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Isolation and Characterization of Rhizosphere Bacterial Community from cultivated plants in Mahikeng, NorthWest Province, South Africa

By

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ABSTRACT

The rhizosphere is characterized by the presence of high microbial activities which are influenced by plant root exudates. This study examined bacterial diversity and physiological functions plants rhizosphere using both culture-dependent and culture-independent techniques of seven cultivated. Physico-chemical properties of soil samples revealed that the rhizobacteria adapted well to pH ranging from 7.5 to 9.1. Macronutrients (carbon, nitrogen, calcium, magnesium, phosphorous, potassium, sodium and iron) had a wide range of concentration between 0 to 4380.1 mg/kg. Concentrations of metal elements (cadmium, cobalt, chromium, copper and zinc) from all rhizosphere samples were below the amount of 3.1 mg/kg, indicating that the samples were free from metal contaminations. Sole carbon substrates utilization of bacteria in rhizosphere samples were measured as Average Well Colour Development (AWCD) and Group-wise Average Well Colour Development ($AWCD_G$) patterns. At seventy two hours, there was no significant difference in AWCD patterns between bacteria in all samples and there was a significant difference in $AWCD_G$ patterns. Biochemical tests showed majority of isolates had similar physiological properties to members of *Bacillus* genus. All the bacterial isolates exhibited positive antifungal trait, fifteen solubilized phosphate and three had cyanide production traits during *in vitro* plant growth promotion assays. *In vitro* plant growth revealed that bacterial isolate RL1 (*Bacillus licheniformis*) produced the highest concentration of indole acetic acid (IAA) at 25 mg/ml. Bacterial isolate RG3 (*Bacillus pumilus*) had the highest amino cyclopropane carboxylase (ACC) deaminase activity indicated by the high production of α-ketobutyrate produced at 4.8 mg/ml. There were significant differences in shoot length at $P \leq 5\%$ level of significance and there was no significant difference in the number of leaves across all three inoculated plants at $P \geq 5\%$ level of significance. Sequence and phylogenetic analysis of

identified culture-dependent bacteria revealed a homologous similarity of 94 to 100% between isolates sequences and GenBank sequences. From this, 81% of the sequences were closely related to Firmicutes, 13% to Actinobacteria and 6% to Proteobacteria. From culture-independent method, only 8 PCR-DGGE bands were detected, the 200 bp sequences in the 16S rRNA fragment showed 91 to 100% homologous similarity to GenBank sequences. Their 16S rRNA sequences was closely related to 50% uncultured bacterium clones, 25% Firmicutes, 13% Proteobacteria and 12% Bacteroidetes sequences. Both culture-dependent and culture-independent techniques were precise in the identification and description of bacterial community in rhizosphere.

Keywords: DGGE, microbial community, plant rhizobacteria, rhizosphere and Root exudates

DECLARATION

I Lorato Modise hereby declare herewith that the dissertation entitled "Isolation and Characterization of Rhizosphere Bacterial Community from cultivated plants in Mahikeng, North West Province, South Africa", 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 all the sources used or quoted have been indicated and acknowledged.

Signed at Mmabatho this 26 day of May 2014 

Declared before me on this.....day of.....2014

Commissioner of Oaths.....

DEDICATION

I dedicate this study to my darling husband, Sakia Radipabe Modise and loving my son, Kganya Lethabo Modise, thank you for the support and love you always give to me.

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LIST OF ABBREVIATIONS

ACC- Amino cyclopropane carboxylase

AWCD- Average well colour development

$AWCD_G$ – Group-wise average well colour development

BLAST- Basic local alignment search tool

Bp- Base pair

CFU- Colony forming unit

CLPP- Community-Level Physiological Profiling

CTAB- Cetyltrimethylammonium bromide

DAS- Days after sowing

DGGE- Denaturing gradient gel electrophoresis

DNA- Deoxyribonucleic acid

DRB- Deleterious rhizobacteria

dNTP- Deoxyribonucleotide triphosphates

EDTA- Ethylenediaminetetraacetic acid

$FeCl_3$- Iron III chloride

GC- Guanine Cytosine

HCl- Hydrochloric acid

$HClO_4$ - Perchloric acid

HCN- Hydrogen cyanide

H_2S- Hydrogen sulphide

IAA- Indole acetic acid

ISR- Induced systematic resistance

LB- Luria Bertani

NA- Nutrient agar

NADH- Nicotinamide Adenine di Nucleotide plus Hydrogen

NaOH- Sodium hydroxide

NCBI- National center for biotechnology

N_2- Nitrogen gas

PCA- Plate count agar

PDA- Potato dextrose agar

PCR- Polymerase Chain Reaction

PGPR- Plant growth-promoting rhizobacteria

rDNA- Ribosomal deoxyribonucleic acid

rpm- Revolutions per minute

RNA- Ribonucleic acid

rRNA- Ribosomal ribonucleic acid

SAR- Systematic Acquired Resistance

Spp- Species

TAE- Tris base, acetic acid and EDTA

DEFINITION OF CONCEPTS

Biofertilizer:	Microorganism's application as fertilizers
Clone:	An organism asexually produced from and having similar genetic make up of one ancestor
Diversity:	Amount of difference between genes, species and region
Exudates:	Substances essential for life released from plant roots
Microbial community:	Microorganisms population in an area
Phylogeny:	Branch of biology addressing evolutionary relationships of organisms
Phytohormone:	Plant hormone
Rhizo:	Root
Rhizosphere:	A soil region associated with plant roots
Rhizoplane:	Root area near the surface
Siderophore:	Iron binding molecules

CHAPTER 1

1. INTRODUCTION

Soil is a complex environment offering a variety of habitats to microorganisms. Many microorganisms inhabit the soil particles pores', rhizoplane and rhizosphere. The rhizosphere of cultivated plants is referred to as the soil region characterised by the presence of high microbial activities which are influenced by plant roots. The high microbial activities and diversity in the rhizosphere result from the presence of highly nutritious substances discharged by the plant roots known as exudates. Plant roots receive close to sixty percent of the net photosynthetic carbon, of this close to forty percent is released into the rhizosphere as exudates (Bais *et al.*, 2004).

The exudates consist of compound molecules like sugars, organic acids, vitamins, alcohols, enzymes and nucleotides (Babalola, 2010). These molecules serve as nutrients and signalling molecules that attract microorganisms into the rhizosphere. Root exudates thus make the rhizosphere a unique area for soil studies as compared to the bulk soil which does not have exudates. Exudate compositions are plant specific and make the rhizosphere of different cultivated plants unique.

Cultivated plants such as maize, spinach, tomato, onion, green peas, beetroot and lettuce are common staple foods which are the standard diet of the majority of people in South Africa. These plants' fruits, leaves and roots contain important nutritious compounds such as carbohydrates, proteins, vitamin A, C and K, calcium, iron and magnesium. Changes in environmental conditions affect the plants productivity hence an increase in food insecurity. Other factors affecting crop productivity are poor farming practices and plant pathogens. Pathogenic organisms causing infections and disease to cultivated plants include *Fusarium*

oxysporum, *Pseudomonas solanacea* which cause tomato wilt, *Erwinia carotovora* causing sugar beet vascular necrosis (Ibrahim *et al.*, 2011). Onion centre rot, sour skin is caused by *Burkholderia cepacia* and *Pantoea ananati*. *Sclerotinia sclerotiorum* causes white mould in green peas, maize ear rot and head blight diseases are caused by *Fusarium sp.* (Gopesh, George, 2012). Other plant pathogens might lead to food-borne illness in both animals and human beings when ingested (Erickson *et al.*, 2010).

Microorganisms are used as indicators of the soil health (Hill *et al.*, 2000). A large number of pathogenic and beneficial microbes are found in cultivated plants rhizosphere region such as some viruses, protozoans, algae, fungi, and bacteria. These microorganisms compete for resources such as space, water and nutrients. Bacteria are the most successful and dominating competitors as compared to the rest of the microorganisms because the majority of exudates signalling molecular components stimulates and attracts bacterial community more as compared to other microorganisms. Bacteria are a large group of typically unicellular microorganisms that comprise the kingdom Prokaryotes, most bacteria are saprotrophs and reproduce by binary fission (Röling *et al.*, 2007). Bacterial communities in the rhizosphere of cultivated plants are a group of bacterial species assemblages which live together within the rhizosphere. These bacteria interact with each other and contribute to soil ecosystem functions (Duineveld *et al.*, 2001).

The physico-chemical properties in the rhizosphere are very beneficial in the soil ecosystem they stimulate and initiate microorgansisms to function as catalyst in the biogeochemical reactions including the decomposition of molecules. Bacterial interactions play a crucial role in ecosystem functions and have huge potential for biotechnological applications. The bacterial community in rhizosphere of cultivated plants is dominated by members of the phyla Proteobacteria, Firmicutes, Actinobacteria and Acidobacteria (Fierer *et al.*, 2007). The majority of these

bacterial species are found in the rhizosphere of plants because they adapt to similar chemical and physical conditions and might not necessarily interact with each other (Young *et al.*, 2008).

There are many interactions occurring in the soil rhizosphere between members of the bacterial community and plant roots through chemical signalling and most of them are mutually beneficial (Bais *et al.*, 2006) but still remain complex. Their complexity occurs because most interactions are not fully understood due to the fact that the whole microbial diversity and stability is not known. Also, it is difficult to understand all the functions of individual organisms within the rhizosphere system made up of such a large population. Continuous modification of abiotic conditions (temperature, pollution, drought, salinity and floods) affect the plant health, age and species type (mutations) and that ultimately influences bacterial community (Saharan, Nehra, 2011).

The bacterial community makes contributions towards the economical, agricultural, medicinal, food, fibre, industrial and ecological sectors (Lauber *et al.*, 2009; Robe *et al.*, 2003). They also play a major role in the biogeochemical cycles of the main elements (carbon, nitrogen and sulphur; trace elements iron, nickel and mercury) and are therefore heavily implicated in energy and food web exchanges within the soil (Ranjard *et al.*, 2000). Bacteria also synthesise antimicrobial agents, auxins and growth factors (Babalola, 2010). The bacterial community members have many adaptation methods which assist them to sense and respond to the stimuli and environmental changes. One method by which bacteria respond to environmental stimuli is through the uptake and secretion of small diffusible signalling molecules such as root exudates. These signalling molecules allow bacteria to monitor their own population, resources, initiate immunity towards pathogens and promote plant growth. Bacteria respond to their population size by regulating the amount of gene expression through quorum sensing (Winzer, Williams, 2001).

Plants' immunity and growth promotion are mainly evident in a group of bacteria known as Plant Growth-Promoting Rhizobacteria (PGPR).

PGPR respond to an exudate signalling molecule by aggressively colonizing plant roots and initiating beneficial effects on the plant (De-Bashan, Bashan, 2010). PGPR are divided into groups based on their beneficial activities towards plants which are the phytostimulating rhizobacteria, mycorrhiza, root nodule symbiosis rhizobacteria and biocontrol rhizobacteria (Frey-klett *et al.*, 2011; Saharan, Nehra, 2011) The bacterial plant growth-promoting traits exuded by PGPR can be quantified and include nitrogen-fixation, phosphate solubilisation, iron sequestration and synthesis of phytohormones which include auxins, cytokinin and gibberellin (Babalola, 2010). Host specificity by rhizobacteria is essential for each of the beneficial processes to occur because exudate compositions are plant host specific. Most of the PGPR members in the rhizosphere belong to genera such as *Arthrobacter, Acinobacter, Bacillus, Burkholderia, Enterobacter* and *Pseudomonas* (Babalola *et al.*, 2003; Babalola, 2010).

The microbial ecology study methods attempt to determine presence, composition and diversity of the bacterial community in the rhizosphere of soil. Initially culture-dependent methods, based on the cultivation of samples on the prepared growth media to enhance their growth were preferred. This allowed for isolation of microorganisms and their identification based on morphology and physiological properties. However, in recent years, the popularity of culture-dependent techniques has declined. Though there are modifications on the culture medium to grow fastidious bacteria in the soil, only one per cent of direct counted cells from 10^9 cultured cells can grow in these modified media (Vartoukian *et al.*, 2010). Failure of these organisms to grow in media is because there is no guarantee that the behaviour of isolated bacteria in the laboratory is similar to that on the range of conditions in natural soils. Another limitation with

these methods is that some bacteria belong to groups whose optimal culture conditions have not yet been defined. Others might be depending on other microbes for growth which might be selective to thrive only in their natural environment. Culture-dependent techniques provide less information about the type of microorganism's active and functioning in the environment (Leckie, 2005).

Despite this, one culture technique which has been used to determine and compare the change over time in the composition, diversity and function of soil microbes between soil samples is Community-Level Physiological Profiling (CLPP) (Preston-Mafham *et al.*, 2002; Stefanowicz, 2006). CLPP is a culture method adopted using a commercial system like Biolog, which identifies bacterial activity based on the usage of 93 different carbon sources (Hill *et al.*, 2000). Their potential to catabolise carbon substrates is measured based on substrate-induced respiration (SIR) and carbon dioxide efflux (Garland *et al.*, 2001) observed by a colour change of the tetrazolium dye. Clear colour change of a positive reaction will appear when a total number of cells using the substrate reach 10^8 cells/ ml (Fang *et al.*, 2001).

A limitation with this technique is similar to culture-dependent plating technique which is whether the data obtained using this technique represents the correct amount of microbes functioning in natural systems (Preston-Mafham *et al.*, 2002). Although culture techniques are not precise in the characterization of soil microbes, they are more useful in allowing us to understand the growth habitat (soil), development and microbial functions in the soil environment. Culture-independent methods reveal more information about the diversity, relationships and composition of soil microbial communities.

As a result of experiencing such challenges with culture-dependent techniques, culture-independent molecular methods that are more reliable to give positive outcome on the identification of bacterial community have been identified and applied. Culture-independent techniques provide an exciting opportunity to understand microbial genetic diversity and functionality directly from the environment without culturing. Unlike the culture-dependent method, culture-independent methods allow the analysis of microbial genetic composition at a species and genus levels.

1.2. Problem statement

Rhizosphere interactions are complex and our understanding of these interactions is limited. This is due to limited studies on methods that facilitate the study of rhizosphere bacterial community and composition. Bacterial density is influenced by unstable habitat factors such as pH, nutrient availability and oxygen. Soil pH is the main habitat factor, which affects other several habitat factors that are difficult to separate from each other because one factor may be dependent on the other. The pH influences abiotic factors such as carbon availability, nutrient availability and metal solubility. In addition, pH may control biotic factors such as bacteria composition. Extreme changes in these habitat factors in the rhizosphere affect soil functions and the latter alters the bacterial community.

1.3. Aim of the study

The aim of this study was to isolate and characterize the bacterial community in the rhizosphere of cultivated plants.

1.4. Objectives

1.4.1. To determine bacterial community activity, diversity and composition using Community-Level Physiological Profiling Method.

1.4.2. To identify and characterize bacterial community using culture-dependent and culture-independent techniques.

1.4.3. To determine plant growth-promoting traits of the selected rhizosphere bacteria.

1.5. Hypothesis

Bacteria dominate the rhizosphere population and are diverse, they differ in catabolic activities and functions but they might share familiar characteristics indicating that they come from a common ancestor.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Bacterial community in plant rhizosphere

Based on culture techniques, the bacterial community dominates the entire population in the soil. Bacteria dominate at about 10^9 cells per gram of rhizosphere soil, followed by viruses and bacteriophages at 10^8, 10^5 for fungi, and 10^3 for both algae and protozoans (Williamson *et al.*, 2005; Ashelford *et al.*, 2003). Besides, the prevalence of bacteria in the rhizosphere especially the ones containing plant-beneficial activities, bacteria are also found in other plant parts such phyllosphere (leaves, fruits, stem, vascular tissues and intercellular space) and spermosphere.

But failure for bacterial dominancy in other plant parts especially the phyllospere is because it is the areas above the plant soil, making the bacterial community vulnerable to continuous environmental changes such as temperature, wind, water availability, humidity and solar radiation. The nutrient makeup and status in this area is hugely affected by these environment changes. Microorganisms in this region are easily washed off from the leaves by rain water, wind and UV light.

Phyllosphere microbes receive their nutrients from exudates derived from the mesophyll, epidermal cells and wound lysates (Leveau, Lindow, 2001) but the thick waxy cuticle interferes with nutrient diffusion, thus reducing the chances of rhizobacterial attachment to and colonization of plant surfaces. The beneficial bacterial community is also found less dominating in spermosphere, because the spermosphere is not a constant zone. Spermosphere is dominated by seed-infecting pathogens such as *Fusarium* and *Phythium* species, these pathogens can easily invade leaves and initiate plant diseases (Babalola, 2010; Nelson, 2004).

2.2. Rhizosphere bacterial community interactions

Rhizosphere has high diversity of rhizobacteria with different structures, taxonomy and purpose. Rhizobacteria are involved in numerous interactive activities within the rhizosphere such as rhizobacteria-plant, rhizobacteria-microbes and rhizobacteria-soil interactions. The relationship between rhizobacteria and soil is a mutually beneficial one. The soil provides habitats for bacteria and in return the bacteria improve the soil structure and health by increasing nutrients through decomposition of organic compounds and cycling of elements. Interactions between rhizobacteria-microbes and rhizobacteria-plant communities are complex and can either be beneficial (mutual symbiotic), non-beneficial (neutral) or harmful (pathogenic).

Bacterial community interactions are influenced by environmental conditions, associated-plant species and plant growth stages. All the interactions in plants' rhizosphere in which the bacteria are involved affect the nutrient levels through changing of biogeochemical cyclic reactions. It is easier to measure biogeochemical property effects by bacteria but it is difficult to determine which taxon is responsible for a specific biogeochemical process (Madsen, 2005); because there are many theoretical explanations on taxons responsible for biogeochemical interactions than verified detailed experiments (Konopka, 2009).

Previous reports showed that metagenomic identification of bacterial gene sequences and protein molecules responsible for biogeochemical processes separate these molecules from their original organisms. As a result the analyses cannot identify specific function with other functional properties of the original organisms (Benincà *et al.*, 2008). Even though the interactions in the rhizosphere are complex, the majority of them remain beneficial. The beneficial interaction between plant and rhizobacteria is evident in the group of bacteria known as PGPR. PGPR are plant growth-promoting rhizobacteria which are able to colonize plant roots and rhizosphere

(Babalola, 2007) aggressively, increase in number and induce plant growth promotion. Growth promotion of a plant by PGPR is initiated by the presence of exudates released by plant root, which act as a signalling molecule attracting PGPR (Berg, 2009). Once the PGPR receive the signal, they respond by chemotaxis driven by flagella to initiate colonization, by producing response regulatory proteins and these proteins allow movement of PGPR towards the plant roots. Upon arrival at the rhizosphere plant roots, bacterial cells attach to root surface or invade root cells (endophytes), multiply to a density of 10^6 cells/ g of soil roots (Dutta, Podile, 2010) and form dense cells known as biofilms (Nihorimbere *et al.*, 2011). Colonization of the plant root by PGPR is a prerequisite to occupy the rhizosphere space and start activities on their association with the plant.

Plant growth promotion caused by PGPR is determined by measuring the yield of the plants, increase of the plant root system, seedling health, strength and prominence (Hou, Oluranti, 2013). PGPR have been divided into two groups based on their mode of action namely; the PGPRs that promotes plant growth through direct association and biocontrol PGPRs that indirectly associate with the plant but still increase plant growth (Ahmad *et al.*, 2008). PGPR are also divided based on their association with the plant, there are PGPR that colonize the rhizosphere and the rhizoplane; while others colonize the inner plant and root cells. In the past twenty years, the direct and indirect methods used by PGPR to promote plant growth have been explained. In the direct association method, the PGPR enhance plant growth and improve crop yield by increasing the plant nutrition and phytohormone production.

PGPR indirectly promote plant growth by suppressing and controlling plant pathogens that inhibit plant growth and health. Direct and indirect influence on plant growth by rhizobacteria has been demonstrated many times in controlled conditions and field trials using crops (Lucy *et*

al., 2004). Common PGPR genera exhibiting plant growth promotion activity are *Pseudomonas, Bacillus, Azospirillum, Azotobacter, Enterobacter, Burkholderia, Rhizobium, Mesorhizobium, flavobacterium, Agrobacterium, Serratia* and *Flavobacteria* (Ahmad *et al.*, 2008), this group of PGPR mainly colonize the rhizosphere and rhizoplane (Bhattacharyya, Jha, 2012). PGPR colonizing the plant and roots cells belong to genera such as *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Allorhizobium* and *Azorhizobium* (Bhattacharyya, Jha, 2012). The PGPR belonging to these genera produce a variety of growth promoting and biocontrol molecules such as Indole acetic acid (IAA), amino cyclopropane carboxylase (ACC)-deaminase, hydrogen cyanide (HCN), and siderophores. The most studied PGPR with many plant growth traits are *Pseudomonas* and *Bacillus* species. PGPR have been identified successfully from maize, wheat, soybean, potato, rice, chickpea, onion, *Arabidopsis* and sugar beet (Neal *et al.*, 2012; Wahyudi *et al.*, 2011; Karnwal, 2009).

2.3. Common PGPR genera

2.3.1. *Pseudomonas* genus

Pseudomonas genus belongs to gamma subgroup of Proteobacteria and it colonizes a variety of niches (Mulet *et al.*, 2010). They also have been characterized as having a unique feature of metabolising a variety of carbon and nitrogen sources and most of the species in this group have been characterized as plant growth promoters except for a few which are classified as pathogens an example is *Pseudomonas syringae*. Pathogenicity of *P. syringae* depends on the function of Type III secretion system, which produces virulent factors causing infections and diseases. The most important *Pseudomonas* spp. containing plant growth-promoting traits are; *P. fluorescens*, *P. putida* and *P. Stutzeri.* These species promote plant growth either directly or indirectly. Indirectly species suppress pathogens by producing antimicrobial agents and induce the plant

systematic immunity (Franche *et al.*, 2009). *Pseudomonas* spp. with plant growth-promoting (PGP) traits directly promotes plant growth by fixing atmospheric nitrogen, solubilising phosphate and phytohormone production (Lugtenberg, Kamilova, 2009).

2.3.2. *Bacillus* genus

It is one of the dominating genera in the rhizosphere and has high plant growth-promoting potential and they are more likely to be used successfully in field applications. *Bacillus* species adapt well to different environmental conditions and that is due to their spore forming characteristics. *Bacillus* genus members are common in the rhizosphere of different plant species and promote their growth using different direct and indirect plant growth routes. *B. subtilis* indirectly promotes plant growth by suppressing plant pathogens; and in the direct route *B. mucilaginous* makes phosphorous available for plants. Co-inoculation of *Bacillus* spp. with other PGPR have shown success in promoting plant growth when applied to plants seeds or roots an example is the combination of *Bacillus* spp. with *Azotobacter* species (Babalola, 2010). Other co-inoculants are *Bacillus* with *Bradyrhizobium japonicum* used to increase fixed nitrogen availability on soybeans; *Bacillus megaterium* with *Azospirillum lipoferum* which increases fixed nitrogen and phosphorous of wheat (Wahyudi *et al.*, 2011).

2.4. Direct mechanisms of growth promotion

PGPR directly promote plant growth using different mechanisms. PGPR increase plant nutrition by input of mineral nutrients into the rhizosphere or by reducing unwanted and harmful chemicals in the soil caused by input of chemical fertilizers. Rhizobacteria carrying these functions can be characterized as rhizo-biofertilizers. These biofertilizers increase nutrient availability, increase plant access to nutrients by increasing the development and cell division of root hairs, which ultimately increase surface area of the roots. Biofertilizers make nitrogen,

phosphorous and potassium available through nitrogen fixation, solubilisation of phosphate and potassium. Other PGPR promote plant growth by producing phyto-hormones; characterization of these mechanisms is challenging because phytohormone production is rhizobacteria and plant host specific (Rubio, Ludden, 2008).

2.4.1. Nitrogen-fixation

About 80% of the atmosphere is made up of unreactive Nitrogen molecule (N_2). Nitrogen (N) is one of the essential substances needed by plants for growth; it is a major component in the amino acids making proteins and nucleic acids (deoxyribose nucleic acid or ribose nucleic acid). Microbes, plant and animals can suffer from nitrogen deficiencies disease and die if nitrogen is unavailable to them. Unfortunately, plants cannot use atmospheric nitrogen (N_2) directly. It must first be converted into suitable plant form by fixation of the atmospheric N_2 into more reactive ammonia (NH_3). There are three forms of nitrogen fixation.

The first form is the industrial nitrogen fixation process which was discovered in the 20^{th} century by Fritz Haber (Rubio, Ludden, 2008). The process involves the usage of a catalyst potassium oxide or aluminum oxide (K_20 and Al_2O_3) at high pressure and temperature (300-500 °C) to break the triple bonds on the unreactive atmospheric nitrogen. This process causes air pollution.

Symbiotic nitrogen fixation is made up of legume symbioses and association with *Frankia*. Legume symbiosis occurs between leguminous plants and rhizobia of the family Rhizobacteriacea (Zahran, 2001). Whilst *Frankia* association symbiotic nitrogen fixation involves *Frankia* species and the non-legume plant (*Actinorhizal)* and their association is referred to as achnorhizal (Mishra *et al.*, 2002). Symbiotic nitrogen fixation between rhizobia and legumes is complex but it results in a mutual benefit between the plant and the microbe (s).

The plant host provides nutrients for the microorganism such as carbon and nitrogenase raw materials from photosynthesized products, which activates the microbes nitrogenase and in return the microbe fix nitrogen for the plant using nitrogenase through nodule formation.

Nodulation process is a prerequisite for successful nitrogen fixation to take place by rhizobia (Chenn, Walsh, 2002). Nodulation is initiated by legumes which release root exudate flavonoids such as diazein and luteolin into plant root and the soil. Flavonoids are attractants and allow root attachment by rhizobia. Flavonoids cause the bacteria to produce *Nod* factors coded for by *Nod* genes. The *Nod* factor causes root hairs curling, which the rhizobium uses to penetrate root hair and forms an infection thread (Chenn, Walsh, 2002).

Rhizobia use the thread to move to the bottoms of the root hair and modify nodule primordia into nitrogen fixing bacteroids whereby nodules and nitrogenase are formed (Kuiper *et al.*, 2001). Once nodules are formed, bacteria reduce oxygen concentration to allow nitrogenase activity to fix N_2 molecule into NH_3 which the plant can use. Nitrogenase structure is made up of dinitrogenase reductase and dinitrogenase components. Dinitrogenase reduces N_2 to NH_3 using electrons provided by dinitrogenase reductase (Compant *et al.*, 2005).

In the non-symbiotic nitrogen fixation there is no formation of nodules, but microbes associate with plant to fix nitrogen. Non-symbiotic nitrogen fixation consists of associative, endophyte and free-living N_2 fixing bacteria such as *Azospirillum*, *Azotobacter*, *Azocarcus* and *Cyanobacteria* (Kennedy *et al.*, 2004). Non-symbiotic fixation process provides a limited amount of fixed nitrogen that is needed by bacteria in association with plants need (Rashid *et al.*, 2012).

In associative nitrogen fixation, bacteria grow in close association with the root rhizoplane, rhizosphere as endophytes. The bacteria in this type of process do not get components to make

nitrogenase from plants because there is no nodules formation but rather the bacteria use root exudates to fix atmospheric nitrogen. The most successful species in this process are *Azospirillum* and they form associations and colonize the rhizosphere of many crops. *Azospirillum* fix N_2 into NH_3 which is not used immediately due to complexity in the cell wall and transport mechanisms of *Azospirillum* (Franche *et al.*, 2009).

Ammonia is transported to plants usually through decomposition when the bacteria die. Other bacteria that are involved in associative nitrogen fixation by colonizing plant tissue have successfully been identified to associate with plants such as cereals, rice and sugar cane and examples of such bacteria include *Azotobacter* and *Burkholderia* (Yan *et al.*, 2008).

2.4.2. Phosphate solubilisation

Phosphorous (P) is the second most important plant growth limiting nutrient after nitrogen. Phosphorous is used by plants acting as an important component of amino acids, root development, stalk and stem strength, flower and seed formation, and crop maturity (Ahemad, Khan, 2010). The amount of phosphorous available for plants to use in the soil is low because phosphorous in the soil is found in insoluble (apatite) or inorganic (inositol, phosphatriester and phosphomoresters) phosphate minerals forms (Khan *et al.*, 2009).

Plants absorb phosphorous in two forms monobasic (H_2PO^{4-}) and diabasic (HPO_4^{2-}), but plants cannot convert insoluble phosphorous into soluble forms themselves. PGPR known as Phosphate Solubilizing Bacteria (PSB) makes P available in the form that plants can use. PSB responsible for phosphate mineralization belong to genera like *Pseudomonas*, *Enterobacter*, *Azotobacter*, *Burkholderia*, *Bacillus*, *Rhizobium* and *Serratia* and they are currently considered to be promising biofertilizers to substitute the usage of chemical phosphorous fertilizers.

Solubilisation of inorganic phosphorous such as calcium phosphate involves the production of low molecular weight organic acids like carboxylic acid which reduce pH in calcareous soils by PSB. In mineralization PSB secret phosphatases which catalyse the hydrolysis of phosphoric esters in organic phosphorous (Rashid *et al.*, 2012). In some cases both phosphate solubilisation and mineralization can be done by the same bacterial strain (Tao *et al.*, 2008).

Usage of PSB inoculants to increase phosphorous intake by plants have been successful, also the combination of PSB inoculants with other PGPR have increased plant available phosphorous (Khan *et al.*, 2009). PSB can also signal nitrogen fixation by other PGPR (Ahmad *et al.*, 2008) and increase availability of nutrients such as iron and zinc (Singh *et al.*, 2001).

2.4.3. Increase of plant growth by phytohormone production

Indole-3-Acetic Acid (IAA) is a quantified vital signal of plant growth produced by a wide range of organisms from plants, animals to bacteria (Kawaguchi, Syōno, 1996). IAA is one of the main plant hormone of auxins which control most of the plant development processes. Auxins are produced in the meristem of shoot tips and move down the plant to interact with certain tissues and cause the physiological response. There are other hormones responsible for plant development such as gibberellins, ethylene, abscisic acid and cytokinins (Mano, Nemoto, 2012).

2.4.3.1. Indole-3-acetic acid

Generally plant indole-3-acetic acid affects plant cell division and cell differentiation but also stimulates seed germination, increased vascular bundle development, controls plant growth, controls response of plants to gravity, light, photosynthesis and stressful conditions. Many bacteria in the rhizosphere have the ability to produce IAA as a secondary metabolite and its outcomes are more evident in plant physiological changes than in bacteria (Patten, Glick, 2002).

Rhizobacteria use IAA to colonize plants, stimulate plant IAA production, other plant hormones and pathogenesis (Spaepen, Vanderleyden, 2011).

Rhizobacteria IAA has beneficial effects on the plant, they increase root surface area allowing plants to take up nutrients and increase root exudates amounts in the plant by making plant cell wall less tight allowing release of rhizodeposits (Rashid *et al.*, 2012). Most rhizobacterial IAA synthesis depends on the precursor tryptophan; it is a product from aromatic acid synthesis and there are five IAA synthesis pathways depending on tryptophan, they are known in both bacterial and plant systems with different intermediates.

The majority of rhizobacteria produce IAA via indole-3-pyruvic acid, indole-3-acetamide and indole-3-aldehyde pathways (Normanly *et al.*, 1995). Other IAA biosynthesis pathways are indole-3-acetonitrile in *Cyanobacterium* (Sergeeva *et al.*, 2002) with tryptophan-independent pathways dominating in plants. IAA production is affected by many factors such as abiotic factors and expression of genes needed in the biosynthesis pathway (Tao *et al.*, 2008).

2.4.3.1.1. Indole-3-Acetamide (IAM) and Indole-3-pyruvic acid (IPyA) pathway

The IAM pathway for the synthesis of IAA, is a two-step process, first tryptophan is converted into indole-3-acetamide (IAM) by enzyme tryptophan-2-monooxygenase (IaaM) encoded by *iaaM* gene. In the second step, IAM is converted into IAA by an IAM hydrolase (IaaH) encoded by *iaaH* gene. IAM hydrolase brings about the conversion of IAM into IAA (Nemoto *et al.*, 2009).

The IPyA pathway involves the deamination of tryptophan to IPyA by aminotransferase. In the second step, the IPyA substrate is decarboxylated (Tsavkelova *et al.*, 2007) into Indole-3-acetaldehyde (IAAid) by indole -3-pyruvate decarboxylase (IPDC) and IAAid is oxidised into

IAA. The IAA produced by these plant-associated bacteria can be a signal molecule; causing stimuli to the plant to express tryptophan which, as a precursor that will initiates IAA production by the plant or the plant-associated bacteria, can be transported into the plant via membrane diffusion mechanisms (Kramer, Bennett, 2006).

2.4.4. Plant growth by Aminocyclopropane-1-carboxylate (ACC) deaminase

Ethylene is a common vital metabolite required by plants in minute quantities for normal plant growth and development. It is produced by almost all plants and besides being a plant growth regulator, ethylene is also a stress hormone. Under stressful conditions created by drought, salinity, heavy metal acquisition and pathogen attack, ethylene concentration increases and high amount of ethylene negatively affect the plant growth and crop productivity (Yoon, Kieber, 2013). PGPR with ACC-deaminase ability promote plant growth and development by decreasing ethylene concentration. PGPR reduce ethylene levels by breaking down the ACC precursor molecule and the detachment of ACC automatically reduces ethylene levels by converting ACC into 2-oxobutanoate and NH_3 (Arshad *et al.*, 2007).

2.5. Indirect mechanisms of growth promotion

As with beneficial microorganisms, exudates may signal the movement of harmful pathogenic microorganisms (bacteria and fungi) into the rhizosphere, which they might attach to, colonize roots and initiate an infection or disease. Diseases caused by these pathogens affect the plant's health and productivity and cause crop losses. The traditional method used to protect plants from pathogens and suppress plant disease caused by pathogens is by using chemical pesticides (Somers *et al.*, 2004). However the pesticides may be harmful to animals and human beings especially during unfavourable conditions like intense rainfall, leading to some pesticides chemicals being leached into ground water systems. As an alternative to chemical pesticides,

biological control agents (biopesticides) made up of bacterial and fungal inoculants are being used (Vessey, 2003).

But, there are still complications with their entire application methods of biopesticides and more still needs to be done concerning their mode of action in different plant species taking into consideration abiotic factor conditions such as temperature, pH and moisture. The method of using microorganisms as biocontrol agents of plant pathogens is more environmentally friendly and economically more viable when compared to using chemical control agents (Bora *et al.*, 2004). So far, the most commonly used biocontrol agents are some members of the PGPR group, which indirectly promote plant growth as biocontrol agents. Biocontrol mechanisms are divided into competition for nutrients and space, production of antifungal pathogens and, induction of plant immunity against pathogens, parasitism and predation (Kuiper *et al.*, 2001).

2.5.1. Competition for nutrients and space

One method by which the biocontrol of PGPR can eliminate and prevent pathogens from attacking the plant host is by creating an unfriendly rhizosphere, acquiring more root surface and reducing exudate nutrients will make pathogens starve and become inactive. Biocontrol agent PGPR attach and, colonize the root system quickly in the area where nutrients are leaking from the roots leaving no space for pathogen colonization. One of the scarce nutrients that both PGPR and pathogens compete for is iron (Fe^{3+}), which is an important nutrient in all forms of life.

Iron in plant soil is oxidised into hydroxide and oxide-hydroxide forms and it is inaccessible to plants (Rajkumar *et al.*, 2010). But PGPR are at an advantage of acquiring more iron than pathogens because they produce iron-chelators known as siderophores, the siderophores chelate Fe^{3+} and form siderophore-Fe complexes which the plant assimilates. The most studied PGPR

producing siderophores is *Pseudomonas* spp. that produces pseudobactins or pyoverdin (Bakker *et al.*, 2007). The inability of pathogens to acquire Fe^{3+} reduces their microbial growth and pathogenesis.

2.5.2. Induced Systematic Resistance

Naturally, plants have their immunity against foreign microorganisms. In the absence of PGPR, plants respond to pathogen attack by accumulating salicylic acid, inducing genes encoding for Pathogenesis Related (PR) proteins and this phenomenon is termed Systematic Acquired Resistance (SAR). When PGPR are present during pathogen attack, they induce plant immunity known as Induced Systemic Resistance (ISR). Once ISR is induced, plants produce defence molecules jasmonic acid and ethylene. ISR and SAR methods differ because in ISR, PGPR are present and PR proteins are absent. The ISR mechanism by PGPR *P. fluroscens* WCS417 has been studied successfully in *Arabdopsis thaliana* ecotype attacked by pathogen *P. syringae* (Meziane *et al.*, 2005)

2.5.3. Production of antifungal metabolites

PGPR interfere with plant root pathogens especially fungi and bacteria using a variety of methods. The methods include production of volatile and non-volatile antibiotics, siderophores, enzymes and secondary metabolites like Hydrogen Cyanides (HCN). Volatile and non-volatile antibiotics affects root colonisation positively in soybean plants by *P. chlororapuis* strain PA23 because of their ability to prevent spore germination by pathogens. Antibiotics that are commonly produced by PGPR include ammonia, butyrolactones, 2, 4-diacetyl phloroglucinol (DAPG), kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide and xanthobaccin (Baehler *et al.*, 2005).

Many of the antibiotics carry of a broad spectrum activity (Whipps, 2001). Molecular genetics and, mutational analysis are some methods that have been used to study antibiotics activity produced by rhizobacteria based on the presence or absence of genes encoding for these antibiotics. Antibiotics have been isolated to determine their regulation both at transcriptional and post transcriptional stages to improve their production and effectiveness (Haas, Keel, 2003). Besides, antibiotics siderophores have also been studied well in *Pseudomonas* genera, they are antibiotic specific and they have been shown to suppress pathogen attack on plants (Whipps, 2001).

Another well studied biocontrol agent produced by rhzobacteria is HCN. HCN is produced by a group of rhizobacteria known as Deleterious Rhizobacteria (DRB). DRB suppress growth of weeds and are plant specific. DRB produce the metabolite inhibitor cyanide that is a toxic chemical used also by algae, fungi, plants and insects to avoid pathogenesis and predation. Host plants are not affected by cyanide producing rhizobacteria inoculant but can be affected by the secondary metabolite HCN that is commonly produced by *Pseudomonas* spp. It is a potential biocontrol agent of weeds and affects plant host roots metabolism and growth.

HCN producing rhizobacteria interfere with the establishment of weeds in crops and overcome the competition for nutrients, space, water and light energy. HCN is a common biocontrol trait in *Pseudomonas* (89%) and *Bacillus* (50%) genera. High concentration of HCN (300 mg/m^3) in the air can cause serious environmental pollutant and it may kill a person but it is produced in small amount by these rhizobacteria. Production of the HCN is activated by the precursor glycine and *in vitro* tests using medium supplemented with glycine have shown HCN production by *P. putida* and *Acidovorax delafieldii* (Wani *et al.*, 2007; Owen, Zdor, 2001). HCN is a better weed biocontrol agent when compared to others chemical control agents because herbicides cannot

affect HCN activity in plants like mustards and it is not supplemented with herbicide chemicals like other biocontrol agents such as glyten meal which is applied with herbicides to increase effectiveness (Zdor *et al.*, 2005). Identified DRB rhizobacteria belong to genera such as *Pseudomonas*, *Bacillus*, *Enterobacter*, *Flavobacterium* and *Citrobacter*. DRB are potential biocontrol agents that benefit the agricultural sector by increasing crop productivity by minimizing weed competitiveness and reducing chemical herbicides usage (Kremer, Kennedy, 1996).

2.6. Root Exudates

Plant roots release rhizodeposits (collection of carbon-containing compounds) like exudates, sloughed off root cells, tissues, soluble lysates and volatiles into the rhizosphere. About 11% of the carbon incorporated in photosynthesis is contained in the rhizodeposits and 5-30% of the rhizo-deposited carbon is taken to the rhizosphere as exudates (Paterson *et al.*, 2007). Root exudates make up a large portion of rhizodeposit ion. They also serve as growth substrates and signalling molecules for some soil microorganisms.

Exudates attract specific microbes to colonize the rhizoplane and the soil immediately influenced by the roots (rhizosphere). Other exudates can repel microbes from the plant (Compant *et al.*, 2005). From the host plant roots, the exudates change the soil chemical properties (minerals and organic contents) to make the environment suitable for microorganisms to colonize the plant roots. In return, the microorganisms modify plant nutrition, cell metabolism and cell leakage and all these changes increase exudate concentration and composition (Puente *et al.*, 2004).

Root exudates are found in new roots, root tip, secondary and lateral root zones. In the root tip zone exudates composition is made up of easily degradable sugars and organic acids, cellulose

and hemicelluloses dominates the exudates in older roots which are made up of sloughed cells (Walker *et al.*, 2003). Lateral roots consist mainly of nutrient rich molecules which are used by the mature organisms.

Root exudate composition differs from one plant to another and they are distributed into different zones within the root system. Root exudates are used by different microorganisms of specific plant hosts. Exudates are made up of a wide variety of chemical compounds (Table 2.1.). The main components of root exudates are amino acids, carbohydrates, organic acids, phenolics, fatty acids, sterols, vitamins, enzymes and nucleotides. Sugars, organic acids and amino acids dominate the composition and are released in large amounts (Farrar *et al.*, 2003).

Exudates components are involved in functions such as attraction of rhizobacteria to the plant, immunity of plant against pathogens and regulation of nutrient availability (Wang *et al.*, 2009; Bais *et al.*, 2004). Composition and concentration of exudates released is dependent on factors such as soil structure, plant species, plant age, plant nutrition and other microorganisms (Carvalhais *et al.*, 2011; Berg, Smalla, 2009).

2.7. Factors affecting soil bacterial community

2.7.1. pH

Plants and microorganisms relate differently towards the requirement of oxygen, light, temperature and pH. A soil with low pH or high pH might have both negative and positive effect on the plant-microbe association, particularly in the soil which is acidic (pH below 5), even though there are some microbes that grow well under acidic conditions the majority of the rhizobacteria are affected by variations in pH.

Low pH negatively affects a large amount of other nutrient elements, PGPR and PGPR-plant associations (Dakora, Phillips, 2002). With continuous exposure to acidic conditions, only a small group of PGPR might become tolerant to acidic conditions. One of the major factors causing this acidic medium in the rhizosphere is organic acids in the root exudate, which are produced by PSB when the phosphorous amounts in the soil are low (Puente *et al.*, 2004). Even though organic acids produced by PSB to solubilize inorganic phosphate to phosphorous are meant to benefit the plants by increasing P and other elements such as iron, manganese and zinc, they are more effective under acidic soil (Welch *et al.*, 2002).

2.7.2. Soil Organic Matter (SOM), temperature and moisture

SOM is all living and dead organic substances in the soil made up of microorganisms, plant residues, humus and detritus. SOM composition, amount and availability determine the bacterial population in the plant host soil. Continuous supply of SOM in plant soil is needed in the bacteria-plant associated relationship because they provide energy and make carbon supply sufficient which makes up the nutrients (Six *et al.*, 2002). High amounts of SOM increases plant productivity, an increase in plant productivity cause an increase in SOM needed by microbes.

SOM increase bacterial diversity and contributes to their pathogen suppression activities. Other environmental factors affecting microbes are pH, temperature and soil moisture. Soil with a high pH decomposes SOM slower than acidic soil. Different microbes require different temperature conditions, some grow well at either high or low temperature; but some growth promoting bacteria are affected by low temperature such as *Bacillus* species which requires 16°C and above to produce antibiotics. Unfavourable temperature might prevent colonization of plant roots by beneficial rhizobia. High temperature reduces soil water quantity and that results in a drought

environment (Diouf, Lambin, 2001), drought causes changes in exudate make up, which interfere with its chemo attractant ability and colonization by bacteria (Henry *et al.*, 2007).

2.8. Molecular studies

Molecular methods rely on the characterization of desired cellular constituents such as nucleic acids, proteins and fatty acids (Hill *et al.*, 2000). With these methods, samples may be extracted directly from the environment without culturing. A variety of molecular techniques are available depending on the cellular constituent of choice e.g. nucleic acids (Deoxyribose or Ribose Nucleic Acids); proteins or fatty acids. Deoxyribose Nucleic Acids (DNA) is one of the most widely used molecules in molecular studies to identify microbial composition (Greene *et al.*, 2003). DNA molecules are present in all prokaryotes; they have highly conserved regions, are easy to amplify using Polymerase Chain Reaction (PCR) and can rapidly be sequenced (Robe *et al.*, 2003).

PCR amplification of the 16S rDNA followed by separation of PCR product on Denaturing Gel Gradient Electrophoresis (DGGE) with urea and formamide is an essential method for analysis of the soil bacterial community (Muyzer *et al.*, 1995). PCR is the preferred molecular method to amplify the bacterial DNA because it has the ability to produce million copies of a small portion of DNA molecule (Malik *et al.*, 2008). It can amplify a desired gene or the entire gene within a short period of time such as, within 4 hours. The PCR technique is the best method to amplify DNA fragments coding for 16S rRNA gene. This gene is part of the DNA, 16S rRNA and 16S rDNA genes are interchangeably used (Clarridge, 2004).

The 16S rRNA gene codes for rRNA and rRNA makes part of the ribosome, PCR amplified products of high yield and quality are dependent on the proper initial step of extraction and

purification of DNA to prevent contamination and false results in further analysis (Muyzer *et al.*, 1995).

The extraction of nucleic acids from a soil sample is by physical or chemical action and the process involves lysis of the cell wall to free the nucleic acids. After the extraction of DNA, PCR takes place and the PCR amplified product serves as the fragments consisting of the 16S rRNA gene. The presence and size of the amplified DNA is determined using agarose gel electrophoresis. PCR product separation by agarose gel electrophoresis result only in a single DNA band that is largely non-descriptive because the DNA fragments are identified and separated based on different DNA fragment lengths.

Denaturing Gradient Gel Electrophoresis (DGGE) overcomes limitation of agarose gel electrophoresis separation because during polyacrylamide gel electrophoresis in DGGE, the DNA amplified fragment separate into same length fragment with different base pair composition and the base pair nucleotides are more descriptive. DGGE as the case with other methods has its own limitations as only less than 500 base pair nucleotides can be amplified and observed on DGGE thus limiting phylogenetic information. Methods that can give precise phylogenetic information involve the cloning of an amplified PCR product into a vector to create a 16S rDNA clone library. Clones of interests are selected from host cells and amplified. The amplified clones have long DNA sequence which allows maximum phylogenetic information (Gonzalez *et al.*, 2003). As such, molecular techniques are more relevant in studying bacterial communities in soil.

2.9. Prospects on soil bacterial community

The common role of bacterial communities as a soil health indicator is very useful especially in agriculture. However, there is still more to understand about their mode of action in promoting growth, host choice, nutrients and applications in the field. Better knowledge on molecular methods determining their beneficial traits must be extended focusing on specific plant growth-promoting agent genes. Molecular studies may help us to genetically modify beneficial genes introducing them into less active bacterial cells.

There is a need to increase the number of combined plant growth traits and inoculants must be identified to better promote plant growth and fight pathogens. A better understanding of this will correlate with an increase in plant growth and crop productivity, which will reduce food insecurity. These beneficial bacterial communities can be used to improve the environment making it more sustainable through the reduction of air pollution and a reduction in public health hazards imposed by chemical fertilizers and pesticides. At the same time constant monitoring of these bacteria in different seasons, environments, and nutrients must be carried out to prevent development of pathogenic resistant strains and cross mutations.

Table 2.1. Root exudate composition in variety of plant host species

Exudate compounds	Examples of element substances in exudate compounds
Sugars	Glucose, galactose, ribose, fructose, arabinose, xylose, rhamnose, maltose, deoxyribose, raffinose and oligosaccharides
Amino acids	Glycine, leucine, lysine, tryptophan, histidine, methionine, serine, asparagine, aspartate, cystein, cystine, proline α-alanine, β-alanine, valine, ornithine, glutamate, threonine, homoserine, Phenylalanine, arginine, α-aminoadipic acid and γ-aminobutyric acid
Organic acids	Citric acid, oxalic acid, malic acid, fumaric acid, succinic acid, acetic acid, butyric acid, valeric acid, glycolic acid, piscidic acid, formic acid, aconitic acid, lactic acid, pyruvic acid, glutaric acid, malonic acid, tetronic acid, aldonic acid and erythronic acid
Enzymes	Protease, acid phosphatase, alkaline phosphatases, amylase and invertase
Vitamins	Biotin, riboflavin, thiamin, niacin and pantothenate
Purines	Adenine, guanine, cytosine and uridine
Inorganic ions and gaseous molecules	HCO_3^-, OH^-, H^+ , gaseous molecules CO_2 and H_2

(Dakora, Phillips, 2002)

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Study area

Soil samples for this study were collected from public schools' vegetable gardens (Figure 3.1.) in two villages; Madibe a Rra Tau and Madibe Makgabane near Mahikeng (25°50'S, 25°38'E), North West Province; South Africa. Mahikeng is a typical semi-arid area characterized by savanna climate and annual mean summer rainfall of 571 mm. The soils of the study area are brown to dark reddish sandy loam.

3.2. Sample collection

Soil samples were collected from the rhizosphere of seven cultivated plants (onion, spinach, lettuce, maize, beetroot, green peas and tomato) using randomised complete block design. Samples were designated as RO (Onion rhizosphere), RS (Spinach rhizosphere), RL (Lettuce rhizosphere) RM, (Maize rhizosphere) RB, (Beetroot rhizosphere), RG (Green peas rhizosphere) and RT (Tomato rhizosphere). Each of the seven plots was divided into three equal sub-plots which were used as replicates of each sample; resulting into a total of twenty one samples. Approximately 200 g of soil samples were randomly collected 5 cm deep from root surface of each replicate and placed into sterile polythene bags using sterile spades and spatulae. Soils were transported on ice to the laboratory and stored at 4°C.

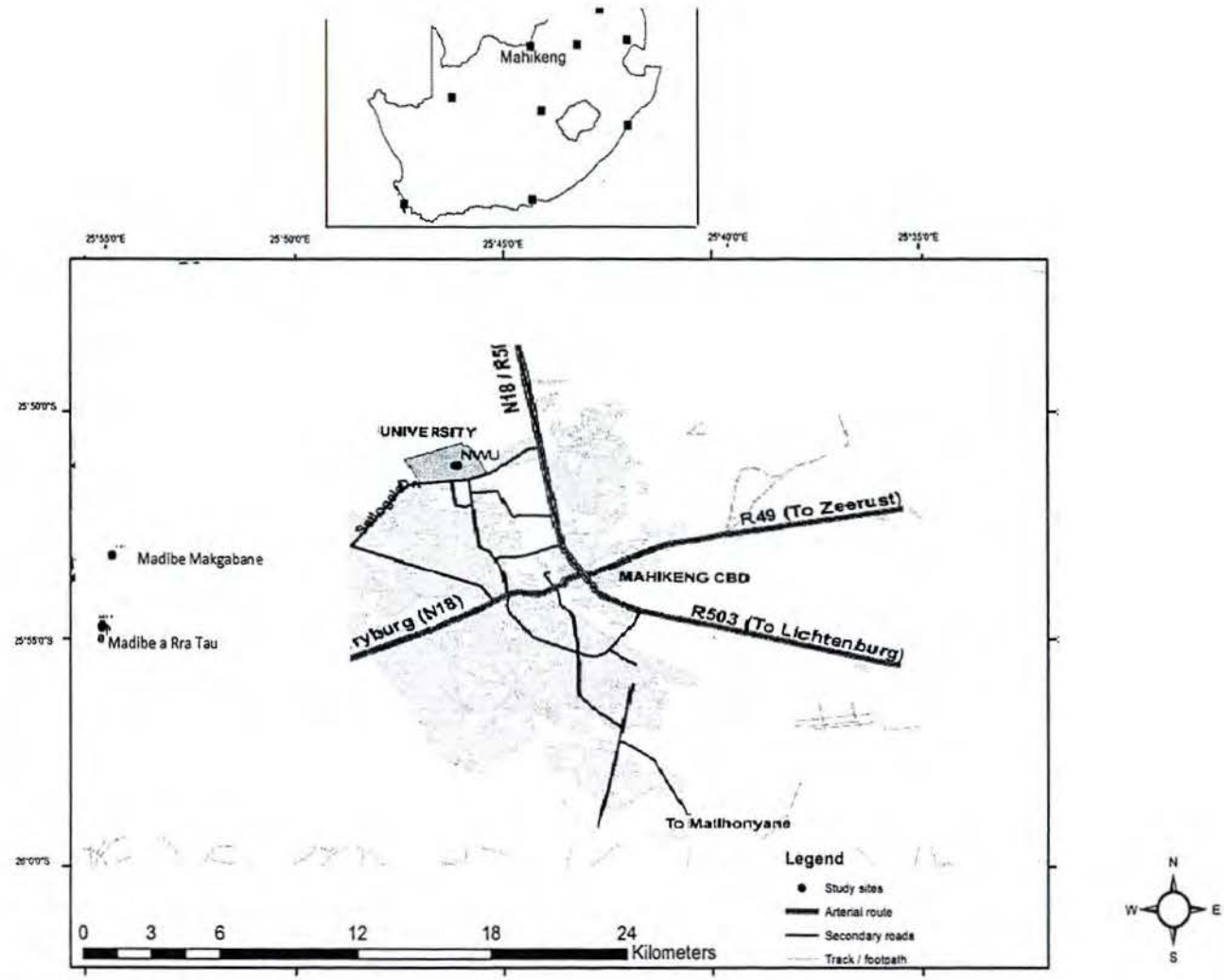


Figure 3.1. Location of villages around Mahikeng where soil samples were collected, samples RS, RM and RT were collected from Madibe Makgabane; samples RO, RL, RG and RB were collected from Madibe a Rra Tau. Greenhouse plant growth assay experiment soil was collected from North-West University (Mafikeng campus).

3.3. Chemical characteristics of soil samples

From three replicates of each sample, 50 g of soil were mixed together to form a homogenous community and chemical analyses were conducted. The soil was air dried for 24 hr and passed through a 2 mm sieve, about 10 g of mixed soil from each plant was placed in 100 ml sterile water and allowed to stand for 1 hr. The pH of each soil sample was taken using a Crison basic pH meter (Lasec, South Africa). From each of the dry soil samples 1 g was mixed with 10 ml of 70% concentrated nitric acid (15.8 M), 3 ml of concentrated 37% hydrochloric acid (12 M) and 3 ml of 30% of 0.04 M hydrogen peroxide. Samples were digested in a multiwave 3000 microwave digesting system (Anton paar) for 45 min and allowed to stand for 24 hr. The digested soil solution was then analysed by Nexion Inductively Coupled Plasma-Mass Spectrometry (PerkinElmer, Inc) for determination of total elements (carbon, nitrogen, calcium, potassium, iron, magnesium, sodium, phosphorous, zinc, cadium, cobalt, chromium and copper) content in the different samples.

3.4. Bacterial community catabolic profiling in rhizosphere

Sole Carbon Substrate Utilization (SCSUP) by the bacterial community in each rhizosphere soil was determined using Biolog GN2 microplate (Cabot Boulenard Hayward, USA) according to manufactures instructions Garland *et al.* (2001). The Biolog GN2 microplate used in this study consists of 93 wells containing carbon sources, tetrazolium dye and a control well with water substrate. Carbon sources included; 30 carbohydrates sources, 26 carboxylic acid sources, 18 amino acids sources, 12 polymers sources and 6 amides/amines sources. Microplates consists of carbon source substrates such as carbohydrates (D-xylose, i-erythritol, alpha-D-lactone, D-mannitol, N-acetyl-D-glucoside, glucose-1-phosphate, and D-cellobiose). Microplate carboxylic acids included 1-hydroxy benzoic acid, D-glucosaminic acid alpha ketobutyric acid, D-

galactomic acid-gamma-lactone, D-galacturonic acid, 2-hydroxy benzoic acid, and itaconic acid. Polymers such as tween 40, glycogen and alpha-cyclodextrin, amino acids such as L-threonine, glycyl-L-glutamic acid, L-phenylalanine and L-asparagine.

Tetrazolium dye is an indicator of a positive reaction it changes to violet formazan when bacterial communities produce nicotinamide adenine dinucleotide plus hydrogen ($NADH + H^+$). The bacterial community members were first isolated from the soil through serial dilution; 4 g of each soil sample were placed in 36 ml of sterile 0.85% sodium chloride (NaCl). The mixture was vortexed for 5 min and 4 ml of the solution was diluted with 36 ml sterile water to make 10^{-1} to 10^{-3} diluted solutions. An aliquot of each sample (140 µl of 10^{-3}) was placed into Biolog GN2 microplate 93 wells and optical density (O.D) was immediately measured by microplate reader at 620 nm. The Biolog microplates were further incubated at 30°C and the O.D was measured after 72 hr to determine average well colour development (AWCD) and Group wise average well colour development ($AWCD_G$).

3.5. Bacterial isolation and characterization

Replicates of soil sample for each treatment (RO, RT, RS, RG, RM, RB and RL) were mixed together and 1 g was weighed and placed into 9 ml distilled water and serially diluted up to 10^{-5}. From the serial dilutions, 1 ml of 10^{-4} solution of each sample was plated onto plate count agar (Merck) in triplicates using spread plate technique and incubated for 48 hr at 37°C. Colony counts were measured to determine colony forming unit (cfu) per g of soil. Dominating colonies were randomly selected and aseptically sub-cultured in Nutrient agar (Merck) to form purified bacterial isolates. Pure bacterial isolates were subjected to cell-wall morphological test and biochemical tests. Pure bacterial isolates were characterized based on cell wall morphology using Gram staining technique and biochemical tests. Biochemical tests performed includes oxidase,

catalase, citrate utilization, sugar fermentation, motility and indole reaction according to Ngoma *et al.* (2013).

3.6. Plant growth-promoting traits assay

3.6.1. Determination of Indole-3-Acetic Acid (IAA) activity

Bacterial isolates were grown in 10 ml Luria Bertani (LB) broth (Merck) for 48 hr at 37°C. After incubation standardized bacterial inoculum was done according to Sadeghi *et al.* (2012), by adjusting 5 ml of bacterial suspension to optical density of 0.5 which is approximate to 10^8 cfu/ml measured at 540 nm using a Thermospectronic spectrophotometer (Merck). Determination of IAA activity was done according to Rashid *et al.* (2012) with modifications, 100 µl of consistent bacterial inoculum was placed into 3 ml of LB supplemented with 1 g of tryptophan (Merck), incubated at 30°C for 48 hr with continuous shaking at 200 rpm on a rotary shaker Ultra rocker rocking platform (Bio-rad). Non inoculated broth was kept as a control.

IAA serially diluted aqueous solutions with different concentrations (6.25 to 100 mg/ml) were prepared and their absorbances were measured at 530 nm using Thermospectronic spectrophotometer (Merck). IAA production was determined, bacteria cells were removed by centrifugation Hermlez Z300 (Lasec, SA) at 10.000 rpm for 10 min, 1 ml of supernatant was mixed with 100 µl Salkowskis colouring reagent (Fluka) consisting of 1 ml of 0.5 M $FeCl_3$, 50 ml of 35% $HClO_4$, 2 drops of orthophosphoric acid and 5 mg/ml IAA solution. Development of a pink colour after 30 min indicated IAA production. The amount of IAA produced by isolates and control were measured by Thermospectronic spectrophotometer (Merck) at 530 nm Karnwal, (2009), measured absorbances of the different IAA concentrations were used to make a standard graph. Unknown concentration of IAA produced by bacterial isolates was determined

using the absorbance of IAA produced by isolates and standard graph. The standard calibration was done using distilled water to set the wavelength to zero optical density.

3.6.2. ACC-deaminase assay

Bacterial cultures were inoculated into 10 ml Nutrient broth (Merck) for 24 hr at 30°C; the 10 ml bacterial suspension was adjusted to form consistent inoculums at optical density of 0.5 by diluting or concentrating the suspension which is equivalent to 10^8 cfu/ml using Thermospectronic spectrophotometer (Merck) at 530 nm Sadeghi *et al.* (2012). The control contained a LB broth without a bacterial inoculants. ACC serially diluted aqueous solutions of different concentrations (0.625 to 10 mg/ml) were prepared and 5 mg/ml ACC was used as a standard reagent. ACC aqueous solutions absorbances were measured at 530 nm with Thermospectronic spectrophotometer (Merck) and a standard graph was made. ACC-deaminase activity was determined as done by Marques *et al.* (2010). The bacterial suspension (10 ml) was centrifuged to remove bacterial cells, 5 ml of the bacterial suspension supernatant was placed into 5 ml of 0.1 M Tris-HCl pH 7.6 and centrifuged Hermlez Z300 (Lasec, SA) at 10,000 rpm for 1 min. Pellets formed were re-suspended with 2 ml 0.1 M Tris-HCl pH 8.5, 30 µl toluene (Fluka) and vortexed. The 200 µl supernatant from suspended culture pellets was placed in tubes with 10 µl of 5 mg/ml ACC (Sigma-Aldrich, USA) solutions, vortexed and incubated for 15 min at 30°C. The solution was placed into 1 ml of 0.56 M HCl, and centrifuged Hermlez Z300 (Lasec, SA) at 10,000 for 1 min and 1 ml supernatant was mixed with 1 ml of 0.56 M HCl. Product was subjected to 2 ml of 2, 4-dinitrophenylhydrazine reagent (Sigma-Aldrich, USA) and vortexed for 5 min, incubated at 30°C for 30 min and 2 ml of 2 M NaOH (Sigma-Alrich, USA) was added to the solution. ACC-deamiase activity was measured by determining α-ketobutyrate production at 540 nm by Thermospectronic spectrophotometer (Merck) using sterile nutrient broth as a control.

The α-ketobutyrate unknown concentration was determined form the standard graph and standard calibration was done using distilled water to set the wavelength to zero optical density.

3.6.3. Detection of Hydrogen cyanide production

The test was done according to Ahmad *et al.* (2008). Isolates were grown in 20 ml of nutrient broth (Merck) for 24 hr at 37°C. Thereafter about 1 ml of the overnight culture was transferred onto the prepared nutrient agar (Merck) supplemented with glycine (4 g/l) and placed on the plate using spread plate technique. Sterile filter paper soaked into 2% sodium carbonate and 0.5% picric acid (Sigma-Aldrich), was placed on the agar plate lid, the plate was sealed with parafilm to prevent oxygen from entering the plate. The agar plate was incubated at 35°C for 72 hr and formation of brown colour in the filter paper indicated a positive test.

3.6.4. Phosphate solubilization activity

Culture isolates were grown on 10 ml nutrient broth (Merck) for 48 hr at 30°C and the two-day old cultures were subjected to serial dilution to form 10^{-2}, 10^{-4}, and 10^{-8} dilutions. The 10^{-8} dilutions were adjusted to 0.5 optical densities (O.D) which was approximately 10^{8} cfu/ml using Thermospectronic spectrophotometer (Merck) to ensure inoculum consistency (Pillay, Nowak, 1997). The consistent inoculum 1 ml solution was plated on a nutrient agar for 24 hr at 37°C using the spread plating technique. After incubation bacterial isolates were spot inoculated in the center of Pivoskaya's agar (SIGMA) plate and incubated at 30°C for 7 days to determine their phosphate solubilizing potential Singh *et al.* (2001). Positive reaction was identified by the formation of a clear zone around the inoculum and the control plate consisted of Pivoskaya's agar without bacterial inoculum.

3.6.5. Biocontrol antifungal activity

Bacteria cultures were placed into 10 ml nutrient broth (Merck) for 48 hr at 37°C, after incubation the bacterial suspensions were adjusted to 0.5 O.D which is equivalent to 10^8 cfu/ml. Adjusted 1 ml was spread on a nutrient agar (Merck) for 48 hr at 37°C, bacterial isolate was inoculated in each quadrant in the potato dextrose agar (PDA) (Merck) plate using sterile toothpicks for 48 hr at 37°C. Antagonism activity of bacterial isolates against *F. oxysporum* was determined according to Ngoma *et al.* (2013) with modifications. *F. oxysporum* was purchased from Davis Diagnostics (Pty) Ltd, (South Africa), cultured on a PDA and incubated at 28°C for 6 days. After the isolates had grown, *F. oxysporum* isolate was placed in the middle of potato dextrose plate with grown bacterial isolates, incubated at 28°C for 46 hr. The control plate contained a fungus only without bacterial inoculation, positive antifungal test was shown by the inability of the fungus to cover over the bacterial isolates. Percentage radial growth inhibition was measured by the formula: RIRG (R1-R2/ RG) x100, where RI is the length of fungus growth in mm from the center of fungus inoculant to the circumference of its growth (R1) minus the radial growth of fungus interacting with bacterial inoculant (R2). RG is the radial growth of fungi in control plate measured.

3.7. Greenhouse plant growth assay

Three bacterial isolates of the species *Bacillus mojavensis* (RT5), *B. pumilus* (RG3) and B. *licheniformis* (RL1) which showed the best plant growth-promoting traits in the preliminary *in vitro* assays were used to assess their ability to initiate and enhance growth parameters of the three crops. Preparation of bacterial inocula and seed inoculation for the greenhouse experiment was done according to Noumavo *et al.* (2013) with modifications. Culture isolates were grown on 20 ml nutrient broth for 48 hr at 30°C and the two-day old cultures were subjected to serial

dilution to form 10^{-2}, 10^{-4}, and 10^{-8} dilutions. The 10^{-8} dilutions were adjusted to 0.5 O.D which was approximately 10^{8} cfu/g using spectrophotometer to ensure inoculum consistency according to (Pillay, Nowak, 1997). Sterilized seeds of tomato, spinach and beetroot were coated with bacterial by putting the seeds into the 10 ml of 10^{8} inocula solutions and the control seeds were coated inside pure sterile nutrient broth for 24 hr at 30°C. Seeds coated with inoculum and inoculum free control seeds were planted into 400 g greenhouse soil (2x sterilized) inside 15 cm diameter pots and placed in the greenhouse at the North-West University. Pots were watered daily and growth parameters shoot length and numbers of leaves were measured at the thirtieth day.

3.8. Molecular characterization

3.8.1. DNA extraction

Isolation of genomic DNA from cultured bacteria was conducted using the ZymoResearch Soil Microbe DNA MiniPrep™ (Zymo Research, USA) extraction kit according to the manufacturer's instructions. Inside ZR bashingbead™ lysis tube with 750 µl lysis solution, 0.25 g of soil was added. Solutions inside the tubes were mixed on a bead beater (Disruptor Genie, Scientific Industries) for 5 min and centrifuged Hermlez Z300 (Lasec, SA) at 10,000 x g for 1 min. The 400 µl supernatant was added to 1,200 µl of soil DNA binding buffer and centrifuged at 10,000 x g for 1 min. The 800 µl supernatant was added to zymo-spin IIC column; pre-wash buffer (200 µl) was placed into the zymo-spin IIC column and centrifuged at 10, 000 x g for 1 min. Also the DNA wash buffer (500 µl) was added into the zymo-spin IIC column and centrifuged at 10,000 x g for 1 min. Zymo-spin IIC column was transferred into a sterile 1.5 ml microcentrifuge tube, followed by the addition of 100 µl DNA eluting buffer and centrifugation

at 10, 000 x g for 30 sec, after which DNA was transferred into 1.5 ml microcentrifuge tube and stored at 4°C until further use.

DNA from soil bacteria was directly extracted using Power soil™ DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, USA) following manufacturer's instructions. Samples 0.25 g was placed separately into power bead tubes with 60 µl lysing solution C (precipitates inorganic materials and cell debris) mixed on a bead beater (Disruptor Genie, Scientific Industries) for 1 min and centrifuged at 10, 000 x g for 30 sec. Supernatant (400 µl) was transferred into a clean 2 ml collection tube and 250 µl of solution C2 was added into supernatant vortexed for 5 min, incubated at 4°C for 5 min and the mixture was centrifuged for 1 min at 10, 000 x g. From the supernatant 500 µl solution was transferred into a clean 2 ml collection tube, 200 µl of solution C3 was added, tube was incubated for 5 min at 4°C and centrifuged for 1 min at 10, 000 x g. From the supernatant 600 µl solution was placed into clean 2 ml collection tube and 1200 µl solution C4 (adjust DNA salt concentration to allow binding of DNA) was added, vortexed for 5 min and centrifuge at 10, 000 x g for 1 min. About 675 µl solution was placed into spin filter column (spin column contains a silica membrane which binds the DNA to the column) centrifuged at 10, 000 x g for 5 min and this step was repeated two times. Solution C5 (500 µl) was added into the spin filter, double centrifuged for 30 sec at 10, 000 x g and the flow was discarded. The spin filter placed on a clean 2 ml collection tube, Solution C6 (100 µl) was added into the collection tube containing a spin filter, tube was centrifuged for 30 sec at 10, 000 x g, spin filter was discarded and supernatant containing DNA was stored at 4°C.

3.8.2. DNA detection and quantification

To determine the presence of extracted bacterial DNA from both cultured isolates and direct soil microbes, 1.0% agarose gel was prepared by adding 2 g agarose (LONZA, USA) into 200 ml 1x

Tris- Acetate EDTA (TAE) buffer (Bio-Rad, USA). Agarose solution was heated for 4 min until it boiled, allowed to cool up to 50°C, followed by the addition of 20 µl of 0.5 µg/ml ethidium bromide into the agarose solution. The solution was poured into electrophoresis tray fitted with, a 20-well comb and allowed to solidify. The comb was removed from solid agarose gel and transferred to a buffer electrophoresis chamber filled with 1x TAE buffer. Ten (10) µl of each DNA solution was mixed with 10 µl of 6x DNA loading dye (Thermo Scientific) made up of bromophenol blue and xylene cyanol FF and loaded into gel wells and electrophoresis was performed using ELITE 300 PLUS (WEALTEC) for 2 hr at 70 V and 400 mA in 1x TAE buffer. After the gel was viewed under UV transilluminator 2000 (Bio-Rad Laboratories, USA) the gel was captured using chemidoc^{TM} MP imaging system (Bio-Rad). For DNA quantification, a drop of DNA solution was placed on the Nanodrop, the concentration of DNA was measured at 260 nm absorbance. The DNA concentration of both cultured and uncultured bacteria are indicated in the (Appendix 5.3. and Appendix 5.4.).

3.8.3. PCR amplification

PCR amplification of the 16S rRNA gene from the cultured bacterial DNA was performed using a Thermal Cycler C1000 Touch (Bio-Rad) in a 50 µl reaction mixture, containing 25 µl master mix (0.05 Unit/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, 0.4 mM of each dNTP, reaction buffer, Thermo Scientific), 0.5 µl of each 100 mM primer F27, R1492, 2 µl DNA templates and 22 µl nuclease-free water. The primers F27 5'-AGAGTTTGATCCTGGCTCAG-3' and R1492 5'-TGACTGACTGAGGCTACCTTGTTACGA-3' Frank *et al.* (2008) were synthesised by (DNA Technologies). The thermal cycling conditions were: 5 min at 94°C for initial denaturation, followed by 30 cycles for 30 sec at 95°C for denaturation, 1 min at 54°C for annealing, 2 min at 72°C for extension, and final extension for 5 min at 72°C.

For amplification of 200 base pair fragment in the 16S rDNA gene, primers F357 GC (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCA G-3' clamp and R518 (5'-ATTACCGCGGCTGCTGG-3') according to Muyzer *et al.* (1995) were used. the 50 µl reaction mixture contained 25 µl master mix (0.05 Unit/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, 0.4 mM of each dNTP, reaction buffer, Thermo Scientific), 0.5 µl of each 100 mM F357 GC and R518 primer, 2 µl DNA template and 22 µl nuclease-free water. The thermal cycling conditions were: 3 min at 94°C for initial denaturation, followed by 30 cycles of 1 min at 94°C for denaturation, annealing at 50°C for 1 min, extension at 72°C for 1 min and final extension for 10 min at 72°C.

To confirm the amplified 16S rRNA gene fragment size, 10 µl of each PCR amplifications product of both cultured and uncultured DNA fragments were mixed with 10 µl 6x loading dye (Thermo Scientific) and the final 20 µl solution was transferred into wells of a 1.2% agarose gel. About 10 µl of 0.1µg/µl 1 Kb plus DNA ladder (Thermo Scientific) was mixed with 10 µl of 6x DNA loading dye, 20 µl of mixed solution was transferred into wells and analysed on gel electrophoresis ELITE 300 Plus (WEALTEC) for 2 hr at 70 V, 400 mA and stained with 20 µl of 0.5 µg/ml ethidium bromide. The amplified 16S rRNA fragments were observed under UV transilluminator 2000 (Bio-Rad) and captured using a Chemidoc™ MP imaging system (Bio-Rad). Exactly 20 µl of amplified fragments of PCR products of cultured bacteria were sent for sequencing at Inqaba Biotechnological Industries (Pty) Ltd, (Pretoria, South Africa).

3.8.4. DGGE analysis

Gel electrophoresis was performed using DGGE D-Code TV 400 system (Bio-Rad Laboratories, USA) as described by Singh and Ramaiah (2011). PCR products (20 µl) from metagenomic DNA amplified using P357F GC-clamp and P518R primers were placed into 8% (w/v)

acryalamide gel (acrylamide/ bis solution, 37.5:1, Bio-Rad) containing a gradient from 40 to 60 % denaturant (100% denaturant contained 7 M urea and 40% v/v formamide). Electrophoresis was performed at a constant voltage of 60 V, at 60°C for 16 hr in a DGGE chamber containing approximately 7 L of 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, at pH 8.0). After electrophoresis, the gel was stained with 0.5 µg/m ethidium bromide for 15 min, and placed in the remaining 500 ml 1x TAE to remove excess ethidium bromide for 45 min at 4 rpm on a rotary spinner. The gel was distained with distilled water, viewed gel on a UV transilluminator 2000 (Bio-Rad Laboratories, USA) and the image was captured sing a Chemidoc^{TM} MP imaging system (Bio-Rad).

3.8.5. Excision of DGGE bands

DGGE gel was placed under UV transilluminator, bands with high intensity were excised from the gel using sterile razor blade and placed into sterile 1.5 ml eppendorf tube and 20 µl of TE buffer (pH 8.0) was added into the tube. The band gel was crushed with a sterile pipette tube, allowed to soak for 24 hr at 4°C to allow the DNA to move from the gel into solution. The product was centrifuged at 5000 x g for 5 min and the eluted DNA (present in the supernatant) was reamplified with F357 without a GC clamp and R518 using thermal cycling conditions similar to those used on F357 with a GC clamp. PCR products were subjected to sequencing at Inqaba Biotechnological Industrial (Pty) Ltd, (Pretoria, South Africa).

3.8.6. Sequencing

Nucleotide sequences of amplified 16 rRNA from PCR products of culture DNA and metagenomic DNA were determined at Inqaba Biotechnological Industries (Pty) Ltd, (Pretoria, South Africa) using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) according to manufacturer's instructions. Nucleotide sequences were first purified and quantification followed

by determining the nucleotides sequences in both forward and reverse direction using F27, R1492, F357 and R518 primers.

3.8.7. Phylogenetic analysis

The identity and evolutionary relationships of sequences were checked by phylogenetic analysis. Sequence information of 1.5 Kb and 200 bp fragments were subjected to Chromatolite 2.10 for nucleotide bases calling, followed by editing the sequences, creating consensus sequences using Bioedit sequence alignment editor. BLAST search was used to retrieve sequence relatedness to the closest identified taxonomy in the national center for biotechnology (NCBI) website (http://www.ncbi.nlm.nih.gov/Blast). Isolate sequences and sequences generated from the GenBank were aligned using Clustal W included in Mega 5.22 (http://www.megasoftware.net) program. The phylogenetic tree was constructed and analysed using MEGA 5.05 software using Neighbor Joining (NJ) method (Nei *et al.*, 1985) of bootstrap settings at 1.000 replicates based on Maximum composite likelihood method. Phylogenetic tree analysing 16S rRNA sequences of all 16 isolates was constructed using 3 sequences from Genbank which are most likely related to the isolate sequences (to increase identity accuracy).

3.8.8. Statistical analysis

One-way Analysis of Variance (AVOVA) was used to determine the significant difference of AWCD and $AWCD_G$ of the bacteria in all rhizosphere samples. For comparing the effect of bacterial inoculants on the plant physiological growth parameters, ANOVA was used to compare the means of the growth parameters with t-test and least significant difference (LSD) at 0.05 significant levels.

CHAPTER 4

4. RESULTS

4.1. Soil sample properties

The pH values of all soil samples ranged from 7.5 to 9.1 and the highest was in rhizosphere of greenhouse (RGH). Physico-chemical properties (Table 4.1.) varied between all soil samples. Macronutrients (carbon, nitrogen, calcium, iron, potassium, phosphorous, sodium and magnesium) had a wide range of average concentrations in all samples from 0 to 4380.1 mg/kg. The trace elements (cadmium, cobalt, chromium, copper and zinc) had the lowest average concentration in all samples between 0 to 3.1 mg/kg.

Table 4.1. Physico-chemical properties of rhizosphere samples

		Average elements concentration (mg/Kg) in three replicates											
Sample	pH	C	N	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Na	P	Zn
RM	8.0	8.1	4380.1	35.6	0	0	0.6	0	39.2	6.4	18.8	0.9	1.1	0.9
RB	8.3	3.6	260.3	18.3	0	0	0.1	0	33.1	5.4	14.2	1.3	0.5	1.9
RL	7.8	5.5	1047.2	24.1	0	0	0.1	0	46.8	7.1	15.2	1.1	0.6	2.6
RG	8.7	9.3	621.3	0.1	0	0	0.2	3.1	65.1	7.8	25.4	1.2	1.3	3.1
RS	8.2	4.6	0	19.3	0	0	0.1	0	9.1	2.4	5.1	1.1	157.3	1.6
RT	8.3	18.1	0	8.4	0	0	0.2	0.1	72.4	9.4	8.6	0.4	0	0.2
RO	7.5	7.7	0	36.1	0	0	0.1	0	40.6	6.6	19.3	1.3	0	0.9
RGH	9.1	13.1	3687.2	31.2	0	0	0	0	44.1	7.2	16.2	1.2	1.1	2.3

C: carbon, N: nitrogen, Ca: calcium, Cd: cadium, Co: cobalt, Cr: chromium, Cu: copper, Fe: iron, K: potassium, Mg: magnesium, Na: sodium, P: phosphorous and Zn: zinc. Recommended metal elements concentrations are 80 mg/kg (Cd), 20 mg/kg (Co), 3 mg/kg (Cr), 100 mg/kg (Cu) and 185 mg/kg (Zn) (Herselman *et al.*, 2005).

4.2. Bacterial community catabolic profiling in rhizosphere

4.2.1. Average well colour development between samples

Average well colour development (AWCD) in microplates by bacterial members was determined between all seven soil samples (Table 4.2.). For AWCD the overall F value = 0.2431, whereas P= 0.068, indicating that P is greater than 5% level of significant and that there is no significant difference in AWCD between all seven samples (Table 4.2.).

4.2.2. Group-wise average well colour development and Sole Carbon Substrate Utilization

Utilization of sole carbon sources by bacteria on the basis of AWCD was compared between groups of carbon sources in this study. Utilization of carbon substrates in each group of carbon source by bacteria differed amongst the samples. That there was a significant difference in the utilization of carbohydrates (F= 7.504 and P= 0.001), carboxylic acid (F= 5.971 and P= 0.003), amino acids (F=5.184 and P= 0.005), polymers (F= 3.565 and P= 0.023) at P less than 5% level of significant. There was no significant difference in the use of amides/amines (F= 2.733 and P= 0.057) at P greater than 5% level of significant. Highest carbon source group used by sample bacteria in microplates were carbohydrates; followed by amino acids, carboxylic acids and polymers. The used amides/ amines were phenylethylamine by bacteria in onion RO (rhizosphere sample) only (Table 4.3.)

Table 4.2. Average well colour development (AWCD) of the soil samples in Biolog GN microplates after 72 hr at 30°C

Sample	AWCD $OD_{620\ nm}$ at 72 hr			Mean AWCD
Rhizosphere of onion	0.51	0.50	0.53	$0.51^{a}\pm 0.03$
Rhizosphere of spinach	0.50	0.49	0.51	$0.50^{a}\pm 0.03$
Rhizosphere of lettuce	0.50	0.52	0.50	$0.50^{a}\pm 0.03$
Rhizosphere of maize	0.51	0.51	0.48	$0.50^{a}\pm 0.01$
Rhizosphere of beetroot	0.50	0.50	0.52	$0.51^{a}\pm 0.04$
Rhizosphere of green peas	0.52	0.53	0.50	$0.52^{a}\pm 0.05$
Rhizosphere of tomato	0.52	0.51	0.51	$0.51^{a}\pm 0.05$

OD: optical density; mean of three replicates followed by the same letter are not significantly different at F= 2.431, P= 0.068 at P greater than 5% level of significant using analysis of variance.

Table 4.3. Group-wise average well colour development in carbon sources groups of bacterial community in rhizosphere soil samples

Sample	Carbohydrate (30)	Carboxylic (27)	Amino acid (18)	Polymer (12)	Amides/amine (6)
Rhizosphere of onion	$0.58^{a}\pm0.02$	$0.42^{b}\pm0.05$	$0.62^{c}\pm0.04$	$0.59^{d}\pm0.03$	$0.29^{e}\pm0.04$
Rhizosphere of spinach	$0.37^{b}\pm0.01$	$0.52^{c}\pm0.01$	$0.43^{d}\pm0.01$	$0.56^{e}\pm0.05$	$0.23^{e}\pm0.01$
Rhizosphere of lettuce	$0.56^{c}\pm0.04$	$0.40^{d}\pm0.04$	$0.56^{e}\pm0.03$	$0.54^{f}\pm0.01$	$0.28^{e}\pm0.05$
Rhizosphere of maize	$0.27^{d}\pm0.01$	$0.26^{e}\pm0.02$	$0.23^{f}\pm0.05$	$0.26^{g}\pm0.04$	$0.28^{e}\pm0.03$
Rhizosphere of beetroot	$0.68^{e}\pm0.05$	$0.61^{f}\pm0.03$	$0.43^{g}\pm0.04$	$0.40^{h}\pm0.05$	$0.25^{e}\pm0.06$
Rhizosphere of green peas	$0.52^{f}\pm0.01$	$0.39^{g}\pm0.04$	$0.42^{h}\pm0.03$	$0.46^{i}\pm0.03$	$0.25^{e}\pm0.02$
Rhizosphere of tomato	$0.36^{g}\pm0.03$	$0.28^{h}\pm0.02$	$0.27^{i}\pm0.05$	$0.29^{j}\pm0.03$	$0.23^{e}\pm0.07$

The values are means of three replicates; means followed by the different letters are significantly different across carbon substrate (carbohydrates, carboxylic acid, amino acids and polymers) groups (columns) at $P \leq 0.05$ using analysis of variance. Means followed by the same letters are not significantly different across amides/ amines at $P \geq 005$ and $F= 2.733$.

4.3. Bacterial population in rhizosphere soil

Total colony counts of bacteria from seven rhizosphere samples were measured on PCA medium in three replicates and cfu/g of soil ranged from $1.25x10^6$ (RM), $1.95x10^6$ (RL), $2.06x10^6$ (RS), $2.20x10^6$ (RB), $2.26x10^6$ (RG), $2.30x10^6$ (RT) to $2.57x10^6$ (RO) in (Figure 4.1). Bacterial population in samples (RO, RM and RL) was significantly different from each other and they were all three significantly different from RS, RB, RG and RT bacterial population. However bacterial population in samples (RS, RB, RG and RT) were not significantly different from each other.

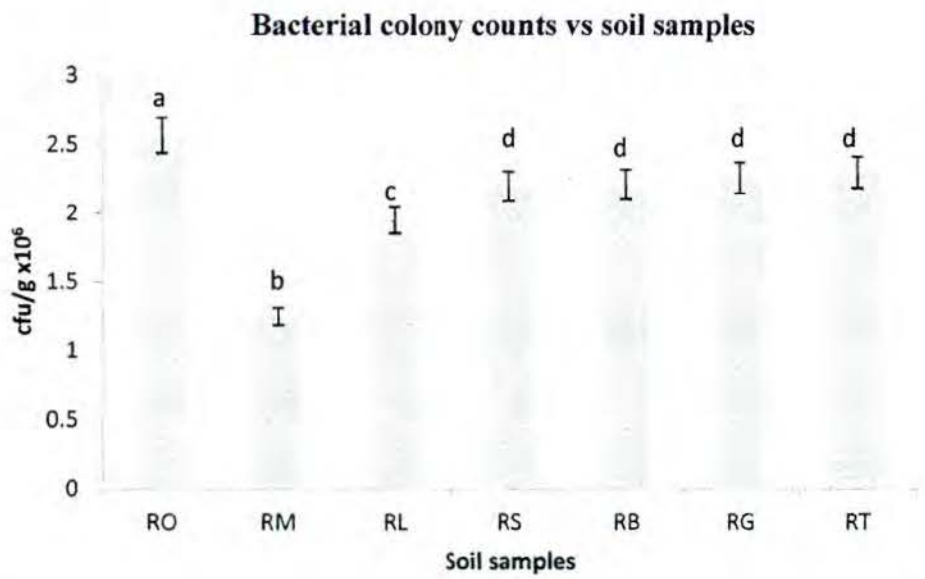


Figure 4.1. Bacterial populations in rhizosphere samples as colony forming unit (cfu/g) on Plate Count Agar (PCA); Bars represent percentage error of the mean at $P \geq 0.05$ using Analysis of variance and bars following with similar letters (d) are not significantly different.

4.4. Morphological and biochemical characterization of rhizosphere bacteria

Morphological characteristics of colonies showed that most colonies were convex, white and cream in colour; they were large with a smooth margin. Morphological and biochemical properties include only 16 isolates that were identified and confirmed at the NCBI. Isolates were designated as RM2, RT5, RO2, RS3, RL1, RG3, RT2, RB3, RO6, RG6, R8, R10, R11, R12, R14 and R15 source of isolates is shown in Appendix 5.7. The results indicate all isolates were gram positive, twelve were oxidase negative, with rod shape; eleven of the isolates were catalase positive. All isolates were indole negative but ten fermented sugar on TSI agar, ten used citrate as sole carbon source and nine were motile in SIM medium (Table 4.4.).

4.5. Preliminary *in vitro* plant growth-promoting traits assay

Plant growth traits in (Table 4.4.), showed that all 16 bacterial isolates have antifungal activity against *F. oxysporum*. The highest inhibition effect against *F. oxysporum* was observed from RT5 (*B. mojavensis*) and RG3 (*B. pumilus).* RT5 (*B. mojavensis*) had 57% percentage inhibition and RG3 (*B. pumilus*) had 43% inhibition percentage calculations showed in appendix 5.1. Phosphate solubilizing test showed that 15 isolates solubilised phosphate on Pikoskaya's agar except for R14 (*Streptococcus agalactiae).* Only three isolates produced HCN namely; RO6 (*B. cereus*), RG6 (*B. megaterium*) and RG3 (*B. pumilus*), highest HCN (Figure 4.2.) was produced by RG3 (*B. pumilus*) followed by RG6 (*B. megaterium*).

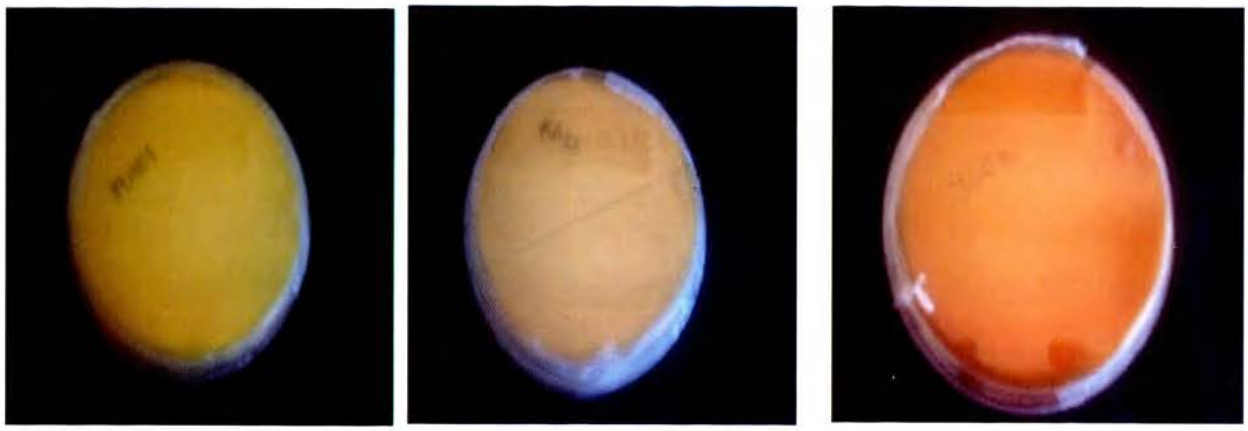
The standard IAA calibration curve was setup by determining the prepared different concentrations (6.25, 12.5, 25, 50 and 100 mg/ml) of IAA produced at 530 nm (Figure 4.3.), the highest IAA produced was 25 mg/ml by isolate RL1 (*Bacillus licheniformis*) with 3.8 absorbance at 530 nm (Table 4.4.). ACC-deaminase activity was determined by measuring the amount of α-ketobutyrate concentration produced by isolates. The α-ketobutyrate concentration was measured

from a prepared standard curve graph (Figure 4.4.) with different ACC concentration (0.625, 1.25, 2.5, 5 and 10 mg/ml). The highest amount of α-ketobutyrate produced was 4.8 mg/ml by RG3 (*B. pumilus*) with1.8 absorbance at 530 nm (Table 4.4.).

Table 4.4. Biochemical characteristics and plant growth traits assay of culture isolates

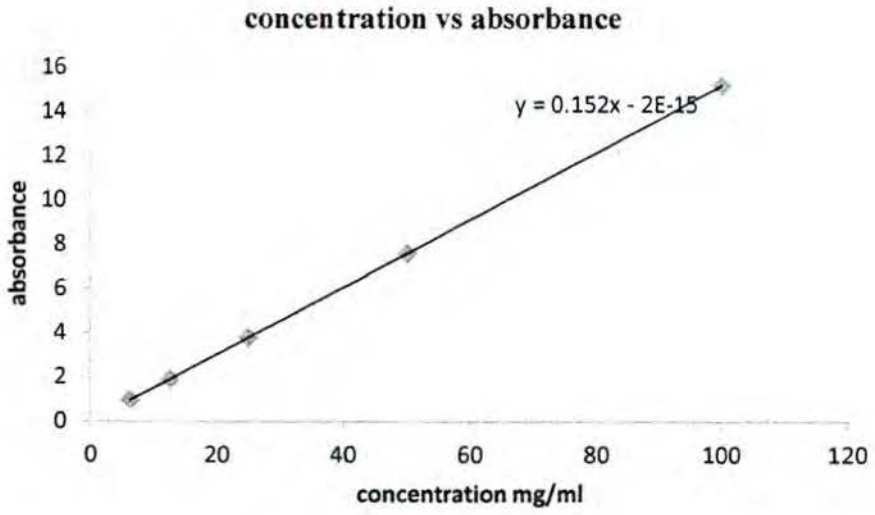
		Biochemical Characteristics							Plant growth traits assay						
Isolate code	Gram-stain	Oxidase	Catalase	TSI	Citrate	Motility	Indole	HCN	PSB	Antifun/ % inhibit		IAA O.D	IAA mg/ml	ACC O.D	ACC mg/ml
RM2	+ Rod	-	+	+	-	-	-	-	+	+	19	1.9	13.0	0.2	0.5
RT5	+ Rod	+	+	+	+	+	-	-	+	+	57	1.5	10.0	0.6	1.6
RO2	+ Rod	-	+	+	-	+	-	-	+	+	08	0.4	2.6	0.5	1.3
RS3	+ Rod	-	+	-	-	-	-	-	+	+	21	0.2	1.3	0.3	1.0
RL1	+ Rod	+	-	+	-	+	-	-	+	+	37	3.8	25.0	1.6	4.2
RG3	+ Rod	-	-	-	-	+	-	+	+	+	48	0. 7	4.6	1.8	4.8
RT2	+ Rod	-	+	+	-	+	-	-	+	+	11	0.7	5.9	0.2	0.5
RB3	+ Rod	-	+	+	+	-	-	-	+	+	28	0.9	1.3	0.3	0.8
RO6	+ Rod	-	+	+	-	+	-	+	+	+	25	0.2	7.2	0.9	2.4
RG6	+ Rod	-	-	-	+	-	-	+	+	+	14	1.1	3.3	1.5	4.0
R8	+ Rod	+	+	+	-	+	-	-	+	+	06	1.2	11.2	0.4	1.0
R10	+ Rod	-	+	+	+	-	-	-	+	+	23	0.5	14.5	0.7	1.8
R11	+ Rod	+	+	+	+	-	-	-	+	+	31	1.7	1.3	1.3	3.4
R12	+ Rod	-	-	-	+	-	-	-	+	+	26	0.2	1.9	0.9	2.4
R14	+ Rod	-	+	-	-	+	-	-	-	+	12	0.2	0.6	1.5	4.0
R15	+ Cocci	-	-	-	-	+	-	-	+	+	30	0.3	0.8	0.5	1.3

+: activity; -: no activity; TSI: triple sugar iron; HCN: hydrogen cyanide; PSB: phosphate solubilisation; Antifun: antifungal test and % inhibition; IAA: indole-3-acetic acid; ACC: 1-aminocyclopropane-1-Carboxylate.



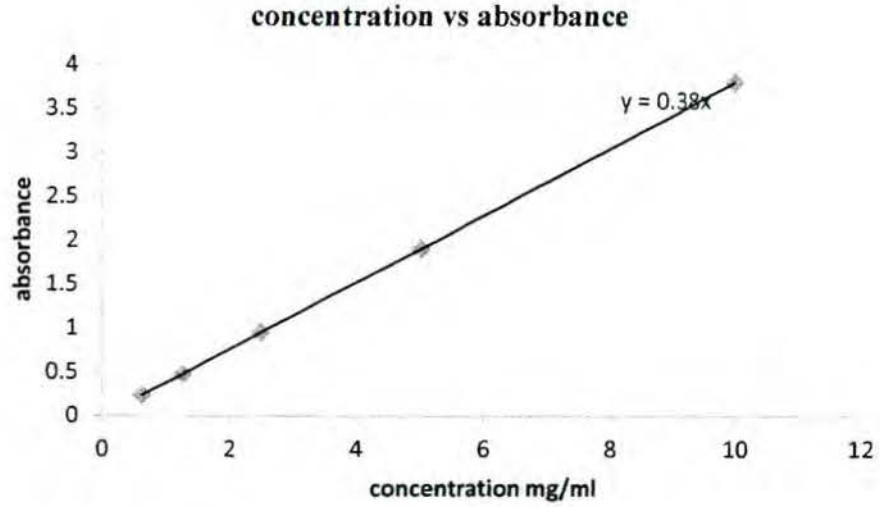
(a) (b) (c)

Figure 4.2. Hydrogen cyanide (HCN) productions by bacterial isolate after 96 hr of incubation as compared to control. (a) Negative HCN production by control without culture isolate. (b) Moderate HCN production by *B. Megaterium* (RG6). (c) High HCN production by *B. Pumilus* (RG3).



y = 0,125x-2E-15, x (concentration of RL1) = 25mg/ml

Figure 4.3. IAA Standard curve graph and the unknown IAA concentration produced by RL1 (*B. licheniformis*) is determined from the standard curve.



y = 0,38x -4E-16, x (concentration of RG3) = 4.74 mg/ml

Figure 4.4. ACC Standard curve graph and the unknown concentration of α-ketobutyrate produced by RG3 (*B. pumilus*) during ACC deaminase activity is determined from the standard graph.

4.6. Greenhouse plant growth assay

The effects of bacterial inocula on plant growth parameters (shoot length and number of leaves) were determined at 30 days after sowing (DAS). Plants of crop seeds inoculated with bacterial isolates grew faster than the control (uninoculated crop seeds) Figure (4.5.) and Figure (4.6.). Using Analysis of Variance (ANOVA) based on least square means. Appendix 5.2 summarizes the ANOVA design. Three levels of bacteria inoculants and 1 control administered to three varieties of crops. For shoot length the overall F value = 4.57 with a P-value = 0.017 indicating that P is less than 5% level of significance and that there was significant differences in shoot length of the plants.

Significant differences in shoot length of plants by inoculums RG3 (*B. pumilus*) were observed using t test (LSD) and Duncan's multiple range tests at the 5% level of significance. Inoculum RG3 caused the highest shoot length, followed by RT5 and RL1 as shown in Figure 4.7. For number of leaves the overall F value = 0.95 and a P = 0.3375 indicating that P is more than 5% level of significant and that there was no significant difference in the number of leaves in plants when RG3, RL1, RT5 and the control were administered as shown by the boxplots in Figure 4.8.

(a)

(b)

(c)

Figure 4.5. Growth of crops inoculated with bacteria and control at 30 DAS. (a) Growth of spinach inoculated with bacterial inoculum from left to right: control, *B. Lichneformis* (RL1), *B. Mojavensis* (RT5) and *B. Pumilus* (RG3). (b) Beetroot inoculated with bacterial inoculum from left to right: control, *B. Lichneformis* (RL1), *B. Mojavensis* (RT5) and *B. Pumilus* (RG3). (c) Tomato inoculated with bacterial inoculum from left to right: control, *B. Lichneformis* (RL1), *B. Mojavensis* (RT5) and *B. Pumilus* (RG3).

(a)

(b)

(c)

Figure 4.6. Growth parameters of crops inoculated with bacterial isolates and the control. (a) Shoots and roots length of spinach inoculated with from left to right: control, *B. Licheniformis* (RL1), *B. Mojavensis* (RT5) and *B. Pumilus* (RG3). (b) Shoots and roots length of beetroot inoculated with from left to right: control, *B. Licheniformis* (RL1), *B. Mojavensis* (RT5) and *B. Pumilus* (RG3). (c) Shoots and roots length of tomato inoculated with from left to right control, *B. Licheniformis* (RL1), *B. Mojavensis* (RT5) and *B. Pumilus* (RG3).

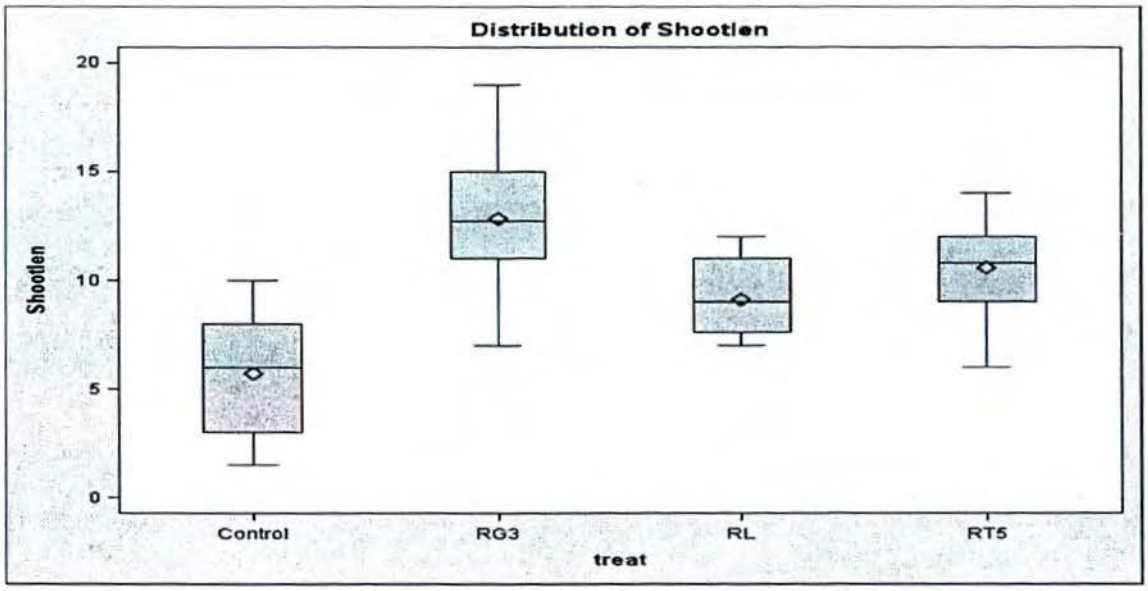


Figure 4.7. Box plot of inoculated plants shoot length by bacterial inoculum and control.

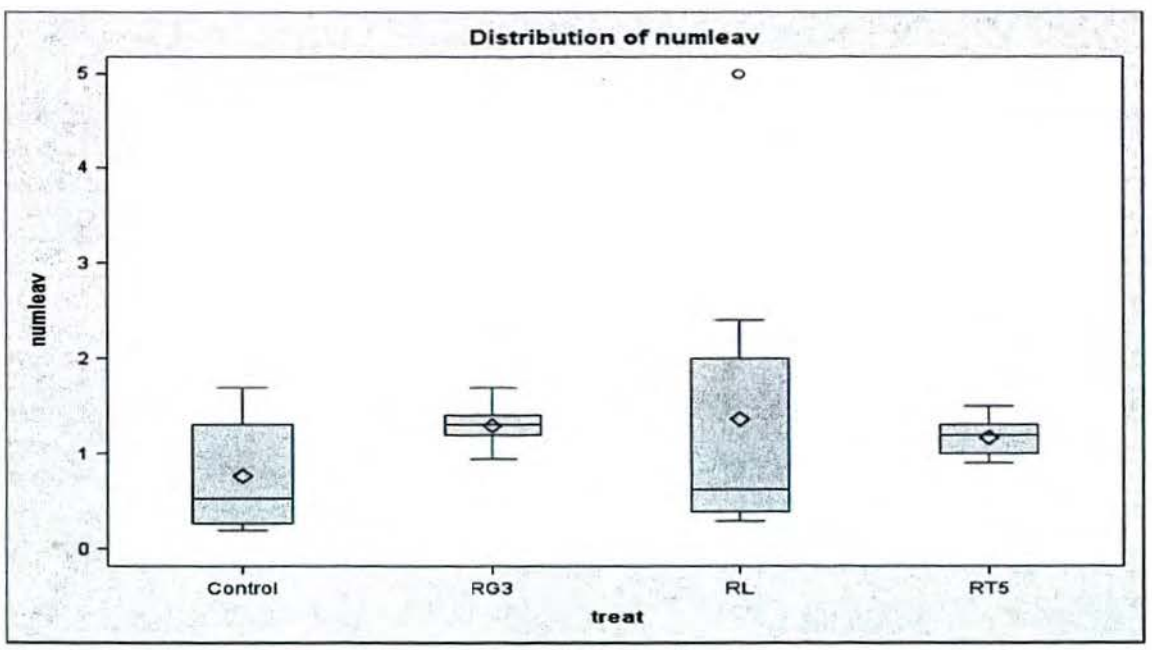


Figure 4.8. Box plot of inoculated plants number of leaves by bacterial inoculum and control.

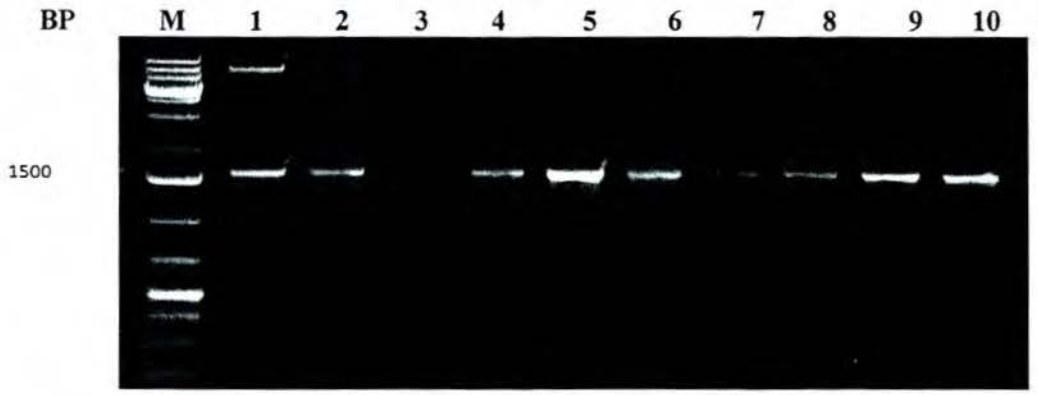
4.7. Molecular characterization

4.7.1. PCR amplification of bacterial isolates DNA

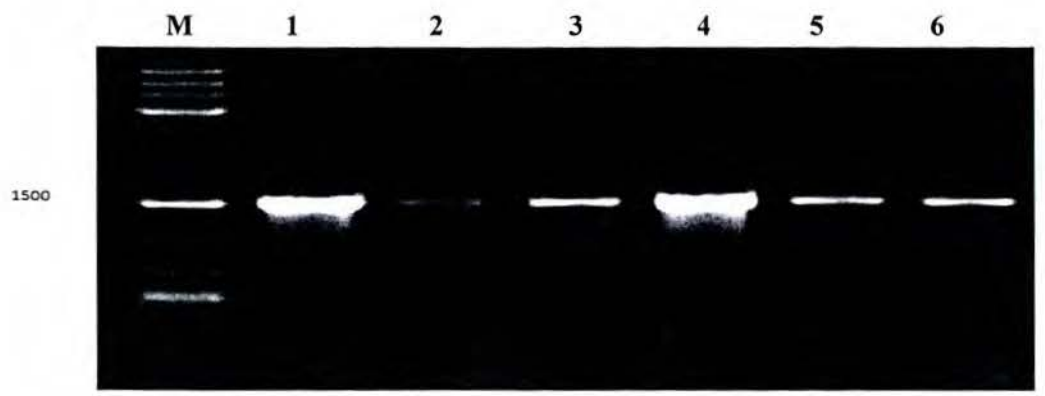
The DNA of bacterial isolates was successfully extracted and the DNA concentrations were quantified based on A260/A280 purity values (Appendix 5.3.) and (Appendix 5.4.) For the 16 bacterial isolates approximately 1.5 Kb within the 16S rRNA gene fragment was amplified by PCR using universal primers F27 and R1492 (Figure 4.9.).

4.7.2. Sequencing and phylogenetic analysis of bacterial isolates

Nucleotides sequences information of 1.5 Kb gene fragment for 16 culture bacteria were compared with the partial 16S rRNA sequences from the GenBank database using Basic Local Alignment Search Tool (BLAST). Bacterial 16S rRNA nucleotides sequences similarity to the closest relative in NCBI was obtained (Table 4.5.). Sequence homologies of the bacterial isolates to sequences of known bacteria in the NCBI ranged from 94 to100% and this indicated that there isolates are highly diverse. Phylogenetic analyses based on identification of evolutionary relationship between isolates and their close related species sequences in the Genbank (Figure 4.10.) indicated that thirteen isolates sequences were closely related to members of Firmicutes. Two isolates sequences were close to members of Actinobacteria and 1 isolate was closely related to member of Proteobacteria phylum. Firmicutes phylum included eleven *Bacillus* species, one *Streptococcus* and a *Lysinibacillus* species. Actinobacteria members were *Promicromonospora*, *Brevibacterium;* and *Alcaligenes* belonged to Proteobacteria.



(a)



(b)

Figure 4.9. PCR amplification of 16S rRNA gene of cultured bacterial isolates using F27 and R1492 primers. (A) From left to right; M - 1 Kb DNA molecular ladder, 1:RM2, 2: RT5, 3: RO2, 4: RS3, 5: RL1, 6: RG3, 7: RT2, 8: RB3, 9: RO6 and 10: RG6. (B) From left to right; M-1 Kb DNA molecular ladder 1: R8; 2: R10; 3: R11; 4: R12; 5:R14; 6: R15

Table 4.5. Sequence similarity of bacterial to the closet Genbank relatives

Isolate name	Assigned codes	Closet related species description	Accession number	% similarity
RM2	KF765930	*Bacillus infantis*	KC751070.1	99
RT5	KF765925	*Bacillus mojavensis*	KF254581.1	100
RO2	KF765927	*Bacillus shackleroni*	JF647661.1	99
RS3	KF765931	*Brevibacterium frigotolerans*	KF254755.1	99
RL1	KF765928	*Bacillus licheniformis*	DQ071561.1	98
RG3	KF765926	*Bacillus pumilus*	KC815305.1	99
RT2	KF765932	*Alcaligenes faecalis*	KC764982.1	100
RB3	KF765924	*Bacillus aryabhattai*	HG424432.1	100
RO6	KF765929	*Bacillus cereus*	KF601957.1	99
RG6	KF765933	*Bacillus megaterium*	JF273850.1	99
R 8	KF737356	*Lysinibacillus xylanilyticus*	KF208475.1	99
R 10	KF737357	*Bacillus tequilensis*	HM854243	99
R 11	KF737358	*Promicromonospora sp.*	KC702696.1	99
R 12	KF737359	*Bacillus anthracis*	JX994147.1	99
R 14	KF737360	*Streptococcus agalactiae*	EU07570.1	94
R 15	KF737361	*Bacillus pumilus*	KF307193.1	99

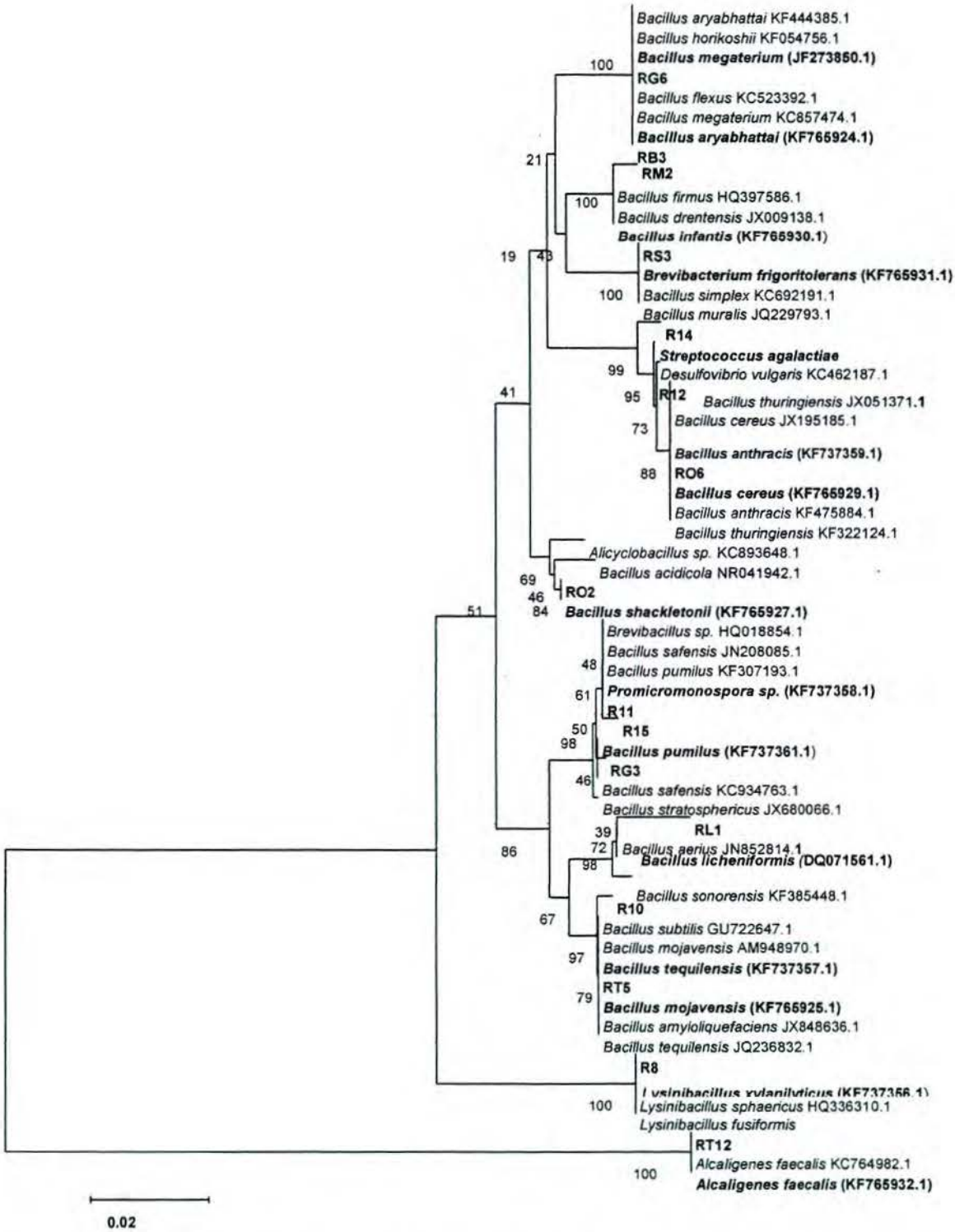


Figure 4.10. Phylogenetic tree based on the relationship between the bacterial isolates. Tree was constructed based on neighbor-joining method, maximum composite likehood distances and 1000 repetition bootstrap analyses. *Alcaligenes* species were used as an outgroup.

4.7.3. PCR-DGGE

The 16S rRNA gene fragment was amplified by PCR using standard PCR-DGGE F357 GC-clamp and R518 primers (Figure 4.11.), the forward primer with a GC-clamp allowed the migration of similar fragment size with different nucleotide sequences. DGGE bands were recovered from all rhizosphere samples and eight visible bands were detectable with high intensity and mobility from 40 to 60% denaturant within the polyacrylamide gel (Figure 4.12.). To gain the identity of bacterial community members, DGGE bands were excised; re-amplified with F357 without GC clamp and R518 primers (Figure 4.13.). The 16S rRNA gene fragment of bacteria from direct soil DNA were amplified in six samples except for rhizosphere of green peas (RG) sample.

4.7.4. Sequence and phylogenetic analysis of DGGE products

Amplified PCR products were used for phylogenetic nucleotide sequence analysis (Table 4.6.). The highest number of DGGE bands was from rhizosphere of green peas (RG) sample and all bands sequences showed highest degree of identity to uncultured clones from soil environments. band 2 (RK2) sequence from RG showed 100% similarity to previously isolated uncultured bacterium clone, band 3 (RK3) from rhizosphere of tomato (RT) showed 99% similarity to sequence of *Alkaliphilus* sp. while band 1 (RK 1) from RG was 96% related to *Flavobacterium* sp. and band 8 (RK8) sequence had 95% similarity to uncultured *Acidobacteria bacterium* sp. Bands sequences that showed to be less similar to previous isolated gene sequences were sequences from band 4, 5, 6 and 7 with 91 to 94% similarity to the related NCBI database sequences. Evolutionary relationships and identification of sequences were done by phylogenetic analysis (Figure 4.14.) and there is no outgroup for isolates whose DNA was directly extracted from environmental samples. DGGE gene sequences of band 1 (RK1), (RK2), (RK3) and (RK5) were closely related to each other and all four bacterial sequences indicated to be closely related

to sequence of uncultured bacterium clone (KF584920.1). Sequences of band 6 (RK6), band 7 (RK7) and band 8 (RK8) were closely related to uncultured bacterium clone (GQ136624.1). Band 4 (RK4) was closely related to *B. thuringiensis*. Other GenBank identified sequences were more related to each other than to the bacterial sequences. Uncultured bacterium clones (GC136624.1 and JX851639.1) were more related to each other, whilst *Alkaliphilus* sp., *Flavobacterium* sp. and uncultured bacterium clone (GU227579.1) were closely related to each other than any bacterial sequences.

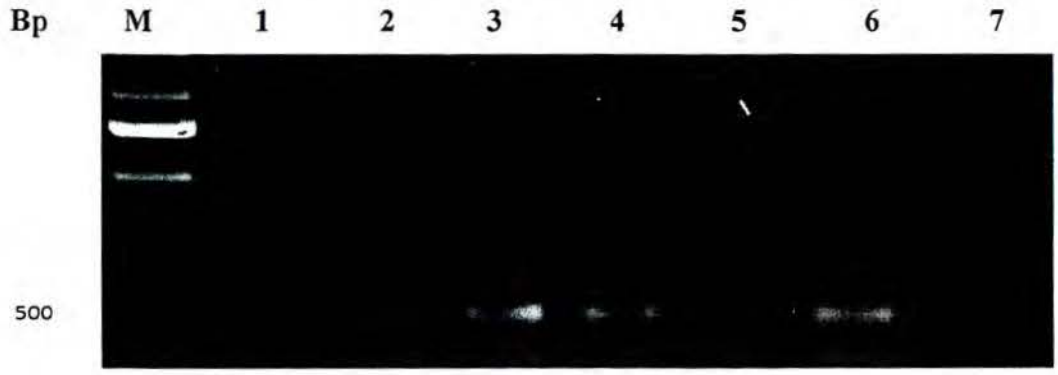


Figure 4.11. PCR amplification products of the 16S rRNA gene from the direct DNA left to right; M- 1Kb DNA molecular ladder, isolates code, 1: RL; 2: RT; 3: RO; 4: RB; 5: RM; 6: RS and 7: RG.

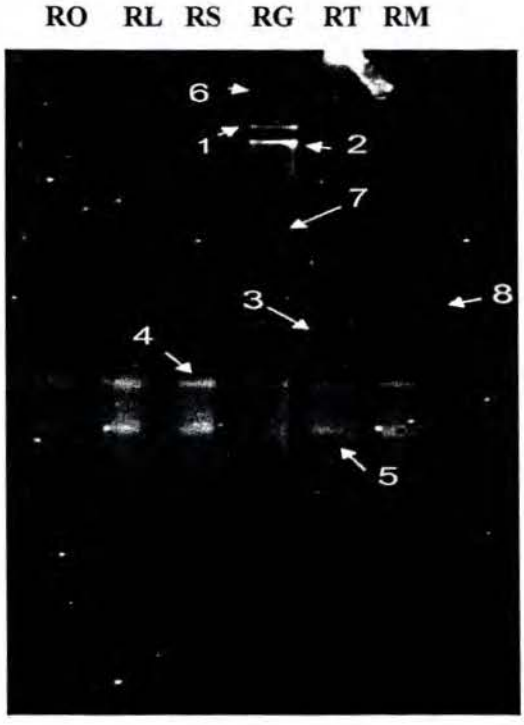


Figure 4.12. Denaturing gradient gel electrophoresis (DGGE) bands profiles of bacterial community in the rhizosphere of six cultivated plants. RO, RL, RS, RG, RT and RM are samples codes, number 1 to 8 and the arrows indicate bands excised from the gel.

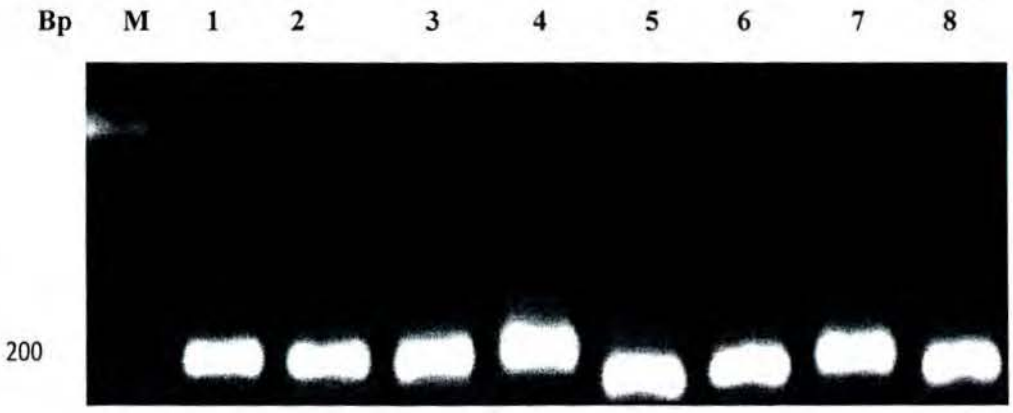


Figure 4.13. PCR amplification products (200 bp) gene fragment of excised DGGE bands; amplified using F357 and R518 primers of direct soil bacterial DNA from rhizosphere soils; left to right; M- 200 bp DNA molecular ladder, lane 1-8 represent sample codes. 1 (RK1, 2 (RK2), 3 (RK3), 4 (RK4), 5 (RK5), 6 (RK6), 7 (RK7) and 8 (RK8).

Table 4.6. Closest relatives of 16S rRNA gene derived from DGGE bands

	Related species		
Band name	**Description**	**Accession number**	**% similarity**
1 (RK1)	*Flavobacterium* sp.	KF556695.1	96
2 (RK2)	Uncultured bacterium clone	KF584920.1	100
3 (RK3)	*Alkaliphilus* sp.	AY554415.1	99
4 (RK4)	Uncultured bacterium clone	JX851639.1	91
5 (RK5)	*Clostridium* sp.	EF589958.1	91
6 (RK6)	Uncultured bacterium clone	GQ136624.1	91
7 (RK7)	*Bacillus thuringiensis*	EF113666.1	94
8 (RK8)	Uncultured Acidobacterium	GU227579.1	95

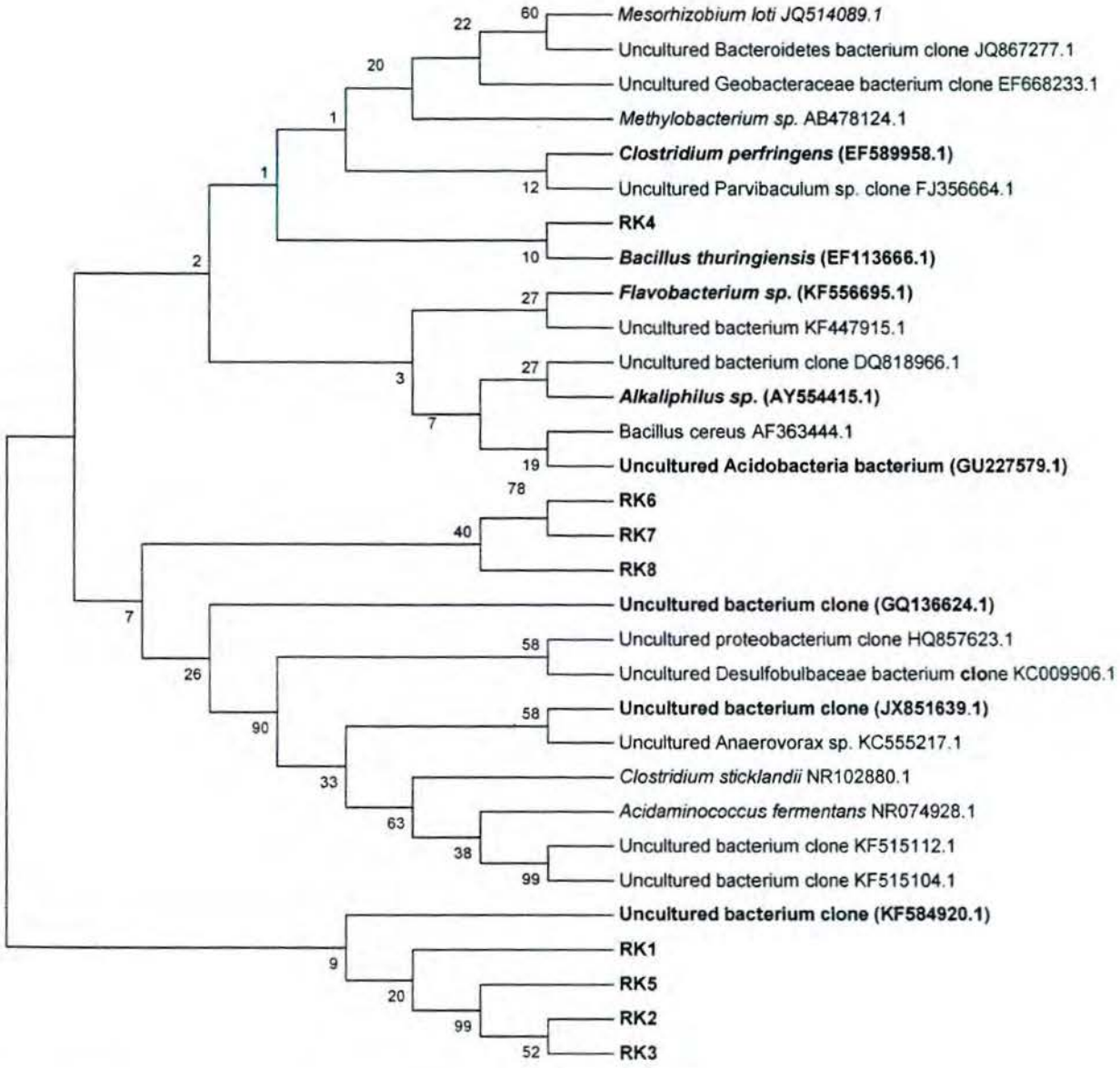


Figure 4.14. Phylogenetic tree showing relationship between the bacteria species sequences of DGGE bands product containing 200 bp genes within the 16S rRNA region amplified by F357 and R518. Tree was constructed based on neighbor-joining method, evolutionary distances were measured using Maximum composite likehood and Bootstrap analyses were performed with 1000 repetition. Species names and Genbank accession numbers are designated in parentheses.

CHAPTER 5

Discussion

In this study both culture-dependent and culture-independent techniques were used to study bacterial community structure and diversity in the rhizosphere of seven cultivated plants. Bacteria were isolated from the soils of the study area which had alkaline pH conditions. It has been reported previously that the majority of rhizosphere bacteria survive well and have high enzymatic performance under high pH (Chaiharn, Lumyong, 2009). High pH also influence physico-chemical properties in the rhizosphere more especially macronutrients, which had low concentration (Garavini *et al.*, 2004). At high pH the macronutrients elements are easily accessible and taken up by plants in large quantity thus reducing their amount in soil solution (Gupta *et al.*, 2002). This supports our findings, as we similarly had the lowest concentration of macronutrients from all samples. Except for phosphorous, which had the highest concentration at 157.3 mg/kg from spinach rhizosphere contradicting the recommended concentration level of phosphorous (20 mg/kg) in rhizosphere of cultivated plants (Herselman *et al.*, 2005). The concentrations of metals elements (Cd, Co, Cr, Cu and Zn) were very low and did not correlate with the recommended metal concentration levels in cultivated plant rhizosphere. Recommended concentrations are 3 mg/kg for (Cr), 20 mg/kg for (Co), 80 mg/kg for (Cd), 100 mg/kg for (Cu) and 185 mg/kg for (Zn) according to total maximum threshold level (TML) (Herselman *et al.*, 2005; Malik, Jaiswal, 2000) on South Africa cultivated plants rhizosphere. Even though the concentrations of metal elements were below the recommended levels they were good indicators that the soils were free of metal contamination (Mcgrath *et al.*, 1995). Physico-chemical parameters measured in all samples varied greatly and such differences might be associated with other environmental factors such as climate and plant species type (Zhu *et al.*, 2011).

Bacterial function and functional diversity were determined by measuring Sole Carbon Substrate Utilization using Biolog microplates (SCSUP). SCSUP was measured by colour change of the tetrazolium dye expressed as AWCD and $AWCD_G$. At 72 hr there was no significant difference in AWCD by bacteria in all rhizosphere samples, this indicated that bacterial community members in these rhizosphere samples had the same pattern in catabolizing carbon substrates supported by (Baudoin *et al.*, 2003); who reported that similar patterns in catabolizing carbon substrates is due the reduced level or absence of carbon substrates in the environment. There was a significant difference in the use of $AWCD_G$ indicated by the difference usage of carbohydrates, carboxylic acids, amino acids, and polymers, indicating that bacteria followed different patterns in utilization of carbon substrates in each carbon source group. Bacteria preferred carbohydrates especially bacteria from sample RG, which showed that bacteria members present in soils are fast growers since they adapted well towards substrate with high carbon contents and used them efficiency within a short period of time. But there was no significant difference in the use of amides amongst bacteria.

$AWCD_G$ patterns indicated that functional diversity differs between bacteria in all samples, indicating that there were high possibilities that bacteria members in soils were different. At the same time similar AWCD patterns by bacteria in all samples indicated that they were diverse and that there were high possibilities that bacteria present in all the samples might be similar. From these results we could not find a conclusion on whether bacteria in all samples might be similar or related. Just like all the other microbial study techniques, Biolog had both advantages and disadvantages; its main disadvantage was the inability to identify heterogeneous bacterial diversity instead of individual members. Results interpretation on a Biolog microplate was a challenge but overall the Biolog technique still contributed in the identification of bacterial

presence and the potential catabolism of carbon sources in the rhizosphere of the cultivated plants.

Bacterial populations were measured and compared between all soil samples using plate count method. Bacterial population ranged from low to high and differences in population size were attributed to physico-chemical properties and plant species type. High bacterial population in this study especially in RO was relevant; since soil samples were from rhizosphere area which is rich in nutrients. Biochemical tests were conducted as classic preliminary diagnostic tests that provide guidance in the identification of the bacteria into genus and species of interest. In this study, initially forty three bacterial isolates were identified but the morphological and biochemical tests result covers only sixteen isolates. Results revealed that the majority of isolates have similar physiological properties to members for *Bacillus* genus and these results are supported by (Parvathi *et al.*, 2009; Mckillip, 2000). Their isolates had similar physiological properties with the isolates identified in this study and assigned them to be *Bacillus* species in rhizosphere. Other bacterial isolates had similar physiological properties to members of genera such as *Brevibacterium, Alcaligenes, Streptococcus, Lysinibacillus* and *Promicromonospora.* Preliminary identification tests failed to identify bacterial isolates into species and strain level and further identification on isolates needed to be done.

In vitro detection of plant growth-promoting traits provided by bacterial isolates showed that, all sixteen cultured isolates had antifungal activities against *F. oxysporum*; the highest activity was from RT5 (*B. mojavensis)* and RG3 *(B. pumilus).* High inhibition effect was been reported to be attributed to potential production of fungicidal surfactins metabolites by these two species; because the *Bacillus* genus has prevailed in the production fungicida surfactins such has surfactins A, B and C followed by only *Serratia* and *Pseudomonas* genera (Saravanakumar *et al.*,

2007). Fungicidal metabolites produced by bacteria play a significant role in biological control of plant pathogens and are becoming a popular alternative to reduce chemical fungicides usage. Both species have been reported successfully to antagonize pathogenic *Fusarium* species (Bacon, Hinton, 2002; Colin *et al.*, 2004).

Three isolates produced HCN, which is important for minimizing soil borne pathogens, the amount of HCN production was indicated by colour change in plates ranging from poor to moderate by isolate RO6 (*B. cereus*), RG6 (*B. megaterium*) and RG3 (*B. pumilus*). HCN is a poisonous toxin but it is produced in minute amount by bacteria able to suppress pathogens attack and the small amount of HCN does not have an effect on plant functions and activities. Fifteen isolates solubilized inorganic phosphates into phosphorous; RG3 (*B. pumilus)* had the highest phosphate solubilizing activity in Pikoskaya's agar and results are in agreement with the findings of (Saravanakumar *et al.*, 2007) who reported *B. pumilus* as one of the prevailing microbes able to solubilize inorganic phosphates in rhizosphere. Only isolate R14 (*Streptococcus agalactiae)* was negative.

The auxin IAA is the most active phytohormone in plants, which affects cell enlargement, cell division, root initiation and growth rates (Ahmad *et al.*, 2008). In this study IAA production was relatively high in some of the bacterial isolates, RL1 (*B. licheniformis*) produced the highest IAA which was 25 mg/ml, in contrast to findings by (Gravel *et al.*, 2007) who reported high IAA values of 46.2 mg/ml produced by prevailing *Pseudomonas putida* in most cultivated plants rhizosphere. IAA productivity results indicate that isolates have the ability to cause root elongations in associated plants when used as inoculants. ACC-deaminase activity was observed in all isolates, isolates RG3 (*B. pumilus*) had the highest ACC- deaminase activity indicated by the highest level of α ketobutyrate production. ACC-deaminase activity by RG3 (*B. pumilus*)

suggests that this isolate if used as inoculants they may be able to control ethylene levels and enhance plant growth.

In vitro plant growth trait tests showed that most of the isolates have plant growth-promoting traits, but their production and concentrations (quantitative traits) can differ under natural environments when using isolates as plants inoculants. Plant growth-promoting traits produced by these bacteria vary among different plant species and in some cases substrate availability (Noumavo *et al.*, 2013).

Bacterial inoculants RG3 (*B. pumilus*), RT5 (*B. mojavensis*) and RL1 (*B. licheniformis*) increased growth parameters in tomato, spinach and beetroot plants when compared to controls. *B. pumilus* had the best plant growth promotion performance over all the inoculants with the highest shoot length and number of leaves in all plants. Plant growth-promoting traits of this *B. pumilus* strain might be attributed to its ability to increase plant nutrients availability by fixing nitrogen, phosphate solubilization and inducing phytohormone production. However *B. subtilis* is generally regarded as the most effective plant growth promoter in many crop plants because it has a variety of plant promotion traits (Melnick *et al.*, 2011; Valenzuela-Soto *et al.*, 2010; Mena-Violante, Olalde-Portugal, 2007). The high plant growth performance by *B. subtilis* is also influenced by the soil physico-chemical properties, plant species type, geographical and environmental factors.

Based on the inconclusive results from the preliminary tests, molecular techniques were conducted to further identify cultured isolates. Phylogenetic analysis revealed that the 16S rRNA sequences homologies between sequences of isolates and sequences of known bacteria in the NCBI ranged from 94 to 100%. It also indicated that 81% of 16S rRNA sequences were classified as Firmicutes, 6% as Proteobacteria and 13% as Actinobacteria. Sequences of isolates

RG6, RB3, RM2, RS3, R14, R12 and R06 were closely related to each other and showed high similarity to Firmicutes nucleotide gene sequences (*B. megaterium, B. aryabhattai, B. infantis, S. agalactiae, B. anthracis* and *B. cereus*) except for RS3 which showed high similarity to Actinobacteria (*Brevi. frigotolerans).* Nucleotide gene sequences of RT5, RG3, RL1, R10, R15, RO2, R11, R8, and RT2 were closer to each other and showed high similarity to Firmicutes sequences (*B. mojavensis*, *B. pumilus*, *B. licheniformis*, *B. tequilensis*, *B. pumilus*, *B. shackletoni* and *Lysinibacillus*); Actinobacteria (*Prominospora*) and Proteobacteria (*Alcaligenes* sp.). Sequences of RL1 and RT5 were closely related to each other which were in agreement with (Ludwig *et al.*, 2009) who reported that in phylogenetic classification of *Bacillus* genus; *B. mojavensis* and *B. licheniformis* are closely related and fall in the same subcluster in a phylogenetic tree of Firmicutes. Sequences of RT2 showed it to be closely related to RG3 more than the rest of the isolates followed by RT5 and R11. The isolate R8 sequence was more related to R12 as supported by Ahmed *et al.* (2007) who reported that *Lysinibacillus* species have pathogenic genes which are similar to *B. cereus*. From the phylogenetic analysis we observed that 16S rRNA gene sequences of bacterial isolates from the different rhizosphere were more closely related to each other than sequences of isolates from the same rhizosphere sample. Similar results observed by (Axelrood *et al.*, 2002) reported that 16S rRNA gene sequences of bacterial isolates from different rhizosphere were more closely related. The result indicated that, even though the bacterial isolates were from the same rhizosphere under the same physico-chemical properties and influence, there are high chances that these bacteria did not interact with each other under the same rhizosphere region. Indicating that, they have different roles in the rhizospheric ecosystem. There is a high likelihood that bacteria have different functions in biogeochemical processes, soil food web and that they preferred different energy sources as

indicated by their dissimilar ways in Sole Carbon Substrate Utilization patterns. The relatedness of isolates' gene sequences was not phylum specific as other Firmicutes affiliated sequences were closely related to other phyla sequences.

The evolutionary phylogenetic similarity between isolates and closely related species sequences followed the same pattern as in Genbank arrangement for most isolates, except for RG3 sequence which appeared to be more related to *B. safensis* during phylogenetic analysis than previously on Genbank output. This indicated that phylogenetic analysis of evolutionary relationship of species was not steady. In this study, altogether the inclusive bacterial isolates 16S rRNA gene sequences from all rhizospheres were closely related to *Bacillus* species that are members of Firmicutes, followed by Actinobacteria and Proteobacteria; these results are in agreement with (Janssen, 2006), who reported that the Firmicutes phyla make up 40% of the rhizosphere whole ribosomal library. But at the same time the results are also in disagreement with studies reported by (Wani *et al.*, 2007; Hamelin *et al.*, 2002) who indicated that most cultivated plants rhizosphere bacterial isolates were closely related to members of Proteobacteria followed by Firmicutes especially (*Bacillus* spp). In this study, the domination of Firmicutes especially Bacilli family throughout the rhizosphere samples is based on their ability to withstand harsh and dry conditions similarly to those found in Mahikeng and its surrounding areas especially our areas of study. Also, as it is generally known that *Bacillus* genera produce a wide range of broad spectrum antibiotics such as bacillomicin, fengicine insurin, mycosubtilin. There is a probability that these *Bacillus* produced antibiotics which might have inhibited growth of other bacteria in plate culture thus reducing the population of other microorganisms.

Phylogenetic analysis of eight DGGE bands gene sequences indicated that 50% of 16S rRNA gene sequences were affiliated to uncultured bacterium clones, 25% to Firmicutes, 13% to

Proteobacteria and another 12% to Bacteroidetes. Firmicutes included *Clostridium* and *Bacillus* species, Bacteroidetes contained *Flavobacterium* sp and Proteobacteria had *Alkaliphilus* sp. High identification of DGGE bands 200 bp sequences to uncultured clones gene sequences correlated to previous reports (Schmalenberger, Tebbe, 2003; Duineveld *et al.*, 2001). The majority of bacterial sequences isolated successfully showed close similarity to previously identified uncultured bacterial clones because the clones have a longer reliable nucleotide bases unlike the DGGE nucleotides fragments which is less than 500 bp (Muyzer *et al.*, 1995). The clone nucleotide sequences give more information which enables the characterization of bacterial species. Sequence of band 2 (RK2) was the only one with an exact match (100 percent) similarity with a previously identified sequence, the rest of sequences had less percentage of similarity. Band 5 (RK5) had the least percentage similarity (91%) to known bacterial sequences of *Clostridium sp*, which can be attributed to the short nucleotide fragment of band 5 sequence which only gave limited information to allow full characterization of the species. *Clostridium sp* is a common foodborne pathogen that can be a potential threat to human health, but it has been identified as biocontrol agent and plant growth enhancer (Polyanskaya *et al.*, 2002). Nucleotide sequences of band 1 (RK1), 2 (RK2), 3 (RK3) and 5 (RK5) were closely related to each other and all of them were more related to uncultured bacterium clone (KF584920.1) in the phylogram and the relationships were different to Genbank sequence arrangements. The Genbank arrangement showed that only the band 2 (RK2) sequence was closely affiliated to the uncultured bacterium clone sequences. Similarly, band 4 (RK4) was more related to *B. thuringiensis, Flavobacterium* and *Alkaliphilus* were more related to the uncultured clone in the phylogram, suggesting that the phylogram did not accurately estimate the evolutionary relationship of the RK2, RK4, *Flavobacterium* and *Alkaliphilus* 16S rRNA nucleotide sequences.

PCR-DGGE yielded different uncultured bacterial strains and *Flavobacterium, Alkaphilus, Clostridium* and *Bacillus* species suggesting that there was high reproducibility level in direct DNA isolation, PCR and DGGE processes. Overall the number of bacterial species retrieved from PCR-DGGE analysis was too small as compared to previous studies done on the rhizosphere of cultivated plants (Duineveld *et al.*, 2001). This outcome could possibly result from the influence of the physico-chemical properties in soils, plant species and other environmental or genetic factors. Only bands with high intensity were able to be sequenced and this indicated that there is a high possibility that other bacterial species present with less band intensity were undetected. Previous studies have highlighted that the small number of DGGE bands indicates that several groups of bacteria present in the rhizosphere were not dominant in metabolic activities in the rhizosphere (Duinevel *et al.*, 2001). The two different culture-dependent and culture-independent methods used in this study to identify and characterize dominating bacterial taxa in the same rhizosphere samples did not corroborate with each other. Both methods had sequences closely associated to Firmicutes and Actinobacteria but they were different species. The culture-dependent methods showed that the dominating bacterial community sequences had the highest similarities to sequences of *Bacillus* species that are members of Firmicutes, followed by Actinobacteria and Proteobacteria whilst in the culture-independent method bacteria were closely related to uncultured bacterial clones, Firmicutes (*Bacillus* and *Clostridium* spp.), Proteobacteria (*Alkaliphilus* sp.) and Bacteroidetes (*Flavobacterium* sp.). *B. thuringiensis* was the only species that affiliated to isolates sequence from both methods. Failure of culture-independent methods to identify some of the sequences related to *Bacillus* and other organisms might be due to heterogeneous lysis of different soil bacteria and primer bias (Pereira *et al.*, 2011). A combination of these two methods is still vital

in microbial ecology studies because they have successfully identified dominating rhizosphere bacteria and have allowed us to meet the study hypothesis, that rhizosphere contains many genetically diverse bacterial members. From both cultured and uncultured phylogenetic analysis we did observe that a few of the 16S rRNA gene sequences did not align well with their previously identified closely related species (from GenBank database) in the phylogenetic tree. This raises uncertainty on whether the phylogenetic trees gave a true representation on estimating phylogeny relationships between isolate sequences and identified bacterial sequences from the GenBank (Sklarz *et al.*, 2009). This can be an indication that there are still some restrictions with either of the molecular techniques (phylogenetic analysis of GenBank or sequences arrangement) in identifying bacterial community composition in the rhizosphere. This suggests that the major limitation from these two methods is based on the incomplete GenBank database, as indicated in the results that most of the 16S rRNA sequences of direct soil DNA were highly related to uncultured bacterial clones. On the other hand we can forecast that these limitations on the identification of bacterial community in complex soil environment will be solved; as more of the full sequences are deposited into the GenBank allowing proper identification of bacterial taxa thus making molecular techniques useful in ecological studies.

Conclusion and Recommendations

Both culture-dependent and independent-technique were successful in identifying the bacterial community in the rhizosphere and those containing plant growth promoting traits. This study also confirms that bacterial isolates identified have plant growth traits and were able to increase root length, shoot length and, the number of leaves when inoculated in crop plant. However the amounts of bacteria retrieved by both methods were very small, this is due to the difficulty of experienced during the extraction of DNA. Optimization of PCR conditions for amplication of the 200 bp was a challenge. Bacteria responsible for plant growth promotion can be very beneficial towards the agricultural sector but further studies still have to be conducted on these plant growth rhizobacteria. To determine if the combination of these bacterial inoculants can improve crops growth further when compared to single inoculations and whether the microbes are harmful to animal and human health when ingested. Further studies on rhizosphere bacterial diversity using both methods are recommended. Soil samples should be collected from different soil horizons on a seasonal basis to explain bacterial diversity and succession under different temperature and moisture regimes. More culture-independent methods such as Terminal Restriction Fragment Length polymorphism (TRFLP) and creating more clone library of 16S rRNA gene sequences will improve our knowledge on what bacteria are doing in the rhizosphere meaning their actual functions in the rhizosphere. Alternative molecular techniques must be used besides the analyses of 16S rRNA such as the analyses of structural genes, functional genes and polymorphisms. Further investigation and monitoring of the 'functional genes' encoding for enzymes and proteins involved in vital ecosystem processes must be conductecd. These enzymes and proteins play major roles in processes such as nitrogen fixation, biocontrol of plant pathogens, bioremediation and can be used in the production of genetically modified food, as

well as to increase crop productivity and secure food production. The challenge on analyzing, interpreting and supporting the data obtained from culture dependent techniques in relation to different environmental conditions must be overcome.

References:

Ahemad, M., Khan, M. S. 2010. Phosphate-solubilizing and plant-growth-promoting *Pseudomonas aeruginosa* PS1 improves greengram performance in quizalafop-p-ethyl and clodinafop amended soil. *Archives of Environmental Contamination and Toxicology,* 58, 361-372.

Ahmad, F., Ahmad, I., Khan, M. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research,* 163, 173-181.

Ahmed, I., Yokota, A., Yamazoe, A., Fujiwara, T. 2007. Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. *International Journal of Systematic and Evolutionary Microbiology,* 57, 1117-1125.

Arshad, M., Saleem, M., Hussain, S. 2007. Perspectives of bacterial ACC deaminase in phytoremediation. *Trends in Biotechnology,* 25, 356-362.

Ashelford, K. E., Day, M. J., Fry, J. C. 2003. Elevated abundance of bacteriophage infecting bacteria in soil. *Applied and Environmental Microbiology,* 69, 285-289.

Axelrood, P. E., Chow, M. L., Radomski, C. C., Mcdermott, J. M., Davies, J. 2002. Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Canadian Journal of Microbiology,* 48, 655-674.

Babalola, O. O. 2010. Improved mycoherbicidal activity of *Fusarium arthrosporioides*. *African Journal of Microbiology Research,* 4, 1659-1662.

Babalola, O. O. 2007. Pectinase and cellulase enhance the control of *Abutilon theophrasti* by *Colletotrichum coccodes*. *Biocontrol Science and Technology,* 17, 53-61.

Babalola, O. O., Osir, E. O., Sanni, A. I., Odhiambo, G. D., Bulimo, W. D. 2003. Amplification of 1-amino-cyclopropane-1-carboxylic (ACC) deaminase from plant growth promoting rhizobacteria in *Striga*-infested soil. *African Journal of Biotechnology,* 2, 157-160.

Bacon, C. W., Hinton, D. M. 2002. Endophytic and Biological Control Potential of *Bacillus mojavensis* and Related Species. *Biological Control,* 23, 274-284.

Baehler, E., Bottiglieri, M., Péchy-Tarr, M., Maurhofer, M., Keel, C. 2005. Use of green fluorescent protein-based reporters to monitor balanced production of antifungal compounds in the biocontrol agent *Pseudomonas fluorescens* CHA0. *Journal of Applied Microbiology,* 99, 24-38.

Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., Vivanco, J. M. 2004. How plants communicate using the underground information superhighway. *Trends in Plant Science,* 9, 26-32.

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

Bakker, P. A., Pieterse, C. M., Van Loon, L. 2007. Induced systemic resistance by *fluorescent Pseudomonas* spp. *Phytopathology,* 97, 239-243.

Baudoin, E., Benizri, E., Guckert, A. 2003. Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biology and Biochemistry,* 35, 1183-1192.

Benincà, E., Huisman, J., Heerkloss, R., Jöhnk, K. D., Branco, P., Van Nes, E. H., Scheffer, M., Ellner, S. P. 2008. Chaos in a long-term experiment with a plankton community. *Nature,* 451, 822-825.

Berg, G. 2009. Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology,* 84, 11-18.

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

Bhattacharyya, P., Jha, D. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology,* 28, 1327-1350.

Bora, T., Özaktan, H., Göre, E., Aslan, E. 2004. Biological control of *Fusarium oxysporum* f. sp. *melonis* by wettable powder formulations of the two strains of *Pseudomonas putida*. *Journal of Phytopathology,* 152, 471-475.

Carvalhais, L. C., Dennis, P. G., Fedoseyenko, D., Hajirezaei, M. R., Borriss, R., Von Wirén, N. 2011. Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *Journal of Plant Nutrition and Soil Science,* 174, 3-11.

Chaiharn, M., Lumyong, S. 2009. Phosphate solubilization potential and stress tolerance of rhizobacteria from rice soil in Northern Thailand. *World Journal of Microbiology and Biotechnology,* 25, 305-314.

Chenn, A., Walsh, C. A. 2002. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science,* 297, 365-369.

Clarridge, J. E. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews,* 17, 840-862.

Colin, M., Bonmatin, J., Moineau, I., Gaimon, C., Brun, S., Vermandere, J. 2004. A method to quantify and analyze the foraging activity of honey bees: relevance to the sublethal effects induced by systemic insecticides. *Archives of Environmental Contamination and Toxicology,* 47, 387-395.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E. A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology,* 71, 4951-4959.

Dakora, F. D., Phillips, D. A. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil,* 245, 35-47.

De-Bashan, L. E., Bashan, Y. 2010. Immobilized microalgae for removing pollutants: review of practical aspects. *Bioresource Technology,* 101, 1611-1627.

Diouf, A., Lambin, E. 2001. Monitoring land-cover changes in semi-arid regions: remote sensing data and field observations in the Ferlo, Senegal. *Journal of Arid Environments,* 48, 129-148.

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

Dutta, S., Podile, A. R. 2010. Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. *Critical Reviews in Microbiology,* 36, 232-244.

Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., Davey, L., Payton, A. S., Liao, J., Ma, L., Doyle, M. P. 2010. Surface and internalized *Escherichia coli* O157: H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. *Journal of Food Protection,* 73, 1023-1029.

Fang, C., Radosevich, M., Fuhrmann, J. J. 2001. Atrazine and phenanthrene degradation in grass rhizosphere soil. *Soil Biology and Biochemistry,* 33, 671-678.

Farrar, J., Hawes, M., Jones, D., Lindow, S. 2003. How roots control the flux of carbon to the rhizosphere. *Ecology,* 84, 827-837.

Fierer, N., Bradford, M. A., Jackson, R. B. 2007. Toward an ecological classification of soil bacteria. *Ecology,* 88, 1354-1364.

Franche, C., Lindström, K., Elmerich, C. 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant and Soil,* 321, 35-59.

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., Olsen, G. J. 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology,* 74, 2461-2470.

Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., Sarniguet, A. 2011. Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiology and Molecular Biology Reviews,* 75, 583-609.

Garavini, G., Folatelli, G., Goobar, A., Nobili, S., Aldering, G., Amadon, A., Amanullah, R., Astier, P., Balland, C., Blanc, G. 2004. Spectroscopic observations and analysis of the *Peculiar* SN 1999aa. *The Astronomical Journal,* 128, 387-404.

Garland, J., Mills, A., Young, J. 2001. Relative effectiveness of kinetic analysis vs single point readings for classifying environmental samples based on community-level physiological profiles (CLPP). *Soil Biology and Biochemistry,* 33, 1059-1066.

Gonzalez, J., Ortiz-Martinez, A., Gonzalez-Delvalle, M., Laiz, L., Sáiz-Jiménez, C. 2003. An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities. *Journal of Microbiological Methods,* 55, 459-463.

Gopesh C, S., George J, V. 2012. Evaluation of expression stability of candidate references genes among green and yellow pea cultivars *(Pisum sativum L.)* subjected to abiotic and biotic stress. *American Journal of Plant Sciences,*3, 235-242.

Gravel, V., Antoun, H., Tweddell, R. J. 2007. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida Trichoderma atroviride* Possible role of indole acetic acid (IAA). *Soil Biology and Biochemistry,* 39, 1968-1977.

Greene, E., Hubert, C., Nemati, M., Jenneman, G., Voordouw, G. 2003. Nitrite reductase activity of sulphate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria. *Environmental Microbiology,* 5, 607-617.

Gupta, R., Beg, Q., Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology,* 59, 15-32.

Haas, D., Keel, C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual Review of Phytopathology,* 41, 117-153.

Hamelin, J., Fromin, N., Tarnawski, S., Teyssier-Cuvelle, S., Aragno, M. 2002. *nifH* gene diversity in the bacterial community associated with the rhizosphere of *Molinia coerulea*, an oligonitrophilic perennial grass. *Environmental Microbiology,* 4, 477-481.

Henry, A., Doucette, W., Norton, J., Bugbee, B. 2007. Changes in crested wheatgrass root exudation caused by flood, drought, and nutrient stress. *Journal of Environmental Quality,* 36, 904-912.

Herselman, J., Steyn, C., Fey, M. 2005. Baseline concentration of Cd, Co, Cr, Cu, Pb, Ni and Zn in surface soils of South Africa: research in action. *South African Journal of Science,* 101, 509-512.

Hill, G., Mitkowski, N., Aldrich-Wolfe, L., Emele, L., Jurkonie, D., Ficke, A., Maldonado-Ramirez, S., Lynch, S., Nelson, E. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Ecology,* 15, 25-36.

Hou, M. P., Oluranti, B. O. 2013. Evaluation of plant growth promoting potential of four rhizobacterial species for indigenous system. *Journal of Central South University,* 20, 164-171.

Ibrahim, A., Musa, K., Sani, A., Aliero, A., Yusuf, B. 2011. Microorganisms associated with the production of volatile compounds in spoilt tomatoes. *Research in Biotechnology,* 2, 14-22.

Janssen, P. H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology,* 72, 1719-1728.

Karnwal, A. 2009. Production of indole acetic acid by *Pseudomonas fluorescent* in the presence of L-tryptophan and rice root exudates. *Journal of Plant Pathology,* 91, 61-63.

Kawaguchi, M., Syōno, K. 1996. The excessive production of indole-3-acetic acid and its significance in studies of the biosynthesis of this regulator of plant growth and development. *Plant and Cell Physiology,* 37, 1043-1048.

Kennedy, I. R., Choudhury, A., Kecskés, M. L. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? *Soil Biology and Biochemistry,* 36, 1229-1244.

Khan, A. A., Jilani, G., Akhtar, M. S., Naqvi, S. S., Rasheed, M. 2009. Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *Journal of Agriculture Biological Sciences,* 1, 48-58.

Konopka, A. 2009. What is microbial community ecology and quest. *The ISME Journal,* 3, 1223-1230.

Kramer, E. M., Bennett, M. J. 2006. Auxin transport: a field in flux. *Trends in Plant Science,* 11, 382-386.

Kremer, R. J., Kennedy, A. C. 1996. Rhizobacteria as biocontrol agents of weeds. *Weed Technology*, 601-609.

Kuiper, I., Bloemberg, G. V., Lugtenberg, B. J. 2001. Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. *Molecular Plant-Microbe Interactions,* 14, 1197-1205.

Lauber, C. L., Hamady, M., Knight, R., Fierer, N. 2009. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology,* 75, 5111-5120.

Leckie, S. E. 2005. Methods of microbial community profiling and their application to forest soils. *Forest Ecology and Management,* 220, 88-106.

Leveau, J. H., Lindow, S. E. 2001. Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proceedings of the National Academy of Sciences,* 98, 3446-3453.

Lucy, M., Reed, E., Glick, B. R. 2004. Applications of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek,* 86, 1-25.

Ludwig, W., Schleifer, K. H., Whitman, W. B. 2009. Revised road map to the phylum Firmicutes. *Bergey's Manual of Systematic Bacteriology.* Springer, 3, 70-98.

Lugtenberg, B., Kamilova, F. 2009. Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology,* 63, 541-556.

Madsen, E. L. 2005. Identifying microorganisms responsible for ecologically significant biogeochemical processes. *Nature Reviews Microbiology,* 3, 439-446.

Malik, S., Beer, M., Megharaj, M., Naidu, R. 2008. The use of molecular techniques to characterize the microbial communities in contaminated soil and water. *Environment International,* 34, 265-276.

Malik, A., Jaiswal, R. 2000. Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. *World Journal of Microbiology and Biotechnology,* 16, 177-182.

Mano, Y., Nemoto, K. 2012. The pathway of auxin biosynthesis in plants. *Journal of Experimental Botany,* 63, 2853-2872.

Marques, A. P., Pires, C., Moreira, H., Rangel, A. O., Castro, P. M. 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. *Soil Biology and Biochemistry,* 42, 1229-1235.

Mcgrath, S. P., Chaudri, A. M., Giller, K. E. 1995. Long-term effects of metals in sewage sludge on soils, microorganisms and plants. *Journal of Industrial Microbiology,* 14, 94-104.

Mckillip, J. L. 2000. Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. *Antonie Van Leeuwenhoek,* 77, 393-399.

Melnick, R. L., Suárez, C., Bailey, B. A., Backman, P. A. 2011. Isolation of endophytic endospore-forming bacteria from *Theobroma cacao* as potential biological control agents of cacao diseases. *Biological Control,* 57, 236-245.

Mena-Violante, H. G., Olalde-Portugal, V. 2007. Alteration of tomato fruit quality by root inoculation with plant growth-promoting rhizobacteria (PGPR): *Bacillus subtilis* BEB-13bs. *Scientia Horticulturae,* 113, 103-106.

Meziane, H., Van Der Sluis, I., Van Loon, L. C., Höfte, M., Bakker, P. A. 2005. Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. *Molecular Plant Pathology,* 6, 177-185.

Mishra, R. S., Gu, Y., Bose, S., Verghese, S., Kalepu, S., Singh, N. 2002. Cell surface accumulation of a truncated transmembrane prion protein in Gerstmann-Straussler-Scheinker disease P102L. *Journal of Biological Chemistry,* 277, 24554-24561.

Mulet, M., Lalucat, J., García-Valdés, E. 2010. DNA sequence-based analysis of the *Pseudomonas* species. *Environmental Microbiology,* 12, 1513-1530.

Muyzer, G., Teske, A., Wirsen, C. O., Jannasch, H. W. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology,* 164, 165-172.

Neal, A. L., Ahmad, S., Gordon-Weeks, R., Ton, J. 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PLoS One,* 7, 35-49.

Nei, M., Stephens, J. C., Saitou, N. 1985. Methods for computing the standard errors of branching points in an evolutionary tree and their application to molecular data from humans and apes. *Molecular Biology and Evolution,* 2, 66-85.

Nelson, E. B. 2004. Microbial dynamics and interactions in the spermosphere. *Annual Review of Phytopathology,* 42, 271-309.

Nemoto, K., Hara, M., Suzuki, M., Seki, H., Muranaka, T., Mano, Y. 2009. The NtAMI1 gene functions in cell division of tobacco BY-2 cells in the presence of indole-3-acetamide. *FEBS letters,* 583, 487-492.

Ngoma, L., Esau, B., Babalola, O. O. 2013. Isolation and characterization of beneficial indigenous endophytic bacteria for plant growth promoting activity in Molelwane Farm, Mafikeng, South Africa. *African Journal of Biotechnology,* 12, 4105-4114.

Nihorimbere, V., Ongena, M., Smargiassi, M., Thonart, P. 2011. Beneficial effect of the rhizosphere microbial community for plant growth and health. *Revue de Biotechnologie, Agronomie, Société et Environnement,* 15, 501-508.

Normanly, J., Slovin, J. P., Cohen, J. D. 1995. Rethinking auxin biosynthesis and metabolism. *Plant Physiology,* 107, 323-329.

Noumavo, P. A., Kochoni, E., Didagbé, Y. O., Adjanohoun, A., Allagbé, M., Sikirou, R., Gachomo, E. W., Kotchoni, S. O., Baba-Moussa, L. 2013. Effect of different plant Growth Promoting Rhizobacteria on maize seed germination and seedling sevelopment. *American Journal of Plant Sciences,* 4, 1013-1021.

Owen, A., Zdor, R. 2001. Effect of cyanogenic rhizobacteria on the growth of velvetleaf *Abutilon theophrasti* and corn *Zea mays* in autoclaved soil and the influence of supplemental glycine. *Soil Biology and Biochemistry,* 33, 801-809.

Parvathi, A., Krishna, K., Jose, J., Joseph, N., Nair, S. 2009. Biochemical and molecular characterization of *Bacillus pumilus* isolated from coastal environment in Cochin, India. *Brazilian Journal of Microbiology,* 40, 269-275.

Paterson, E., Gebbing, T., Abel, C., Sim, A., Telfer, G. 2007. Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytologist,* 173, 600-610.

Patten, C. L., Glick, B. R. 2002. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology,* 68, 3795-3801.

Pereira, P., Ibáñez, F., Rosenblueth, M., Etcheverry, M., Martínez-Romero, E. 2011. Analysis of the bacterial diversity associated with the roots of maize (*Zea mays L.*) through culture-dependent and culture-independent methods. *ISRN Ecology,* 1, 1-10.

Pillay, V., Nowak, J. 1997. Inoculum density, temperature, and genotype effects on *in vitro* growth promotion and epiphytic and endophytic colonization of tomato (*Lycopersicon esculentum L.*) seedlings inoculated with a *Pseudomonad* bacterium. *Canadian Journal of Microbiology,* 43, 354-361.

Polyanskaya, L., Vedina, O., Lysak, L., Zvyagintsev, D. 2002. The growth-promoting effect of *Beijerinckia mobilis* and *Clostridium sp.* cultures on some agricultural crops. *Microbiology,* 71, 109-115.

Preston-Mafham, J., Boddy, L., Randerson, P. F. 2002. Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles–a critique. *FEMS Microbiology Ecology,* 42, 1-14.

Puente, M., Bashan, Y., Li, C., Lebsky, V. 2004. Microbial populations and activities in the rhizoplane of rock-weathering desert plants. I. Root colonization and weathering of igneous rocks. *Plant Biology,* 6, 629-642.

Rajkumar, M., Ae, N., Prasad, M. N. V., Freitas, H. 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends in Biotechnology,* 28, 142-149.

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

Rashid, S., Charles, T. C., Glick, B. R. 2012. Isolation and characterization of new plant growth-promoting bacterial endophytes. *Applied Soil Ecology,* 61, 217-224.

Robe, P., Nalin, R., Capellano, C., Vogel, T. M., Simonet, P. 2003. Extraction of DNA from soil. *European Journal of Soil Biology,* 39, 183-190.

Röling, W. F., Van Breukelen, B. M., Bruggeman, F. J., Westerhoff, H. V. 2007. Ecological control analysis: being (s) in control of mass flux and metabolite concentrations in anaerobic degradation processes. *Environmental Microbiology,* 9, 500-511.

Rubio, L. M., Ludden, P. W. 2008. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Annual Review of Microbiology,* 62, 93-111.

Sadeghi, A., Karimi, E., Dahaji, P. A., Javid, M. G., Dalvand, Y., Askari, H. 2012. Plant growth promoting activity of an auxin and siderophore producing isolate of *Streptomyces*

under saline soil conditions. *World Journal of Microbiology and Biotechnology,* 28, 1503-1509.

Saharan, B., Nehra, V. 2011. Plant growth promoting rhizobacteria: a critical review. *Life Science Medicine Research,* 21, 1-30.

Saravanakumar, D., Vijayakumar, C., Kumar, N., Samiyappan, R. 2007. PGPR-induced defense responses in the tea plant against blister blight disease. *Crop Protection,* 26, 556-565.

Schmalenberger, A., Tebbe, C. C. 2003. Bacterial diversity in maize rhizospheres: conclusions on the use of genetic profiles based on PCR-amplified partial small subunit rRNA genes in ecological studies. *Molecular Ecology,* 12, 251-262.

Sergeeva, E., Liaimer, A., Bergman, B. 2002. Evidence for production of the phytohormone indole-3-acetic acid by *Cyanobacteria*. *Planta,* 215, 229-238.

Singh, S. K., Ramaiah, N. 2011. Denaturing gradient gel electrophoresis profiling of bacterial communities composition in Arabian Sea. *Journal of Environmental Biology,* 32, 339-346.

Singh, S., Ma, L., Harris, W. 2001. Heavy metal interactions with phosphatic clay. *Journal of Environmental Quality,* 30, 1961-1968.

Six, J., Conant, R., Paul, E., Paustian, K. 2002. Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. *Plant and Soil,* 241, 155-176.

Sklarz, M. Y., Angel, R., Gillor, O., Soares, M. I. M. 2009. Evaluating amplified rDNA restriction analysis assay for identification of bacterial communities. *Antonie van Leeuwenhoek,* 96, 659-664.

Somers, D. J., Isaac, P., Edwards, K. 2004. A high-density microsatellite consensus map for bread wheat (*Triticum aestivum L.*). *Theoretical and Applied Genetics,* 109, 1105-1114.

Spaepen, S., Vanderleyden, J. 2011. Auxin and plant-microbe interactions. *Cold Spring Harbor Perspectives in Biology,* 3, 4-38.

Stefanowicz, A. 2006. The Biolog Plates Technique as a Tool in Ecological Studies of Microbial Communities. *Polish Journal of Environmental Studies,* 15, 669-676.

Tao, G. C., Tian, S. J., Cai, M. Y., Xie, G. H. 2008. Phosphate-solubilizing and-mineralizing abilities of bacteria isolated from soils. *Pedosphere,* 18, 515-523.

Tsavkelova, E. A., Cherdyntseva, T. A., Botina, S. G., Netrusov, A. I. 2007. Bacteria associated with orchid roots and microbial production of auxin. *Microbiological Research,* 162, 69-76.

Valenzuela-Soto, J. H., Estrada-Hernández, M. G., Ibarra-Laclette, E., Délano-Frier, J. P. 2010. Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting *Bacillus subtilis* retards white fly *Bemisia tabaci* development. *Planta,* 231, 397-410.

Vartoukian, S. R., Palmer, R. M., Wade, W. G. 2010. Strategies for culture of 'unculturable' bacteria. *FEMS Microbiology Societies,* 309, 1-7.

Vessey, J. K. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil,* 255, 571-586.

Wahyudi, A. T., Astuti, R. P., Widyawati, A., Meryandini, A., Nawangsih, A. A. 2011. Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. *Journal of Microbiology Antimicrobodies,* 3, 34-40.

Walker, T. S., Bais, H. P., Grotewold, E., Vivanco, J. M. 2003. Root exudation and rhizosphere biology. *Plant Physiology,* 132, 44-51.

Wang, F., Zhou, H., Meng, J., Peng, X., Jiang, L., Sun, P., Zhang, C., Van Nostrand, J. D., Deng, Y., He, Z. 2009. GeoChip-based analysis of metabolic diversity of microbial communities at the Juan de Fuca Ridge hydrothermal vent. *Proceedings of the National Academy of Sciences,* 106, 4840-4845.

Wani, P. A., Khan, M. S., Zaidi, A. 2007. Effect of metal tolerant plant growth promoting *Bradyrhizobium* sp. on growth, symbiosis, seed yield and metal uptake by greengram plants. *Chemosphere,* 70, 36-45.

Welch, S., Taunton, A., Banfield, J. 2002. Effect of microorganisms and microbial metabolites on apatite dissolution. *Geomicrobiology Journal,* 19, 343-367.

Whipps, J. M. 2001. Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany,* 52, 487-511.

Williamson, K. E., Radosevich, M., Wommack, K. E. 2005. Abundance and diversity of viruses in six Delaware soils. *Applied and Environmental Microbiology,* 71, 3119-3125.

Winzer, K., Williams, P. 2001. Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. *International Journal of Medical Microbiology,* 291, 131-143.

Yan, Y., Yang, J., Dou, Y., Chen, M., Ping, S., Peng, J., Lu, W., Zhang, W., Yao, Z., Li, H. 2008. Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501. *Proceedings of the National Academy of Sciences,* 105, 7564-7569.

Yoon, G. M., Kieber, J. J. 2013. 14-3-3 regulates 1-aminocyclopropane-1-carboxylate synthase protein turnover in *Arabidopsis. The Plant Cell Online,* 25, 1016-1028.

Young, I. M., Crawford, J. W., Nunan, N., Otten, W., Spiers, A. 2008. Microbial distribution in soils: physics and scaling. *Advances in Agronomy,* 100, 81-121.

Zahran, H. H. 2001. Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *Journal of Biotechnology,* 91, 143-153.

Zdor, R. E., Alexander, C. M., Kremer, R. J. 2005. Weed suppression by deleterious rhizobacteria is affected by formulation and soil properties. *Communications in Soil Science and Plant Analysis,* 36, 1289-1299.

Zhu, W. L., Cui, L. H., Ouyang, Y., Long, C. F., Tang, X. D. 2011. Kinetic adsorption of ammonium nitrogen by substrate materials for constructed wetlands. *Pedosphere,* 21, 454-463.

APPENDIX

Appendix 5.1. Percentage inhibition of fungus growth

Percentage inhibition of RT5 (*B. mojavensis*)

R1= 50 mm

R2= 10 mm

RG= 70 mm

RI= R1-R2

Percentage of inhibition = $\frac{RI\ (R1-R2)}{RG} \times 100$

$= \frac{50-10}{70} \times 100$

$= 57\%$

Percentage inhibition of RG3 (*B. pumilus*)

R1= 60 mm

R2= 30 mm

RG= 70 mm

RI= R1-R2

Percentage inhibition = $\frac{RI\ (R1-R2)}{RG} \times 100$

$= \frac{60-30}{70} \times 100$

$= 43\%$

Appendix 5.2. One Way Analysis of variance

Source	DF	Sum of squares	Mean square	F value	Pr> F
Model	7	277. 9491067	39. 7070152	4.57	0.0017
Error	28	243. 2183933	8. 6863712		
Corrected total	35	521. 1675000			

Appendix 5.3. DNA concentration of culture isolates by Nanodrop at A260/280 purity value

Isolate name	Purity (A260/A280)		
	A260	A260/A280	Concentration µg/µl
RM2	4.112	2.389	1.72
RT5	5.222	2.506	2.08
R02	0.545	0.235	2.32
RS3	1.17	0.555	2.11
RL1	0.82	0.421	1.95
RG3	2.422	1.232	1.17
RT2	3.2	1.56	2.05
RB3	0.612	0.386	1.59
R06	3.191	1.649	1.11
RG6	2.665	1.447	1.84
RL2	4.571	2.216	2.06
RG10	3.291	1.521	2.16
RG11	1.057	0.533	1.98
RG12	1.435	9.515	1.06
RT14	3.69	1.935	1.91
RT15	2.875	1.454	1.77

A: absorbance value used to measure DNA amount, good DNA quantity measured by nanodrop for downstream application it should be > 1.5 µg/µl.

Appendix 5.4. DNA concentration of direct soil bacterial by Nanodrop at A260/280 purity value

	Purity (A260/A280)		
Sample	A260	A260/280	Concentration µg/µl
RO	6.701	1.454	1.77
RS	2.301	3.777	1.98
RL	0.771	1.112	2.09
RM	2.942	0.335	1.99
RB	1.698	1.58	1.86
RG	2.369	0.994	1.71
RT	1.59	1.342	1.77

A: absorbance value used to measure DNA amount, A: absorbance value used to measure DNA amount, good DNA quantity measured by Nanodrop for downstream application it should be > 1.5 µg/µl

Appendix 5.5. Mean Comparison – t Tests (LSD) for Shoot length

	Mean with same letter are not significantly different			
	t grouping	Mean	N	Treat
	A	12.856	9	RG3
	A			
B	A	10.533	9	RT5
B				
B		9.122	9	RL1
	C	5.722	9	Control

Appendix 5.6. Mean Comparison – Tukey's Studentized Range (HSD) Test for Shoot Length

	Mean with same letter are not significantly different			
	t grouping	Mean	N	Treat
	A	12.856	9	RG3
	A			
	A	10.533	9	RT5
	A			
B	A	9.122	9	RL1
B				
B		5.722	9	Control

Appendix 5.7. Original source of designated bacterial isolates

RM2	Rhizosphere of maize
RT5	Rhizosphere of tomato
RO2	Rhizosphere of onion
RS3	Rhizosphere of spinach
RL1	Rhizosphere of lettuce
RG3	Rhizosphere of green peas
RT2	Rhizosphere of tomato
RB3	Rhizosphere of beetroot
RO6	Rhizosphere of onion
RG6	Rhizosphere of green peas
R8	Rhizosphere of onion
R10	Rhizosphere of green peas
R11	Rhizosphere of maize
R12	Rhizosphere of tomato
R14	Rhizosphere of onion
R15	Rhizosphere of beetroot