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Identification and Molecular Characterization of the Bacterial Community Structure in Mafikeng Soils

By

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ABSTRACT

Soil is a complex environment and a hotspot of microbial diversity with millions of different bacterial species in a 1-g sample. In this study, bacteria from rhizosphere soil in Mafikeng were isolated and characterized using morphological, physiological, biochemical as well as culture-dependent and culture-independent molecular techniques. The soil revealed an alkaline pH range (7.54-9.8) and most of the soil samples were rich in microbial loads 1.5×10^6 (cabbage and spinach soil) to 3×10^6 cfu/ml (maize1 soil). The various elements in the soil samples include carbon (3.8-18.04 $mg^{-1}kg$), iron (3.1-72.44 $mg^{-1}kg$), chromium (0.01-06 $mg^{-1}kg$), magnesium (4.5-2.98 $mg^{-1}kg$), cadmium (0.001-0.002 $mg^{-1}kg$), zinc (0.22-44 $mg^{-1}kg$) and phosphorus (0.3-1.22 $mg^{-1}kg$). Soil samples from the maize fields showed no phosphorus. Calcium was more pronounced from cabbage soil sample. The bioavailability of cobalt (0.01-0.03 $mg^{-1}kg$), sulphur (12-17 $mg^{-1}kg$), nitrogen (32-100 $mg^{-1}kg$), potassium (1.7-17 $mg^{-1}kg$), manganese (1 $mg^{-1}kg$) and copper (0.03-0.4 $mg^{-1}kg$), were noted. Green peas, maize 2, onion and lettuce soil samples indicated no copper detection. The 16S rRNA gene products were amplified using universal primers which resulted in approximately 1500 bp DNA segments by PCR. The partially sequenced amplicons were used in reconstructing phylogenies. The majority of the 16S rRNA showed close similarity to those of *Bacillus* (11 species), and one each to *Paenibacillus* sp., *Ensifer adhaerens*, *Aquamicrobium* sp., *Lactobacillus* sp., *Alcaligenes* sp., *Brevibacillus* sp. *Sinorhizobium* sp., *Pseudaminobacter* sp. and *Proteus vulgaris*. This analysis revealed that *Bacillus* sp. were the dominant population in all the rhizosphere soil samples collected from Mafikeng. The 16S rRNA from sequences obtained in this study have been submitted to the GenBank database and assigned accession numbers. Bacterial community structure was studied in nine rhizosphere soil samples representing varying crop rhizosphere, total community DNA was extracted and purified by a direct method. A variable region of the 16S rRNA gene was then amplified by PCR with

bacterial primers, resulting in a mixture of amplicons separable via denaturing gradient gel electrophoresis (DGGE). The DGGE profiles of soil were indicative of dominant soil bacterial types. Since rhizosphere associated bacteria play a crucial role in plant health, knowledge of their community structure is imperative for the proper understanding of their individual roles. Metagenomics holds the promise to reveal several important questions regarding the unculturable fraction of the rhizosphere community. DGGE analysis of the metagenomic soil DNA revealed some percentage identity with uncultured *Bacillus* sp., Bacterium *Rubrobacter* sp, Rhizobiales bacterium, Soil bacterium as well as *B. megaterium* and *Cohnella* sp. The potential of the bacteria to function as plant growth-promoting bacteria (PGPR) was examined *in vitro*. All the 29 bacterial isolates tested were found to produce ammonia, while several (38%) produced indole acetic acid (IAA) and hydrogen cyanide (HCN). Forty eight percent of the isolates were capable of phosphate solubilisation. Twenty-one percent (21%) also exhibited antifungal activity against test pathogen *Fusarium solani*. All the HCN- producing bacteria belong to the genus *Bacillus*. *B. amyloliquefaciens* indicated high cyanogenic potential compared to other strains. Four bacterial inoculants (*B. pumilus*, *B. amyloliquefaciens*, *L. xylanilyticus* and *Bacillus* sp) that exhibited more *in vitro* PGPR traits were selected for use in screenhouse studies of plant growth-promotion in tomato and spinach. The treatment of both crops with the bacterial inoculants promoted plant growth in terms of increased shoot length at $P<0.05$. *Bacillus amyloliquefaciens* MR16 had significantly higher growth at $P<0.05$ compared to other treatments.

DECLARATION

I, Kedibone Masenya declare herewith that the dissertation entitled Identification and Molecular Characterization of the Bacterial Community Structure of Mafikeng Soil which I herewith submit to the North-West University upon completion of the requirements set for the degree of Master of Science degree, is my own work and has not been submitted to any other university

Signed at MAFIKENG this 25 day of SEPT 2013

Declared before me on this 25 day of SEPT 2013

KEDIBONE MASENYA

DEDICATION

I dedicate this thesis

To my mom, Ms Rebecca Masenya

For her steadfast support, love, advice and encouragement which have been invaluable to me.

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LIST OF ABREVIATIONS

ACC –Amino cyclopropane carboxylase

CFU – Colony forming units

DGGE– Denaturing gradient gel electrophoresis

dNTP – deoxyribonucleotide triphosphates

FA – Fatty acid

$Fecl_3$ – Iron III chloride

GC – Guanine- Cytosine

H_2S – Hydrogen sulphide

HCl – Hydrochloric acid

$HClO_4$ – Perchloric acid

HCN – Hydrogen cyanide

IAA – Indole acetic acid

MR – Methyl red

N_2 – Nitrogen gas

NaOH – Sodium hydroxide

NCBI – National centre for biotechnology Information

PCR – Polymerase Chain Reaction

PLFA- Phospholipid-derived fatty acids

PGPR – Plant growth-promoting Rhizobacteria

RNA – Ribonuceic acid

rRNA – Ribosomal ribonucleic acid

TAE – Tris base, acetic acid and EDTA

DEFINITION OF CONCEPTS

Biogeography	: The study of the geographical distribution of organisms throughout the landscape.
Diversity	: The state or quality of being different or varied
Edaphic factors	: An 'ecological influences properties of the soil brought about by its physical and chemical characteristics'
Oxidizers	: Is a substance that accepts or receives electron from another substance
Phylogeny	: The evolutionary development of a species or of a taxonomic group of organisms
Primers	: Short polynucleotide chain to which new deoxyribonucleotides can be added by DNA polymerase
Rhizodeposition	: Organic substances released from living roots into the soil during plant growth
Rhizosphere	: The soil zone that surrounds and is influenced by the roots of plants

CHAPTER 1
INTRODUCTION

Microbial diversity is a vast frontier and a potential goldmine for the biotechnology industry because it offers a variety of new genes and biochemical pathways to probe for enzymes, antibiotics and other useful molecules (Gurung *et al.*, 2010). The choice of natural materials like soil in research for enzymes and biochemical pathways, is based on the assumption that samples from widely diverse locations are more likely to yield novel microorganisms and, therefore hopefully, novel metabolites as a result of geographical variation (van Elsas *et al.*, 2006). The assessment of the structure within the microbial communities is one of the fascinating aspects of microbiology and the number of prokaryotic species of microorganisms described so far is remarkably low (van Elsas *et al.*, 2006).

Many prokaryotic species of microbes cannot easily be isolated from complex environmental matrices or cannot be grown *in vitro*. To appreciate their true functional diversity and the activities they express *in situ* (in soil) in response to different environmental constraints, it is necessary to develop new experimental approaches adapted to these microorganisms. One such approach, developed in recent years, is the use of molecular techniques (Bailly *et al.*, 2007). Historically, this was achieved through cultivation and subsequent characterization of strains. Decades later, culture-independent methods have provided new tools to study the microbial world (Rappé and Giovannoni, 2003). Molecular techniques can be used to study the structure and activity of soil microbial communities.

It has been estimated that less than one percent of bacterial species are currently known (van Elsas *et al.*, 2006). van Elsas and Boersma (2010) suggest that differences in soil bacterial communities can be detected using molecular methods. For more than a century,

microbiologists have sought to determine the species' richness of bacteria in soil, but without success, as more than 99% of the bacteria do not respond to conventional culturing, and are thus unculturable (Schloss and Handelsman, 2006). In the last decade, this limitation has been partially overcome through the application of molecular ecological techniques.

Metagenomic approaches have been applied to study a range of soil environments (Demaneche *et al.*, 2009; Schloss and Handelsman, 2006). Direct amplification and analysis of 16S rRNA genes have been carried out to examine the predominant sequences in mixed PCR products amplified from environmental samples such as soil samples (Nakatsu *et al.*, 2000). Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified genes from environmental samples, is a useful tool in environmental microbiology (Muyzer, 1999). The PCR-DGGE method targeting the 16S rRNA gene is most widely applied for the studies of bacterial community structure in the environment, because this gene is essential to all living prokaryotes and helpful in tracing phylogenetic relationships. In particular, comparative analysis of the 16S rRNA genes derived from nucleic acids extracted directly from soil has revealed the presence of many new groups of bacteria that were previously undetected in cultivation studies (Sait *et al.*, 2002).

Therefore, molecular characterization of bacteria has been shown to be effective as compared to traditional cultivation techniques such as plate counting methods, which are now increasingly considered inadequate. Thus, new and more sophisticated techniques have been developed for the isolation of bacteria from complex microbial habitats (Sait *et al.*, 2002). The only avenues currently available for the study of uncultured bacteria are cultivation-independent molecular ecological techniques that have proven to be a very powerful tool for the study of bacteria in their natural settings such as soil (Sait *et al.*, 2002; Gray and Head,

2001). Thus, the use of molecular approaches for describing microbial diversity is still an important thrust of research.

Research Problem

Agricultural producers are becoming more dependent on agrochemicals as a relatively reliable method of crop protection. However, increasing use of chemical inputs causes negative effects, i.e., development of pathogen resistance to the applied agents and their nontarget environmental impacts (Gerhardson, 2002). In addition, the growing cost of chemical fertilizers, particularly in less-affluent regions of the world has led to a search for substitutes for these products. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Welbaum *et al.*, 2004; Postma *et al.*, 2003). Therefore, this study will help to clearly understand the bacterial community structure in soil, because the extent of the diversity of microorganisms in soil is seen to be of critical importance in the maintenance of soil health and quality, as a wide range of microorganisms are involved in important soil functions such as bioremediation, biodegradation, biogeochemical cycles and plant growth-promoting abilities (Maron *et al.*, 2011).

Significance of the Study

This study will help in clearly understanding the bacterial community structure in soil as the extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality. It will help in addressing the problem of Mafikeng soil health and quality, for increased crop production since a wide range of bacteria are involved in biogeochemical cycles and as plant growth promoters. Thus, this study aims at contributing to the existing knowledge on the occurrence of bacteria in soil for maintenance of soil health and quality.

Objectives of the Study

This study is designed to:

- Isolate bacteria from rhizosphere soil collected from Mafikeng.
- Identify and characterize the bacterial isolates using standard methods.
- Assess the composition of the soil bacterial community using both culture-dependent and culture-independent molecular techniques.
- Screen for *in vitro* and *in vivo* rhizosphere bacteria plant growth-promoting properties.

CHAPTER 2
LITERATURE REVIEW

2.1. Soil as a habitat for microbes

Soil is a complex environment and a dynamic biological system of microbial diversity, with several thousands of different bacterial species in one gram soil sample. The majority of bacterial species are unknown and uncultivable on standard microbiological media (Demaneche *et al.*, 2009; Fierer *et al.*, 2007; Rappé and Giovannoni, 2003). The soil microbial community is relatively diverse (Robe *et al.*, 2003; Curtis *et al.*, 2002) with arguably the highest prokaryotic diversity of any environment (Roesch *et al.*, 2007; van Elsas *et al.*, 2006). It is inhabited by many bacteria from phylogenetic groups that are globally distributed and abundant in terms of the contributions of individuals of those groups to total soil bacterial communities (Rappé and Giovannoni, 2003; Buckley and Schmidt, 2002). Bacterial communities are poorly studied because representatives are rarely isolated in cultivation studies. Part of the reasons for failure to cultivate these bacteria is the low frequency with which bacterial cells form visible colonies when inoculated on standard microbiological media, resulting in low viable counts (McCaig *et al.*, 2001). Only about 1% of bacterial cells in each gram of soil are able to form colonies on laboratory media (Fierer *et al.*, 2007; Rappé and Giovannoni, 2003). This means that many groups of soil bacteria cannot be studied due to the inability of microbiologists to grow representatives in the laboratory. Some isolates of these groups have recently been cultured by the use of new culture media, extended incubation periods to increase the numbers of colonies formed, and by the selection of isolates from plates receiving only small inocula (Joseph *et al.*, 2003; Sait *et al.*, 2002). This soil species pool represents a goldmine for genes involved in pharmaceutical and industrial applications and in the biodegradation of man-made pollutants (van Elsas *et al.*,

2006). However, cultured soil microorganisms are the most common source of antibiotics and other medicinal agents of any group of organisms (Joseph *et al.*, 2003; Janssen *et al.*, 2002; Sait *et al.*, 2002). Therefore, soil is arguably the most useful and valuable habitat on earth. It has been used by humans for planting crops, for mining minerals, for building on and for discovering medicinal chemicals (Srivastava *et al.*, 2013).

2.2. Importance of soil bacteria

Soil biodiversity performs ecosystem services beyond production of food, fibre, fuel and income. Soil organisms are assumed to be directly responsible for soil ecosystem processes and these include decomposition of soil organic matter and the cycling of nutrients that contribute to the sustainability of life on earth (Högberg *et al.*, 2002; Kowalchuk and Stephen, 2001). These processes are considered to be major components in the global cycling of materials, energy and nutrients. For example, the soil biomass (25 cm top soil layer) is known to process over 100,000 kg of fresh organic material each year per hectare in many agricultural systems (Chiurazzi, 2008). This processing includes the decomposition of dead organic matter by the microbes as well as consumption and production rates in the soil community food web. Bacteria serve useful roles in transforming organic materials, decomposing toxic wastes and protecting plant roots from attack by diseases and pests (Waldrop *et al.*, 2000). They are also involved in many important functions such as soil formation, toxin removal and elementary cycles of carbon, nitrogen and phosphorus (Waldrop *et al.*, 2000).

Bacteria maintain critical and key processes such as carbon storage, nutrient cycling, plant species diversity, soil fertility, soil erosion, nutrient uptake by plants, formation of soil organic matter, nitrogen fixation, bio-degradation of dead plant and animal materials,

reduction of hazardous waste, production of organic acids that weather rocks and control of plant and insect populations through natural bio-control (De Deyn *et al.*, 2003; Cragg and Bardgett, 2001; Wolters, 2001). These renewal processes and ecosystem services are largely biological; therefore their persistence depends upon maintenance of biological diversity (Balser *et al.*, 2002; Cavigelli and Robertson, 2001). The biotic elements (micro- and macro-life forms) within soil interact with the soil abiotic elements (chemical and physical properties) to maintain the diverse, multi-functional value of soils (Hafez and Elbestawy, 2009).

2.3. Bacteria found in soil

Examples of bacterial population types found in soil are: decomposers, nitrogen fixers, nitrifying bacteria, disease suppressors, sulphur oxidizers, aerobes and anaerobes. A number of decomposers can breakdown pesticides and pollutants in the soil. These microbes are important in retaining nutrients within the plant cell, thereby preventing the loss of nutrients such as nitrogen from the rooting zone (Eisenhauer *et al.*, 2010). The *Bacillus* sp. WD23 has been found to produce spore laccases which are active in the alkaline pH range and can be used for bioremediation or application in membrane reactions. In a study conducted by Murugesan *et al.* (2010), it was found that *P. aeruginosa*, *Bacillus species* and *Corynebacterium species* were active in utilizing cypermethrin found in pesticides, as pesticides are a problem because of toxicity and carcinogenicity.

Lithoautotrouphs or chemoautotrophs obtain the energy from compounds of nitrogen, sulphur, iron and hydrogen instead of carbon compounds and most soil bacteria fall within this group. Sulphur oxidizers include *Thiobacillus* bacteria which can convert sulfides into sulfates and form sulfur which can be utilized by plants (Berthelin, 2010). Many soil minerals

contain sulfides but this form of sulphur is largely unavailable to plants. Actinomycetes help to slowly breakdown humic acids in soil and are responsible for earthly odor of freshly plowed field. They are also able to degrade many complex substances such as cellulose and chitin (Hayakawa, 2008). They are mostly of the genera *Nocardia*, *Streptomyces* and *Micromonospora*. Blue-green algae are photosynthetic (photoautotrophs) bacteria and transform sunlight into energy and release oxygen as a by-product which can be used by obligate aerobes (Singh *et al*., 2011).

Disease suppressors such as *Bacillus megaterium* have been used on some crops to suppress the disease-causing fungus *Rhizoctonia solani* (Dorrestein, 2009). Nguyen and Ranamukhaarachchi (2010) suggested that antagonists isolated from soil *B. megaterium*, *Enterobacter cloacae*, *Pichia guillermondii* and *Candida ethanolica* indicated high potential for disease suppression and also increased fruit weight, biomass and plant height. Studies have also shown that Streptomycetes suppressed root pathogenic fungi and promoted plant growth (El-Tarabily *et al*., 2006). Streptomycetes act as direct antagonists or growth promoters of soil microorganisms due to production of secondary metabolites. They enhance plant root colonization through suppression of plant defense responses (Van Loon, 2007). In addition, plant growth and local defense responses are enhanced by unknown factors through direct root contact. The induced defenses include changes in cell wall composition and expression of defense-related genes. Pathogen resistance in distal plant parts is enhanced by Streptomycetes, and this is accompanied by strain-specific changes in plant gene expression levels (Schrey and Tarkka, 2008). Soil solarisation, alone or in combination with other disease management practices, has been shown to be effective in reducing the inoculum density of many soil-borne disease causing organisms (Berg and Smalla, 2009).

The soil-borne potato pathogen, *R. solani* AG3, is of great distubance in the production of potatoes and other cash crops such as sugar beets. Natural soil suppressiveness against these pathogens has been observed for several sites. However, its nature is little understood, although it is known to be based on microbiological mechanisms. Thus, it is highly relevant to improve the understanding of the complexity of factors that drive natural antagonistic functions in soil, and the rhizosphere, in particular, and the key role that microbes play in natural suppressiveness (Berg and Smalla, 2009).

The increasing interest in the biological control of soil-borne plant pathogens requires a proper understanding of the antagonistic potentials of root-associated bacteria. Several studies have demonstrated the ability of a wide variety of rhizobacteria to suppress diseases caused by soil-borne plant pathogens (Garbeva *et al.*, 2006; Berg *et al.*, 2002). Aerobes and anaerobes are also found in the soil. The aerobic bacteria need oxygen so they tend to dominate where soil is well drained and anaerobic bacteria do not need oxygen, they favor wet, poorly drained soil and can produce toxic compounds that can limit plant root growth (Pratscher *et al.*, 2011). Anaerobic microenvironments within soil aggregates also seem to allow for both anaerobic and aerobic-based metabolism, further highlighting the complexity and heterogeneity impacting microbial community structure and metabolic potential within soils. Soil bacteria are subjected to considerable seasonal fluctuations in environmental conditions (Yao *et al.*, 2011).

Rhizobia are the soil bacteria with the ability to induce nitrogen fixing nodules on roots in lentil as well as other legume crops (Laguerre *et al.*, 2003). To select highly effective bacterial strains for a particular host is an important objective in microbial inoculants research. The bacteria used as inoculants for crops have to compete with indigenous flora for

establishment in a specific host. Rhizobial strains differ in their nodulating competitiveness, as estimated by the percentage of nodules formed when host legumes are inoculated with a mixture of strains or when they are applied as a single inoculant in soil containing indigenous rhizobial population (Laguerre *et al.*, 2003). Isolates of the same species can significantly differ in their different characters like 16S rRNA, N_2-fixing efficiencies and in their abilities to occupy nodules in competition with other closely related strains. Mutations, over expression of desired genes and inter-species transfer of desired traits have been accomplished in rhizobia (Harun-or Rashid *et al.*, 2009). Therefore, it is imperative to have a sensitive and reliable method to detect and quantify introduced rhizobia and it is also important to understand the dynamics of the microbial community and to develop ways of detecting its presence and measuring its activity.

Table 2: Diversity of bacteria in rhizosphere soil and their ecological and industrial importance

Genus	Description	Ecological importance	Industrial importance	References
Rhizobium spp	Gram-negative, motile non sporulating rods	Form an endosymbiotic nitrogen-fixing association with the roots of legumes and paraspona	Production of biofertilizers And has disease suppressive activity	Lai *et al.*, 2004 Brewer and Larkin, 2005
Bacillus spp	Gram-positive, rod-shaped bacteria and a member of the phylum Firmicutes	Promote plant growth	Production of biofertilizers	Ryu *et al.*, 2003
Serratia marcescens	Gram positive, rod shaped and member of the phylum Proteobacteria	Decreased the number of diseased plants caused by Phytophthora	Production of biofertilizers	Lavania *et al.* 2006

Bradyrhizobium	Gram negative member of the phylum Proteobacteria	Promote plant growth	Production of biofertilizers	Shaharoona *et al.*, 2006
Azotobacter spp	Gram-negative bacteria, usually motile, oval or spherical bacteria forming thick wall cysts	Play a role in the nitrogen cycle in nature binding atmospheric nitrogen inaccessible to plants	Production of biofertilizers food additives and some biopolymers	Castañeda *et al.*, 2000
Azospirrilum spp	Gram-negative aerobic bacteria can live as a free living or can be associated with the roots of the cereal	Is important for nitrogen fixation and plant nutrient	Used in production of fertilizers	Babalola, 2010; Saikia *et al.*, 2012
Agrobacterium spp	Gram-negative bacteria	Infects the roots of plants to cause crown gall disease	Genetic engineering for plant improvement	Pitzschke and Hirt, 2010; Chhikara *et al.*, 2011

Herbaspirrilum spp	Gram-negative bacteria comprised of vibrioid or sometimes helical cells. They archemoorganotrophic nitrogen fixers found free-living in the soil	Colonize intracellular spaces of grasses such as rice and sugar	Production of fertilizers	Vilchez and Manzanera, 2011,
Pseudomonas spp	Gram-negative rod-shaped spore-producing bacteria	Metabolize chemical pollutants in the environment	Bioremediation	Su *et al.*, 2009; Vojtová *et al.*, 2011
Thiobacillus spp	Colorless, rod-shaped, Gram-negative bacteria with polar flagella. They possess an iron oxidase, which allows them to metabolize metal ions such as ferrous iron	Convert sulfides into sulfates and form sulfur utilized by plants	Pest control and bioleaching	Beller *et al.*, 2012; Torrentó *et al.*, 2012.
Alcaligenes spp	A genus of non-spore forming Gram-negative bacteria which are obligate aerobic, and facultative	Colonize intracellular spaces of grasses such as rice and sugar	Production of biofertilizers	Sayyed *et al.*, 2010; Ercisli *et al.*, 2010.

Nitrospira spp	Spiral non-motile bacteria	Play an important role in nitrification	Bioremediation	Attard *et al.*, 2010; Foesel *et al.*, 2008;
Nitrobacter spp	Short to long rods occasionally motile or non-motile	Convert nitrites into nitrates	Bioremediation	Attard *et al.*, 2010
Nitrosomonas spp	Gram-negative short to long rods, motile or non-motile	Oxidize ammonium salts into nitrous acid, nitrites in the soil.	Bioremediation and removal of soil pollutants	Yuichi *et al.*, 2011; Wahman *et al.*, 2011.

2.4. Factors affecting microbial diversity

Several biotic and abiotic factors influence the structural and functional diversity of bacterial communities, for example, pesticide treatments, soil structure and plant health and developmental stage (Jousset *et al.*, 2008; Rasche *et al.*, 2006; Garbeva *et al.*, 2006; Granér *et al.*, 2003; Siciliano *et al.*, 2001), and different soil types are assumed to harbour specific microbial communities, as recently shown in a continental-scale study of soil bacterial communities (Fierer *et al.*, 2007).

2.4.1. Edaphic factors

Because of the enormous importance of plant–microorganism interactions in the rhizosphere, for ecosystem functioning and nutrient cycling in natural ecosystems as well as in agricultural and forest systems (Singh *et al.*, 2011), it is crucial to understand the factors influencing the microbial communities in this habitat. Edaphic factors that are presumed to be significant drivers of soil microbial community include availability of nutrients, moisture, temperature, pH, soil aeration and soil texture. These factors can vary considerably with soil depth, soil aggregates, the quantity, quality and availability of soil carbon and mineralogy (Maron *et al.*, 2011; Rousk *et al.*, 2010; Wakelin *et al.*, 2008; Fierer *et al.*, 2007; Drenovsky *et al.*, 2004; Hackl *et al.*, 2005; Waldrop *et al.*, 2000). The absence of these abiotic factors can alter the composition of the microbial community (Fierer *et al.*, 2007; Waldrop *et al.*, 2000). In contrast to what is known about the biodiversity of macro organisms, the microbial biogeography is controlled primarily by edaphic variables, especially pH.

2.4.2. Agriculture

Agricultural practices that maintain adequate soil organic matter content favour the production of soil biota (Ehrenfeld *et al.*, 2005; Tilman *et al.*, 2002). For example, the simple practice of the addition of straw mulch on the soil surface increased soil organic matter and the number of living organisms as much as threefold (Palmroth *et al.*, 2007; Enwall *et al.*, 2005).

2.4.3. Tillage

Physical disturbance of the soil caused by tillage and residue management is a crucial factor in determining soil biotic activity and species diversity in agro ecosystems. Tillage usually disturbs at least 15-25 cm of the soil surface and substitutes stratified surface soil horizons with a tilled zone being more homogeneous with respect to physical characteristics and residue distribution (Alvear *et al.*, 2005). The loss of a stratified soil microhabitat causes a decline in the density of species that inhabit agro ecosystems. Such soil biodiversity reductions are negative because the recycling of nutrients and proper balance between organic matter, soil organisms and plant diversity are necessary components of a productive and ecologically balanced soil environment (Altieri, 1999). Reduced tillage (with surface placement of residues) creates a relatively more stable environment and encourages development of more diverse decomposer communities and slower nutrient turnover (Alvear *et al.*, 2005). Available evidence suggests that conditions in no-till systems favour a higher ratio of fungi to bacteria, whereas in conventionally tilled systems bacterial decomposers may predominate (Govaerts *et al.*, 2008; Govaerts *et al.*, 2007). As opposed to conventional tillage, in reduced tillage, nutrient reserves are stratified, with concentrations of organic matter and microbial populations being greatest near the soil surface. Stratification of crop residues, organic matter, and soil organisms often slows cycling of nitrogen as compared with

conventional tillage with the moldboard plow. Increased microbial immobilization of soluble nitrogen in the surface of reduced tillage soils may need modified fertility or tillage management practices for optimal growth and yield of grain crops (Ceja-Navarro *et al.*, 2010).

2.4.4. Crop rotation system

A crop-rotation system with grass and other suitable plant associations included may well be in the position to make the best use out of the soil by mobilizing and, at the same time, renewing continuously its biotic potential (Chiurazzi, 2008). For instance, microbial diversity was significantly higher under wheat preceded by red clover green manure or field peas than under wheat following wheat (continuous wheat) or summer fallow (Larkin *et al.*, 2012). These results indicate that legume-based crop rotations support diversity of soil microbial communities and may affect the sustainability of agricultural ecosystems (Giller, 2001; Bagayoko *et al.*, 2000). The diversity index and richness of the microbial community were reduced by monocropping (Chiurazzi, 2008).

2.4.5. Type of farming

Modern agriculture entails the simplification of the structure of the environment over vast areas, replacing nature's diversity with a small number of cultivated plants and domesticated animals. A number of management techniques are identified to sustain soil biodiversity, increasing, in turn, soil quality. Organic farming is becoming a major tool for sustaining the soil quality degraded by intensive use of synthetic chemicals for increasing crop production and therefore, use of bio-agents as biofertilizers or biopesticides is an integral part of organic farming, especially in vegetable cultivation (Srivastava *et al.*, 2007). A comparative study of organic and conventional arable farming systems was conducted in the Netherlands

determining the effect of management practices on chemical and biological soil properties and soil health (van Diepeningen *et al.*, 2006) and organic management resulted in higher numbers of bacteria as well as larger species richness.

2.4.6. Fertilizers

As an important anthropogenic management practice for crop yields, the use of fertilizers can also change the abundance and composition of soil functional microorganisms such as ammonia-oxidizers, which play an important role in the nitrogen cycle (Shen *et al.*, 2008). Therefore, given the nutrient cycling processes, soil microorganisms influences global climate change by significantly shaping the effects that global climate change has on terrestrial ecosystems (Xu *et al.*, 2009; He *et al.*, 2008), it is important to monitor the effects of fertilizer application on soil microbial composition and diversity. Many studies have focused on the influence of long-term fertilizer applications on soil physical properties (Pernes-Debuyser and Tessier, 2004), soil fertility (Mallarino and Borges, 2006), soil organic matter, and crop yield (Cai and Qin, 2006). The biological component of soils usually responds more rapidly to changing soil conditions than chemical or physical properties (Šimek *et al.*, 1999). Microbial activities have found to be promoted by organic fertilizers, such as swine or cow manure, and even bio waste compost (Palmroth *et al.*, 2007; Enwall *et al.*, 2005; Šimek *et al.*, 1999). Application of chemical fertilizers has also been shown to have effects on soil microbial biomass (Zhong and Cai, 2007; Herai *et al.*, 2006). However, there are not so many studies simultaneously investigating the bacterial and fungal composition and diversity of soils receiving long-term fertilizer applications (He *et al.*, 2008; Pernes-Debuyser and Tessier, 2004). Studies have shown that pesticides and herbicides can also decrease microbial respiration biomass and diversity in the soil (Kirk *et al.*, 2004). These factors may exert an influence on microbial community structure simultaneously and produce

interactive and feedback effects (Allison and Treseder, 2011). Thus, microbial community structure measures could be conceptualized as an integrated assessment of numerous soil and ecosystem characteristics. However, comprehensive characterization of soil microbial community dynamics during ecosystem restoration has been limited by the enormous microbial diversity within soils (Torsvik and Ovreas, 2002). Soil bacteria are essential components of the biotic community in natural forests and they are largely responsible for ecosystem functioning because they participate in most nutrient transformations (Hackl *et al.*, 2005).

2.4.7. Pesticides and herbicides

Studies have shown that pesticides and herbicides can also decrease microbial respiration, biomass and diversity (Atlas *et al.*, 1991). Efficient strains of nitrogen-fixing bacteria can save a lot of resources being spent on nitrogen fertilizers and also prevent the degradation of the environment besides improving the yield (Dogra, 2010). More than 50% of the land used for agricultural production in developing countries uses about 26% of the total pesticides produced in the world (Gerhardson, 2002). Extensive and improper use of chemicals leads to greater health risk to plants, animals and human populations which have been reviewed time to time by several workers (Gerhardson, 2002). One of the major problems aside from toxicity and carcinogenicity of pesticides is their long persistence in nature that amplifies the toxicity and health risk problems in the area of contamination. A variety of physical and chemical methods available to treat the soil contaminated with hazardous chemical compounds are bound in a modified matrix or transferred from one phase to another, hence biological treatment is essential because it involves the transformation of complex or simple chemical compounds into non-hazardous forms (Gerhardt *et al.*, 2009). For biodegradation,

the target pesticide will be able to serve as the sole carbon source and energy for microorganisms including the synthesis of appropriate enzymes.

2.4.8. Soil quality

Soil quality is a measure of the condition of the soil relative to the requirements of one or more biotic species and or to any human need or purpose and has been used as an important indicator of ecosystem health and sustainability of agroecosystems (Karlen *et al.*, 2004). More definitely, soil quality is the capacity of a specific kind of soil to function within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and to support human health and habitation. People have different ideas of what a quality soil is. For people active in agricultural products, it may mean highly productive land, sustaining or enhancing productivity, maximizing profits, or maintaining the soil resource for future generations. For consumers, it may mean plentiful, healthy, and inexpensive food for present and future generations. Indicators can be physical, chemical, and biological properties, processes, or characteristics of soils. They can also be morphological or visual features of plants (Karlen *et al.*, 2004). Good indicators are relevant, sound and cost-effective. Soil quality indicators are advantageous to policy makers to monitor the long-term effects of management practices on soil quality. They assess the economic impact of alternative management practices designed to improve soil quality, such as cover crops and minimum tillage practices. Soil quality examines the effectiveness of policies addressing the agricultural soil quality issue; and improve policy analysis of soil quality issues by including not only environmental values (Karlen *et al.*, 2004) but also taking into account economic and social factors (Fließbach *et al.*, 2009). Since soil quality is strongly influenced by microbe-mediated processes, and function can be related to diversity, it is likely that the microbial community structure will have the potential to serve as an early indication of soil degradation or soil improvement. Therefore, there is growing evidence that

soil microbiological and biological parameters may function as early and sensitive indicators for soil ecological stress. This was the case when soil enzyme activities, exo polysaccharides, soil microbial biomass and composition of soil microflora were used as biochemical/biological indicators of soil quality (Bending *et al.*, 2004).

2.4.9. Biomass

Similarly, the application of organic matter or manure, enhanced earthworm and microorganism biomass as much as fivefold (Palmroth *et al.*, 2007). Also, when organic manure was added to agricultural land in Hungary, soil microbial biomass increased tenfold (Palmroth *et al.*, 2007; Enwall *et al.*, 2005). Owing to the fact that increased biomass generally is correlated with increased biodiversity (Zhong and Cai, 2007; Herai *et al.*, 2006), it is logical to assume that the increase in biomass of microbes represents an increase in biodiversity (Herai *et al.*, 2006).

Biomass, community structure, and specific functions of soil microorganisms seem to be of major importance for general soil functions and, if detectable, could serve as sensitive soil quality indicators. Since microbial soil communities strongly depend on the conditions of the habitat they colonize, microbiological characteristics of soil may provide indicators, which integrate short, middle and long term changes in soil quality. As soils display a multitude of biological characteristics and many of them may not be accessible, specific indicators have to be chosen. Oberholzer and Höper (2000) have proposed a reference system for the evaluation of agricultural soil based on the most applied soil microbial parameter (Fierer *et al.*, 2007).

2.4.10. Environmental pollution

Many levels of the ecosystem organization are seriously affected by environmental pollution, which might affect the efficiency of the usage of available resources. Therefore, making the system more sensitive to subsequent stress might lead to the development of community tolerance, hence making the system more resistant to additional stresses (Tobor-Kaplon *et al.*, 2006). Soil biodiversity is endangered by pollution which results in biological invasions, endangering endemic fauna and flora and brings about changes in microbial diversity and function. Of the industrial contaminants, polycyclic aromatic hydrocarbons (PAHs) are common soil and groundwater contaminants and are highly carcinogenic chemicals. The soil microbial community structure and composition measures are increasingly being used to assess ecosystem responses to anthropogenic disturbances and provide an indicator of ecosystem recovery (Monciardini *et al.*, 2003). However, in comparison to plant communities, there is limited experimental evidence that predictable patterns in microbial community structure or composition occur during secondary succession (Kuramae *et al.*, 2010; Felske *et al.*, 2000) or ecosystem restoration (Jangid *et al.*, 2010; Gros *et al.*, 2006). Microbial communities are able to respond more rapidly than plant communities to changes in environmental conditions and may provide an early indication of the recovery trajectory (Harris, 2009). However, the high level of sensitivity to numerous environmental factors can also result in long term shifts (in the order of decades or more) in microbial community structure in rehabilitated ecosystems (Jangid *et al.*, 2010). Rehabilitation programs may be expected to leave a soil legacy in terms of some alteration to the soil organo-physico-chemical environment.

2.4.11. Soil profiles

Most studies in soil microbiology have focused exclusively on the surface 25 cm of soil where the densities of microorganisms are highest. However, soil profiles are often many

meters deep and large numbers of microorganisms reside in subsurface horizons (Blume *et al.*, 2002; Fritze *et al.*, 2000). These subsurface microbes play an important role in soil formation, ecosystem biogeochemistry, contaminant degradation, and the maintenance of groundwater quality (Singh *et al.*, 2011), yet little is known about the microbial communities residing in the deeper soil horizons/levels. Microbial community composition may be one important influence on soil processes (Balser *et al.*, 2002; Cavigelli and Robertson, 2001). If the microbial communities residing at depth are simply diluted analogs of the surface microbial communities and exhibit minimal differentiation, the characteristics and properties of microbial processes should be fundamentally similar in the surface and subsurface horizons. However, deeper layers of soil may contain microbial communities that are specialized for their environment and are fundamentally distinct from the surface communities (Blume *et al.*, 2002; Fritze *et al.*, 2000). In this case, the microbial communities in the soil subsurface may function differently from those at the surface and their metabolic properties could not be inferred by studying the microbial communities found in the surface horizons.

2.4.12. Soil properties as well as plant species

The bacterial community composition changed with the age of soil that developed over 77,000 years of intermittent Aeolian deposition. The overall diversity, richness and evenness of the communities' increased (Tarlera *et al.*, 2008). Plants affect these indigenous microbial populations in soil and, each plant species is thought to select specific microbial populations. The existing huge diversity of plant species with an estimated range of from 310,000 to 422,000 species (Pitman and Jørgensen, 2002) and corresponding secondary metabolites of plants (Long, 2001) affects the below-ground diversity. Interestingly, invasive plants can have major on microbial communities in soil by forming symbioses with rhizosphere

microbes (Van der Putten *et al.*, 2007; Garbeva *et al.*, 2006). However, little is understood about which factors are the key drivers of root colonization and rhizosphere microbial community structure.

There is no doubt that factors such as, soil properties, as well as plant species, influence the structure and function of microbial communities. However, the extent to which both factors contribute to microbial communities is not fully understood. There are several contrasting reports in the literature indicating plant or soil type as a dominant factor (Nunan *et al.*, 2005; Girvan *et al.*, 2003). Based on differences in rhizodeposition, rhizosphere microbial communities can differ in structure and species composition, depending on plant species, plant age, root zone and soil type (Kowalchuk *et al.*, 2002; Yang and Crowley, 2000). Analyses of microbial communities (using both culture-dependent and culture-independent methods) showed clear effects of the rhizosphere on species composition (Smalla *et al.*, 2001; Yang and Crowley 2000). In several instances, a relatively high abundance of Gram positive bacterial species was found (Smalla *et al.*, 2001; Picard *et al.*, 2000), especially in the last stage of plant growth. This was in contrast with earlier findings that Gram-negatives were the most dominant rhizosphere colonizers. The work of Smalla *et al.*, (2001) has provided evidence that different plant species select different bacterial communities. Therefore these plant specific enrichments can be increased by repeated cultivation of the same plant species in the same field (Smalla *et al.*, 2001). Based on differences in rhizodeposition, analyses of microbial communities (using both culture-dependent and culture-independent methods) showed clear effects of the rhizosphere on species composition (Smalla *et al.*, 2001; Yang and Crowley, 2000). Recently, Berg *et al.*, (2002) observed differences in the proportion and phenotypic diversity of antagonistic rhizobacteria from different host plants of *Verticilium dahliae*. They concluded that the abundance and composition of *Verticillium* antagonists was

plant species dependent. Considerable diversity among diacetylphloroglucinol (DAPG) producing bacteria was detected depending on plant species and plant growth stages (Picard *et al.*, 2000). Furthermore, soil type has been identified as being another important factor determining the structure of microbial communities present in the vicinity of plant roots (Araújo da Silva *et al.*, 2003). Garbeva *et al.*, (2006) identified soil type as the most important factor affecting fluorescent *Pseudomonas* populations in the rhizospheres of flax and tomato. Information on the effect of soil or plant type was recently reviewed by Garbeva *et al.*, 2006. The relationship between species composition and ecosystem functioning is difficult to quantify. However, the use of polyphasic approaches; combining novel cultivation-independent and more traditional techniques to study microbial communities, led to a significantly better understanding of community structure and function in the rhizosphere in the last decade.

2.5. Molecular methods

For over 80 years, it has been known that there is a large discrepancy between the number of bacterial colonies that form on solid media, when soil is used as an inoculum (Fierer *et al.*, 2007; Rappé and Giovannoni, 2003). This discrepancy has limited the understanding of the species diversity of soil bacterial communities. However, until recently, no representatives of many of these groups were available for detailed study due to their apparent inability to grow in or on laboratory media. It has been established that the genetic diversity of soil bacteria is high and that soils contain many bacterial species of lineages for which no known cultivated isolates are available (Joseph *et al.*, 2003). Many soil bacteria are referred to as uncultured or even unculturable. In the past decade, this limitation has been partially overcome through the application of molecular ecological techniques. In particular, comparative analysis of 16S rRNA genes derived from nucleic acids extracted from soil has revealed the presence of

many new groups of bacteria that were previously undetected in cultivation studies (Axelrood *et al.*, 2002; Felske *et al.*, 2000). A range of methods have been developed to study these organisms directly in their habitats (Lynch *et al.*, 2008; Ranjard *et al.*, 2001) These methods are extremely useful for studying the ecology of microorganisms as parts of communities, but initial physiological and genetic studies of pure cultures should greatly facilitate such synecological studies. There is a certainty that many of these bacteria are in fact culturable using relatively simple recent technologies. The techniques provide ways to screen for a broad range of agents in a single test. It has truly come of age that the range of molecular applications is expected to broaden in the near future speculation. Molecular methods vary with respect to discriminatory power, reproducibility, ease of use and ease of interpretation (Lasker, 2002). Less than 1% of bacterial diversity is considered to be culturable by traditional techniques (Schloss and Handelsman, 2006), a problem that can be avoided by molecular approaches. Molecular approaches have been applied to study a range of soil environments (Demaneche *et al.*, 2009; Rajendhran and Gunasekaran, 2008; Schloss and Handelsman, 2006; Ginolhac *et al.*, 2004; Curtis *et al.*, 2002) and comparisons with cultivation techniques should include biases in the methods used to extract DNA from soil.

Traditionally, estimates of microbial diversity were based solely on culturable microorganisms. However, microscopic observations and mathematical modelling estimate that 99% of bacteria are unculturable under standard laboratory conditions (Schloss and Handelsman, 2006; Stach and Bull, 2005). Recently-developed technologies provide relatively quick and deep sequencing of DNA samples at a moderate cost (Kahvejian *et al.*, 2008; Shendure and Ji, 2008), although DNA sequencing depends on the DNA extracted. Deciphering soil function based on soil DNA sequencing (Vogel *et al.*, 2009) requires extracting the DNA from all members of the soil microbial community. The difficulty is that

every protocol facilitates the extraction of part of the diversity to the disadvantage of the rest. With the DNA approach, total DNA is extracted directly from the soil (van Elsas *et al.*, 2006; Bürgmann *et al.* 2005; Stach and Bull, 2005). Specific genes can be isolated and their DNA sequences determined which allows identification of the organisms at the genus level and in certain cases even to the species or sub-species level (Widmer and Lexer, 2001). The result of these analyses is a GC profile with identified and quantified peaks for specific FAs (e.g. PLFA fingerprint). Some of these groups appear to be important within soils, at least in terms of relative abundance of 16S rRNAs or 16S rRNA genes. However, these numerically abundant bacteria are rarely, if ever, isolated in cultivation experiments, which instead tend to result in the isolation of bacteria that appear to be minor components of the soil bacterial community (Axelrood *et al.*, 2002; Furlong *et al.*, 2002; Dalevi *et al.*, 2001; Rheims *et al.*, 1998). As a consequence, traditional cultivation techniques such as plate counting methods have been increasingly considered inadequate, and new, more sophisticated techniques have been developed for the isolation of novel bacteria from complex microbial habitats.

These culture-independent techniques have proven to be powerful in detecting soil microbial composition and diversity (Babalola *et al.*, 2009), providing an insight into the response of soil ecosystems to environmental changes or anthropogenic disturbance., Irrespective of the method used to quantify the species (diversity in a soil), the total microbial diversity of soil might still be underestimated. Indeed, the relative dominance of certain groups in DNA extracted from soil will mask less abundant species, thus confounding estimates of the soil microbial community structure. Nucleic acids provide information about an organism's genetic composition allowing its classification. Prokaryotes offer limited and indecisive classification criteria based on morphology and biochemical traits. The molecular approach is very useful. It can be used to detect fastidious microorganisms that are difficult or dangerous

to culture *in vitro* and to determine the fates of selected or genetically engineered microorganisms and of particular genes disseminated by transfer to indigenous microbes (Hirsch *et al.*, 2010). This technique can also be used to study the natural bacterial diversity in these complex environments, from which only a small percentage of the indigenous microorganisms can be isolated *in vitro* (Zhao *et al.*, 2011). A select number of bacterial taxa have been well studied and their ecological characteristics are reasonably well defined. This is the case for those taxa with specific physiological capabilities, such as the ammonia-oxidizing nitroso-genera, nitrogen-fixing *Rhizobium*, and the methane-oxidizing methylo-genera (Fierer *et al.*, 2007); however, these taxa are the exception. The majority of soil bacterial taxa, even those that are numerically dominant, have not been extensively studied and their ecological characteristics remain largely unknown. For example, the phylum Acidobacteria is one of the most abundant taxonomic groups of soil bacteria and 95% are yet to be identified (Lee *et al.*, 2008).

CHAPTER 3
MATERIALS AND METHODS

3.1. Area of study

Nine rhizosphere soil samples were collected from different locations in Mafikeng. Mafikeng is located in the North West Province (25.8500° S, 25.6333° E), Republic of South Africa. This region has an average annual precipitation of 559 mm, annual average temperature of 18.3°C and annual rainfall of 539 mm. The sample site collection is represented in Fig 3.1.

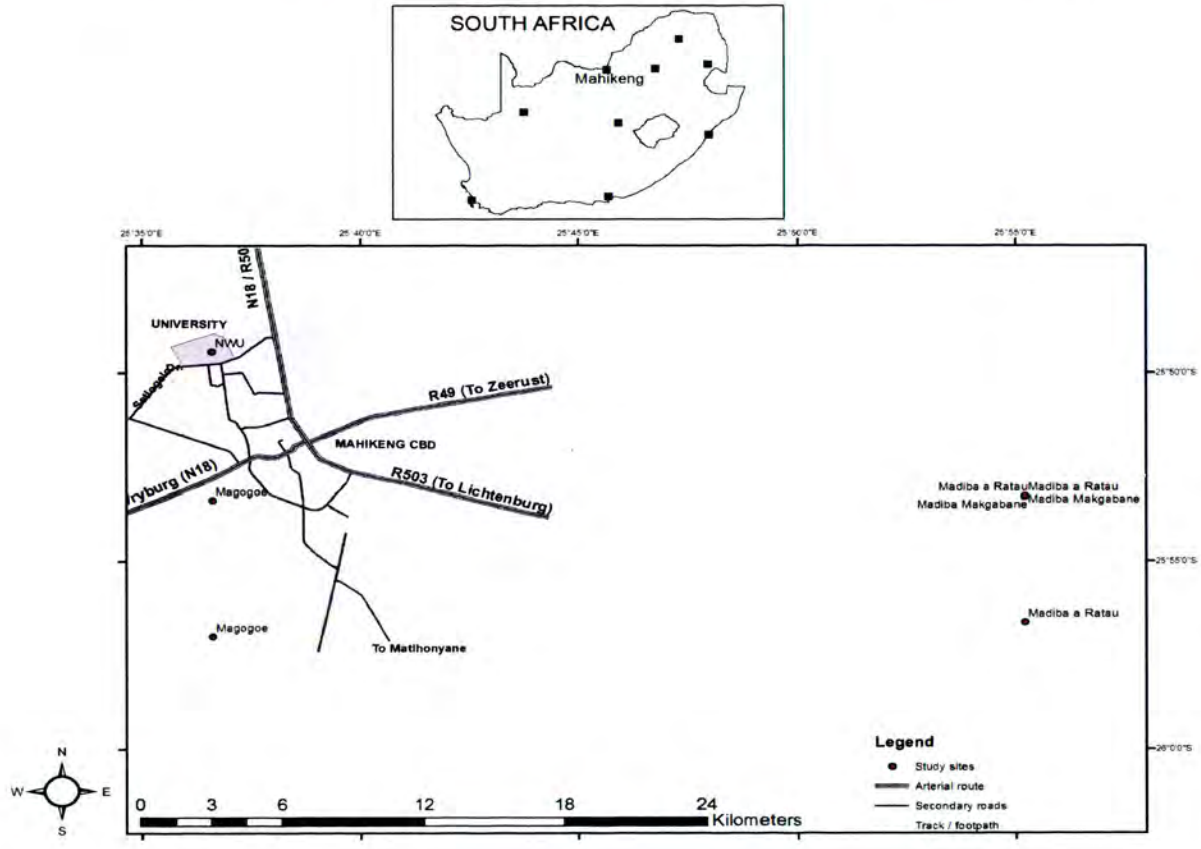

Figure 3.1: The study sites in Mafikeng where the rhizosphere soil samples of cabbage, tomato, maize, beetroot, onion, spinach, lettuce and greenhouse soil were collected

3.2. Sample collection

Rhizosphere soil samples were collected randomly from North West Province, South Africa. Eight crops were selected in this study, viz onion, maize (2 samples), spinach, beetroot,

lettuce, green peas, tomato and cabbage grown in Mafikeng. Table 4 shows the location, and sample code of the nine rhizosphere soil samples (100-200 g), which were collected aseptically in sterile polythene bags. The samples were placed in a cooler box for transportation and stored at 4°C.

3.3. Measurement of pH and chemical analysis of soil

Ten grams of each sample were bathed in 20 ml of distilled water and allowed to stand for 20 minutes with occasionally stirring to maximize the mixing. The pH of all the samples was determined by using a Crison Basic 20 pH meter (Shanghai, China). After standing the pH of the soil was measured. Digestion of the soil samples was done prior to chemical analysis adopting the method of Yuan and Xu (2011) where 1 g of soil samples was added to a mixture of 3 ml nitric acid (HNO_3), 9 ml HCL and 1 ml hydrogen peroxide (H_2O_2). The mixture was digested in a microwave reaction system multiwave 300 (Perken Elmer, USA). The clear digested samples were then transferred into 25 ml calibrated flasks with deionized water. Blanks and a standard reference material were also analysed concurrently for accuracy. The digested samples were analysed using Perkin-Elmer NexION 300 ICP-MS (Inductively Coupled Plasma Mass Spectrometry) instrument where 1 ml of the digested sample was filtered using Whatman 13 mm Syringe Filter to remove silica content and other fine materials that may be present after digestion. The filtered sample was then transferred into a pre-cleaned centrifuge tube and adjusted to 10 ml with 0.14 M HNO_3. Samples were then introduced into the nebulizer of ICP-MS to determine chemical properties of the soil.

3.3 Culture-dependent techniques

3.3.1. Isolation of bacteria

Isolation and enumeration of soil organisms present in the soil samples was performed by serial dilution plate technique using Nutrient agar, as previously described (Saha and Dhanasekeran, 2010). To obtain pure cultures the colonies were streaked on the fresh agar

plates and were incubated. Pure cultures were identified on the basis of their morphological and cultural characteristics (Seshadri and Ignacimuthu, 2002) and were used for the identification of isolated organisms and for the PGPR assays.

3.4. Morphological characterization

3.4.1 Shape and Gram reaction of recovered isolates were studied.

Bacteria were characterised according to colony pigmentation, cell and colony morphology and Gram staining. The morphology of the isolates was assessed by macroscopic and microscopic characterization using standard methods. Cultural characterization was carried out as previously described by Sunanda *et al.* (2009). The observed morphology of the isolates was compared with the organisms morphology provided in Bergey's manual for the presumptive identification of the isolates (Goodfellow *et al.*, 2012).

3.5. Biochemical characterization

Various biochemical tests such as catalase test, oxidase test, starch hydrolysis, citrate utilization test, indole test, triple sugar iron (TSI) test, indole test, urea utilization test, aesculin production, gelatin liquefaction test, nitrate reduction test, methyl red and voges-proskauer test were carried out using standard methods as described by (Reddy *et al.*, 2010).

3.6. *In vitro* plant growth-promoting assays

3.6.1. Ammonia production

Determination of ammonia production was done by a method previously described by Cappuccino and Sherman, (1992). Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 h at 37°C. Nessler reagent (0.5 ml) was added into each tube. Development of a brown to yellow color indicated a positive test for ammonia production.

3.6.2. Determination of indole acetic acid produced

Indole acetic acid production (IAA) was determined by a method previously described by Patten and Glick (1998); freshly grown cultures were inoculated into 10 ml nutrient broth in each tube and incubated at 30 °C for 48 h. A 4 ml culture was removed from each tube and centrifuged at 10,000 rpm for 15 min. An aliquot of 1 ml supernatant was transferred into a fresh tube to which 50 µl of 10Mm orthophosphoric acid and a 2 ml of reagent comprising of (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated at room temperature for 25 min. The development of a pink color indicated the presence of IAA.

3.6.3. Detection of Hydrogen Cyanide (HCN) production

All the isolates were screened for the production of HCN by adapting the method of (Ahmad *et al.*, 2008). Production of HCN was observed where freshly grown cells were spread on a tryptone soy agar (30 g) containing glycine 4.5 g/l and a sterilized filter paper saturated with 1% solution of picric acid and 2% sodium carbonate was placed in the upper lid of the petri dish. The petri dish was then sealed with parafilm and incubated at 30°C for 4 days. A change in color of the filter paper from yellow to reddish brown was an indication of cyanogenic activity.

3.6.4. Assessment of antifungal activity *in vitro*

Fusarium solani ATCC 36031 was obtained from Davies Diagnostic, SA. Fungal strains and inoculum preparation were prepared by cultivation on Potato Dextrose agar for 10 days in petri dishes. Pure culture was isolated by single spore isolation according to the method described by Choi *et al.* (1999). The Microconidial suspension was prepared by pouring

sterile water onto the fungal culture and the surfaces of the culture were scrapped to dislodge the conidia from the mycelium. The conidia suspensions were 10^7 using Weber BS742 haemocytometer (Adebayo and Ekpo, 2005). Selected fungi (*Fusarium solani*) and a test culture were spread on the Potato Dextrose agar. Antibiosis of the test strain was assessed on the basis of the inhibition zone sizes after 4 days of incubation at 30°C.

3.6.5. Phosphate solubilisation

The phosphate solubilizing efficiency of the study isolates were carried out by performing an experiment of halozone formation where bacteria were first screened on Pikovskaya's agar plates for phosphate solubilization as described by Gaur (1990). Pikovskaya's agar medium was compounded by adding 0.5 g Yeast Extract, 10 g Dextrose, 5 g calcium phosphate, 0.5 g ammonium sulphate, 0.20 g potassium chloride, 0.10 g magnesium sulphate, 0.0001 g manganese sulphate, 0.0001 g ferrous sulphate and 15 g agar in 1000 ml distilled water. Test bacteria were inoculated on Pikovskaya's agar; the plates were incubated for 48 h. A halozone around the bacterial colony when incubated was an indication of a positive test while the absence of the halozone indicated a negative test. The principal mechanism for phosphate solubilization in bacteria is the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorous in soil

3.6.6. ACC -Deaminase assay

Amino cyclopropane carboxylase (ACC)-deaminase activity was assayed according to a modification of the method of Honma and Shimomura (1978), in this method, bacterial isolates were grown at 30°C in 10 ml nutrient broth (supplemented with 15 µg/ml of tetracycline) to late log phase, after which the cells were harvested by centrifugation at 9000 rpm for 10 min at room temperature. The cells were washed twice with 5 ml of Tris-HCl

buffer (pH 7.5). To induce ACC deaminase activity, the cells were suspended in 5 ml of M9 medium (1M MgSO4, 1M $CaCl_2$, 20% Glucose and sterilised H_2O) containing 3 mM ACC and then incubated for 18 h at 30°C in a rotary shaker. The bacterial isolates were harvested by centrifugation, washed twice with Tris-HCl buffer (pH 8.0), and resuspended in the same buffer solution. A 200 µl aliquot of bacterial suspension was removed and 10 µl of toluene was added. The cells were vortexed vigorously to facilitate permeabilization, and then 200 µl of 3 mM ACC was added to 50 µl of bacterial lysates. A concentration of 0.1 M Tris-HCl buffer (pH 8.5) was added to the reaction mixture and incubated for 30 min at 30°C, 0.5 ml of 0.56 M HCl was added, and the mixtures were centrifuged at 9000 rpm for 5 min. Then 200 µl of 0.56 M HCl and 75 µl of 0.2% 2, 4-dinitrophenylhydrazine in 2 M NaOH were added to 500 µl of the supernatant. The absorbance of the samples was read at 540 nm optical density, the values greater than 0.5 indicated a positive production of ACC Deaminase.

3.7 Molecular characterization

The table below illustrates the PCR oligonucleotides used in this study for both culture-dependent and culture-independent molecular studies.

Table 3: List of PCR primers used in this study

Primer	Sequence	Amplicon length (bp)	Reference
27f[a]	5'-AGA GTT TGA TCC TGG CTC AG-3'	1500	Khamis *et al.*, 2001
1492r[a]	5'-TGA CTG ACT GAG GCT ACC TTG TTA CGA-3'		
357f-GC[b] clamp	5'-GC CGC CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG CCT -3'	500	Muyzer, 1999
518r[b]	5'-ACG GGA GGC AGC AG-3'		
357f[b]	5'-CCT ACG GGA GGC AGC AG-3'	200	

Primer abbreviations: f, forward; r, reverse. Primers [a] used in the study of the culture-dependent bacterial diversity; primers [b] used in the study of PCR-DGGE culture-independent bacterial diversity.

3.7.1. DNA extraction from bacterial isolates

The isolation of genomic bacterial DNA was performed using the ZR Soil Microbe DNA MiniPrep™ (Zymo Research, USA) extraction kit according to the manufacturer's instructions. Briefly, overnight cultures were prepared in Luria Bertani Broth. Bacterial cells (50-100 mg wet weight) were added to ZR BashingBead™. The bacterial cells were secured in a bead beater fitted with a 2 ml tube holder assembly and the bead beater was run at a maximum speed for 5 min. A volume of 500 µl 100% beta-mercaptoethanol was added to the DNA Binding Buffer in the ratio of 500 µl in 100 ml. The ZR BashingBead™ Lysis Tube containing bacterial cells was centrifuged in a microcentrifuge at 10,000 rpm for 1 min and 400 µl of the supernatant was transferred to a Zymo-Spin ™ IV Spin Filter in a collection tube and centrifuged at 7,000 rpm for 1 min. A volume of 1,200 µl Soil DNA Binding Buffer was added to the filtrate in the collection tube. Eight hundred (800) µl of the mixture from the prior step was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10,000 rpm for a min. The flow through from the collection tube was discarded and the remaining 800 µl of the mixture was transferred into a collection tube and centrifuged again at 10,000 rpm for 1 min and 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000×g for 1 min. Five hundred (500) µl DNA Wash Buffer was added to the Zymo-Spin ™ IIC column on a new collection tube and centrifuged at 10,000×g for 1 min. The Zymo-Spin™ IIC column was transferred to a clean 1.5 ml microcentrifuge tube 25 µl DNA elution buffer was added directly to the column matrix and centrifuged at 10,000 rpm for 30 s to elute the DNA. The eluted DNA from the step above was transferred to a Zymo-Spin™ IV-HRC spin Filter in a clean 1.5 ml microcentrifuge tube and was then centrifuged at 8,000 rpm for 1 min.

3.7.2. 16S rRNA gene amplification

Polymerase Chain Reaction (PCR) amplification of the 16S rRNA gene of the isolates was conducted using the universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-TGA CTG ACT GAG GCT ACC TTG TTA CGA-3') (Khamis *et al.*, 2001). The amplification reaction was performed with a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA), in a total volume of 50 µl containing 30-50 ng DNA, 100 mM of each primer (Integrated DNA Technology), 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. The thermal cycling conditions were: 5 min at 94°C for the initial denaturation, 30 cycles of 30 s at 95°C for denaturation, 1 min at 54°C for annealing, 2 min at 72°C for extension, and further final extension for 5 min at 72°C. For each reaction, a negative control lacking DNA template was included.

3.7.3. Gel electrophoresis

The PCR amplicons were analysed by electrophoresis in 1% (w/v) agarose gel stained with 10 µg/ml ethidium bromide (Sigma-Aldrich, USA). DNA amplicons were viewed under the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, California, USA) to confirm the amplification and expected size of the PCR products. The remaining mixture was purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany).

3.7.4 Nucleotide sequence determination

PCR purified products of the 16S rRNA gene of the strains were analysed for nucleotide sequence determination by using the ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. Nucleotide sequences of the 16S rRNA of the strains were determined and compared for similarity level with the reference species of bacteria contained in genomic database banks, using the 'NCBI

Blast' website (Altschul *et al.*, 1990). Identification of the bacterial cultures to their nearest species using 16S rRNA sequence data was obtained.

3.7.5 Molecular bacterial taxonomy determined by sequences and phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were analyzed and edited using Chromaslite 2.0 software and sequences were carried to process raw data into high quality sequences (Pappas *et al.*, 2005). Nucleotide sequences were analyzed and edited using BioEdit software (Hall, 1999). After this initial analysis, the sequences were compared to the basic local alignment search tool database of sequences deposited at the National Centre for Biotechnology (NCBI) using the BLASTN website (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignments of the sequences were carried out using the Mafft software 6.864 (http://mafft.cbrc.jp/alignment/server/index.html) against corresponding nucleotide sequences retrieved from GenBank. Then evolutionary distance matrices were generated as described by Jukes and Cantor, (1969). After which phylogenetic analyses were conducted using MEGA Version 5.22 (Tamura *et al.*, 2011). The evolutionary history was then inferred using the Neighbor-Joining method and the evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000). The robustness of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Putative chimeric sequences were identified using the ChimeraBuster 1.0 software. Finally manipulation and tree editing were carried out using TreeView (Page, 1996).

3.7.6 Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study were submitted to the GenBank database and assigned accession numbers indicated in parentheses, MR1 (JX971518), MR2 (JX971519), MR3 (JX971520), MR4 (JX971521), MR5 (JX971522), MR6 (JX971523),

MR7 (JX971524), MR8 (JX971525), MR9 (JX971526), MR10 (JX971527), MR11 (JX971528), MR12 (JX971529), MR13 (JX971530), MR14 (JX971531), MR15 (JX971532), MR16 (JX971517), MR17 (JX971533), MR18 (JX971534), MR20 (KF738827), MR21 (KF38828), MR22 (KF712533), MR23 (KF712534), MR24 (KF712535), MR25(KF712536), MR26 (KF712537), MR27 (KF71238) MR28 (KF71240), MR29 (KF712529), MR30 (KF712540).

3.8. Culture-independent techniques

3.8.1. Isolation of soil DNA directly from soil

Total nucleic acid was extracted from the soil without prior culturing. Isolation of DNA directly from soil was conducted using the PowerSoil® DNA Isolation Kit (MOBIO laboratories, USA) according to the manufacturer's instructions. Briefly, a total of 0.25 g of soil sample was added to the powerbead tubes, the mixture was gently vortexed and 60 µl of solution C1 was added. The mixture was inverted several times and the powerbead tubes were secured horizontally using the adapter tube holder. The mixture was vortexed at a maximum speed for 10 min. The powerbead tubes were centrifuged at 10,000 rpm for 30 s at room temperature. The supernatant was transferred to a clean 2 ml collection tube and 250 µl of solution C2 was added. The mixture was vortexed for 5 s and incubated at 4°C for 5 min, the tubes were centrifuged at room temperature for 1 min at 10,000 rpm. A total of 600µl of supernatant was transferred to a clean 2 ml collection tube. Two hundred (200) µl of solution C3 was added, and the mixture was vortexed briefly. The collection tube was incubated at 4°C for 5 min. The tubes were centrifuged at room temperature for 1 min at 10,000 rpm and 750 µl of supernatant was transferred into a clean 2-ml collection tube. 1200 µl solution C6 was added to the supernatant and the mixture was vortexed. Approximately 675 µl was loaded onto a spin filter and centrifuged at 10,000 rpm for 1 min at room temperature. The flow-through was discarded and an additional 675 µl of supernatant was added to the spin filter and centrifuged at 10,000 x g for 1 min at room temperature. The remaining supernatant was loaded onto the spin filter and centrifuged at 10,000 rpm for 1 min at room temperature. A total volume of 500 µl solution C5 was added to the spin filter and the spin filter was centrifuged at room temperature for 30 s at 10,000 rpm. The flow was discarded and the spin filter was centrifuged again at room temperature for 1 min at 10,000

rpm. The spin filter was placed in a clean 2 ml collection tube. And a total volume of 100 µl of solution C6 was added to the center of the white filter membrane to elute the DNA. The filter membrane was centrifuged at room temperature for 30 s at 10,000 rpm. The DNA was collected in the collection tube and stored at -20 °C till needed.

3.8.2. PCR amplification and DGGE analysis of the bacterial community structures

The 16S rRNA gene fragments were PCR amplified using the universal primer pair f357-GC clamp (5'-GC CGC CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG CCT -3') and r518 (5'-ACG GGA GGC AGC AG-3') (Muyzer, 1999). Amplification was performed in a total volume of 50 µl containing 30-50 ng DNA, 100 mM of each primer (Integrated DNA Technology), 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. Thermal cycling was carried out in a Bio-Rad C1000 Touch^{TM} Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) with an initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min. annealing at 56°C for 1 min and extension at 72°C for 1 min. Final extension was performed at 72°C for 10 min. PCR products were evaluated by electrophoresis on a 2% agarose gels and visualised by 10 µg/ml ethidium bromide (Sigma-Aldrich, USA) staining and UV illumination.

PCR products were analysed by DGGE using a DCode Universal Detection System (Bio-Rad Laboratories, Hercules, California, USA). DGGE analysis was conducted at a denaturing gradient of 40–60% in 1 mm vertical polyacrylamide gels (8% (wt/vol) acrylamide in 1 × TAE). 20 µl of amplification product were mixed with 5 µl of loading buffer (6× Orange Loading Dye, Fermentas Life Sciences, Maryland, USA) and loaded into the gel. Electrophoresis was performed at a constant temperature of 60°C for 16 h at 100 V in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Polyacrylamide gels were

stained with 10 mg/l ethidium bromide (Sigma-Aldrich, USA) for 45 min and visualised with a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, California, USA). Bands of interest were excised from gels with a sterile scalpel on a UV transilluminator and eluted in 20 µl of sterile nuclease-free water for 2 h at 4°C. A volume of 2 µl of the eluent was used as DNA template in PCR amplification reactions with primer pair f357 and r518 (Muyzer, 1999) and the cycling conditions described above were used. PCR purified products of the 16S rRNA of the strains sequenced by population based sequencing using the ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa, where a chromatogram was generated and the raw data was edited by Chromaslite 2.0 and Bioedit softwares (Hall, 1999). The sequences were edited and blasted using BLASTN searches to determine their identity to other 16S rRNA sequences in the National Center of Biotechnology Information Database (NCBI) .

3.9 Pot experiments for plant growth-promoting activity

3.9.1 Study design

Based on the *in vitro* plant growth activities, four bacterial species exhibiting *in vitro* plant growth-promoting capabilities were selected i.e. (*Bacillus* sp, *B. amyloliquefaciens, L. xylanilyticus* and *B. pumilus)*. Screen house pot experiments were carried out in 4000 ml plastic pot with 30 cm diameter and 30 cm depth. The soil for potting was collected at North-West University (Mafikeng) in a screenhouse. The collected soil was passed through a 10-mesh sieve and thoroughly mixed for potting. The soil for use was sterilized twice at 120°C for 45 min with 24 h intervals (Gholami *et al*., 2009). Four hundred (400 g) of sterilized soil was weighed in sterilized plastic pot. The experiment was set in triplicates and a control. Tomato (*Solanum lycopersicum*) and spinach (*Spinacia oleracea*) seeds from the commercial market were used. Pots were distributed randomly in a randomized block design in the

screenhouse to minimize the experimental errors and watering was done once a day in order to maintain moisture content.

3.9.2 Preparation of an inoculum and seed coating

Bacteria were grown on Tryptic Soy agar plates overnight at 30 °C. Bacterial colonies were removed from the plates, inoculated into nutrient broth, and allowed to grow with shaking at 37°C at 120rpm for 48 h. Serial dilutions were conducted to the test tubes and bacterial suspension optical density were of 0.5 (10^6-10^7 CFU ml^{-1}) measured at 540 nm. The dilution of the bacterial suspension was conducted using an uninoculated nutrient broth. (Natsch *et al.* 1997). Tomato and spinach seeds were coated with the inoculant. Only one isolate was added per pot. Non inoculated pots served as controls.

3.9.3 Data collection after plant growth

Plants were harvested 30 days after planting and the data regarding the growth parameters (number of leaves, root and shoot length, dry and fresh mass weight) were recorded.

3.9.4 Statistical analysis

Data were statistically analyzed by analysis of variance (ANOVA) using SPSS software version 10 for Window (SPSS, Chicago, IL, USA). Each bacterial treatment was replicated 3 times. Means were separated by T-test at the significance level of $P \leq 0.05$.

CHAPTER 4
RESULTS AND INTERPRETATION

4.1 Isolation of soil bacteria and determination of the sample pH

In this study, a total of 52 isolates were obtained from 9 different soil samples collected in Mafikeng. However a total of 29 isolates were used in this study based on differences in the colonial morphology and sequence data (Table 4.2). The results presented in the Table 4.1 below indicate pH and the chemical properties of the soil samples. Soil pH revealed an alkaline soil ranging from 7.54-9.8. This parameter may significantly affect the survival of microbial activity and population. The chemical properties of the samples collected from Mafikeng indicated the presence of a variety of elements such as the carbon content ranging from 3.8-18.04 $mgkg^{-1}$. The bioavailability of potassium ranged from 1.7-17 $mgkg^{-1}$. An elevated concentration of 17 $mgkg^{-1}$ in potassium was indicated in soil sample collected from the screenhouse. Almost all samples indicated the same concentration of approximately 1 mg kg^{-1} of manganese. Calcium concentration was more pronounced in samples from soils carrying cabbage. Cadmium concentration in the soil samples ranged from 0.001-0.002 $mgkg^{-1}$and a wide range of 3.1-72.44 $mgkg^{-1}$ iron was observed. Copper bioavailability in all the samples ranged from 0.03-0.4 $mgkg^{-1}$. However green peas, maize, onion and lettuce soil samples indicated no copper detection. The bioavailability of cobalt ranged from 0.01-0.03 $mgkg^{-1}$ in all the soil samples. Sulphur bioavailability in the samples ranged from 12-17 $mgkg^{-1}$. Maize 2 soil sample did not have any phosphorus detected and the phosphorus concentration in other soil samples ranged from 0.3-1.22 $mgkg^{-1}$. Nitrogen bioavailability in the soil samples ranged from 100-321.3 $mgkg^{-1}$. Chromium was detected in all the samples

and its concentration ranged from 0.01-06 $mgkg^{-1}$. Magnesium concentration in the soil samples ranged from 4.5-2.98 $mgkg^{-1}$ and zinc concentration ranged from 0.22-44 $mgkg^{-1}$.

Table 4.1: Physico-chemical properties of soil samples collected in this study

Soil samples	Elements in a sample $mgkg^{-1}$													
	pH	C	N	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	S	P	Zn
Cabbage	8.03	5.5	170	24.1	0.001	0.02	0.6	0.05	46.8	15.2	15.2	0.7	17	1	2.6
Maize1	8.32	18.04	150	8.48	0.001	0.02	0.23	0.08	72.44	9.64	8.66	0.58	N/D	0.33	0.27
Beetroot	7.99	10	321.3	0.1	0.001	0.01	0.1	0.07	33.1	5.4	14	0.4	12	1	1.9
Spinach	8.23	4.56	163	19.34	0.001	0.01	0.07	0.06	9.11	2.24	5.19	0.6	15.7	1.32	1.61
Green peas	8.32	10	421.3	0.1	0.001	0.02	0.02	0.2	3.1	6.51	25	1	N/D	1.2	3.1
Maize2	9.8	17.2	293.4	146	0.001	0.01	0.01	0.1	42	12.3	33	1	N/D	N/D	1.2
Onion	7.54	7.7	163	36.1	0.001	0.02	0.1	0.03	40.65	6.6	19.3	0.6	N/D	0.99	0.9
Lettuce	9.31	9.3	120	54.2	0.001	0.03	0.18	0.41	67.3	8.11	25.98	1.05	N/D	1.01	4.4
Tomato	8.3	8.1	408.0	35.6	0.001	0.02	0.60	0.03	39.16	6.4	18.7	0.60	1.30	0.95	0.9
Screenhouse	9.7	16.92	169	10.7	0.002	0.03	0.13	0.07	55.02	17.0	25.19	1.39	N/D	0.74	0.83

Keys: N/D-Not detected, SH-Screen house

Table 4.2: Areas in North West (Mafikeng) where the soil samples were collected and the bacterial cultures isolated from each soil sample.

Location	GPS Coordinates	Crop	Isolate number
Makogoe	S25°53. 275' E25°36.45'	Cabbage	MR1, MR2
Makogoe	S25°56. 946' E25°36.45'	Maize 1	MR3, MR4, MR5, MR6, MR7
Madiba Makgabane	S25°5. 318' E25° 55. 16'	Maize 2	MR8, MR9, MR10
Madiba Makgabane	S25°53. 296' E25° 55. 16'	Spinach	MR11, MR12
Madiba Makgabane	S25°53. 275' E25° 55. 16'	Green Peas	MR13, MR14, MR15
Madiba Makgabane	S25°53. 285' E25° 55. 16'	Beetroot	MR16, MR17, MR18, MR20
Madiba Makgabane	S25°56. 657' E25° 55. 16'	Onion	MR21, MR22, MR23, MR24, MR25
Madiba Makgabane	S25°53. 275' E25° 55. 16'	Lettuce	MR26, MR27, MR28
Madiba Makgabane	S25°53. 275' E25° 55. 16'	Tomato	MR29, MR30

4.2 Morphological and biochemical characterization

Colonial morphological characterization revealed that the colonies were elevated and mainly convex, the color ranged from cream to red, optical density for all the isolates was opaque and the margins were largely smooth or entire. Gram staining was conducted on all the isolates and, 79% of bacteria were found to be Gram-positive while 21% of the bacteria were Gram-negative. These isolates were subjected to biochemical tests to determine genus-level identification (Table 4.3). On the basis of morphological, cultural and biochemical characteristics, the bacterial isolates were identified as mainly members of the genus *Bacillus*., based on the rod structure and strictly aerobic nature of the Gram-positive bacteria, then followed by *Proteus* spp and *Rhizobium* spp according to, Bergey's Manual of Systematic Bacteriology (2012). Ninety three (93%) of the bacteria possessed catalase enzyme, 59% of the isolates were oxidase negative, 62% of the isolates were able to utilize citrate as their sole carbon source. Thirty eight (38%) were able to liquefy gelatin, 62% of the isolates were able to hydrolyze starch, while 83% of the isolates reduced nitrate. For triple sugar fermentation, 44% utilized both glucose and sucrose and only 2% of the isolates lactose. Production of H_2S was observed in 16% of bacterial isolates, and 79% of the bacteria produced aesculin. The similarity in the soil samples was that all samples had *Bacillus* species which was due to their endospore producing abilities that make them survive harsh conditions. Phylogenetic analysis was also conducted and one representative of the various isolates with a high percentage similarity with the 16S rRNA sequences from the GeneBank was selected for phylogenetic characteristics on the basis of 16S rRNA (Table 4.3). The isolates indicated a high percentage identity of 80% to 100% with the sequences from the GenBank. The 16S rRNA gene sequences obtained in this study have been submitted to the GenBank database and assigned accession numbers, which give the community unique, accession number.

Table 4.3: Species identification using biochemical characterization and molecular methods on the basis of 16S rRNA gene sequences, centred on similarity of BLAST results returned from GenBank databases of rhizobacteria isolated from crop soil samples of cabbage, onion, maize, spinach, beetroot, lettuce, tomato and green peas in Mafikeng.

Isolate code	Gram reaction	Catalase test	Oxidase test	Citrate utilization	Starch hydrolysis	Gelatin hydrolysis	Motility	Nitrate reduction	Glucose test	Lactose test	Sucrose test	H_2S production	MR test	V.proskauer test	Indole test	Aesculin test	Assigned Accession no	Sequence Length (bp)	Closest relatedorganisms from the GenBank	Accession no	% similarity
MR1	+	+	-	-	+	-	-	+	-	-	+	-	+	-	-	+	JX971518	1500	*B. subtilis*	JX094283	100%
MR2	-	+	-	+	+	-	+	+	+	-	-	-	+	-	-	-	JX971519	1442	*Ensifer adhaerens*	EU221356	99%
MR3	+	-	-	+	-	-	+	+	+	+	-	-	+	-	-	-	JX971520	1410	*B. pumilus*	JX293286	99%
MR4	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	+	JX971521	1259	*Brevibacillus* sp	HQ01884	99%
MR5	+	+	-	-	-	-	-	+	-	-	-	+	-	+	-	-	JX971522	1428	*B. vallismortis*	JX144955	99%
MR6	+	+	-	-	-	-	V	+	+	-	-	-	-	+	-	-	JX971523	1397	*B. thuringiensis*	JQ988062	99%
MR7	+	+	-	-	-	-	+	+	+	-	-	-	+	-	-	+	JX971524	1513	*Bacillus* sp	AF406633	99%
MR8	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	+	JX971525	1470	*B. mojavensis*	JQ236831	99%
MR9	+	+	+	+	+	-	+	+	-	-	-	-	+	-	-	+	JX971526	1418	*B. methylotrophicus*	JX094955	99%
MR10	-	+	+	+	+	-	-	+	-	-	-	-	+	-	-	+	JX971527	1501	*Lactobacillus* sp	JN987182	99%
MR11	-	+	+	+	+	-	+	+	-	-	-	-	+	-	-	+	JX971528	1346	*Aquamicrobium* sp	FM210786	96%
MR12	+	+	+	+	+	-	+	+	-	-	-	-	+	+	-	+	JX971529	1466	*B. aerius*	JX009139	99%
MR13	+	+	+	+	+	-	+	+	+	-	-	-	+	-	-	+	JX971530	1424	*Paenibacillus* sp	HM352396	99%
MR14	-	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	JX971531	1447	*Proteus vulgaris*	JN409462	99%
MR15	-	+	-	-	+	-	-	+	-	-	+	-	+	-	-	+	JX971535	1502	*Alcaligenes* sp	AB118220	80%
MR16	+	+	+	+	-	+	+	+	+	-	+	-	+	-	-	+	JX971532	1466	*B. amyloliquefaciens*	FJ889051	100%
MR17	+	+	-	-	+	+	-	-	-	-	+	+	+	-	-	+	JX971533	1396	*B. cereus*	JX293290	99%
MR18	+	+	-	-	+	-	-	+	-	-	-	+	-	-	+	+	JX971534	1519	*B. clausii*	EU117277	89%
MR20	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	KF738827	1592	*B. aerophilus*	KF053054	96%
MR21	+	+	-	-	-	+	+	-	+	+	+	-	-	-	-	+	KF738828	1516	*L. sphaericus*	JX489610	87%

MR22	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	+	KF712533	1426	*L. xylanilyticus*	KF208475	87%
MR23	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	KF712543	1427	*Brevibacterium halotorens*	KC511538	99%
MR24	+	-	+	-	-	-	+	-	+	+	+	+	+	+	-	-	KF712535	1473	*Streptococccus agalactiae*	EU075070	98%
MR25	+	+	-	-	+	-	-	+	-	-	+	-	+	-	-	+	KF712536	1524	*Bacillus sp. 2*	KF114419	99%
MR26	-	+	-	+	+	-	+	+	+	-	+	+	+	-	-	+	KF712537	1419	*Endophytic bacterium*	JF901377	99%
MR27	+	+	-	-	-	+	+	+	+	+	+	-	-	+	-	+	KF712538	1441	*B. altitudinis*	KF254585	98%
MR29	+	+	-	+	+	+	-	V	+	-	-	+	+	+	-	+	KF712540	1493	*B. mycoides*	EU285664	99%
MR30	+	+	-	+	+	+	+	V	-	-	-	-	-	+	-	+	KF738829	1512	*Lysinibacillus* sp	KF423357	87%

Key: + = Positive; - =Negative; V = Variable

4.3 *In vitro* plant growth-promoting bacteria Assays

The results of the qualitative test for HCN production showed that a low percentage of the isolated bacteria were capable of producing HCN. *B. subtilis*, *Proteus vulgaris and B. amyloliquefaciens* sp. indicated high cyanogenic potential due to the color change of the filter paper from yellow to dark brown to red when compared to other strains and was scored as positive (Table 4.4). Further tests indicated that the HCN-producing isolates belong to the genus *Bacillus, Proteus vulgaris* and *Brevibacterium halotorens.* However, other bacterial strains indicated no production of HCN based on the color development. Ammonia production was positive for 100% of the bacterial isolates. *B. amyloquefaciens*, *Aquamicrobium aerolotum*, *Alcaligenes* sp. and six *Bacillus* spp indicated antifungal activity over *Fusarium solani* and this is indicated by figure 4.1 which shows the antibiosis of the test strain was assessed on the basis of the inhibition zone. Eleven *Bacillus* spp, *Brevibacterium* and *Ensifer adhaerens* bacterial isolates showed an ability to solubilise the insoluble phosphate; and three bacterial isolates, *Paenibacillus, B. aerius* and *Proteus vulgaris* produced IAA. ACC deaminase production was observed in nine (9) *Bacillus* spp, *Aquamicrobium* sp. and *Alcaligenes* sp.

Table 4.4: *In vitro* plant growth-promoting activities of rhizosphere soil of tested crops[a].

Isolate name	Ammonia production	ACC deaminase	Anti-fungal activity	Phosphate solubilization	Hydrogen Cyanide	Indole Acetic Acid
B. subtilis	+	+	-	+	+	+
Ensifer adhaerens	+	-	-		-	-
B. pumilus	+	+	-	+	+	-
Brevibacillus sp	+	-	-	+	-	-
B. vallismortis	+	+	-	-	-	-
B. thuringiensis	+	-	-	-	-	-
Bacillus sp	+	-	-	-	-	-
B. mojavensis	+	-	-	-	-	-
B. methylotrophicus	+	-	-	-	-	-
Lactobacillus sp	+	-	-	-	-	-
Aquamicrobium sp	+	+	-	-	-	-
B. aerius	+	-	-	-	-	+
Paenibacillus sp	+	-	-	+	-	+
Proteus vulgaris	+	-	-	-	+	+
Alcaligenes sp	+	+	+	-	-	-
B. amyloliquefaciens	+	+	+	+	+	-
B. cereus	+	-	-	+	-	-
B. clausii	+	-	-	-	-	-
B. aerophilus	+	+	+	+	-	+
L. sphaericus	+	-	-	+	+	+
L. xylanilyticus	+	+	+	+	+	+
Brevibacterium halotorens	+	+	-	+	+	+
Streptococccus agalactiae	+	-	-	-	-	-
Bacillus sp2	+	-	-	-	-	-
Endophytic bacterium	+	+	-	-	-	+
B. altitudinis	+	+	+	+	+	+
B. safensis	+	+	+	+	+	-
B. mycoides	+	+	-	+	+	-
Lysinibacillus sp	+	+	-	+	+	-

[a]onion, maize, cabbage, spinach, green peas, tomato, beetroot and lettuce

Key; +=Positive, -=Negative

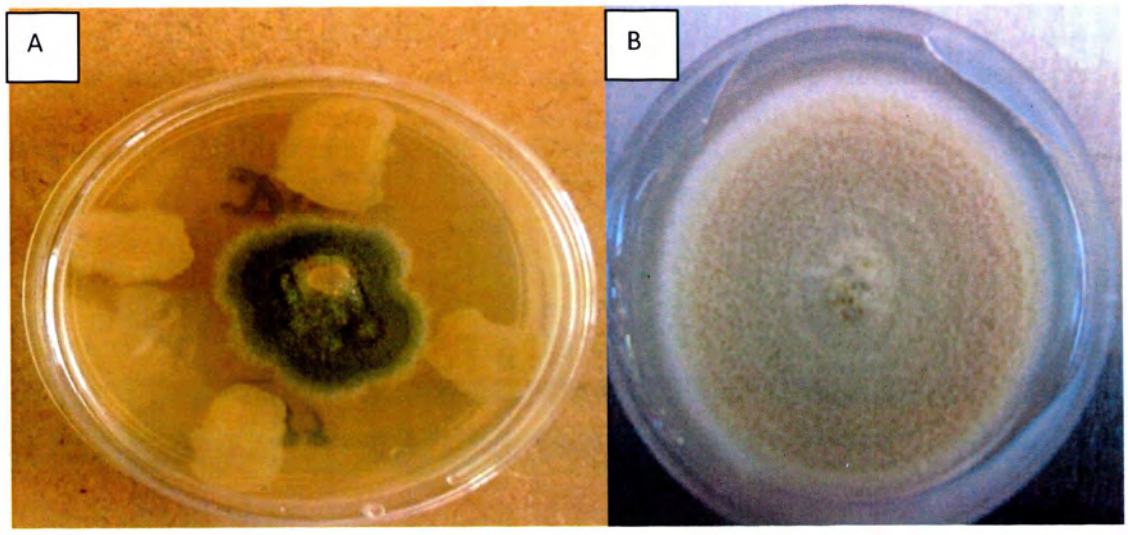

Figure 4.1: Bacterial antifungal activity in the potato dextrose agar. *F. solani* growth was completely inhibited in the presence of the bacteria streaked in the plates (A), as compared to the control (B), which had no bacteria.

4.4 16S rRNA gene amplification

Results presented in Figs: 4.1 and 4.2 indicate that 1500 bp ribosomal gene product was successfully amplified using the 16S ribosomal primers.

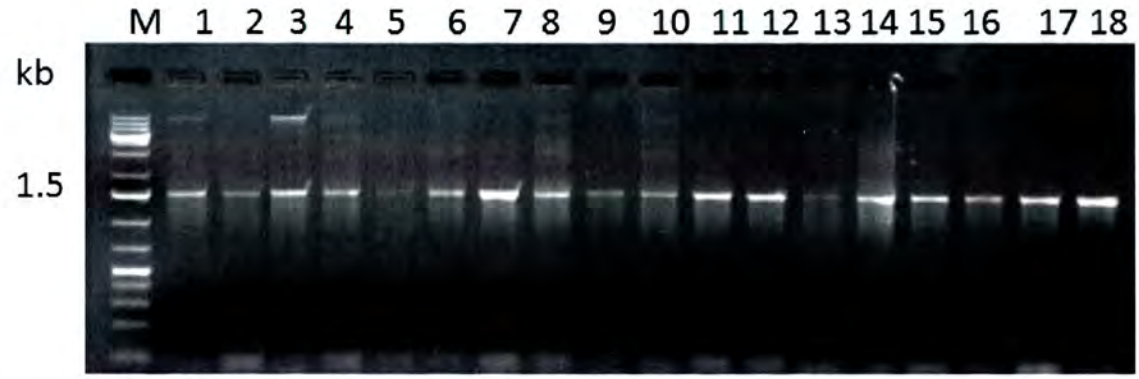

Figure 4.2: Ethidium bromide stained agarose gel (1%) showing PCR amplification of 16S rRNA of bacterial isolates obtained from the farming sites of Mafikeng. M= DNA marker (1.5 Kb); Lanes 1-4; =PCR amplification of the 16 rRNA gene fragments from isolates obtained from soil samples in the rhizosphere of cabbage; Lanes 5-7; = rhizosphere of maize1, Lanes 8-11; = rhizosphere of maize2, Lanes 12-15; = rhizosphere of spinach. Lanes 16-18; = rhizosphere of green peas

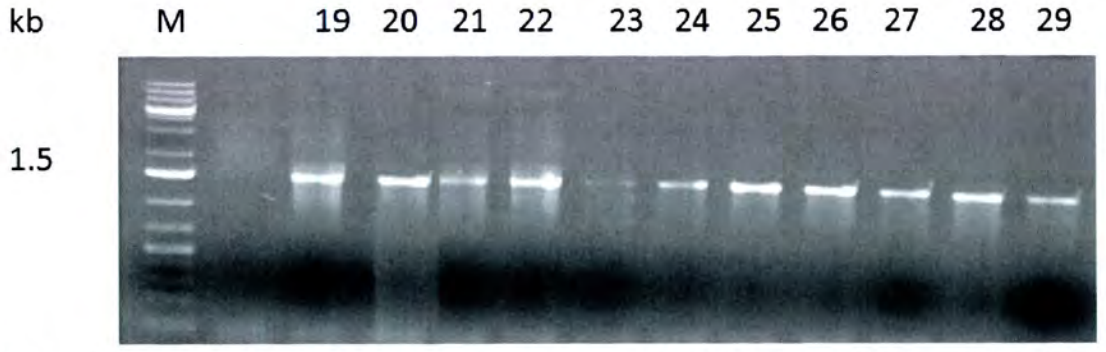

Figure 4.3: Ethidium bromide stained agarose gel (1%) showing PCR amplification of 16S rRNA of bacterial isolates obtained from the farming sites of Mafikeng. M= DNA marker (1.5 Kb); Lanes 19-21; = PCR amplification of the 16S rRNA gene fragments from isolates obtained from the rhizosphere of beetroot. Lanes 22-24 = rhizosphere of onion. Lanes 25-27; = rhizosphere of lettuce; Lanes 28-29; = rhizosphere of tomato

4.6.1 Phylogenetics analysis on the basis of 16S rRNA

A phylogenetic tree based on the 16S rRNA sequences of the total of 29 representative sequences was constructed. Evolutionary analyses by Neighbor-joining revealed the presence of groups that fell in several of the established bacterial groups. Evolutionary relationships of taxa derived from analysis of the 16S rRNA sequences of strains MR1-MR18 and MR20-MR30 using more than one related sequences obtained from the NCBI GenBank was conducted, to discover the evolutionary relationship among this group of organisms. Sequences of the phyla Firmicutes, Proteobacteria, gamma Proteobacteria and Actinobacteria were isolated and identified in this study. Seventy seven (77%) of the isolates were affiliated with the Firmicutes, while 15% of isolates were affiliated with the family Proteobacteria and 4% were affiliated with Actinobacteria. The phylum Firmicutes were closely related to the members of named and characterized genus *Bacillus*, while 5% were closely related with *Paenibacillus*. Evolutionary analyses by Neighbor-Joining method (Fig. 4.3) revealed the presence of groups that fell in several of the established bacteria; the results suggest the nucleotide sequences are highly diverse. *Clonopsis felicitatis* was selected as an outgroup which is a sequence not contained within the group under study but is closely related to the species. About 89-100% sequence identity was observed between the 16S rRNA gene sequences. The 16S rRNA genes were mostly related to 16S rRNA gene sequences obtained from the GenBank. MR1, MR5, MR16, MR9, MR8, MR12, MR10, MR3, MR28, MR7, MR25 and MR29 clustered with referenced *Bacillus* spp from the phylum Firmicutes sequences in the GenBank. MR23 and MR11 clustered with other referenced sequences in the GenBank related to the phylum Proteobacteria while MR23 branched with referenced sequences from the GenBank belonging to the phylum Actinobacteria. These suggest that the isolates have the same sequence from the GenBank because they clustered with the closely related organisms in the GenBank.

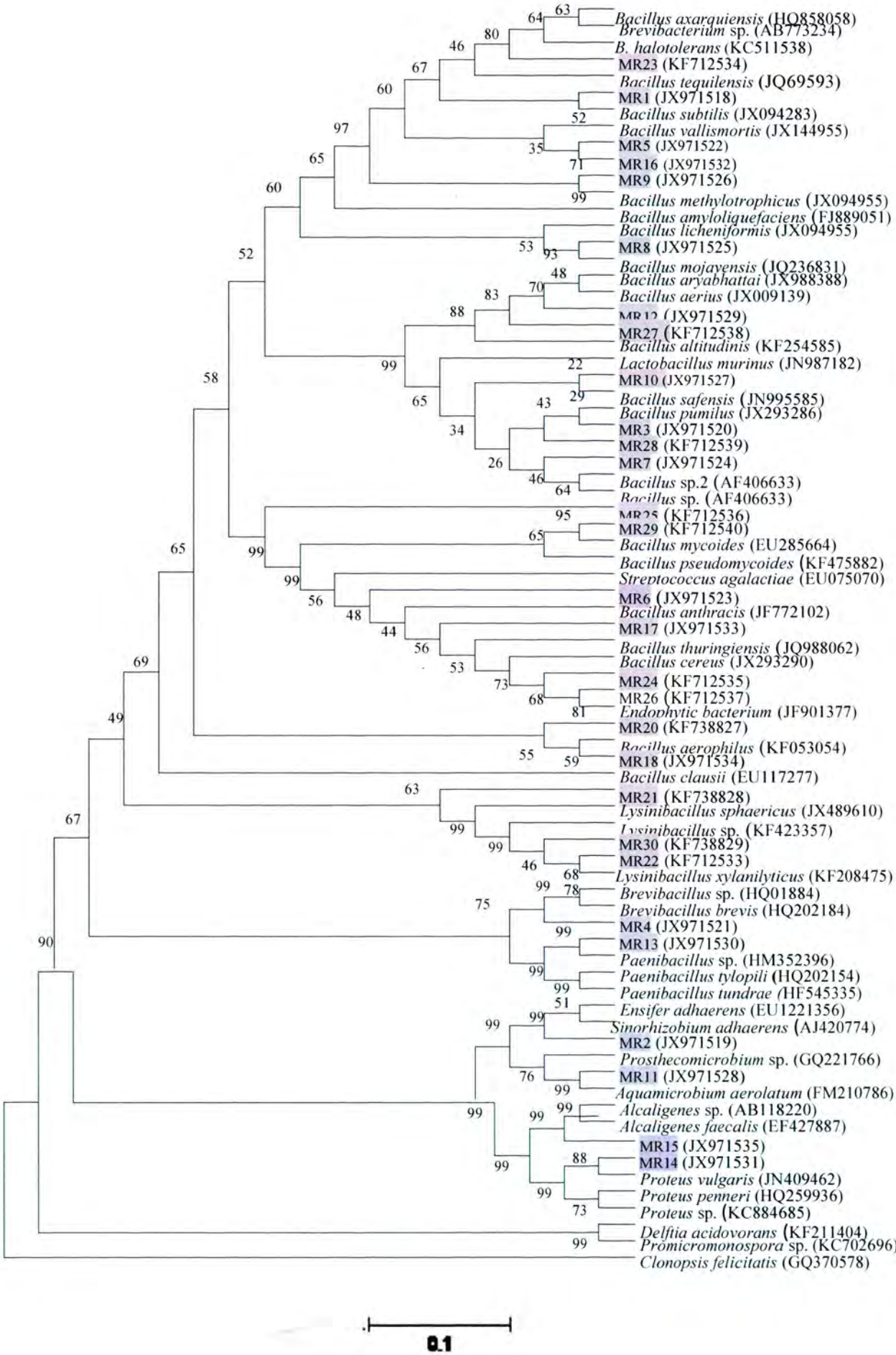
Bacillus axarquiensis (HQ858058)
Brevibacterium sp. (AB773234)
B. halotolerans (KC511538)
MR23 (KF712534)
Bacillus tequilensis (JQ69593)
MR1 (JX971518)
Bacillus subtilis (JX094283)
Bacillus vallismortis (JX144955)
MR5 (JX971522)
MR16 (JX971532)
MR9 (JX971526)
Bacillus methylotrophicus (JX094955)
Bacillus amyloliquefaciens (FJ889051)
Bacillus licheniformis (JX094955)
MR8 (JX971525)
Bacillus mojavensis (JQ236831)
Bacillus aryabhattai (JX988388)
Bacillus aerius (JX009139)
MR12 (JX971529)
MR27 (KF712538)
Bacillus altitudinis (KF254585)
Lactobacillus murinus (JN987182)
MR10 (JX971527)
Bacillus safensis (JN995585)
Bacillus pumilus (JX293286)
MR3 (JX971520)
MR28 (KF712539)
MR7 (JX971524)
Bacillus sp.2 (AF406633)
Bacillus sp. (AF406633)
MR25 (KF712536)
MR29 (KF712540)
Bacillus mycoides (EU285664)
Bacillus pseudomycoides (KF475882)
Streptococcus agalactiae (EU075070)
MR6 (JX971523)
Bacillus anthracis (JF772102)
MR17 (JX971533)
Bacillus thuringiensis (JQ988062)
Bacillus cereus (JX293290)
MR24 (KF712535)
MR26 (KF712537)
Endophytic bacterium (JF901377)
MR20 (KF738827)
Bacillus aerophilus (KF053054)
MR18 (JX971534)
Bacillus clausii (EU117277)
MR21 (KF738828)
Lysinibacillus sphaericus (JX489610)
Lysinibacillus sp. (KF423357)
MR30 (KF738829)
MR22 (KF712533)
Lysinibacillus xylanilyticus (KF208475)
Brevibacillus sp. (HQ01884)
Brevibacillus brevis (HQ202184)
MR4 (JX971521)
MR13 (JX971530)
Paenibacillus sp. (HM352396)
Paenibacillus tylopili (HQ202154)
Paenibacillus tundrae (HF545335)
Ensifer adhaerens (EU1221356)
Sinorhizobium adhaerens (AJ420774)
MR2 (JX971519)
Prosthecomicrobium sp. (GQ221766)
MR11 (JX971528)
Aquamicrobium aerolatum (FM210786)
Alcaligenes sp. (AB118220)
Alcaligenes faecalis (EF427887)
MR15 (JX971535)
MR14 (JX971531)
Proteus vulgaris (JN409462)
Proteus penneri (HQ259936)
Proteus sp. (KC884685)
Delftia acidovorans (KF211404)
Promicromonospora sp. (KC702696)
Clonopsis felicitatis (GQ370578)
0.1

Figure 4.4: The Phylogenetic tree of culture dependent 16S rRNA from rhizosphere using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method; Evolutionary analyses were conducted in MEGA 5.22

4.7 Culture-independent techniques

4.7.1 16S rRNA gene amplification

Results presented in Fig. 4.4 indicate that 500 bp ribosomal of rRNA gene product was successfully amplified using the GC clamped forward primer f357 and r518.

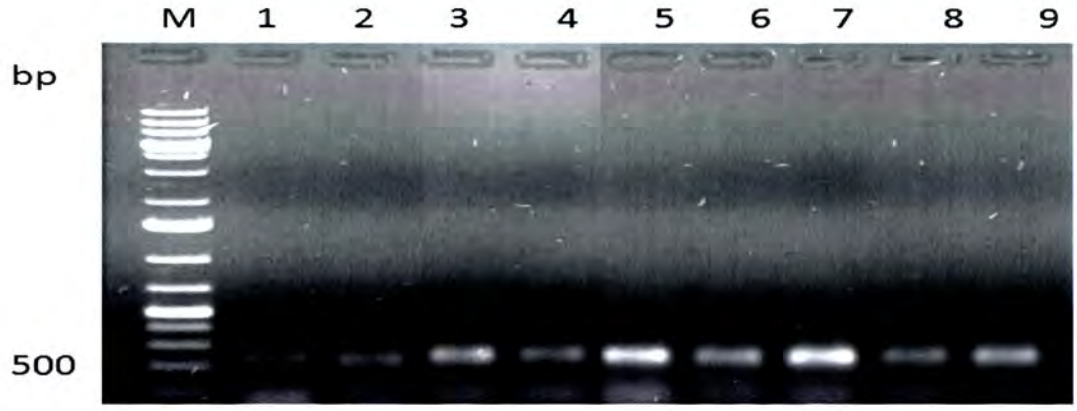

Figure 4.5: Ethidium bromide stained agarose gel (2%) showing PCR amplification of 16S rRNA gene of bacterial isolates obtained from the farming sites of Mafikeng. M= DNA marker (500 bp); Lanes 2; = rhizosphere of cabbage; Lanes 3; = rhizosphere of tomato, Lanes 4; = rhizosphere of green peas, Lanes 5; = rhizosphere of beetroot, Lanes 6; = rhizosphere of maize1, Lanes 7; = rhizosphere of maize 2, Lanes 8; = rhizosphere of onion, Lanes 9; = rhizosphere of spinach

4.7.2 PCR-DGGE

Different 16S rRNA gene band patterns were obtained from soil samples by PCR-DGGE. The amplifiable DNA by 16S rRNA gene-based bacterial primers was obtained from the rhizosphere of the cultivated plants. The 16S rRNA gene amplicons generated from the field soil samples were then run using DGGE. A total of 8 bands that were prominent in the gel were excised and sequenced. Fig 4.5 shows the DGGE patterns of PCR products from the rhizosphere of cultivated plants. Profiling a number of bacterial types detectable by DGGE was observed with consistent concentration of amplicons in a few bands. The soil samples 1-9 had similar profiles, with about 5-10 dominant bacterial types per lane. Three strong bands were present in the DGGE profiles of all samples, and strongly dominated the profiles of the rhizosphere of cultivated plants.

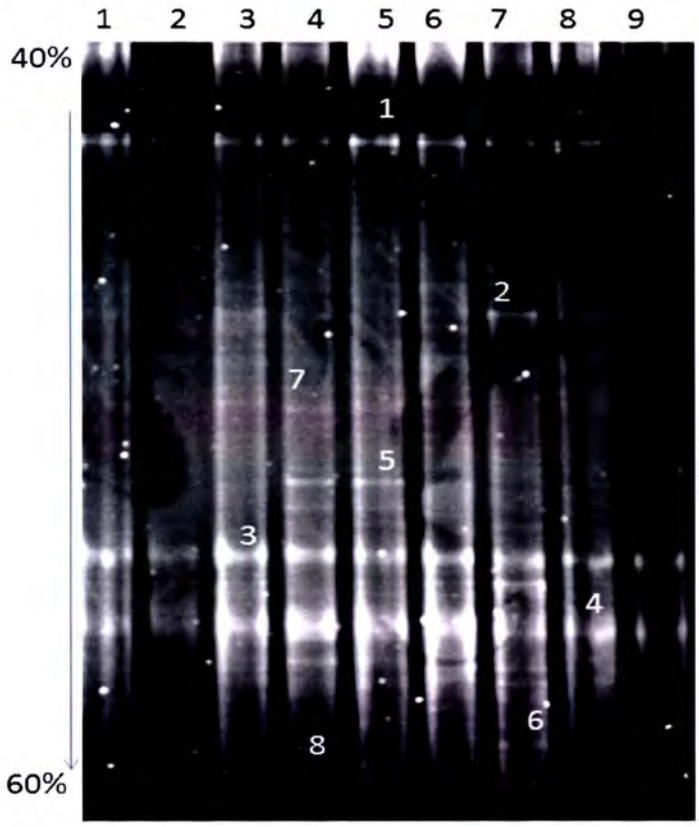

Figure 4.6: 16S rRNA gene PCR-DGGE patterns of 16S ribosomal DNA (rRNA) fragments profiles of rhizosphere bacteria on a polyacrylamide gel with a 40-60% denaturation gradient of Urea-Formamide DGGE patterns fragments from samples of rhizosphere plants collected at different crop plants. Lanes 1-9, represents bacterial community of the rhizosphere of maize1, spinach, tomato, cabbage, maize2, lettuce, onion, beetroot and green peas respectively and numbers 1-8 indicate the excised bands sent for sequencing

4.7.3 PCR Amplification of 200 bp rRNA gene

Results presented in Fig 4.6 indicate that 200 bp ribosomal gene product was successfully amplified using the 16S ribosomal primers f357 and r518.

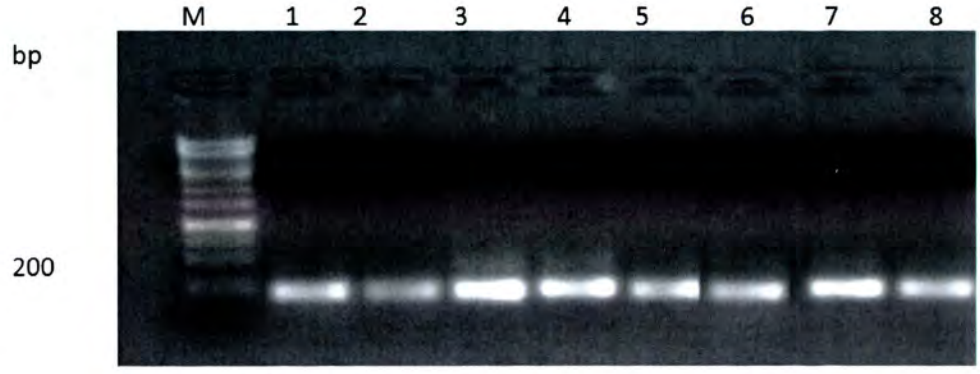

Figure 4.7: Amplified 200 bp ribosomal gene product from excised bands on the DGGE profile, Lanes 1-8; =PCR amplification of the fragments from isolates excised from DGGE gel

4.7.4 Sequence analysis of bands excised from DGGE gels

The DNA bands that were closely related to the individual principal components are presented in Table 4.5. Taxonomic names were estimated for the eight bands through verification of the sequencing results with BLAST data. DGGE bands 1-8 were closely related to uncultured *Bacillus* sp, *Bacillus megaterium*, uncultured delta proteobacterium, *Cohnella* sp, uncultured *Rubrobacter* sp, uncultured Rhizobiales bacterium and uncultured Soil bacterium respectively with a percentage similarity ranging from 80% to 95%.

Table 4.5: Sequence analysis of bands excised from DGGE gels derived from bacterial 16S rRNA extracted from rhizosphere samples of cultivated plants of Mafikeng

Isolate code	Band(s)	Mostly related bacterial sequence	% identity	Accession no
K1	1	Uncultured *Bacillus* sp.	94%	KF504397
K2	2	*Bacillus megaterium*	97%	KF358455
K3	3	Uncultured Delta proteobacterium	86%	JN596749
K4	4	Uncultured Bacterium	80%	HF565418
K5	5	*Cohnella* sp.	84%	AM900791
K6	6	Uncultured *Rubrobacter* sp.	90%	JF706679
K7	7	Uncultured Rhizobiales bacterium	95%	KF509797
K8	8	Uncultured Soil bacterium	87%	HM164438

4.7.8 Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA5.22 (Tamura *et al*., 2011). Excised bands K1, K3, K4, K6 and K8 showed maximum similarity with sequences of uncultured strains in the database, the majority of the uncultured strains belonged to the phylotype Firmicutes followed by the Proteobacteria then Actinobacteria.

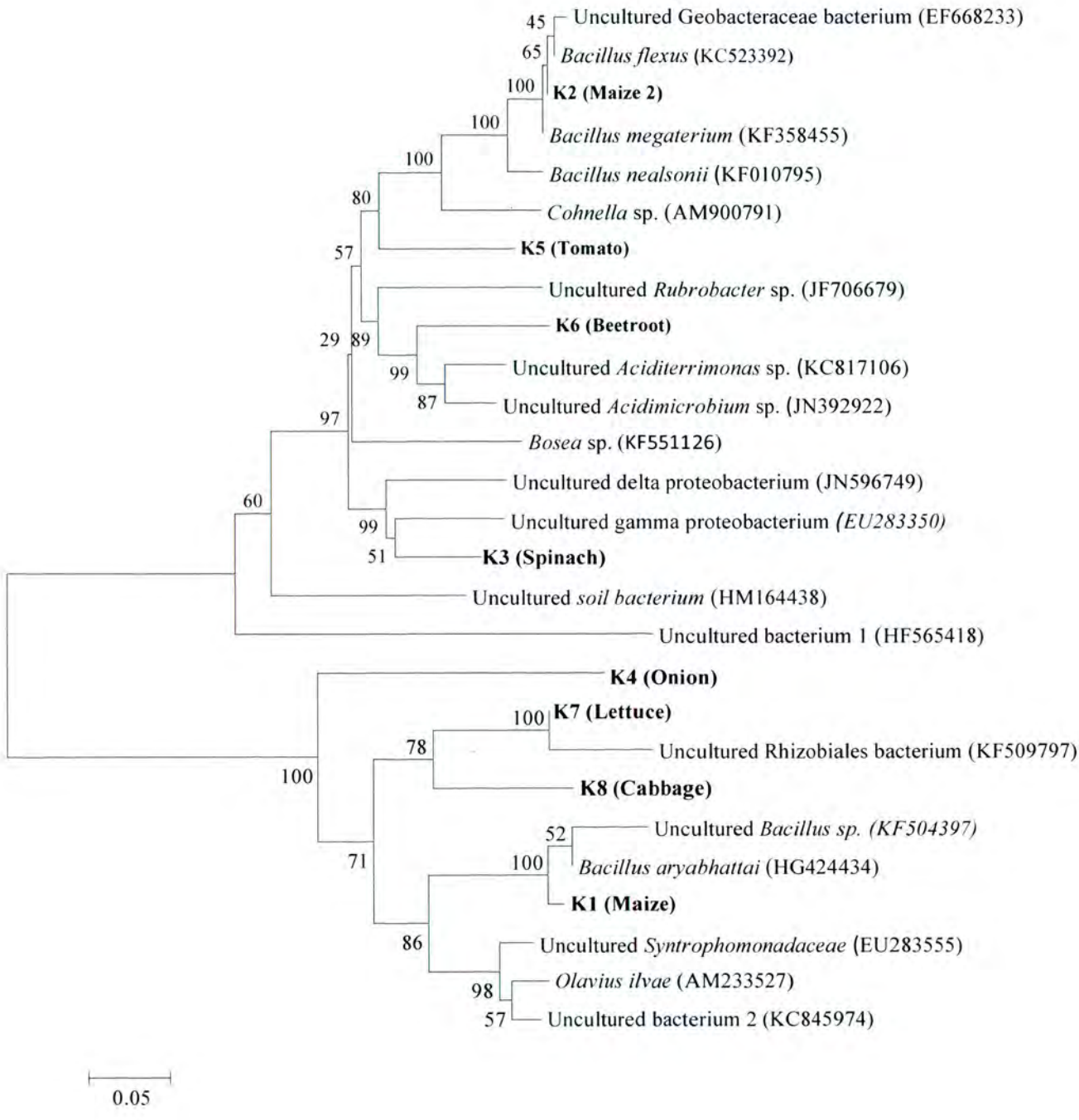

Figure 4.8: The Phylogenetic tree of culture- independent of 16S rRNA of soil rhizosphere bacteria fron North-West using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method; Evolutionary analyses were conducted in MEGA 5.2

4.8 *In vivo* studies of plant growth-promoting bacteria

4.8.1 Pot experiment of tomato

The selected strains (MR3, MR7, MR16 and MR22) were evaluated in a plant growth promotion experiment with tomato in a screenhouse (Fig 4.8). The inoculation of tomato with MR16 bacterial strain resulted in a significant increase in root length, shoot length, dry weight and fresh weight when compared with control and other bacterial treatments. The exception was with MR22 and MR3 because they did not promote growth significantly with respect to other treatments at $P<0.05$.

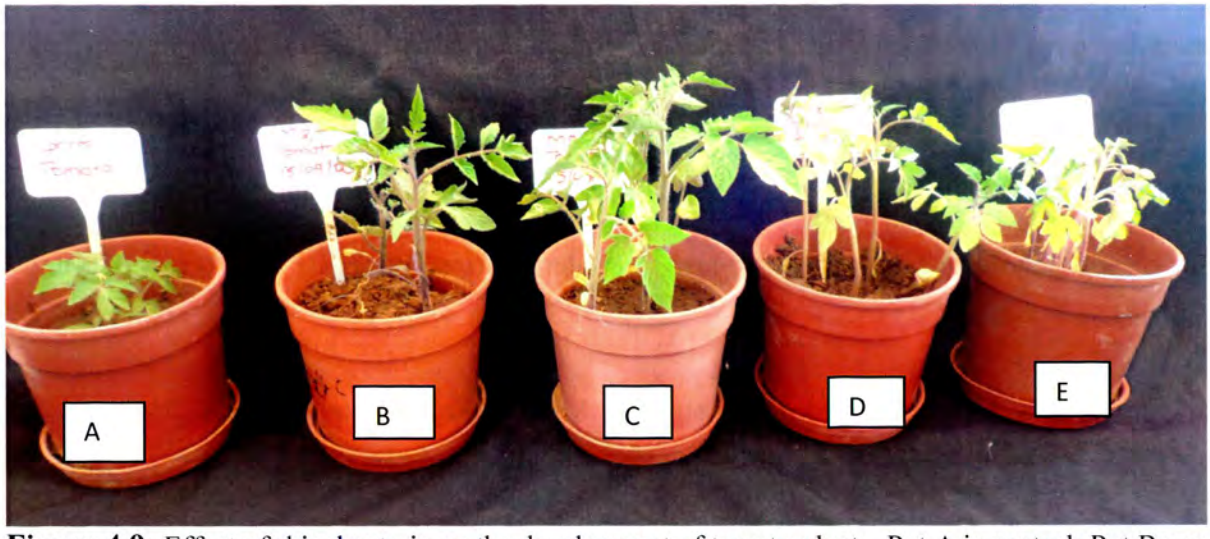

Figure 4.9: Effect of rhizobacteria on the development of tomato plants. Pot A is control; Pot B was treated with MR3-*B. pumilus*, Pot C was treated with MR16- *B. amyloliquefaciens*, Pot D was treated with MR22-*L. xylanilyticus*, Pot E was treated with MR7-*Bacillus* sp

4.8.2 Statistical analysis

Plant growth parameters show the potential of different bacterial isolates to promote plant growth of tomato seeds. The inoculation of tomato resulted in a significant increase in root length, shoot length, number of leaves, dry weight and fresh weight when compared to the control (Figs. 4.9-4.13). Both the root and shoot length significantly improved due to the inoculation with *Bacillus amyloliquefaciens* (MR16) when compared to the control, *Bacillus pumilus* (MR3) was at par with the control in respect to root length but had a significant difference $P<0.05$ in relation to the shoot length. All the inoculants had a signilficant difference on the number of leaves when compared with the control. MR22, MR16 and MR7 bacterial inoculants had a significant difference on the fresh weight except that *Bacillus pumilus* (MR3) was at par with the control in respect to dry weight. The treatment of tomato crops with the bacterial inoculants promoted plant growth in terms of increased shoot length (8.1±9.5 cm), root length (5.2±11.9 cm) number of leaves (8.3±9.3), fresh weight (1.69±3.2 g) and dry weight (0.46±1.9 g) at $P<0.05$.

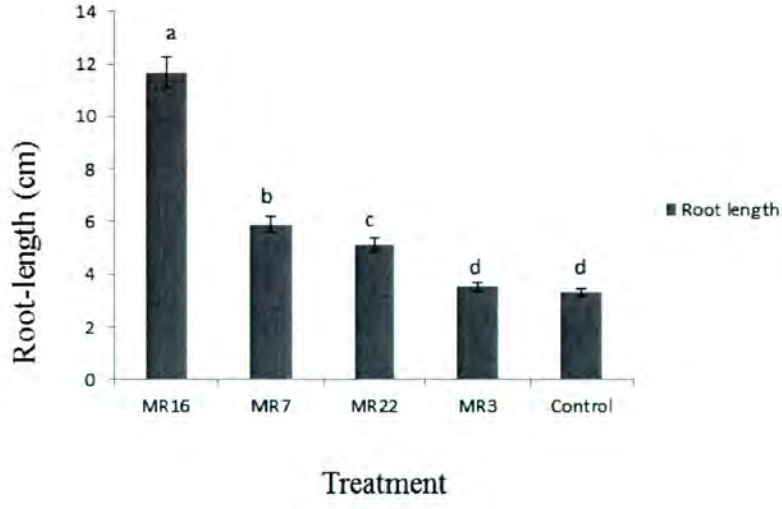

Figure 4.10: Effects of rhizosphere isolates on tomato leaf development of different treatments (MR16, MR7, MR22 and MR3) in tomato looking at root length 30 days after sowing. The bars with similar letters are not significantly different at $P\leq0.05$

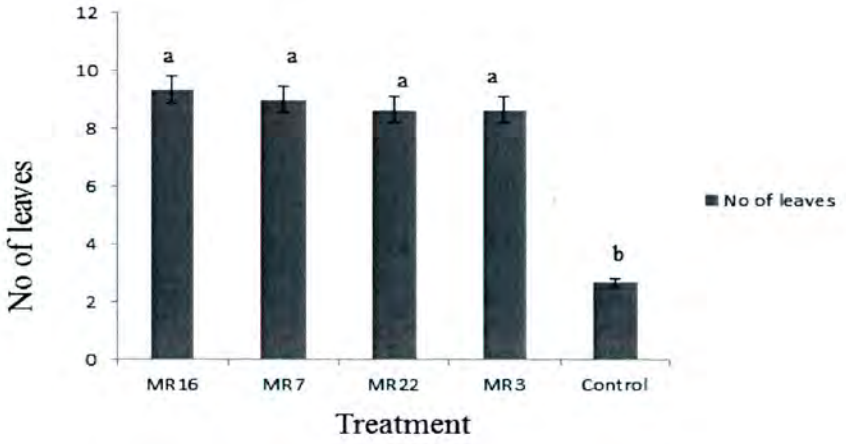

Figure 4.11: Effects of rhizosphere isolates on tomato leaf development of three replicates of different treatments (MR16, MR7, MR22 and MR3) in tomato looking at no of leaves parameter 30 days after sowing. The bars with similar letters are not significantly different at $P \leq 0.05$

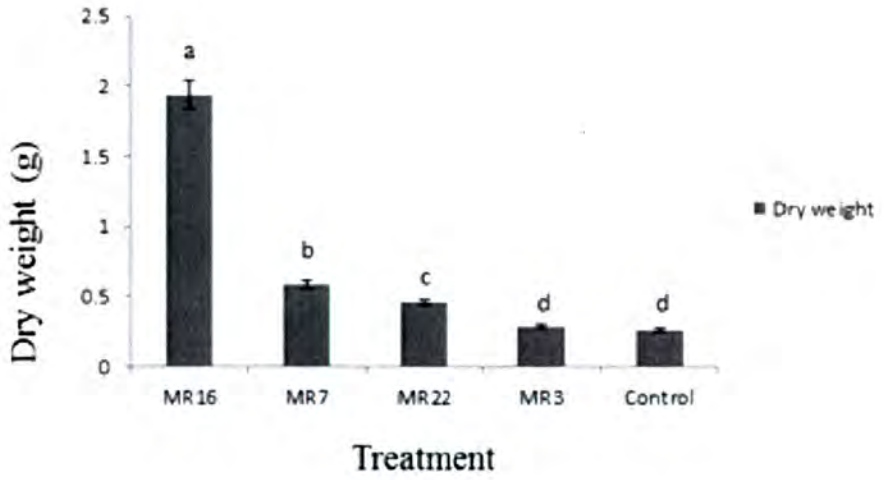

Figure 4.12: Effects of rhizosphere isolates on tomato leaf development of three replicates of different treatments (MR16, MR7, MR22 and MR3) in tomato looking at dry weight parameter 30 days after sowing. The bars with similar letters are not significantly different at $P \leq 0.05$

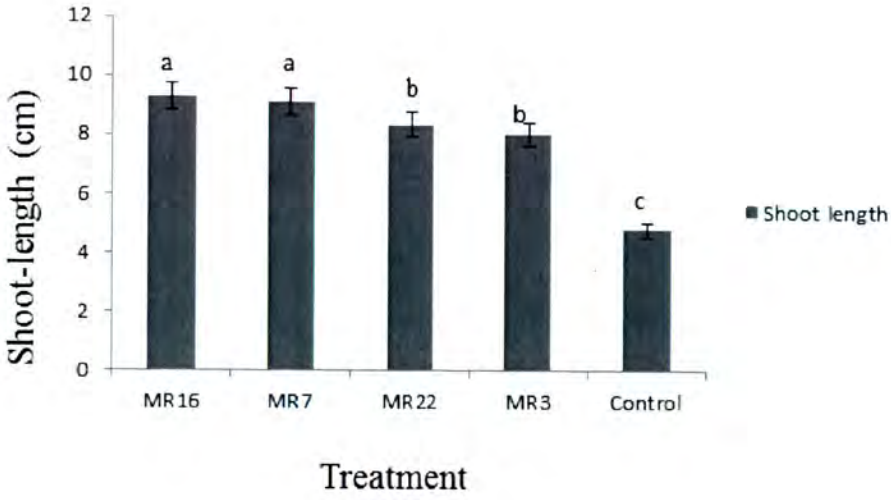

Figure 4.13: Effects of rhizosphere isolates on tomato leaf development of three replicates of different treatments (MR16, MR7, MR22 and MR3) in tomato looking at shoot length parameter 30 days after sowing. The bars with similar letters are not significantly different at $P \leq 0.05$

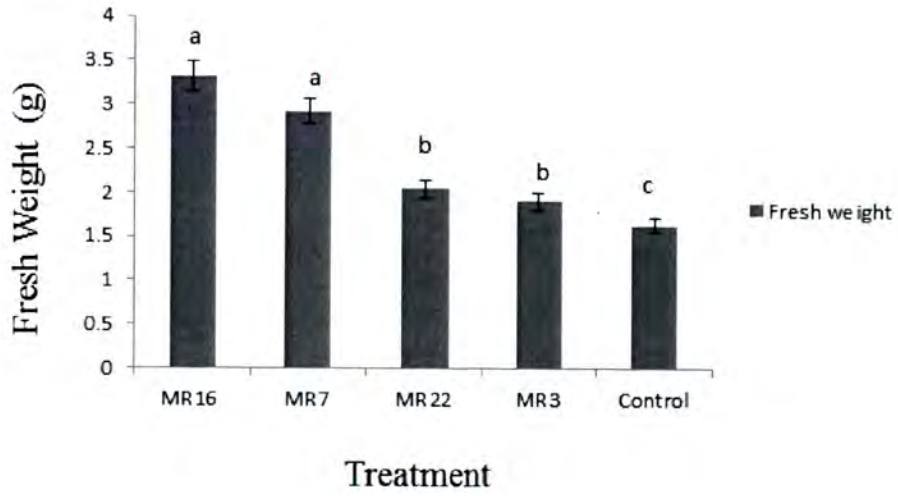

Figure 4.14: Effects of rhizosphere isolates on tomato leaf development of three replicates of different treatments (MR16, MR7, MR22 and MR3) in tomato looking at fresh weight parameter 30 days after sowing. The bars with similar letters are not significantly different at $P \leq 0.05$

4.8.4 Pot experiments of spinach

The selected strains (MR3, MR7, MR16 and MR22) were evaluated in a plant growth promotion experiment with spinach in a screenhouse (Fig. 4.14). The inoculation of spinach with MR16 bacterial strain resulted in a significant increase in root length, shoot length, dry weight and fresh weight when compared with control and other bacterial treatments. The exception was with MR22 and MR3 because they did not promote growth significantly with respect to other treatments.

Figure 4.15: Effect of rhizobacteria on the development of spinach plants. Pot A is control; Pot B was treated with MR16- *B. amyloliquefaciens*, Pot C was treated with MR7- *Bacillus* sp, Pot D was treated with MR22-*L. xylanilyticus*, Pot E was treated with MR3-*Bacillus pumilus*

4.8.5 Statistical analysis

The PGPR treatment led to a significant increase in plant growth as measured by the shoot and root length, number of leaves and dry and fresh weight of spinach (Figs. 4.15-4.19) Significant root growth was observed in treatment/inoculant with *Bacillus amyloliquefaciens* (MR16) when compared to the control and a significant increase in the shoot length was observed with the treatment *Bacillus pumilus* (MR3). *Bacillus* sp. (MR7) and *L. xylanilyticus*

(MR22) did not show any significant difference with respective to each other regarding the root length. The number of leaves significantly increased with the inoculation of *Bacillus amyloliquefaciens* (MR16) comparing to other treatments and the control. All the treatments had a significant difference on the number of leaves with regard to the control. The significant increase in the both dry weight and fresh weight was observed in *Bacillus amyloliquefaciens* (MR16) compared to other treatments and control. The treatment of spinach crops with the bacterial inoculants promoted plant growth in terms of increased shoot length (5±8.9cm), root length (6±10.9 cm), number of leaves (7.9±9.33), fresh weight (0.7±3.32 g) and dry weight (0.5±1.95 g) at $P<0.05$.

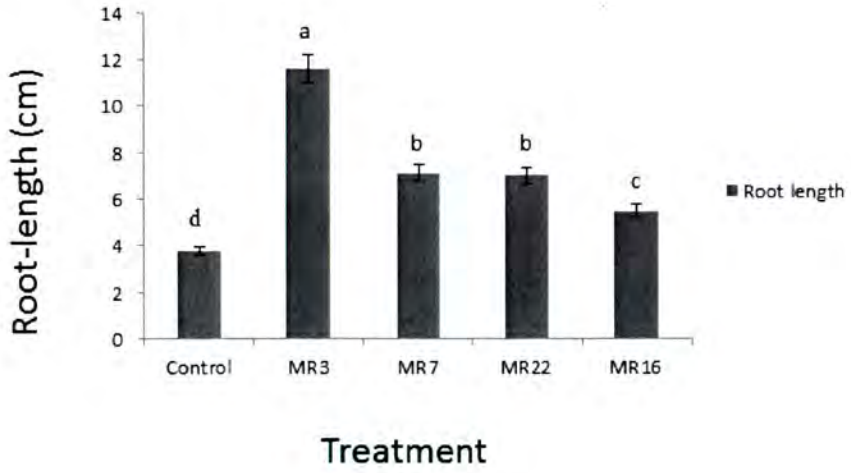

Figure 4.16 Effects of rhizobacteria on the development of roots of different treatments (MR16, MR7, MR22 and MR3) in spinach looking at root length parameter 30 days after sowing. The bars with similar letters are not significantly different at $P\leq0.05$

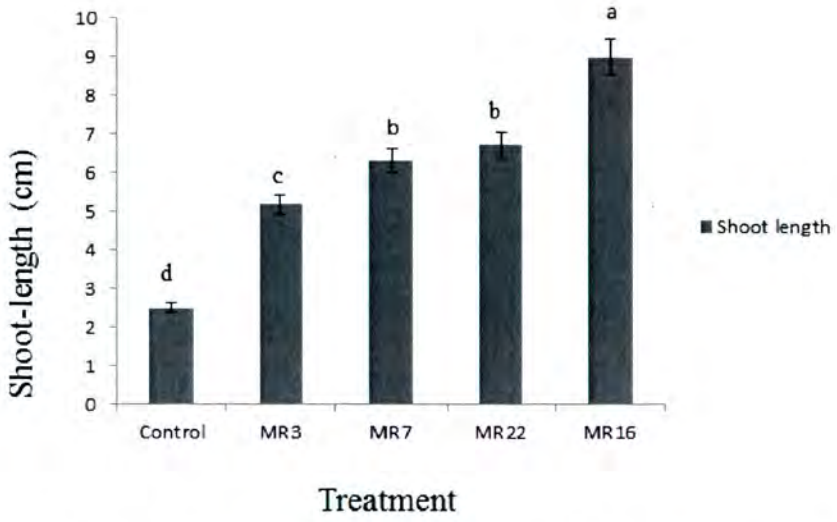

Figure 4.17: Effects of rhizobacteria on the development of roots of different treatments (MR16, MR7, MR22 and MR3) in spinach looking at shoot length weight parameters 30 days after sowing. The bars with similar letters are not significantly different at P≤0.05

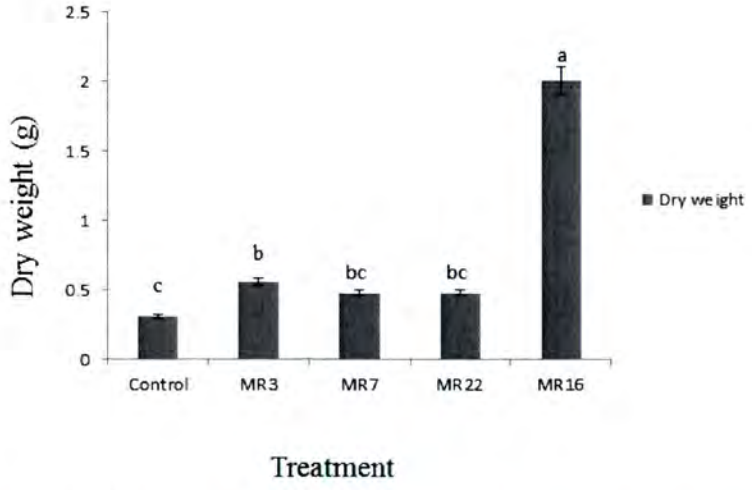

Figure 4.18: Effects of rhizobacteria on the development of roots of different treatments of different treatments (MR16, MR7, MR22 and MR3) in spinach looking at dry weight parameters 30 days after sowing. The bars with similar letters are not significantly different at P≤0.05

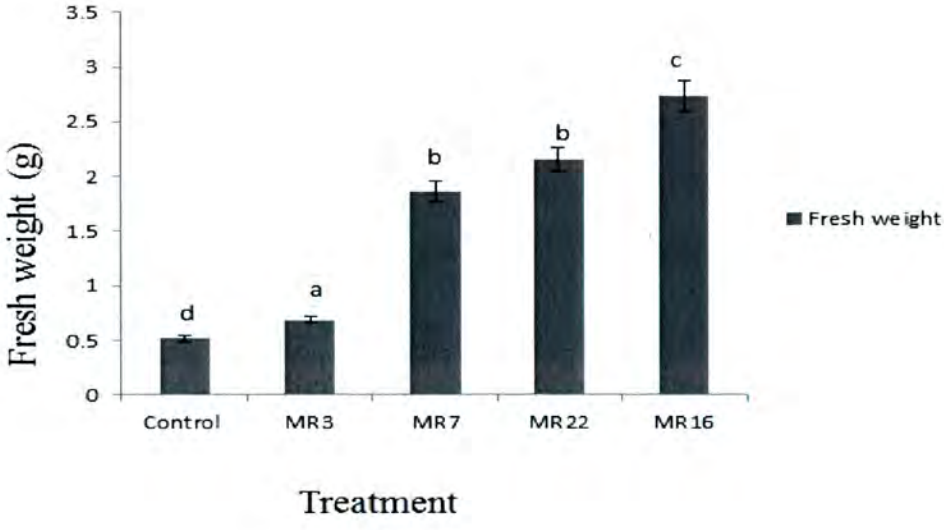

Figure 4.19: Effects of rhizobacteria on the development of roots of different treatments of different treatments (MR16, MR7, MR22 and MR3) in spinach looking at shoot length, root length, no of leaves, dry weight and fresh weight parameters 30 days after sowing. The bars with similar letters are not significantly different at $P \leq 0.05$

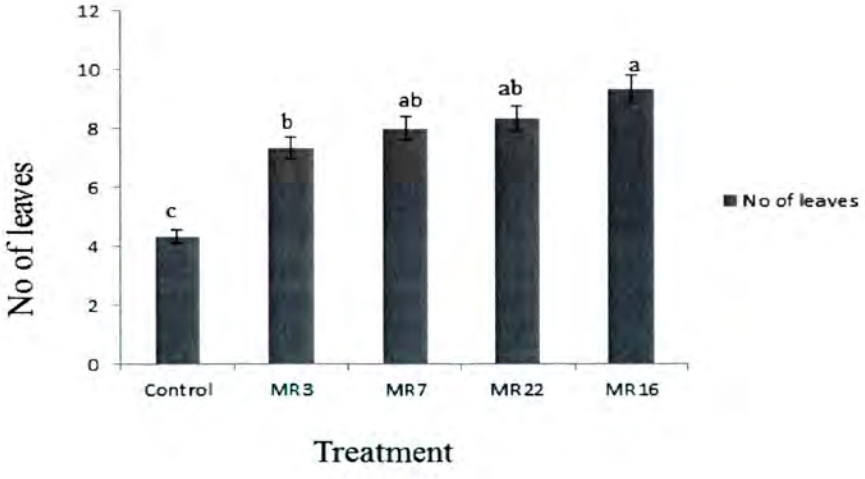

Figure 4.20: Effects of rhizobacteria on the development of roots of different treatments of three replicates of different treatments (MR16, MR7, and MR22) in spinach looking at no of leaves parameter 30 days after sowing. The bars with similar letters are not significantly different at $P \leq 0.05$

CHAPTER 5
DISCUSSION

Bacteria associated with the rhizosphere are fundamentally important in growth promotion, for this reason there have been considerable interest in characterizing the diversity of rhizosphere soil bacteria. Analysis of the bacterial communities present in the rhizosphere of onion, maize, cabbage, lettuce, tomato, beetroot, green peas and spinach was addressed by both culture-dependent and culture-independent molecular methods. The pH value of the soil samples measured was found to be alkaline which correlated with literature (Rousk *et al.*, 2010) that the neutral to slightly alkaline pH is good for microbial existence and microbial activities such as enzymatic activities. The pH affects ionization and the binding and interaction of molecular processes. It also affects the solubility of many substances that the bacteria need. Furthermore, it has been reported that soil properties such as pH, can directly or indirectly affect rhizosphere communities (Garbeva *et al.*, 2006).

Calcium, magnesium and potassium measured in this study showed elevated concentrations, and according to Ashman and Puri (2002), a soil with pH range of 6.5-9.5 will have high concentrations of Ca, Mg and K as compared to an acidic soil (4.0-5.5), which they reported as being a characteristic of agricultural soil. Chemical properties are appropriate indicators of soil quality and the growth of microorganisms (Fierer *et al.*, 2007). Phosphate was not detected in Mm2 and according to Zhong and Cai (2007) phosphate deficiency directly affects the growth of culturable bacteria resulting in less pronounced growth of microorganisms. Most of the soil processes such as biological pumping of nutrient and nutrient uptake by roots are more strongly expressed in the rhizosphere. The soil chemical properties measured in this work were indicative of the pool of available nutrients at the

individual level. The values of the heavy metals were well below the required standards according to the national soil environmental quality standards concentration of elements in agricultural soil samples and therefore conformed to the required standards. The required standards for cadmium, zinc, chromium and lead in agricultural soil are 7.5, 240, 46,000 and 20 $mgkg^{-1}$ respectively according to the Department of Environmental Affairs (South Africa), 2008.

The population study revealed that the samples were rich in microbial diversity. Increased microbial activity was expected as there is a higher concentration of microorganisms in the rhizosphere than in a soil away from the plant and the increase is more pronounced with bacteria (Singh and Mukerji, 2006). The rhizosphere is considered to be a hotspot of bacterial diversity, it harbours bacterial flora whose diversity is mainly expressed in terms of functions adapted to root presence and in particular to favour plant growth (Rawat *et al.* 2011). The poor yield of cabbage, tomato and spinach soil samples could be attributed to the inability of bacterial cells to move toward roots in response to root exudates as explained by the study conducted by Dennis *et al.* (2010).

Bacterial diversity detected from Mafikeng, were predominated by Gram-positive, rod-shaped, aerobic and spore-forming organisms of the genus *Bacillus* which are most widely represented in the soil. This is due to their ability to form spores and withstand a range of variable environmental conditions such as low nutrient availability, desiccation and chemical disinfection (Babalola and Akindolire, 2011; Parvathi *et al.*, 2009). Isolation of *Bacillus* also has been reported earlier from various crops plants (Vessey, 2003; Minkwitz and Berg, 2001; Chan e*t al.*, 1994)

The plant rhizosphere is a preferential niche for various types of microorganisms in the soil. Some of the isolated bacteria were able to produce IAA, *Bacillus* spp, *Paenibacillus* and *Proteus vulgaris* isolated from different crops in this study had a great potential to synthesize IAA. The common traits of PGPR include production of plant growth regulators which include indole acetic acid a product of tryptophan metabolism that stimulate plant growth (Cassán *et al.*, 2013).

Soil microorganisms play an important role in making phosphorus available to plants by mineralizing the organic phosphorus in the soil (Khan *et al.*, 2009; Ryu *et al.*, 2003). Different *Bacillus* spp, *Paenibacillus* and *Ensifer adhaerens* showed the ability to solubilize phosphate. Earlier reports showed that some strains of *B. subtilis* and *B. amyloliquefaciens* produced certain volatile compounds such as 2-3, butanediol and acetoin that stimulated plant growth (Ryu *et al.*, 2003).

All bacterial strains were found to be able to synthesize ammonia. Ammonium and nitrate are believed to be the principal sources of nitrogen for plant growth in agricultural and most natural environments. They are required in greater amounts than any other mineral nutrient (Howitt and Udvardi, 2000). Production of ammonia is explained by symbiotic nitrogen fixing bacterial species which are able to specifically interact with host plants and convert atmospheric nitrogen to ammonia (Hardoim *et al.*, 2008).

Bacillus spp showed production of ACC deaminase, these results are consistent with a previous study conducted by Sgroy *et al.* (2009) where ACC deaminase production was more pronounced in the *Bacillus* spp. ACC deaminase reduces the potentially inhibitory effects of high ethylene concentrations (Glick *et al.*, 1998), which is of extreme importance when plants

are exposed to stressful conditions such as heavy metal contamination of the soil (Grichko *et al.*, 2000). According to the report of authors such as Shaharoona *et al.* (2006), this influence is real for other plants.

The genus *Fusarium* comprises a wide and heterogeneous group of fungi that causes economically harmful diseases in many crops, such as soybean, tobacco and common beans reducing both the quality and the quantity of their products (Matarese *et al.*, 2012). *Fusarium solani* is a well-known soil-borne fungus and some of its strains are pathogenic to plants and are difficult to control. However, biological methods may be a reliable alternative to chemical methods for controlling soil-borne fungal growth. For applications in agriculture, *Bacillus* species are considered important biological control agents (Yuan *et al.*, 2012). *B. amyloliquefaciens* isolated from the rhizosphere soil, was found to efficiently antagonise *F. oxysporum* (Yuan *et al.*, 2012). Bacterial isolate, *B. amyloliquefaciens* inhibited the growth and spore germination of *Fusarium solani* in this study as a result of the production of volatile compounds (Acetylaldehyde, Methanethiol, Ethanol and Phenyl Isothiocyanate (Xu *et al.*, 2009). The release of volatile compounds by soil microbes has been described to promote plant growth (Ryu *et al.*, 2003). *Bacillus* bacterial isolates were found to be highly inhibitory of *F. solani* growth whereas others showed mild activity or no activity at all. This suggested that the mode of action exerted and the type of antifungal metabolites produced by the isolates varied (Williams and Asher, 1996). Reduction of fungal growth by certain PGPR and formation of inhibition zones were due to the materials (antifungal substances and/or cell wall degrading enzymes) released by the bacteria into the culture medium (Suslow and Schroth, 2009). Applications of bacteria to plants resulted in significant growth measured in terms of root and shoot length, fresh and dry weights in potted plants. In this study, *Bacillus* spp significantly promoted growth of both spinach and tomato. The ability of *Bacillus* spp to

promote growth in plants was previously reported by Marulanda-Aguirre *et al.* (2008). An increase in root growth by the application of PGPR has also been reported by Hall *et al.* (1996). The improved root growth is important due to the resulting increase in the volume of soil explored. For example, treatment of clipped soybean with *Azospirillum brasilense* caused a 63% increase in root dry mass and resulted in more than 10 fold increase in total root length in a study conducted by Molla *et al.* (2001). The magnitude of growth promotion varied among the strains in this study, as *B. amyloliquefaciens* had significantly higher ($P<0.05$) plant growth-promoting capabilities compared to the other strains. In one study, some bacilli PGPR strains promoted growth of maize seedlings through the production of extracellular phytase, degrading phytate under conditions of limited phosphate availability (Idriss *et al.*, 2002). Several studies also reported that the production of phytohormone is implicated in the plant growth promoted by PGPR and are believed to increase root growth and root length resulting in greater root surface area which enables the plants to access nutrients from the soil (Beneduzi *et al.*, 2008). However enhanced growth is dependent on the strain, method of application and amount of inoculum used (Nandakumar *et al.*, 2001).

From the analyses of the 29 amplicons, the soil samples were found to be phylogenetically diverse, with representatives from 3 different groups of bacteria. The majority of the amplicons had sequences with 80-100% similarity to the sequences obtained from the current database. These results suggest that these amplicons are diverse in phylogeny. Different diversity and phylogenetic analyses supported the hypothesis that bacterial communities associated with the rhizosphere of soils are different but share some genera in common, such as *Bacillus*. BLAST analysis of partial 16S rRNA gene sequences showed that the isolates were closely affiliated with members of the genus *Bacillus*. A higher percentage of bacteria affiliating with the Firmicutes, indicates that Firmicutes are considered to be a dominant

bacterial family in the soil tested by molecular methods, because Firmicutes are characterized by the formation of heat and desiccation resistant spores thus explaining the lower percentage of Proteobacteria and Actinobacteria. Microbial diversities have been analysed in Wisconsin soil using sequence based method and according to findings by Borneman *et al.*, (1996), our results are very different from their findings, because the majority of the sequences that we obtained were from Firmicutes group, followed by the Proteobacteria and Actinobacteria. Contrary to the Winconsin soil, most of the phylotypes in the soil sample were members of Proteobacteria (60.5%) and Fibrobacter (16%). Such differences can most likely be explained by differences in temperature, moisture, pH and vegetation.

The homology tree based on sequence alignment of the isolated bacterial 16S rRNA gene in this study permitted rapid phylogenetic analysis. However, strains isolated from different geographic locations shared similar DNA homology. Phylogenetic analysis on the basis of 16S rRNA gene sequence provided better understanding in evaluation of genetic diversity of the bacteria. Phylogenetic analysis of 16S rRNA gene has shown the existence of wide bacterial diversity (Clarridge III, 2004).

The partial 16S rRNA gene sequences (1500 bp) of these strains revealed 80-100% similarity with *Bacillus*, *Proteus*, *Rhizobium*, *Aquamicrobium* and *Brevibacterium* 16S rRNA gene sequences in the GenBank database. Thus, a combination of conventional test and genetic analysis enabled identification of rhizosphere bacteria and it has led to a significantly better understanding of the bacterial community structure, phylogeny and function in the rhizosphere from the Mafikeng soil.

Both biochemical characterization and molecular methods were carried out to determine the conformity between these two methods. The methods were reliable, however distinguishing *Bacillus* species from each other using the classical biochemical test is difficult because of the fact that most *Bacillus* species only differ by one biochemical property and it makes the biochemical identification at the species level difficult. For instance, *B. subtilis* and *B. pumilus* were only distinguished by their ability to hydrolyse starch (Thompson *et al.* 1998) and that can alter the identification of the isolate. Molecular methods were found in this study to be an excellent alternative to classical biochemical identification procedures. The dominance of gram positive spore forming *Bacillus* sp. was observed using both methods; however a shift towards the use of molecular methods is needed in order to provide a more robust classification and differentiation (McCartney, 2002).

Culture -independent DGGE fingerprinting of PCR-amplified 16S rRNA genes was used to study bacterial populations in the rhizosphere. Sequencing of DGGE bands revealed that the majority of dominant populations detected, had 16S rRNA gene sequences that were mostly related to those of previously described bacterial species. And a proportion of dominant populations in the rhizosphere belonging to Gram-positive bacteria, and interestingly the sequences of one dominate band from the rhizosphere shared a similarity of more than 97% with that of cultured isolate. There is increasing evidence that Gram-positive bacteria might be more dominant in the rhizosphere than previously supposed (Normander and Prosser, 2000). *Bacillus* species were found to be the dominant population in the rhizosphere of chrysanthemum and barley (Normander and Prosser, 2000). In this study Proteobacteria were the second most abundant group after the Firmicutes followed by the Actinobacteria contrary to the study by McCaig *et al* (1999) whose bacterial diversity from grass rhizosphere was studied. *Actinomyces* sp. were the second most abundant group after the most frequently

found Alpha-proteobacteria. *Arthrobacter* spp were found as dominant population in the molecular fingerprint of 16S rRNA fragments amplified from the rhizosphere DNA of maize grown in tropical soil (Gomes *et al.*, 2001) and also from the rhizosphere DNA of *M. sativa* and *C. album* (Schwieger and Tebbe, 2000). The bands showing similarity to the uncultured bacterial strains in this study also revealed the possibility of more unknown bacteria in the rhizosphere (Atlas and Bertha, 1999). Representatives of the phylum Firmicutes were more prevalent; a possible explanation to this is that some of the representatives of this phylum such as bacteria of the genus *Bacillus* use a survival strategy which includes a rapid growth when nutrients are in abundance (Atlas and Bertha, 1999).

Our results indicated that combination of culture-dependent and culture-independent techniques can be used to profile bacterial microbiota and diversity of the natural rhizosphere. On the overall, this study confirmed dominance of *Bacillus* species and occurrence of other bacteria with plant growth-promoting capabilities. The results also showed that a significant fraction of the taxa might be overlooked by the culture-dependent method. It was reported that PCR-DGGE was the most suitable technique to investigate the microbial community in different soil samples where the cultivation of many microorganisms is difficult or is thought to be (Araújo da Silva *et al.*, 2003). Ideally, both approaches, culture-dependent and culture-independent, should be complementary.

CONCLUSION

The use of both culture-dependent and culture-independent molecular techniques has enabled identification of the bacterial community structure in Mafikeng soil. According to phylogenetic analysis the bacterial isolates obtained from roots of various rhizosphere soils were found to be similar to the classes Firmicutes, Proteobacteria and Actinobacteria using

both culture-independent and culture-independent molecular techniques. *Bacillus* spp constituted the bulk of the rhizosphere bacteria communities owing to their diverse and flexible physiological properties. *Bacillus* spp isolated from the rhizosphere were meaningful as *Bacillus* spp bacteria perform defined roles in the soil such as ammonia oxidation. The PGPR are relevant for achieving sustainable agriculture since one of the importances of PGPR is to make reliable and accessible products such as biofertilizers for farmers. Furthermore these rhizobacteria can be used for the isolation of genes for exploitation. The plant growth-promoting abilities were correlated with IAA, HCN, ACC-deaminase activity and ammonia production of the isolates, which is considered as important plant growth-promoting traits of rhizobacteria. Such isolates have potential in future field applications as plant growth promoters. Using both culture-dependent and culture-independent molecular techniques, it is possible to identify bacteria up to the species level and thus enables us to detect the presence and dominance of bacteria performing specific functions. Profiling of bacterial communities by DGGE has proved to be a powerful method allowing a cultivation–independent analysis of rhizosphere soil samples. Therefore the culture-independent molecular analysis of prokaryotic communities from widely diverse locations will augment our knowledge about these organisms and help in formulating strategies for improved soil quality, enhanced crop production and protection to conserve natural resources and ultimately create more sustainable agriculture.

RECOMMENDATIONS

Further studies, are required to assess the antagonistic and plant growth-promoting activities of these strains in more natural conditions. Consequently continued research is needed to develop new approaches to improve the efficiency of rhizobacteria and also to further understand the ecology, genetics and biochemistry of their habitat. Understanding the responses of microbial diversity to seasonal variations and cultivar effect is also a prospect for future studies.

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