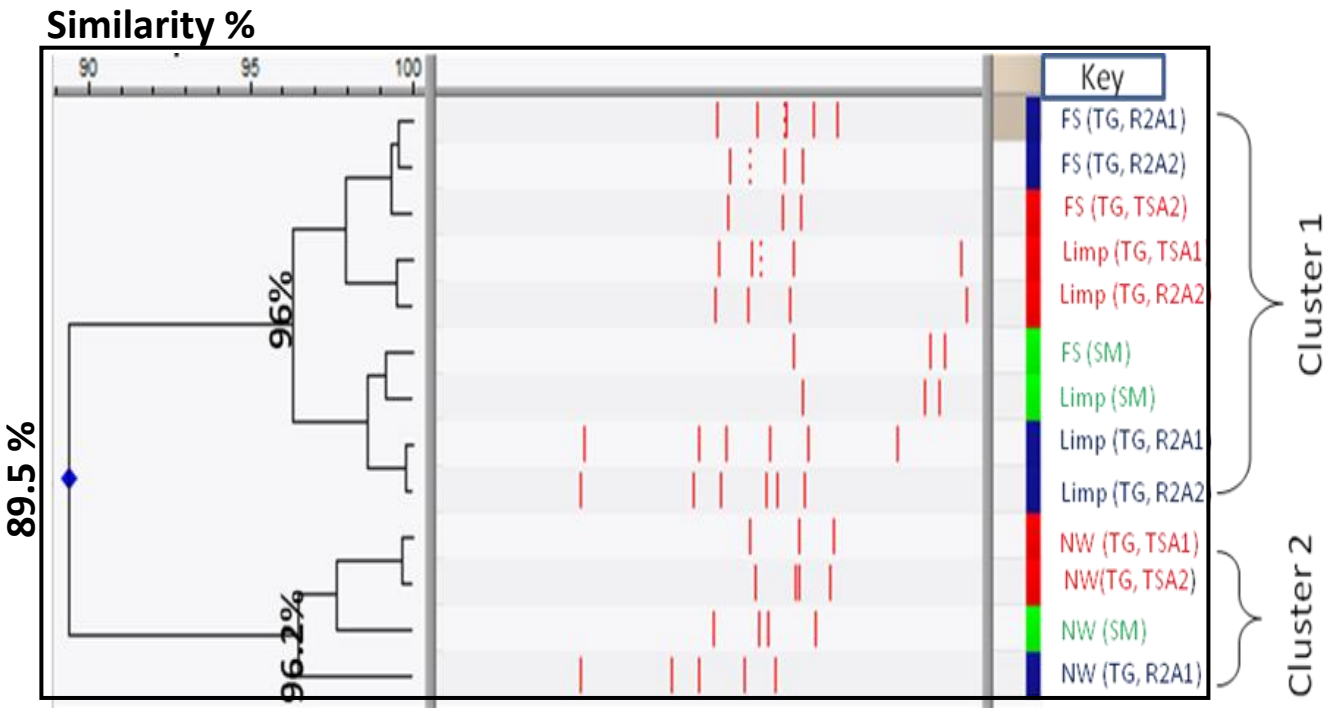


Figure 3.8b Cluster analysis of the DGGE fingerprints of the stem associated endophytic bacterial communities of the three sampling sites. FS: Free State. Limp: Limpopo. NW: North West. SM: sorghum metagenomic (green) DNA. TG: total genomic DNA of the total fast growing (TSA) culturable microbial communities (red). TG: total genomic DNA of the total slow growing (R2A) culturable microbial communities (blue). 1, 2: replicates. Uncertain band.

3.2.4.5 Conclusion

Analysis of the DGGE fingerprints of the root, shoot and stem associated endophytic bacterial communities of sorghum plants growing in the three South African provinces yielded a maximum of 51 distinct bands from root samples, 101 bands from shoot samples and 42 from the stem samples. The cluster analysis in this study illustrates that the endophytic bacterial community associated with shoots (Figure 3.7b) appears to be more diverse than those of the roots (Figure 3.6b) and stems (Figure 3.8a). Two common endophytic bacterial species (*Pantoea* sp. and *Erwinia* sp.) were revealed in the shoots (Figure 3.7a). Members of these species have been reported to function as biofertilizers (Loiret *et al*., 2004) and produce

phytohormones (Kuklinsky-Sobral *et al*., 2004) respectively. Their presence in the shoots of the sorghum plants sampled in the study suggests that they are potential plant growth promoting endophytes in sorghum. Endophytic cyanobacteria which are plant promoting endophytes have been isolated from the stems of sweet potato plants (Terakado-Tonooka *et al*., 2008), playing a role in nitrogen fixation. The presence of cyanobacterium closely related to *Synechococcus* sp. (Table 3.3) in the roots of the sorghum plants sampled in this study suggests that *Synechococcus* has the potential to be used as a plant growth-promoting endophyte.

3.2.5 Microbial community fingerprinting using T-RFLP

Terminal restriction fragment length polymorphism is a culture independent fingerprinting method used to study microbial community structure (Malik *et al*., 2008). In this technique one or both of the PCR primers are fluorescently labeled and the resultant PCR products are digested with specific restriction endonucleases. This produces different terminal restriction fragments (T-RFs) which may be quantified by automated electrophoresis systems (Liu *et al*., 1997).

3.2.5.1 Comparative analysis of the root, shoot and stem endophytic bacterial communities

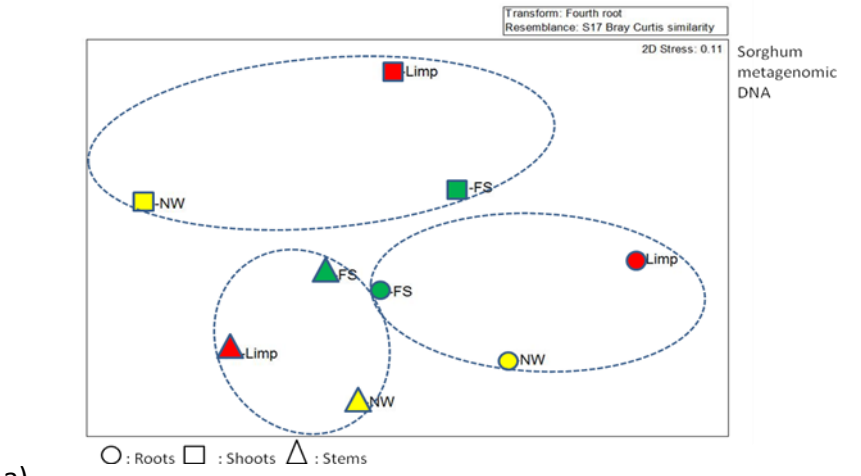
The three (meta)genomic DNA samples (sorghum metagenomic DNA, genomic DNA isolated for fast growing (TSA) and slow growing (R2A) species) of the different sorghum plant organs (root, shoot and stem) of the three provinces were analysed separately by T-RFLP in order to view the relationship between the samples. The MDS analysis of the sorghum metagenomic DNA showed three clusters (Figure 3.9a). Cluster one was formed by the shoot samples from the three South African provinces. Cluster two was formed by the

stem samples from the three provinces and cluster three was formed by the root samples from the three provinces.

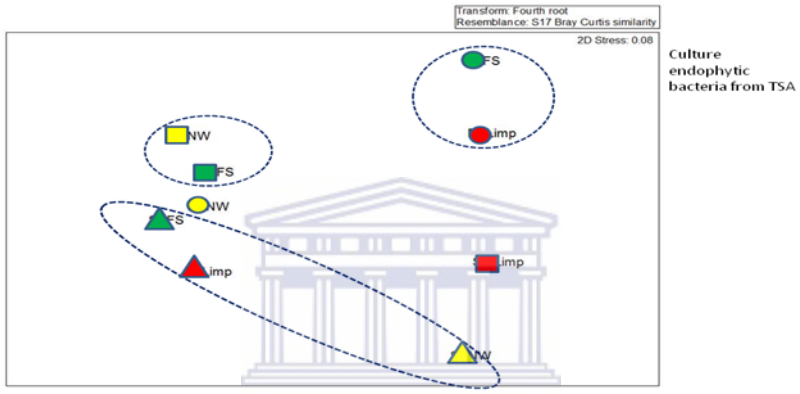
MDS analysis of the genomic DNA of the total fast growing (TSA) communities showed three clusters (Figure 3.9b). Cluster one was formed by the stem samples from the three provinces. Cluster two was formed only by the shoot samples from Free State and North West. Cluster three was formed only by the root samples from Free State and Limpopo.

MDS analysis of the genomic DNA of the total slow growing (R2A) communities showed two clusters (Figure 3.9c). Cluster one was formed by the shoot samples from the three provinces. Cluster two was formed by the stem samples from the three provinces.

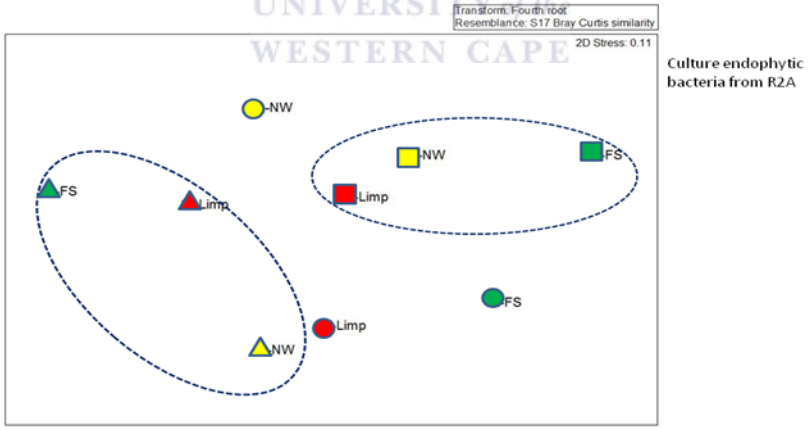
To conclude, the different sorghum plant organs from the three South African provinces harbour different endophytic bacterial communities. The endophytic bacterial communities of the stem sorghum plants are highly conserved therefore are not affected by the different agricultural practices.



a)



b)



c)

Figure 3.9 MDS ordinations showing the relationship between endophytic bacterial communities of the sorghum plant samples (roots, shoots and stems): a) Sorghum metagenomic DNA, b) genomic DNA of the total fast growing (TSA) culturable microbial communities, c) genomic DNA of the total slow growing (R2A) culturable microbial communities. FS: Free State (green). Limp: Limpopo (red). NW: North West (yellow).

3.2.5.2 Root microbial communities

A total of 330 T-RFs were identified (Table 3.5). The relationship between the endophytic bacterial communities from the MDS (Figure 3.10) of the 12 root samples was not close. This indicates that the endophytic bacterial communities isolated using the three approaches to analyzing community diversity were different, again validating the choice of using the three (meta)genomic approaches. The replicate metagenomic DNA samples FS1 and FS2, Limp1 and Limp2, NW1 and NW2 clustered together, showing that T-RFLP analysis is a reproducible molecular tool to study sorghum microbial community diversity (Figure 3.10). The samples FS4, Limp4 and NW3 clustered together as did samples FS3 and Limp3 (Figure 3.10). Although some clustering is observed between some samples, the communities identified in root tissue using T-RFLP seem to be dependent on the extraction method used.

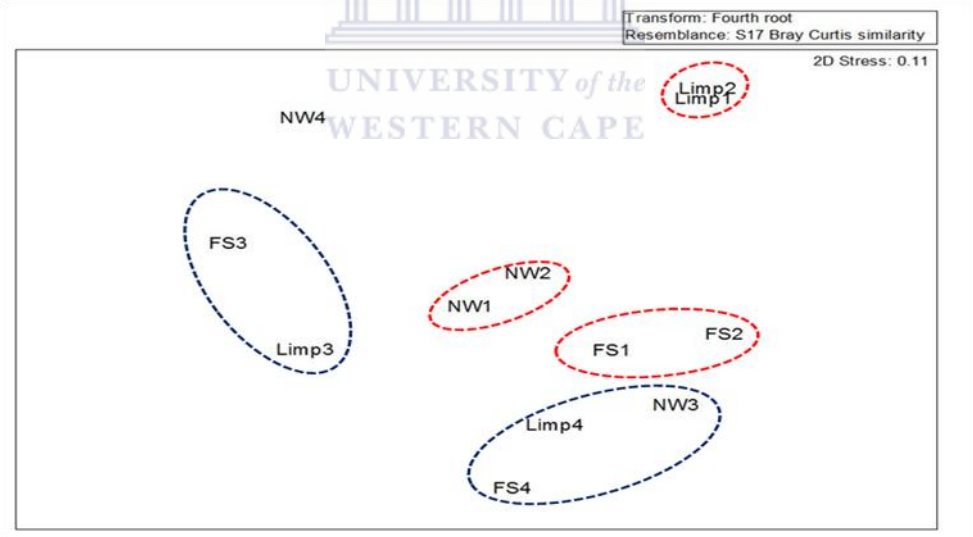


Figure 3.10 MDS ordinations showing the relationship between endophytic bacterial communities of the root samples. 1, 2: sorghum metagenomic DNA. 3: genomic DNA of the total fast growing (TSA) culturable microbial communities. 4: genomic DNA of the total slow growing (R2A) culturable microbial communities. FS: Free State. Limp: Limpopo. NW: North West.

Table 3.5 Summary of the total number and common T-RFs in the root samples.

Total (meta) genomic DNA	Location	Total no of T-RFs	Common T-RFs (bp)
Sorghum metagenomic DNA	Free State	52	197
	Limpopo	30	
	North West	70	
Genomic DNA of the total fast growing (TSA) culturable microbial communities	Free State	18	222
	Limpopo	70	
	North West	22	
Genomic DNA of the total slow growing (R2A) culturable microbial communities	Free State	21	309
	Limpopo	25	
	North West	22	

Common T-RFs were observed in the sorghum root endophytic T-RFLP profiles: 197 bp, 222 bp, and 309 bp (Table 3.5). *In silico* putative assignments were performed for the 197 bp, 222 bp and 309 bp signals. Based on these assignments the potential common endophytic bacteria identified in the sorghum root are presented in Table 3.6.

Table 3.6 Potential common endophytic bacteria in sorghum root as determined by *in silico* assignment.

T-RFs	Accession	Predicted taxonomic assignment
197	GQ464395	Uncultured *Pseudomonas* sp. clone 2-38
222	GQ464397	Uncultured *Aminomonas* sp. clone 2-37
309	GQ456062	*Staphylococcus saprophyticus*

3.2.5.3 Shoot microbial communities

As observed previously with the root endophytic bacterial communities (Section 3.2.5.2), different extraction approaches revealed different bacterial community data (Figure 3.11). The replicate metagenomic DNA samples FS1 and FS2, Limp1 and Limp2, NW1 and NW2 clustered together, again indicating that T-RFLP analysis is a reproducible molecular tool to study sorghum microbial community diversity. Although clustering is observed from some samples, the communities identified in root tissue using T-RFLP also seem to be dependent

on the extraction method used. To conclude, only the Limpopo and North West shoot endophytic bacterial communities of sorghum were similar.

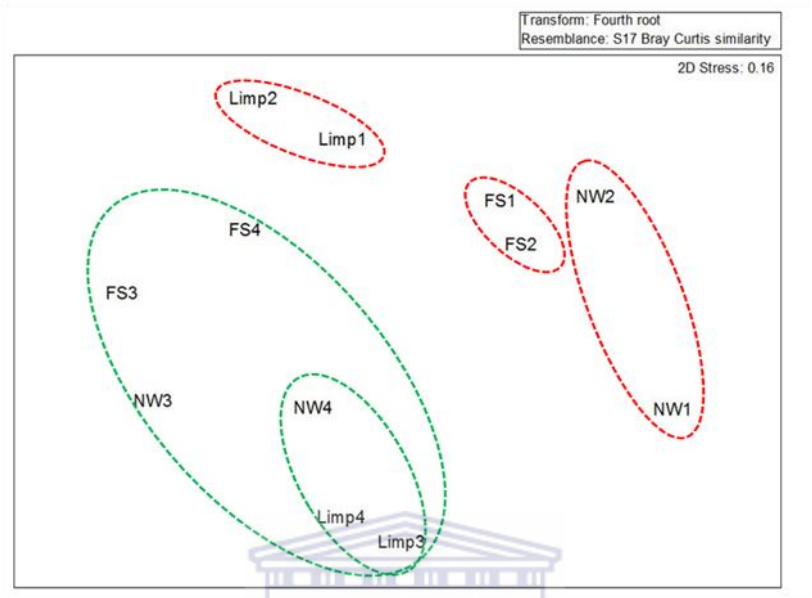


Figure 3.11 MDS ordinations showing the relationship between endophytic bacterial communities of the shoot samples. 1, 2: sorghum metagenomic DNA. 3: genomic DNA of the total fast growing (TSA) culturable microbial communities. 4: genomic DNA of the total slow growing (R2A) culturable microbial communities. FS: Free State. Limp: Limpopo. NW: North West.

Table 3.7 Summary of the total number and common T-RFs in the shoot samples.

Total (meta) genomic DNA	Location	Total no of T-RFs	Size of the common T-RFs
Sorghum metagenomic DNA	Free State	25	38
	Limpopo	9	
	North West	17	
Genomic DNA of the total fast growing (TSA) culturable microbial communities	Free State	15	250
	Limpopo	37	
	North West	15	
Genomic DNA of the total slow growing (R2A) culturable microbial communities	Free State	10	309
	Limpopo	45	
	North West	41	

Common T-RFs were observed in the sorghum shoot endophytic T-RFLP profiles: 38 bp, 250 bp and 309 bp. *In silico* putative assignment were performed for the 38 bp, 250 bp and 309 bp signals. Based on these assignments the potential common endophytic bacteria identified in sorghum shoot are presented in Table 3.8.

Table 3.8 Potential common endophytic bacteria in sorghum shoot as determined by *in silico* assignment.

T-RFs	Accession	Predicted taxonomic assignment
38	EU545402	*Klebsiella* sp. XW 721
250	GQ464393	*Enterobacter sp.* XW 122
309	GQ4456062	Uncultured *Psychrobacter* sp. clone 2-7 Subsp

3.2.5.4 Stem microbial communities

The endophytic bacterial communities of the sorghum stem samples were analysed as described for the root and shoot samples. The dispersed positioning of the samples in the MDS plot revealed the dissimilarity of the stem endophytic bacterial communities among the stem samples (Figure 3.12). The replicate metagenomic DNA samples (FS1 and FS2, Limp1and Limp2, NW1 and NW2) clustered together as observed previously with the root and shoot samples. These results reconfirmed the reproducibility of T-RFLP analysis and validated its use as a molecular tool to study sorghum microbial community diversity. Samples NW3 and NW4 clustered together, while samples FS3 and FS4 and Limp3 and Limp4 generated a separate cluster (Figure 3.12). This data suggests that the endophytic communities of the sorghum stem samples from the Free State and Limpopo provinces were similar, but distinct from those derived from the North West province samples.

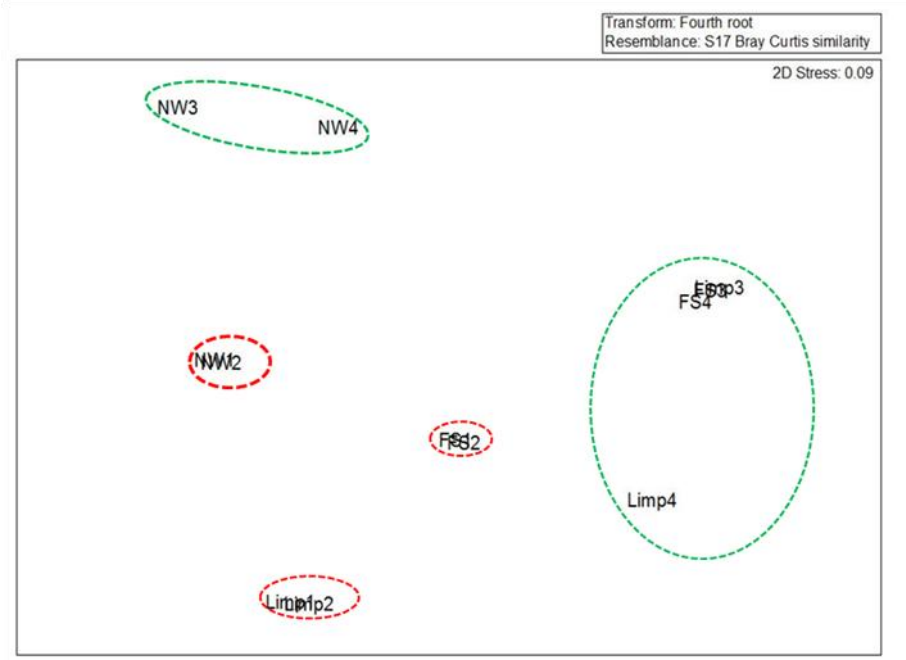


Figure 3.12 MDS ordinations showing the relationship between endophytic bacterial communities of the stem samples. 1, 2: sorghum metagenomic DNA. 3: genomic DNA of the total fast growing (TSA) culturable microbial communities. 4: genomic DNA of the total slow growing (R2A) culturable microbial communities. FS: Free State. Limp: Limpopo. NW: North West.

Table 3.9 Summary of the total number and common T-RFs in the stem samples.

Total (meta) genomic DNA	Location	Total no of T-RFs	Size of the common T-RFs
Sorghum metagenomic DNA	Free State	22	0
	Limpopo	9	
	North West	10	
Genomic DNA of the total fast growing (TSA) culturable microbial communities	Free State	12	0
	Limpopo	10	
	North West	15	
Genomic DNA of the total slow growing (R2A) culturable microbial communities	Free State	10	0
	Limpopo	13	
	North West	16	

No common T-RFs were observed in the sorghum stem endophytic T-RFLP profiles.

3.2.5.5 Conclusion

Little similarity among the endophytic bacterial communities of the different plant organs (roots, shoots and stems) from the three South African provinces was observed. However, three common T-RFs were found in the root samples, one from the uncultured and two from the cultured approaches. The common T-RFs were putatively assigned to *Pseudomonas* sp., *Aminomonas* sp. and *Staphylococcus saprophyticus***.** Three common T-RFs were found in the shoot samples, one from the uncultured and two from the cultured approaches. They could be assigned putatively to *Klebsiella* sp., *Enterobacter* sp. and *Psychrobacter* sp. No common T-RFs were found in the stem samples. Organisms from these genera are well known plant growth-promoting endophytic bacteria. *Klebsiella* sp. and *Pseudomonas* sp. have previously been described as potential plant growth-promoting bacteria (Lugtengberg *et al*., 1991; Elbeltagy *et al*., 2000). *Staphylococcus* sp. have found as endophytes in carrots (Surette *et al*., 2003). *Enterobacter asburiae* has been found inside the stem of sweet potatoes although a plant growth-promoting function has not been demonstrated (Asis and Adachi, 2003).

3.2.6 Isolation of common sorghum-associated endophytic bacteria

Using molecular biology tools (DGGE/T-RFLP), we were able to identify potential endophytic bacteria present in sorghum plant organs (roots and shoots only) cultivated in various South African provinces. Thus, the next step of this study was to attempt the isolation of these organisms using selective media or semi-selective media (Table 3.10).

A selective medium for the isolation of cyanobacteria (nitrogen free medium BG-11) was used for the isolation of *Synechococcus* sp., which have been found in the roots of South African sorghum (Porta *et al*., 2003). LGI-P medium was used to isolate *Pantoea* sp. (Loiret *et al*., 2004), and NG medium to isolate *Erwinia* sp. (Lee and Yu, 2006). As most of the

endophytic bacteria identified in this study have been reported to have a nitrogen fixing activity, a nitrogen deficient medium was used. Also, as endophytic *Pseudomonas* species have been reported to be potential plant hormone producers (Lugtenberg *et al*., 1991), a *Pseudomonas* selective medium was used.

Table 3.10 Endophytic bacteria isolated from root and shoot samples of sorghum plants (Free State, Limpopo and North West) on different selective media.

Plant organ	Selective media	Potential common endophytic bacteria isolated
Roots	Nitrogen free medium (BG-11)	60
Shoots	*Pseudomonas* selective agar medium	90
	Nitrogen deficient medium	75
	NG medium	30
	LGI-P medium	30

A total of 225 endophytic bacteria were isolated (Table 3.10). ARDRA was used to segregate these isolates and to identify those common to all the province samples (Rodas *et al*., 2003). Only bacteria displaying the same ARDRA patterns with two different restriction endonucleases (*Hae*III, *Alu*I) were further identified by sequencing. For example, the isolated bacteria m2, m4, n5, n7, and p8 showed similar ARDRA patterns with *Hae*III and *Alu*I restriction endonuclease digestion and thus their 16S rRNA genes were sequenced for further identification (Figures 3.13 and 3.14).

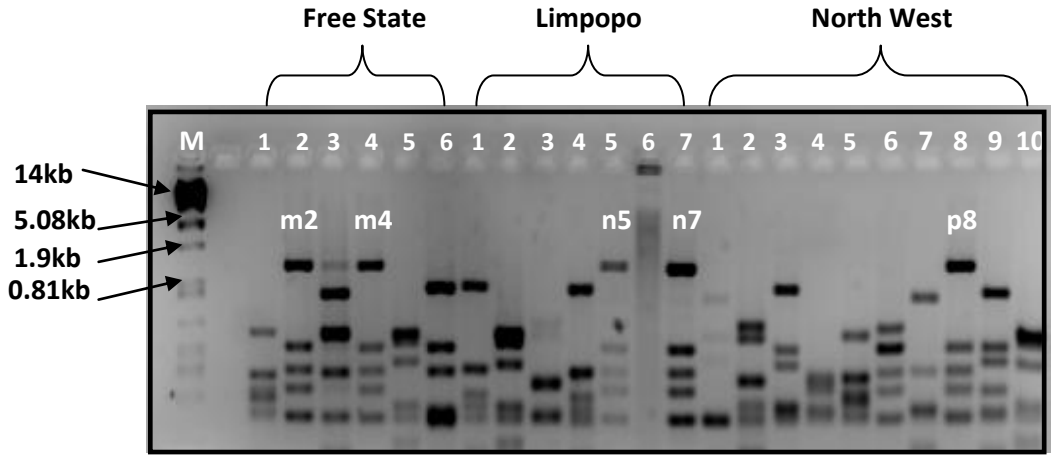


Figure 3.13 *Hae*III ARDRA analysis of 16S rRNA PCR gene amplicons of selected bacteria isolated on nitrogen deficient agar. A total of 75 isolates were obtained on the medium: results for 23 are presented.

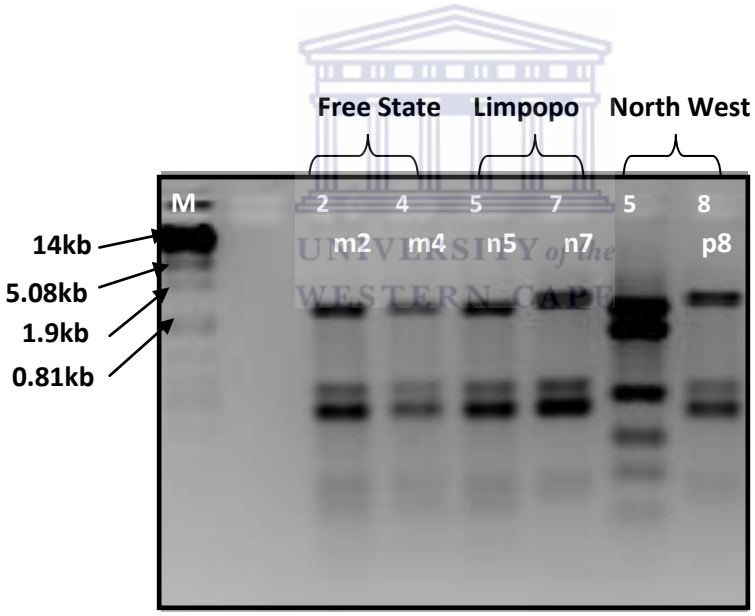


Figure 3.14 *Alu*I ARDRA analysis of 16S rRNA PCR gene amplicons of selected bacteria (m2, m4, n5, n7, and p8) isolated on nitrogen deficient agar.

The procedure to identify common endophytic cyanobacteria was complicated due to the long time (4 weeks) required to culture cyanobacteria (Jezberova and Komarkova, 2007). Thus 16S rRNA amplicons of mixed colonies were cloned (Figure 3.15). Clones containing the

appropriately sized insert were further analyzed by ARDRA (Figure 3.16) and sequenced (Table 3.11).

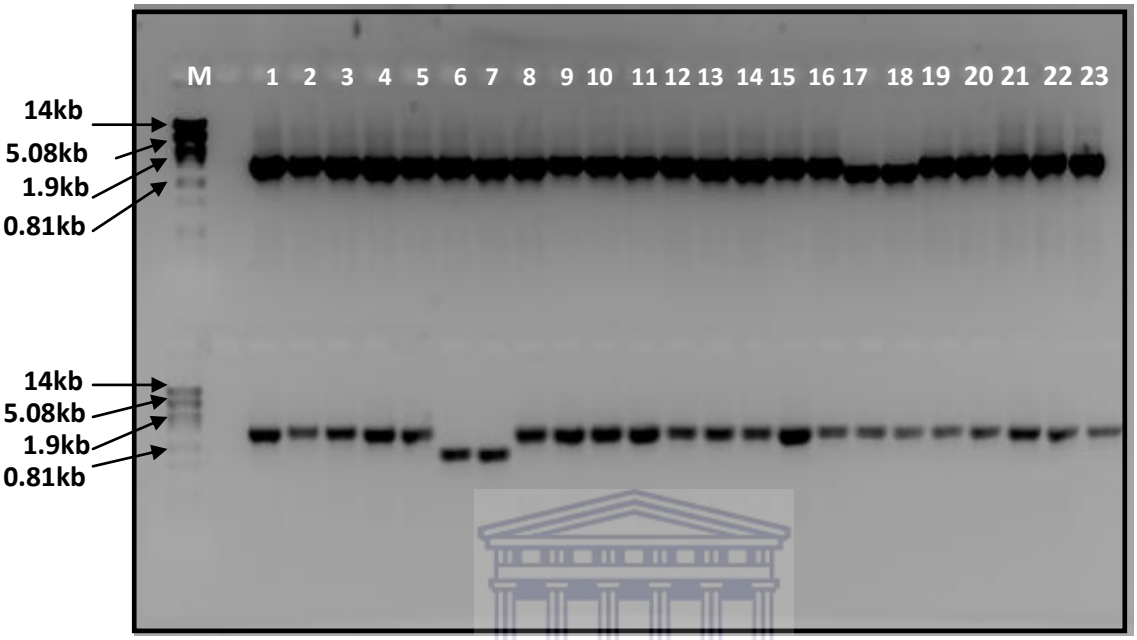


Figure 3.15 16S rRNA gene colony PCR of the cyanobacterial mixed-culture clones.

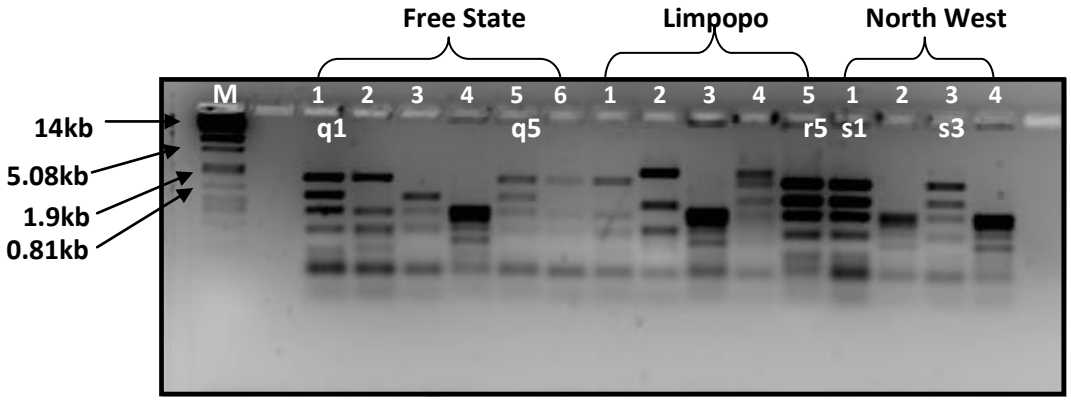


Figure 3.16 ARDRA analysis of 16S rRNA PCR gene amplicons obtained from the cyanobacterial mixed-culture clones.

Table 3.11 BLAST analysis of bacterial 16S rRNA gene fragments recovered from the isolated bacteria.

Selective media	Plant organ	Province	Accession	Description	Isolation source	E-value	Max-ident (no.of bp)
BG-11	Roots	FS	FJ812377	*Pedobacter* sp. AR-138	Forest soil	0	97% (701 bp)
		FS	DQ984206	*Stenotrophomonas* sp. VA-15	Soil	0	96% (810 bp)
		Limp	FJ812377	*Pedobacter* sp. AR-138	Forest soil	0	96% (703 bp)
		NW	DQ984206	*Stenotrophomonas* sp. VA-15	Soil	0	96% (603bp)
P S B	Shoots	FS	GU325690	*Pseudomnas* sp. DQ-01	China	0	99% (820bp)
		Limp	JN082269	*Pseudomnas hibiscicola* strain cp17	Magnetite mine drainage	0	92% (900bp)
		NW	FJ233849	*Pseudomonas* sp.SC-NO5O	Plant tissue and rhizosphere Soils of sugarcane	0	98% (801)
NDMSA	Shoots	FS	EU593589	*Nocardia fluminea* strain 173590	Xinjiang, China	0	99% (900bp)
		Limp	EU593589	*Nocardia fluminea* strain 173590	Xinjiang, China	0	98% (991bp)
		NW	EU593589	*Nocardia fluminea* strain 173590	Xinjiang, China	0	99% (1043bp)
NG	Shoots	FS	HM104658	*Bacillus cereus* strain 84-5	Soil	0	99% (899bp)
		Limp	HM104658	*Bacillus cereus* strain 84-5	Soil	0	98% (938bp)
		NW	HM104658	*Bacillus cereus* strain 84-5	Soil	0	98% (966bp)
LGI-P	Shoots	FS	HQ202813	*Microbacterium oxydans* strain OL-5	Soil	0	98% (991bp)
		Limp	HQ202813	*Microbacterium oxydans* strain OL-5	Soil	0	99% (708 bp)
		NW	HM222654	*Microbacterium* sp. 0702P1-2	Deep-sea sediment	0	99% (603 bp)

NFM: Nitrogen free medium, PSB: *Pseudomonas* selective medium,
NDMSA: nitrogen deficient medium salt agar, NG: Nutrient agar plus glycerol, LGI-P agar FS: Free State, Limp: Limpopo, NW: North West.

BG-11 medium was used to target common nitrogen fixing endophytic cyanobacteria such as *Synechococcus* sp. We were unsuccessful in isolating *Synechococcus* but *Pedobacter* sp. were isolated from Free State and Limpopo samples, while *Stenotrophomonas* sp were isolated from the Free State and North West samples (Table 3.11). *Pedobacter* sp. have previously been described in potato roots (*Solanum tuberosum*) but no potential plant growth-promoting action has been reported (Manter *et al*., 2010). Endophytic *Stenotrophomonas* sp. have been observed on surface-sterilized roots of *Lasiurus sindicus* (a drought-tolerant perennial grass), and appear to be a potential nitrogen fixer (Chowdhury *et al*., 2007).

No common endophytic *Pseudomonas* sp. were isolated, but different strains were isolated from the sorghum shoots samples of the three provinces (Table 3.11). *Pseudomonas aeruginosa* has been shown to produce a plant hormone (Guineth *et al*., 2000).

On NDMSA medium, common endophytic bacteria showing 99 % sequence similarity with *Nocardia fluiminea* strain 173590 were isolated from sorghum shoot samples from the three South African provinces (Table 3.11). Endophytic *Nocardia* species have previously been isolated in citrus plants but are not known to play a role as plant growth-promoters (Araujo *et al*., 2002).

On NG medium, the targeted endophytic bacteria were *Pantoea* species. No *Pantoea* isolates but were recovered but *Bacillus cereus*-like strains were recovered (Table 3.11). *B. cereus* has been characterized as a plant growth-promoting endophyte in the pneumatophores of *Avicensia marina* and this species acts as a biofertilizer in other plant species (Janarthine *et al*., 2010).

On LGI-P medium the targeted endophyte was *Erwinia* sp., but only *Microbacterium* species were isolated on this medium (Table 3.11). These endophytic bacteria have been isolated from tomato tissues (Rashid *et al*., 2011) where their growth promoting activities were characterized. *Microbacterium* species produce phytohormones (auxins) which are responsible for root elongation of canola (*Brassica rapa*) (Rashid *et al*., 2011).

Figure 3.17 shows the phylogenetic tree of the 16S rRNA sequences of the bacteria isolated on the different selective media. Sequences of known 16S rRNA species retrieved from the Genbank database were added to the tree in order to allocate the experimental clusters to specific taxonomic groups. The archaea *Ignicoccus pacificus* was used as outgroup. The sequences of the isolated bacteria from PSB NW and BG-11 FS, with bootstrap values of 98 %, were closely related to *Pseudomonas* sp. and *Stenotrophomonas* sp. The sequence of the bacterium isolated from PBS Limp, with a bootstrap value of 91 %, was closely related to the sequence of *Pseudomonas hibiscicola*. The sequence of the bacterium isolated from PBS FS, with bootstrap value of 100 %, was related to the sequence of *Pseudomonas* sp. The sequence of the bacterium isolated from BG-11 Limp, with a bootstrap value of 100 % was related to the sequence of *Pedobacter* sp.

The sequences of the isolated bacteria from NG (Free State, Limpopo and North West), with bootstrap values of 100 %, were related to the sequence of *B. cereus*. The sequences of the isolated bacteria from NDMSA (Free State, Limpopo and North West), with bootstrap values of 99 %, were related to the sequence of *Nocardia fluiminea*. The sequences of the isolated bacteria from LGI FS and LGI NW, with bootstrap values of 98 %, were related to the sequences of *Microbacterium oxydans* and *Microbacterium* sp.

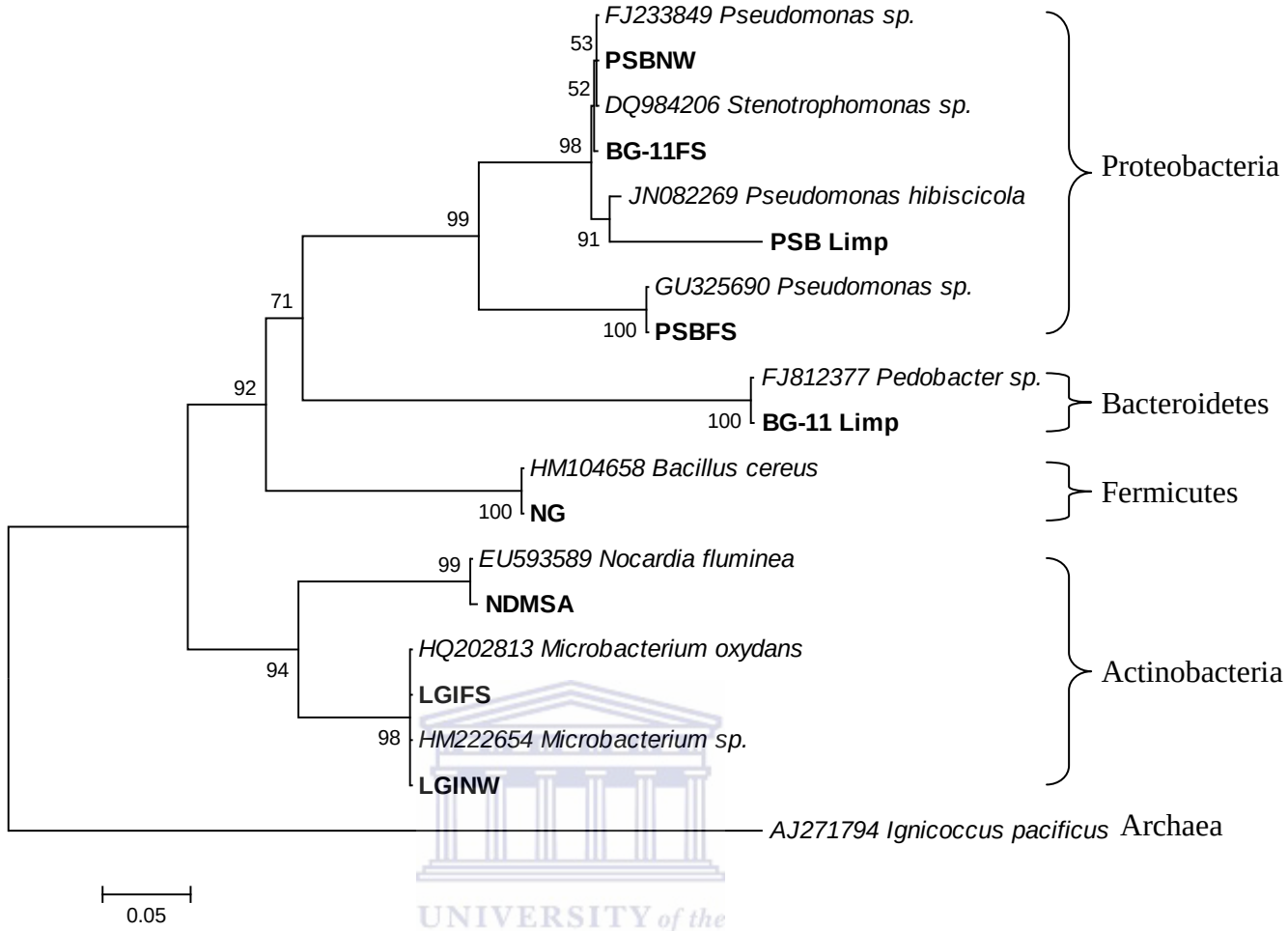


Figure 3.17 Phylogenetic tree of partial 16S rRNA gene sequences obtained from the isolated bacteria constructed with MEGA 5.05 software. The sequences obtained in the present study are shown in bold letters.

3.2.7 Conclusion

Isolation of the common endophytic bacteria identified previously in the roots and shoots of sorghum through DGGE/T-RFLP analysis using a variety of selective media was not achieved. Nevertheless three potential common endophytic bacteria (*Nocardia fluminea*, *B. cereus* and *Microbacterium* sp.) were isolated using three different selective media.

CHAPTER FOUR

General discussion

Sorghum plants (*Sorghum bicolour* L.) are native African cereals that are well adapted to grow in African countries with semi-arid and sub-tropical agronomic conditions. Africa remains the largest area where sorghum plants are cultivated with 55 % of the world's cultivation occurring in Africa (Belton and Taylor, 2004). Sorghum is used as a source of dietary carbohydrate and is consumed by 1 billion people in the semi-arid tropical regions of Africa (Belton and Taylor, 2004). In South Africa sorghum is economically valuable as it is used as a food crop for humans and for animal fodder (Taylor, 2003).

Microorganisms are present in the rhizosphere and endosphere of all plants (Loiret *et al*., 2004). The subsets of "useful" bacteria are termed plant growth-promoting bacteria. PGPBs contribute to the well-being of plants in a number of ways: they may produce fertilizers (such as assimilable forms of nitrogen) (Vessey, 2006), inhibit the growth of plant pathogenic bacteria (Bais *et al*., 2004) and/or produce plant hormones (Lugtenberg *et al*., 1991). The endophytic bacteria found within plant tissues also contribute to plant growth-promotion (Germaine *et al*., 2006).

Plant growth promoting endophytic bacteria are microorganisms that colonize the internal part of the plants without harming their hosts (Azevedo *et al*., 2000). They originate essentially from the rhizosphere but may also be transmitted by the seed or may enter through the arial parts of the plant (Bressan *et al*., 2004). Studies of endophytic bacteria have mainly focused on cultivation based methods (Bell *et al*., 1995; Sturz *et al*., 1998). These methods have yielded useful information but provide information only on those microorganisms that are able to grow on growth medium, which are estimated to represent 0.1 to 1 % of all existing microorganisms (Torsvik *et al*., 1998). However, culturing is still valuable, enabling the isolation of pure colonies. To circumvent the selectivity resulting from cultivation

conditions, culture-independent methods have been developed providing the possibility of studying entire microbial communities (Rintala *et al*., 2001). Thus, these two approaches were considered in this study.

The goal of the present research was to characterize the endophytic bacterial communities associated with South African sorghum plants, and to identify whether key endophytic PGPBs are always associated with South African sorghum plants. Thus, healthy sorghum plant samples were harvested from three South African provinces (namely the Free State, Limpopo and North West provinces) where different agricultural practices, such as the use of organic (cow feces) or synthetic fertilizers are used. Organic fertilizers are derived from animal and plant sources and microorganisms are required to assimilate these, resulting in the release of nutrients benefiting plant growth and for structuring the soil texture. Synthetic fertilizers are ready to be released, therefore work faster and can easily be over applied and "burn" roots by creating toxic concentration of salts. It was previously demonstrated that the intensive use of the synthetic fertilizers substantially changes the composition of the soil microbial community and therefore will also affect the endophytic bacterial communities (Marschner *et al*., 2003). Geographical distance, climate and the different agricultural practices employed can lead to differences in soil composition. These factors play an important role in determining the structure and composition of microbial communities present in soil (Girvan *et al*., 2003) and therefore in the communities present in the endosphere (Seghers *et al*., 2004).

A chemical analysis of the rhizospheric and bulk soil from the three provinces was undertaken. The results showed dissimilarities in soil characteristics among the three South African provinces, and this may be influenced by the different agricultural practices, which

likely impact the sorghum endophytic bacterial communities. For example, the low pH in the Free State soils (pH 4.2) might select for the growth of acidic rhizobacteria, whereas slightly more neutrophilic organisms would be promoted in the Limpopo (pH 6.3) and North West (pH 6) soils. As the endophytic bacteria originate essentially from the rhizosphere (Bressan *et al*., 2004), it could be hypothesised that sorghum grown in the low pH soils of the Free State would harbour more acid tolerant endophytic bacteria than the sorghum plants grown in Limpopo and North West soils.

Where synthetic fertilizers are used there is a decrease in the nitrate in the rhizosphere soils compared to that in the bulk soil. Where organic fertilizer is used the above is not observed but there is a decrease in the ammonium in the rhizosphere (Table 3.1). This could be an indicator that sorghum plants and microorganisms absorbed more nitrogen in the form of ammonium than in form of nitrate. If this is the case, it could be expected that sorghum plants from the Free State and Limpopo select for organisms exhibiting nitrogen-fixing activity such as *Pantoea* sp (Loiret *et al*., 2004), compared to the sorghum plants growing in the North West. Since most of the endophytic bacteria from the root and shoot samples identified in this study had been reported to be potential plant growth-promoting endophytic bacteria with nitrogen-fixing activity, there would be a possible correlation between soil nitrogen contents and South African sorghum endophytic bacterial communities.

The validity of this study largely depends on the extraction of endophytic metagenomic DNA from sorghum plant organs and the genomic DNA of the total culturable microbial communities. There are difficulties associated with the extraction of DNA from plant tissue which include the compactness of the cell wall (composed of large amounts of complex carbohydrates) (Hattori *et al*., 1987) and the contamination of the extracted DNA by

polysaccharide which severely reduces the quality of DNA extracted from plant tissues (Demeke and Adams, 1992). In this study a modified version of the Miller *et al*. (1999) extraction procedure was developed to extract metagenomic DNA from sorghum. The method produced high yields of DNA suitable for downstream applications (section 3.3.2). In order to investigate the sorghum endophytic bacterial diversity, the DNA extracted from sorghum plant organs (root, shoot, stem) harvested in three South African provinces and from the total culturable bacterial communities was used for PCR amplification of a fragment of the 16S rRNA gene, the commonly used bacterial phylogenetic marker used to study environmental microbial communities (Malik *et al*., 2008).

DGGE and T-RFLP are two molecular techniques used to study the microbial diversity in the environments (Malik *et al*., 2008). DGGE can separate amplified DNA of the same length with one base-pair difference (Miller *et al*., 1999) therefore, in theory, it can be used in the identification of common species in environments. T-RFLP analysis is based on the restriction endonuclease digestion of fluorescently end labelled PCR products such as the 16S rRNA gene. These molecular fingerprinting techniques are still some of the best techniques to easily explore the dynamic diversity of soil bacterial communities. If deep detailed phylogenetic information is needed, DGGE provides the advantage that individual bands can be excised, cloned and sequenced (Sekiguchi *et al*., 2002), although it possesses limitations such as co-migration of bands with similar sequence composition (Gafan and Spratt, 2005). Despite the high resolution and sensitivity, T-RFLP has limitations that must be taken into consideration such as the use of fluorescently labelled primers that limits the analysis to only the terminal fragment of the digestion (Marsh, 1999). Different species might have the same T-RF, thus one T-RF may represent multiple related species, resulting in a lower estimation of the microbial community in the given environment. Both techniques rely on PCR

amplification (in this case of the 16S rRNA gene) which is dependant on/or affected by DNA extraction method, PCR biasis and the choice of universal primers (Kirk *et al*., 2004).

Plant associated ecosystems, i.e. the rhizosphere and the endosphere, are environments affected by factors such as agricultural practices, over-use of pesticides and synthetic fertilizers (Girvan *et al*., 2003; Seghers *et al*., 2004). Previous studies on plant/endophytic bacterial community interactions have shown that the endophytic bacterial diversity decreased from the roots to the stems (Fisher *et al*., 1992; Quadt-Hallman *et al*., 1997) and is affected by interactions (or associations) with other endophytes such as fungi or plant pathogenic bacteria (Quadt-Hallman *et al*., 1997). In this study, using DGGE, the sorghum endophytic bacterial diversity in the shoot was richer in terms of number of species than the one in its stems. These results were similar to previous findings that showed that lower plant parts (such as roots) possessed more diverse endophytic bacteria than higher plant parts (such as stems) (Aravind *et al*., 2009). The similarity among the endophytic bacterial communities of the sorghum plant organs from the three South African provinces was high. These results indicated that South African sorghum plants recruit similar endophytic bacteria in each of its plant organs. This highest similarity of the endophytic bacterial communities was found in the stem (89.59 %) compared to the root (80.65 %) and the shoot (76.30 %) organs independently to the geographical locations and different agricultural practices. This highest similarity between plants isolated from different geographies might be due to the plant genotype, the type of plant tissue (Hardoim *et al*., 2008). But the interactive colonization processes, communication between the plant and bacterium (and vice versa) possess a vital role. Bacterial root colonizers usually recognize the specific compounds released by the plants from its root exudates. Plants then communicate with commensalistic, mutualistic and symbiotic via compounds exuded by their roots (Hardoim *et al*., 2008).

Contrastingly the T-RFLP results showed a low similarity of the sorghum endophytic bacterial communities. This low similarity might be due to the high sensitivity of the technique. It may also result from the fact that the diversity may be underestimated because different species may have identical restriction terminal fragments and therefore restriction fragments of the same length are generated. The use of only one restriction endonuclease (such as *Hae*III used during this analysis) can compromise the results. In order to increase the accuracy of results more than two restriction endonucleases may be used in the analysis (Tiedje *et al*., 1999).

In this study various common sorghum endophytic bacteria species were identified by the two molecular methods. From the DGGE analysis, *Synechococcus* sp., *Pantoea* sp. and *Erwinia* sp. were identified. *Synechococcus* is a cyanobacterium that has previously been reported to be associated with the rhizosphere of rice plants (Megharaj and Venkateswarly, 1989) but never as an endophyte. They have also been isolated in marine environments and they showed potential plant growth-promoting bacteria activity as they participated in nitrogen fixation (Philips *et al*., 1989). Indeed, endophytic cyanobacteria, such as *Anabaena* sp. have been found to be potential PGPBs in the stem of sweet potatoes (Terakado-Tonooka *et al*., 2007). *Pantoea* sp. was found to be a potential plant growth-promoting endophytic bacterium from its nitrogen fixating activity (Loiret *et al*., 2004). *Erwinia* sp. was found as a soybean endophyte which produces plant hormones (Kuklinsky-Sobral *et al*., 2004). From T-RFLP analysis the different common potential plant growth-promoting endophytic bacteria identified were *Pseudomonas* sp., *Aminomonas* sp., *S. saprophyticus, Klebsiella* sp., *Enterobacter* sp. and *Psychrobacter* sp. *Staphylococcus* sp. was found as an endophyte in carrots (Surette *et al*., 2003). *Klebsiella* sp. have been found inside growing shoot tips of banana plants (*Musa* sp.) and have been reported to be potential PGPBs for their biocontrol

activity (Piuos and Soly, 2009). Using the two molecular techniques different common endophytic bacteria species were identified. This indicates that both molecular techniques are important tools which may be used to gain an understanding of the microbial diversity present in the environment.

Considering some of the common organisms were identified using enrichment cultures and since the endophytic bacterial communities identified were definitely method dependant, a more extensive culturing approach was employed to further identify other common endophytes and to attempt to isolate those identified in the culture-independent studies. However, except for *Pseudomonas* sp., none of the other bacteria identified in this study could be isolated. Similar results were obtained by Tian *et al*. (2007) who detected *Mycobacterium* sp. as endophytes in the roots of rice plants using a molecular approach. This suggests that the media used in this study were not sufficiently selective to specifically target these endophytic bacteria. *Nocardia fluiminea* was a common endophytic bacterium isolated from sorghum cultivated in the three provinces studied. These bacteria have previously been isolated from citrus trees, but the plant growth-promoting activity of these endophytes is still unknown (Araujo *et al*., 2002). *Pedobacter* sp. AR-138 was isolated from sorghum tissue in both the Free State and the Limpopo provinces. *Pedobacter* sp. had been found to be an endophyte living within the roots of potato (*S. tuberosum*) (Manter *et al*., 2010). *Stenotrophomonas* sp., which was common to the Free State and the North West samples has been found to be a potential plant growth-promoting endophyte (Chowdhury *et al*., 2007). *Microbacterium* sp. was found as a common endophytic bacterium in the three provinces. *Microbacterium* sp. has been found to be a potential plant growth-promoting endophyte bacterium as it has the ability to express plant hormones (auxins) (Rashid *et al*., 2011). *B. cereus* was also found to be common to the Free State, Limpopo and North West samples.

The association of *B. cereus* with the pneumatophores of *Avicensia marina* was reported to lead to root elongation (Janarthine *et al*., 2010). Previous studies have reported that agricultural practices and soil types have an impact on microbial communities (Girvan *et al*., 2003; Marschner *et al*., 2003). The common endophytic bacterial species isolated from the healthy sorghum plants farmed in the three South African provinces where different agricultural practices are used can be seen to be a selective choice made by the sorghum plants and may contribute to plant growth and well being.

After investigating the activity (whether it be as a biofertilizer, a biocontrol agent or a plant hormone producer) and effect on the plant growth, it would be possible to genetically engineer selected endophytic bacteria for the systematic delivery of antibiotics, biofertilizers and plant hormone and/or biopesticides to the tissues of the host plant without genetic manipulation of the host genome. This action would be an excellent vehicle to enhance the yield of the sorghum plants despite the ethics of the use of genetically engineering organisms in agriculture.

Conclusions and perspectives

In summary, the main aim of this study, which was the isolation of the common endophytic bacteria among the sorghum plants farmed in three South African provinces using different agricultural practices, has been successfully achieved. Endophytic bacterial communities were found to be different in the three South African provinces. The use of different agricultural practices which is always seen as a factor that affects bacterial diversity (Girvan *et al*., 2003; Marschner *et al*., 2003) has been found to have an effect on the endophytic bacterial communities of South African sorghum plants based on DGGE and T-RFLP analysis of samples. DGGE and T-RFLP analysis revealed that the sorghum bacterial communities from the three provinces shared some similarities. However, they were certainly not homogenous. As most of the common endophytic bacteria identified in this study have been reported to be PGPBs with nitrogen fixing potential, these results can lead to the development of a hypothesis for the studies of the interactions between the endophytic bacterial communities and South African sorghum plants. In light of this, it would be interesting to focus directly on the analysis of the nature of the isolated common endophytic bacteria and the mechanisms involved in the plant growth process.

Congress and Contibutions

- Biotechnology open day. 02 September 2011. Oral presentation.
  Characterisation of the endophytic bacterial communities associated with South African sorghum plants: looking for potential plant growth-promoting endophytes.
- SASM/SAVM (South African Society for Microbiology). 6-9 November 2011. Oral presentation. Characterisation of sorghum-associated microbial communities in South Africa.

Appendices

Appendix 1: General laboratory chemicals and reagents

Table 1: Chemicals reagents used in this study.

Chemicals	Suppliers
Actidione (Cyclohexamide)	Fluka, Germany
Agar	Merck, Darmstadt, Germany
Agarose	Bioline, England
Ampicillin	Fluka, Germany
Ammonium sulfate	Merck, Darmstadt, Germany
Ammonium acetate	Sigma Aldrich, Deisenhofen, Germany
Ammonium peroxodisulfate (APS)	Bio Rad, München, Germany
Chloroform	Sigma Aldrich, Deisenhofen, Germany
EDTA disodium salt	Merck, Darmstadt, Germany
Ethanol (70%)	Kimix, South Africa
Ethidium bromide	Sigma Aldrich, Deisenhofen, Germany
Formamide	Merck, Darmstadt, Germany
Isoamyl alcohol	Merck, Darmstadt, Germany
Isopropanol	Kimix, South Africa
IPTG	Fermentas, Vilnius, Lithuania
Magnesium chloride	Saarchem, South Africa
Methanol	Kimix, South Africa
Na-salicylate	Fluka, Germany
Na-nitroprusside	Fluka, Germany
Na-dichloroisocyanurate	Fluka, Germany
Orange G loading buffer	Promega, Madison, Wis. USA
Polyvinylpolypyrrolidone (PVPP)	Sigma Aldrich, Deisenhofen, Germany
Potassium chloride	Merck, Darmstadt, Germany
X-Gal	Fermentas, Vilnius, Lithuania
Sodium phosphate	Fluka, Germany
Sodium hydroxide	Saarchem, South Africa
Sodium chloride	Kimix. South Africa

Sodium chloride	Kimix, South Africa
Tris (hydroxymethyl) aminomethane	Merck, Darmstadt, Germany
Tryptone	Fluka, Germany
TEMED (N,N,N,N Tetramethyllethylene diamine)	Sigma Aldrich, Deisenhofen, Germany
Triton X-100	BDH, England
Yeast extract	Merck, Darmstadt, Germany
Enzymes	
Taq DNA polymerase	Fermentas, Vilnius, Lithuania
*Hae*III	Fermentas, Vilnius, Lithuania
*Alu*I	Fermentas, Vilnius, Lithuania
λ*Pst*I	IMBM Laboratory
Kits	
QIAquick® gel extraction kit	Qiagen Hilden, Germany
TA cloning kit	Fermentas, Vilnius, Lithuania
GFX^{TM}	GE Healthcare, UK

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