Determination and comparison of intraspecific variation in the bacterial strains resident in the rhizoplane and rhizosphere of Bambaranut (*Vorandzeia subterranean L. thouars*)

By

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DECLARATION

I declare that, this work submitted for the degree of Masters in Biology at the North-West University, Mafikeng Campus, has not been submitted by me for a degree at this or any other University. This is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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DEDICATION

I dedicate this work to my family, who inspired and encouraged me to push forward in my studies, and to all the academics for their support in planting more to the body of knowledge of Science. I will forever cherish that.

Motlagomang

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ABSTRACT

The bacterial community found in the rhizosphere has been found to have more diversity compared to the rhizoplane due to the interaction of microbes and plants in the soil environment. The intraspecific variations of bacterial strains resident in the rhizosphere and rhizoplane of the bambaranut were identified, characterized and compared in the study. Soil and root samples were collected from the North-West University agricultural farm located in the Ngaka Modiri Molema district, North West Province, and from Vhembe district in Thohoyandou, Limpopo Province in South Africa. Bacteria were then isolated on fourteen cultivated sites. A total of twenty eight samples from both the rhizosphere and rhizoplane samples were studied. Isolates were biochemically and culturally characterized using conventional methods. Several bacterial plant growth-promoting (PGPR) traits were tested and revealed that 93% were positive for phosphate solubilzation, in both rhizosphere and rhizoplane isolates, 64% siderophore production for the rhizosphere and 57% for the rhizoplane, 64% indole acetic acid production for the rhizoplane and 79% of the rhizoplane, 64% chitinolytic activity for rhizosphere and rhizoplane, both the rhizosphere and rhizoplane isolates yielded 64% of ACCD activity. Moreover 64% of isolates tested positive for hydrogen cyanide production for both the rhizosphere and rhizoplane. Interestingly all isolates (100%) produced ammonia from both rhizosphere and rhizoplane isolates, which is a good indication that rhizobacteria can fix nitrogen, hence bambaranut is well known to make the soil around it fertile even for the other plants grown near it or intercropped with it. Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) revealed high bacterial diversity across the rhizosphere since it is directly influenced by root secretions (exudates) and competition in soil microorganisms is very high compared to the rhizoplane where soil particles and microbes attach. The bacterial taxa observed were consistent with

findings from other studies that used culture-independent techniques to describe taxa abundances. Selected genomic DNA samples were subjected to Next Generation Sequencing (NGS); MS8, TS6 from the rhizosphere and MP7, TP5 from the rhizoplane were identified by the 16S rDNA as clusters of most abundant bacterial communities. All sequences clustered into groups (phyla or classes) according to the taxonomic classification. The NGS identified them as phyla belonging to *Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria* and *Acidobacteria*. A synthesis of available data suggests a two-step selection process by which the bacterial microbiota of roots is differentiated from the surrounding soil biome. Rhizodeposition appears to fuel an initial substrate-driven community shift in the rhizosphere, which converges with host genotype–dependent fine-tuning of microbiota profiles in the selection of root endophyte assemblages. Substrate-driven selection also underlies the establishment of phyllosphere communities but takes place solely at the immediate rhizoplane surface. Both the rhizoplane and rhizosphere contain bacteria that provide indirect pathogen protection, but the rhizosphere members appear to serve additional host functions through the acquisition of nutrients from soil for plant growth. Thus, the plant rhizosphere emerges as a fundamental trait that includes mutualism enabled through diverse biochemical mechanisms, as revealed by studies on plant growth–promoting and plant health–promoting bacteria. The rhizosphere and rhizoplane communities do not possess much of the variation though the degree of intimacy is most likely to be different. The study suggests that bacteria found in both rhizosphere and rhizoplane in agriculture might prove beneficial towards crop production and conservation. Moreover, they might prove beneficial in agriculture towards crop production and conservation in addition, the isolates are likely to be potential candidates for biofertilizers, biocontrol and biotechnological application of commercial value. This, along with other innovations could prove to be an environmentally friendly strategy to ensure sustainable agriculture. Population diversity and genomics

combined with NGS will be the key for understanding how adaption and horizontal gene transfer come about, how it takes place and where it leads. Future efforts will be focused on studying these bacteria in defined communities in the soil with plants simulated based on next generation sequencing results to understand the impact of one species on the whole community, their metabolites production and impact on the plant itself.

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Chapter 1

1.1 Introduction

Food security is a complex issue, faced with a number of interlinking challenges (Mayes et al., 2011). Agriculture needs to stand the effects of climate change, increasing competition for water, loss of productive land and competition for available land (Muhammad, 2014). Previous research, indicated that sustainable agricultural practices are the answer to serious problems that have resulted due to prolonged use of chemical based agronomic tools to improve crop development and productions for many decades (Prashar et al., 2013). The search for suitable environment-friendly options to replace or even to supplement the chemical fertilizers has thus been aggravated. By exploring the microorganisms that reside in close proximity to the plant, this will be a justified move in the quest for achieving the objectives of this research.

Soil appears to be a major reservoir of microbial genetic diversity and may be considered as a complex environment (Brevik et al., 2015). This extreme complexity results from multiple interacting parameters including soil texture and structure, water content, pH, climatic variations and biotic activity (Nannipieri et al., 2003). Soil is the medium for growth and survival of plants together with the microbes; therefore it is bound to affect the bacterial populations directly on their growth and/or indirectly by influencing the host plant by providing as a substrate as indicated by Berg (2009).

A variety of soil factors are known to increase nutrient availability and plant productivity. The most influential might be the organisms comprising the soil microbial community of the rhizosphere, which is the soil surrounding the roots of plants where complex interactions

occur between the roots, soil, and microorganisms (Chaparro et al., 2012). The Rhizosphere as explained by Pii et al. (2015) is subdivided into three zones, (a) the endorhizosphere; that consists of the root tissue including the endodermis and cortical layers, (b) the ectorhizosphere; that consists of soil immediately adjacent to the root and (C) the rhizoplane which is the root surface which attaches the soil particles and microbes. It also encompasses the epidermis, cortex and mucilaginous polysaccharide layer, the area of interest in this study.

Research shows that the plant is the most important factor in determining the resident bacterial strains in the rhizosphere and the rhizoplane because of the important role of the root exudates in the set-up of bacterial populations. Related features in plants such as the cultivars, ageing of plant and root characteristics have been found to rule the bacterial diversity and the predominant species in the rhizosphere (Smalla et al., 2007). Age and developmental stage of the plant play a critical role in deciding the bacterial nature of the rhizosphere community (Andreote et al., 2009b). However, it is very important to understand the composition, ecology, dynamics and activities of rhizosphere microbial communities, before we can exploit the rhizosphere microflora as a tool for developing sustainable agricultural practices. According to previous researches such as of Pham and Kim (2012), it is indicated that the majority of the soil microorganisms (approximately 99 %) are not yet culturable, on the contrary, Streit and Schmitz (2004) showed alternatives to culture-dependent techniques to identify and quantify organisms and more advancements have been made according to Otlewska et al. (2014), proving that biochemical and molecular genetics techniques for isolation of yet uncultured bacterial strains have enabled scientists to generate important information with respect to the rhizosphere bacterial communities.

According to Babalola (2010), plant bacteria residing in the rhizosphere and exerting beneficial effects on the plants are called plant growth promoting rhizobacteria (PGPR). The PGPR inoculants can fulfill diverse beneficial interactions in plants leading to promising solutions for sustainable and environment-friendly agriculture. The applications of rhizosphere soil of agricultural crops with desirable bacterial populations have established considerable promise in both the laboratory and greenhouse experiment (Bhattacharyya and Jha, 2012).

In this work, bambaranut (*Vorandzeia subterranean L. thouars*) was used as a trap plant on soils for the molecular comparison and identification of the intraspecific variation of resident bacterial strains in the rhizosphere and rhizoplane. Bambaranut is a seed of African origin used locally as a food source (Muhammad, 2014, Okonkwo and Opra, 2010). It is the third most important leguminous crop south of the Sahara (Mathews, 2010), after cowpeas and groundnut (Omoikhoje, 2008). Okonkwo and Opra (2010) indicated that the bambaranut plant is also used to sustain the plant habitat as it increases the fertility of the soil and brings about the high yields of other crops cultivated around it without the application of fertilizers.

The relationship between intraspecific residents of rhizoplane and rhizosphere bacterial populations was determined and compared using PCR-amplified 16S rRNA genes employing fingerprinting methods: terminal restriction fragment length polymorphisms (T-RFLP) and next generation sequencing (NGS). Profiling with polymerase chain reaction-based culture independent techniques, targeting bacteria, gives a more objective analysis (Rastogi and Sani, 2011). T-RFLP is the gold standard of several molecular analyses aimed to generate a fingerprint of an unknown microbial community (Desai et al., 2010), it also has greater potential resolving power than other fingerprinting methods. However, the introduction of

NGS for this work has revitalized my research in environmental DNA. Genomics and molecular biology have already revolutionized the study of ecology and evolution. For example, the ability to identify individuals by their DNA (Jeffreys et al., 1985), to perform taxonomic studies on DNA sequence information (Cann et al., 1987, Woese et al., 1990) to study gene flow within wild populations e.g. Ellstrand et al. (1999) and to identify unculturable microorganisms from their DNA sequence as reviewed by Handelsman (2004) have all allowed great advances in their respective fields and in many cases had much broader impacts on society. However, all these techniques are based on the analysis of a tiny fragment of an organism's total gene complement, usually one gene or variable region (Hudson, 2008).

1.2 Problem statement

The soil has been over utilized resulting in the depletion of essential soil nutrients. The threat to global food security due to climate change has been recognized as one of the greatest challenges facing humanity in this twenty-first century. Climate change contributes to food scarcity all over the globe due to stress conditions such as, high temperature and water shortages in agricultural sites, leading to low agricultural productivity. The quest for crops with nutritional properties also continues to receive attention. The use of chemicals to enhance plant growth is expensive with varying degree of side effects on the environment.

There is a need for a more environmentally friendly approach to sustain our agriculture. This research is aimed at determining and exploiting rhizobacteria that is common in both rhizosphere and rhizoplane which has plant growth promoting traits and can maintain soil fertility which will help enhance plant growth and development especially alleviating stress induced by the presence of drought.

1.3 Aim

The aim of the study was to determine and to compare intraspecific variation in the resident bacterial strains of the rhizosphere and rhizoplane of bambaranut.

1.4 Objectives

This study was designed to:

- Isolate bacteria of the rhizoplane and rhizosphere from bambaranut of Ngaka Modiri Molema district, Mahikeng municipality in the North West province and from a different climate in Vhembe district, Thulamela municipality in Limpopo province, South Africa.

- Characterize the bacterial strains resident in the rhizoplane and rhizosphere of bambaranut.
- Assay for the plant growth promoting traits from the isolates.
- Molecularly identify bacterial strains resident in the rhizoplane and rhizosphere of the bambaranut.
- Compare intraspecific variation in the bacterial strains resident in the rhizoplane and rhizosphere of bambaranut.

Chapter 2

2.1 Literature review

Soil microorganisms are part of the food chains, thus serving as sources of nutrients to one another and frequently serve as the primary members of food chains in soil biota (Ritz et al., 2009). Rhizosphere is the immediate vicinity of plant roots with increased microorganisms where the biology and chemistry of the soil are influenced by the root (Andreote et al., 2009a). In the rhizosphere, very important and intensive interactions take place between the plant, soil, microorganisms and soil microfauna feeding on these compounds. All this activity makes the rhizosphere the most dynamic environment in the soil (Kumar and Dubey, 2012). Nannipieri et al. (2007) have distinguished rhizosphere fractions which include the rhizoplane which is the surface of the root and rhizosphere soil that adheres to the root when the root system is shaken manually.

Soil bacteria found around the plant root that are capable of exerting beneficial effects (Babalola, 2010) are called plant growth promoting rhizobacteria (PGPR). On the basis of relationship with plants, PGPR can be divided into group: symbiotic bacteria and free-living rhizobacteria (Ahemad and Khan, 2011, Khan, 2005). Moreover, PGPR can also be divided into groups based on their residing sites: Intracellular PGPR (iPGPR) or symbiotic bacteria which live inside the plant cells, produce nodules and localized structures (Zhuang et al., 2007) and extracellular PGPR (ePGPR) or free living rhizobacteria which live outside the plant cells and do not produce nodules but still promote plant growth (Gray and Smith, 2005). Previous studies have focused on rhizosphere soil communities defined as those influenced by plant roots and few have investigated rhizoplane communities those colonizing the root surface as a distinct ecological niche (Nunan et al., 2005). However, any influence of plants

is likely to be mediated through root exudates and it is therefore likely to be greatest on the rhizoplane where specific nutritional selection will occur prior to diffusion of less plant-specific breakdown products into the rhizosphere soil (Kowalchuk et al., 2002). This is depicted in Figure 2.1.

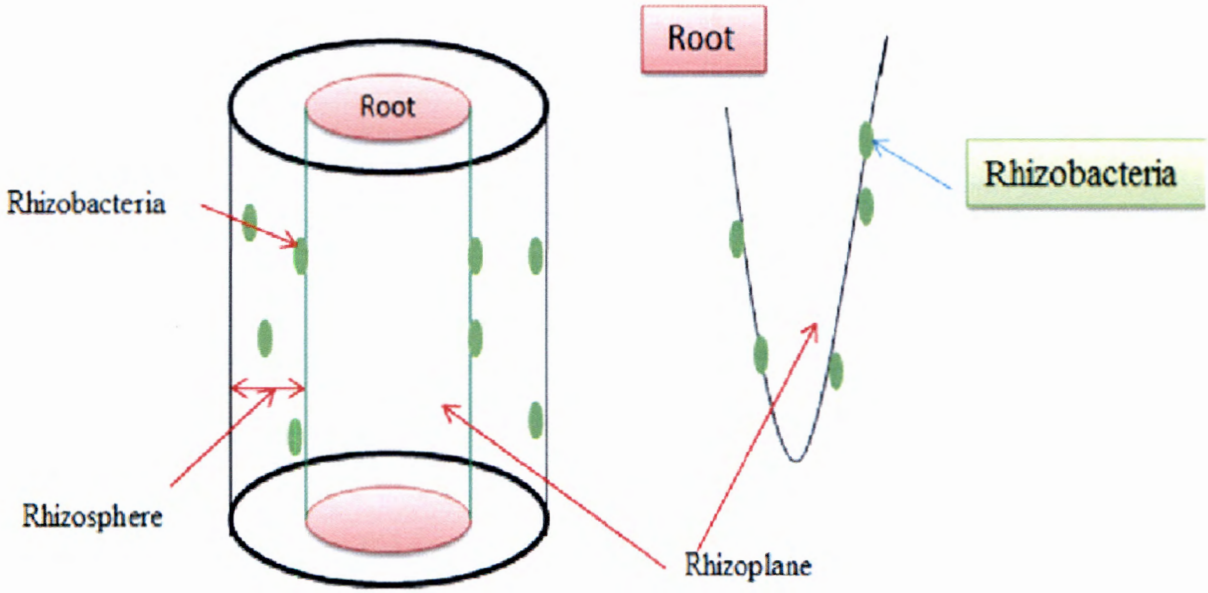


Figure 2.1 The rhizosphere, rhizoplane the root and attachment of microbes

2.2 Plant Growth Promoting Bacteria

In soil, plant growth is influenced by either abiotic or biotic factors. The region around the roots is rich in nutrients because as much as 40% of plant photosynthates are lost from the plant roots (Nelson, 2004). These nutrients attract bacteria and fungi. The microorganism may inhibit or enhance plant growth (Kloepper and Schroth, 1978). The bacteria that colonize the roots of plants following the inoculation onto seed and that enhance plant growth are plant growth promoting rhizobacteria (PGPR) (Zahir et al., 2003).

Plant growth is influenced by a number of biotic and abiotic factors which may promote growth or cause stress to plants. Soil bacteria and other microorganisms contribute greatly to the Earth's biomass as they form the bottom of the food chain and orchestrate the cycling of carbon, nitrogen, and flow of other nutrients through the ecosystem. PGPR have the potential to contribute in the development of sustainable agricultural systems such as nitrogen fixation in leguminous plants ability to produce phytohormones, siderophores, antimicrobial agents and hydrogen cyanide, and solubilize phosphate. It has been proposed that many plant growth promoting bacteria may promote plant growth by lowering the levels of ethylene in plants. This is attributed to the activity of enzyme 1- aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyzes ACC, the immediate biosynthesis precursor of ethylene in plants. The products of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth (Hayat et al., 2010, Khantsi et al., 2013).

2.3 PGPR in phytoremediation and bioremediation

Plant growth-promoting and other inoculant bacteria have many potential uses, most notably in agriculture, forestry and phytoremediation. For instance, they may be used to prevent

damage caused by plant pathogens, to prevent flowers from senescing to increase their shelf-life, and to facilitate the clean-up of soils that are contaminated with pollutants (Lucy et al., 2004). The use of synthetic chemicals such as fumigants is gradually decreasing due to concern over environmental impact and safety issues, including residues in food. Several microorganisms are currently produced commercially as biological control agents to protect against pathogens. Phytoremediation, which is the planting of trees, grasses, or other vegetation to remove or neutralize contaminants, as in polluted soil or water of organic compounds involves the absorption of microbial degradation of pollutants by the plant. For example, an ACC-deaminase containing strain *Kluyvera ascorbata* SUD165, stimulated canola plants to have higher chlorophyll content and attain a moderate amount of biomass in nickel-polluted soils, but a wildtype strain does not have the ability to enhance that. Siderophores and ACC deaminase enables a plant to obtain iron, thus reducing iron contamination and at the same time decrease the levels of stress ethylene (Nie et al., 2002).

Microbial methylation of inorganic Hg is an important process, as Hg is dominantly found in its inorganic form in the environment (Poulain et al., 2007). The emerging methylmercury (MeHg) is neurotoxic and can accumulate in the biota. On the other hand, microbial production of Me_2Hg does not receive much attention, because the concentrations of Me_2Hg are usually very low in nature. Dimethyl mercury is only little accumulated in soils or waters, because it is water insoluble and volatile. For this reason the transformation of MeHg to Me_2Hg can be a detoxifying mechanism. Microbes responsible for mercury methylation are methanogens, anaerobic microorganisms, aerobic microorganisms like *Pseudomonas* spp., *Bacillus megaterium*, *Escherichia coli* and *Enterobacter aerogenes*, fungi like *Aspergillus niger, Scopulariopsis brevicaulis* or *Saccharomyces cerevisiae* (Ehrlich, 2002).

Commercially, petroleum hydrocarbons are widely used sources of energy. However the release of large amounts of these compounds into the soil, for example, in the case of oil spill or leakage of petrol tanks, results in pollution. A number of micro-organisms belonging to the bacteria, fungi, microalgae are known to degrade such hydrocarbons to assist in environmental clean-ups. The diverse nature of microbes and their ability to excrete several enzymes provides a potential and considerable opportunity for sustainable use in the cleaning of organic pollutants, pesticide, dyes, heavy metals and agricultural waste from the environment (Lucy et al., 2004).

Bacteria with tiny wire like appendages, not only digest waste- including PCBs and chemical solvents- they produce electricity while they are at it, example, Shewanella (deep sea bacteria that grow these oxygen-seeking nanowires when placed in low oxygen environments). Researchers discovered that, when the microbes' nanowires are pricked with platinum electrodes, they carry a current, if these capabilities are harnessed effectively, they could one day be used in sewage treatment plants to simultaneously digest waste and power the facilities (Pandey et al., 2011).

Plant growth promoting rhizobacteria have different means to enhance plant growth and development, such as production of hormones and metabolites are other ways to promote plant growth. Eighty per cent of micro-organisms isolated from the rhizosphere of various crops have the ability to produce auxins as secondary metabolites. Various metabolic pathways such as indole-3-acetamide pathway, indole-3-pyruvic acid pathway, tryptophan side chain pathway, tryptamine pathway and indole-3-acetonitrile pathway are involved in the production of IAA (Patten and Glick, 2002). Bacterial production of IAA suggests that the pathways involved in IAA production may play an important role in defining the effect of the

bacterium on the plant. The beneficial bacteria such as PGPR, synthesize IAA via indolepyruvic acid pathway and the IAA secreted is thought to be strictly regulated by the plant regulatory signals. Some of the plant responses to auxin include: cell enlargement, cell division, root initiation, root growth inhibition, increased growth rate, phototropism and apical dominance (Frankenberger Jr and Arshad, 1995).

Cytokinins represent another class of phytohormones produced by micro-organisms (Persello-Cartieaux et al., 2003). There are fewer studies on cytokinin synthesis by micro-organisms than on microbial biosynthesis of auxins. One of the common biosynthetic pathways utilizes adenine as the precursor of free cytokinin production in the micro-organisms such as *Corynebacterium fascians* and *Azotobacter* spp. Plant responses to exogenous application of cytokinin result in either one of the following effects a) enhanced cell division, b) enhanced root development, c) shoot initiation and certain other physiological responses (Frankenberger Jr and Arshad, 1995).

Apart from production of phytohormones, direct mechanisms of action have been associated with PGPR for enhancing plant growth. These include: lowering of ethylene concentration by production of ACC deaminase, phosphate solubilzation and production of siderophores. ACC deaminase converts ACC to α-ketobutyrate and ammonia- thereby lowering the amount of ACC in a developing seedling or plant (Glick, 2005). Lowering of ACC concentrations within the plant also reduces the amount of ethylene produced in the plant, decreasing the inhibitory effect of ethylene on the root elongation and leading to longer roots (Patten and Glick, 2002).

Bacteria isolated from the rhizosphere are capable of increasing availability of phosphorus to plants either by mineralization of organic phosphate or solubilzation of inorganic phosphate by production of acids. The increase in dry weight is directly correlated to phosphorus uptake by seedlings. Siderophores are low-molecular weight iron-binding molecules that are synthesized by many micro-organisms under low-iron conditions. Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots. Plants such as peanuts, oats, cotton, and sunflower demonstrated the ability to use radiolabeled microbial siderophore as a sole source of iron (Wang et al., 2000). *Kluyvera ascorbata*, a siderophore-producing PGPR, was able to protect plants from heavy metal toxicity (Burd et al., 1998). Plants have a way to cope with stress in their developmental stages and ethylene production is their way to stress response.

2.4 Why bambaranut?

Bambaranut was used as a trap plant to conduct our study. The common names of bambaranut are Okpa (Nigeria Igbo), Congo groundnut (Congo), Njugo bean (South Africa), Nzama (Malawi) Ntoyo or Katoyo (Zambia). Bambaranut (*Vorandzeia subterranean* L. *thouars*) is believed to have originated from the African continent, especially Central Africa, long before the introduction of groundnuts (peanuts). It belongs to the family Leguminosae and sub-family Papilionoideae (Hillocks et al., 2012). Bambaranut is an underutilized leguminous species, which is of interest for its reported high levels of drought tolerance in particular, and which contributes to environmental resilience in semi-arid environments. The climatic requirements are those required for peanuts. However, it survives in conditions which are more arid, that is, in the drier savannah areas with short periods of scant rainfall of up to 750 mm per annum. The crop prefers, average day temperatures of 20°C - 28°C and full sun (Muhammad, 2014). It is adapted to a wide range of soils especially light or sandy loam and does well on very poor soils where other crops fail. Bambaranut is a traditional

indigenous crop mainly cultivated as a subsistence crop, usually for the resource poor men and women farmers (Figure 2.2). This is normally done on soils which are poor to support the growth of other crops, and to some degree, for income generation (Okonkwo and Opra, 2010). The crop has the potential to contribute to food security in view of its ability to withstand drought (Hillocks et al., 2012, Okpuzor et al., 2010).

Bambaranut is an inexpensive source of high quality protein, it contains 6% oil, 60% carbohydrates and 25% protein which is higher as compared to other legumes (Okonkwo and Opra, 2010), despite this its use is still limited. It grows underground and the seed is spherical and hard, when dry. It has a deep taproot surrounded by lateral profuse roots bearing N-fixing nodules. However, it is highly adaptable and tolerates harsh weather conditions better than most crops. Evidence has shown that based on the root nodules, the plant supports land care provision in Africa (Hillocks et al., 2012).

The bambaranut can be eaten raw when immature because it is soft and pleasant. It can be boiled and eaten as nut and can also be grounded into flour for preparing fufu maize (Nigeria, Middle Belt). The extract from the nut, particularly the protein extracts can be used directly in cosmetic formulations and provides specific properties and notable particular effects. The nut can be used quite freely to replace the high-prized lumps of meat without sacrificing adequate nutrition. The fatty acid present in the nut oil is among the essential fatty acids needed in the body. These fatty acids are primarily used to produce hormone like substance that regulates the wide range of functions (Hillocks et al., 2012). The unique properties and composition of bambaranut make it serve as a balanced food which contains almost all the vital nutrients that promotes good health for people mostly living in Africa (Okonkwo and Opra, 2010).

Figure 2.2 Bambaranut field cultivated as a subsistence crop usually for the resource of poor men and women farmers. (A) Screen house experiment, (B). Bambaranut can traditionally be intercropped with major commodities such as maize, millet, sorghum, cassava, yam, peanut and cowpea, (C) rhizosphere soil of the Bambaranut, (D). The crop has well adapted nodules are best known for their nitrogen fixing noodles (E). The seeds can be consumed in different forms but at maturity, the seeds (F) become very hard and therefore require boiling before any specific preparation can be carried out.

2.5 PGPR as primary producers

Some of the microorganisms, especially soil bacteria have an important role in the economy, because they are the initial biological CO_2/O_2 exchangers, and the most important primary producers of biomass and one of the most variable ecological groups of organisms. The rhizosphere exists because of complex soil-plant-microorganism interactions. It is a unique soil environment found closer to the plant roots, where nutrients are more abundant because of the plant itself, where some of these nutrients are recycled by microorganisms (Jones Jr, 2012). Antimicrobial drugs kill or prevent the growth of other microbes. However, with the development of antimicrobials, microbes have adapted and become resistant to previous antimicrobial agents. There are natural sources such as antibiotics or protein synthesis inhibitors; which are synthetic agents, examples are antivirals, antifungals, and antiparasitics. Among other microorganisms, there are bacterial species such as Actinomycetes that are particularly rich sources of metabolites, with essential biological activities, including antibiotics production. Antibiotics are compounds produced by microorganisms that inhibit or kill other microorganisms; they are naturally, products obtained from microorganisms making them to be primary producers with respect to natural products. The involvement of microorganisms in cycling of nutrients, confirms their role as primary producers in the food chain (Schmidt and Lipson, 2004). Table 2.1 depicts some of the nodulating bacteria, enhancing plant growth and development.

Table 2.1 Examples of various legume nodulating bacteria involved in improving plant growth and development

Soil fertility parameters	PGPR	Comments	References
Nitrogen fixation	*Burkholderia* spp. and *Bacillus* spp.	*Burkholderia* in association with papilionoid forage legumes from South Africa adapted to infertility and acidity, have the potential to play an important role in symbiotic N-fixation on intractable soils. Bacillus from family rhizobiaceae living in the soil infect plant root to form nodules and fix nitrogen in this structure through complex enzymes system, the activity of enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyzes ACC, the immediate biosynthesis precursor of ethylene in plants. The products of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth.	(Wong-Villarreal and Caballero-Mellado, 2010, Rashid et al., 2016)
Phosphate solubilization	*Burkholderia* spp.	Phosphate-solubilizing bacteria employ different strategies to convert unavailable forms of phosphate into available forms. In most bacteria, production of organic acids is shown to be related to the dissolution of mineral	(Adhya et al., 2015, Ghosh et al., 2016)
Iron (Fe) solubilization	*Pseudomonas aeruginosa BS8*	Production of siderophores which has affinity to chelate and solubilize iron from mineral or organic compounds.	(Goswami et al., 2015)

2.6 Resident bacteria in rhizosphere soil

On account of the biodiversity of indigenous soil bacteria and the population densities involved, it is not surprising that it has proven difficult to make any long lasting structural changes to the composition of bacteria within any given soil-community. Prashar et al. (2013) state that the rhizosphere has a very wide range pool of soil microorganisms and hence is considered as the "plague spot" for microbial activity and colonization. A challenge in soil and rhizosphere ecology has been developing effective methods that can be used to describe the diversity, function and abundance of soil and plant associated microbial populations (Thies, 2008). One strategy which may help contribute to the establishment of pre-selected beneficial organisms in root zone soils, and which has until recently been excluded from the research equation, is through fostering the early establishment of selected communities of endophytic microorganisms within root systems.

2.7 Resident bacteria in rhizoplane

Many studies of non-agricultural plant-microbial communities have focused on rhizosphere soil communities, defined as those influenced by plant roots (Brodie et al., 2002, Broughton and Gross, 2000), whereas relatively few research studies have investigated rhizoplane communities, that is those colonizing the root surface, as a distinct ecological niches (Kowalchuk et al., 2002). Nonetheless, any influence of plants is likely to be mediated through root excretion (exudates) and is therefore likely to be greatest on the rhizoplane, where specific nutritional selection will occur before diffusing of less plant-specific breakdown products into rhizosphere soil. The plant kingdom is colonized by a diverse array of bacteria which form non-pathogenic relationships with their hosts. When beneficial, such associations can stimulate plant growth, increase disease resistance, improve the plant's ability to withstand environmental stresses (e.g. drought), or enhance N_2 fixation (Yang et al., 2009). Crop sequences can favor the build-up of advantageous associations of rhizoplane

bacterial populations leading to the development and maintenance of beneficial host. Utilization of rhizobacteria in sustainable crop production systems will require strategies to create and maintain beneficial bacterial populations within crops and as well in the soils surrounding those crops (Verma et al., 2010).

Table 2.2 Alleviation of impact of various stresses by PGPR to promote plant growth

Sources of stress	PGPR	Comment	Reference
Temperature	*Burkholderia phytofirmans*	Potato plants maintained normal growth under heat stress	(Naveed et al., 2014)
Water	*Pseudomonas putida* UW4	Tomato plants showed substantial tolerance to flooding stress	(Grover et al., 2011)
Salinity	*Achromobacter piechaudii*	Significant increase in fresh and dry weights of tomato seedlings in the presence of NaCl salt	(Mayak et al., 2004)
Pathogenicity	*Pseudomonas putida* UW4	The strain was more effective in biocontrol of cucumbers disease caused by *Pythium ultimum.*	(Lugtenberg and Kamilova, 2009)

2.8 Molecular methods for DNA analysis

The advances made in research to define and understand microbial diversity have informed biologists to the fact that the number of prokaryotic species may well exceed that of all other life forms on the planet. These advances allow identification and characterization which show the origin and the evolution of species involved. Molecular identifications of micro-organisms are the more abroad approaches used for identification compared to the morphological characterization. Despite their technical limitations and biases, various approaches have proven to be quite useful to describe the structure of microbial communities (Forney et al., 2004). One such approach, namely the construction and analysis of clone libraries, provides detailed phylogenetic information about the members of communities. However, this approach is generally not well suited for the analysis of numerous samples because of the time and cost associated with the analysis of numerous clone libraries.

According to Schütte et al. (2008) several molecular approaches now provide powerful adjuncts to the culture-dependent techniques. One approach in particular that couples PCR and rRNA-based phylogeny has been effective in the exploration of microbial environments and the identification of uncultured organisms. The stepwise strategy of this approach is to isolate total community DNA and use this DNA as a template for PCR amplification of 16S rRNA genes with universal or domain-specific primers. Fingerprinting techniques such as Terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) have been successfully used in numerous studies to explore microbial diversity of the predominant populations in various habitats and offer the advantage that they are more amenable to high throughput and more comprehensive than cultivation-dependent methods (Smalla et al., 2007, Muyzer, 1999, Liu et al., 1997).

2.8.1 Polymerase chain reaction- Denaturing gradient gel electrophoresis

Polymerase chain reaction-Denaturing gradient gel electrophoresis (PCR-DGGE) analysis is a microbial fingerprinting technique that separates amplicons of roughly the same size (length) based on sequence properties (different base pairs) (Muyzer et al., 2004). These properties dictate the threshold at which DNA denatures. Separation is based on the electrophoretic mobility of a partially melted DNA helix in polyacrylamide gels, which is decreased, compared to that of a completely helical form of the molecule (Hill et al., 2000). The major factor influencing DGGE analysis is the inability to run all samples on a single gel and the potential for variation between gels (Green et al., 2010). As a result, each dimension will account for a greater percentage of total distance for DGGE profiles patterns, despite modification of statistical analysis to account for integral variation. Therefore, in DGGE analysis, each dimension may represent the effect of a number of different factors or influences, reducing resolution or masking some information (in particular of weaker effects). A major advantage of DGGE analysis is putative identity of the origin of bands of interest, through sequence analysis. The DGGE is restricted by PCR biases, difficult sample handling (Wintzingerode et al., 1997) which have probable impact on the microbial community and may result in variable DNA extraction efficiency (Theron and Cloete, 2000). In addition, the bacterial diversity revealed by the DGGE only reflects the numerically dominant species not the total number of the species in the sample (Liu et al., 2010).

2.8.2 PCR-TRFLP

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a polymerase chain reaction (PCR)-fingerprinting method that is commonly used for comparative microbial community analysis. The method can be used to analyze communities of bacteria, archaea, fungi, other phylogenetic groups or subgroups, as well as functional genes (Powell et al., 2015). The method is rapid, highly reproducible, and often yields a higher number of

operational taxonomic units than other, commonly used PCR-fingerprinting methods. The T-RFLP approach has been used successfully to analyze the composition of microbial communities in soil, water, marine, and lacustrine sediments, biofilms, faeces, in and on plant tissues, and in the digestive tracts of insects and mammals. The T-RFLP method is a user-friendly molecular approach to microbial community analysis that is adding significant information to studies of microbial populations in many environments. T-RFLP has been found five times better than DGGE in the detection of specific ribotypes (Tiedje et al., 1999). The major advantage of T-RFLP is its ability to analyze large numbers of samples rapidly, which will be of benefit in the present study.

2.8.3 Next Generation Sequencing

Sequencing technologies have significantly improved since the first genome was read (Sanger et al., 1977). They remain at the core of genomics and have many practical applications. They are of course used to determine the genome sequence of a new species or of an individual within a population. However, they also provide tools for readout assays in molecular biology, for example when studying RNA transcripts (Adams et al., 1991), gene expression (Velculescu et al., 1995) and protein to DNA binding (Kim et al., 2005).

Determining the complete genome sequence of a species is still an important application of sequencing. Next-generation sequencing, although reliable and considerably optimized, the complete Sanger process is quite long and involves major costs, in terms of infrastructure, reagents, sample DNA and laborious. Important breakthroughs have recently been made to streamline the sequence pipeline. Many instrument makers have entered this lucrative market (Metzker, 2010, Hudson, 2008). Among the techniques which are currently automated, are 454, Illumina and SOLiD. They produce shorter reads with higher error rates, but are much

faster and cheaper than Sanger sequencing. Moreover, they generally require less DNA sample to function.

In contrast to techniques based on a single gene (usually 16S rDNA, like T-RFLP or DGGE), metagenomics gives much more information. Analysis of microbes' physiology is possible and biodiversity can be studied in more detail. Metagenomics is less biased than PCR and it gives information about relative abundance of different organisms and the community structure. Metagenomics captures different variants present in natural communities, which makes sequence assembly even more difficult but contains additional information (Sjöling et al., 2007). The use of Next Generation Sequencing was used for further analysis on this work. The main advantage of Next Generation Sequencing (NGS) over classical Sanger sequencing is that it needs significantly less DNA and is more accurate and reliable than Sanger sequencing (Eck, 2014). For Sanger sequencing, a large amount of template DNA is needed for each read. Several strands of template DNA are needed for each base being sequenced (i.e. for a 100bp sequence you'd need many hundreds of copies, for a 1000bp sequence you'd need many thousands of copies), as a strand that terminates on each base is needed to construct a full sequence. In NGS, a sequence can be obtained from a single strand. In both kinds of sequencing multiple staggered copies are taken for contig construction and sequence validation (Kothari et al., 2016).

NGS is quicker than Sanger sequencing in two ways. Firstly, the chemical reaction may be combined with the signal detection in some versions of NGS, whereas in Sanger sequencing these are two separate processes. Secondly and more significantly, only one read (maximum ~1kb) can be taken at a time in Sanger sequencing, whereas NGS is massively parallel, allowing 300 Gb of DNA to be read on a single run on a single chip (Stanton, 2010). The

reduced time, manpower and reagents in NGS mean that the costs are much lower. Sanger sequencing can be used to give much longer sequence reads. However, the parallel nature of NGS means that longer reads can be constructed from many contiguous short reads (Peng, 2015). Repeats are intrinsic to NGS, as each read is amplified before sequencing, and because it relies on many short overlapping reads, so each section of DNA or RNA is sequenced multiple times. Also, because it is so much quicker and cheaper, it is possible to do more repeats than with Sanger sequencing. More repeats means greater coverage, which leads to a more accurate and reliable sequence, even if individual reads are less accurate for NGS.

However advantageous NGS is, massive sequencing projects are still expensive. Data analysis can be hard. Assembling reads is impossible for medium or high diversity communities, chimeric sequences can easily be produced. Fragment recruitment methods depend on available genomes. Matching interesting genes with taxonomic groups is hard and can only be done if a gene marker (like 16S rRNA) is present on the same fragment of DNA. Also studying low-abundance members of the community is impossible (Shokralla et al., 2012).

2.8.3.1 NGS Illumina (MiSeq)

The NGS technologies are different from the Sanger method in aspects of massively parallel analysis, high throughput, and reduced cost. Although NGS makes genome sequences handy, the followed data analysis and biological explanations are still the bottle-neck in understanding genomes. In 2006, Solexa released the Genome Analyzer (GA), and in 2007 the company was purchased by Illumina. The sequencer adopts the technology of sequencing by synthesis (SBS). The library with fixed adaptors is denatured to single strands and grafted to the flowcell, followed by bridge amplification to form clusters which contains clonal DNA fragments. Before sequencing, the library splices into single strands with the help of

linearization enzyme (Mardis, 2008), and then four kinds of dideoxynucleotides (ddATP,ddGTP, ddCTP, ddTTP) which contain different cleavable fluorescent dye and a removable blocking group would complement the template one base at a time, and the signal could be captured by a (charge-coupled device) CCD. MiSeq which still uses SBS technology was launched by Illumina. It integrates the functions of cluster generation, SBS, and data analysis in a single instrument and can go from sample to answer (analyzed data) within a single day (as few as 8 hours). The Nextera, TruSeq, and Illumina's reversible terminator-based sequencing by synthesis chemistry was used in this innovative engineering. The highest integrity data and broader range of application, including amplicon sequencing, clone checking, ChIP-Seq, and small genome sequencing, are the outstanding parts of MiSeq. It is also flexible to perform single 36 bp reads (120 MB output) up to 2 × 150 paired end reads (1–1.5 GB output) in MiSeq. Due to its significant improvement in read length, the data (shown in this work) is a sequencing result of MiSeq.

Chapter 3

3. Materials and methods

3.1 Rhizosphere and rhizoplane sampling and sample preparation

The study area covered two different geographical environments (Figure 3.1). Ten (10) samples were collected from North-West University Agricultural Farm located in Mafikeng, Ngaka Modiri Molema district, North West Province 25,789149 latitude, 25,618401 longitudes, South Africa. Temperatures range from 17° to 35 °C (62° to 95°F) in the summer and from 3° to 21 °C (37° to 70°F) in the winter. The average rain fall is 360 mm. In addition, another ten samples were collected from the area where the bambaranut is a sustenance crop to most poor farmers, Tshiulungoma village of Vhukati ha Manini, Vhembe district in Thohoyandou, Limpopo Province 22,878541 latitude, 30,481845 longitudes in South Africa. Temperatures range from 25° to 40 °C (77° to 104°F) in summer and from 10 °C to 28 °C (50° to 82°F) in winter. The average rainfall is 650mm. A total of twenty (20) Samples were collected at random in the fields.

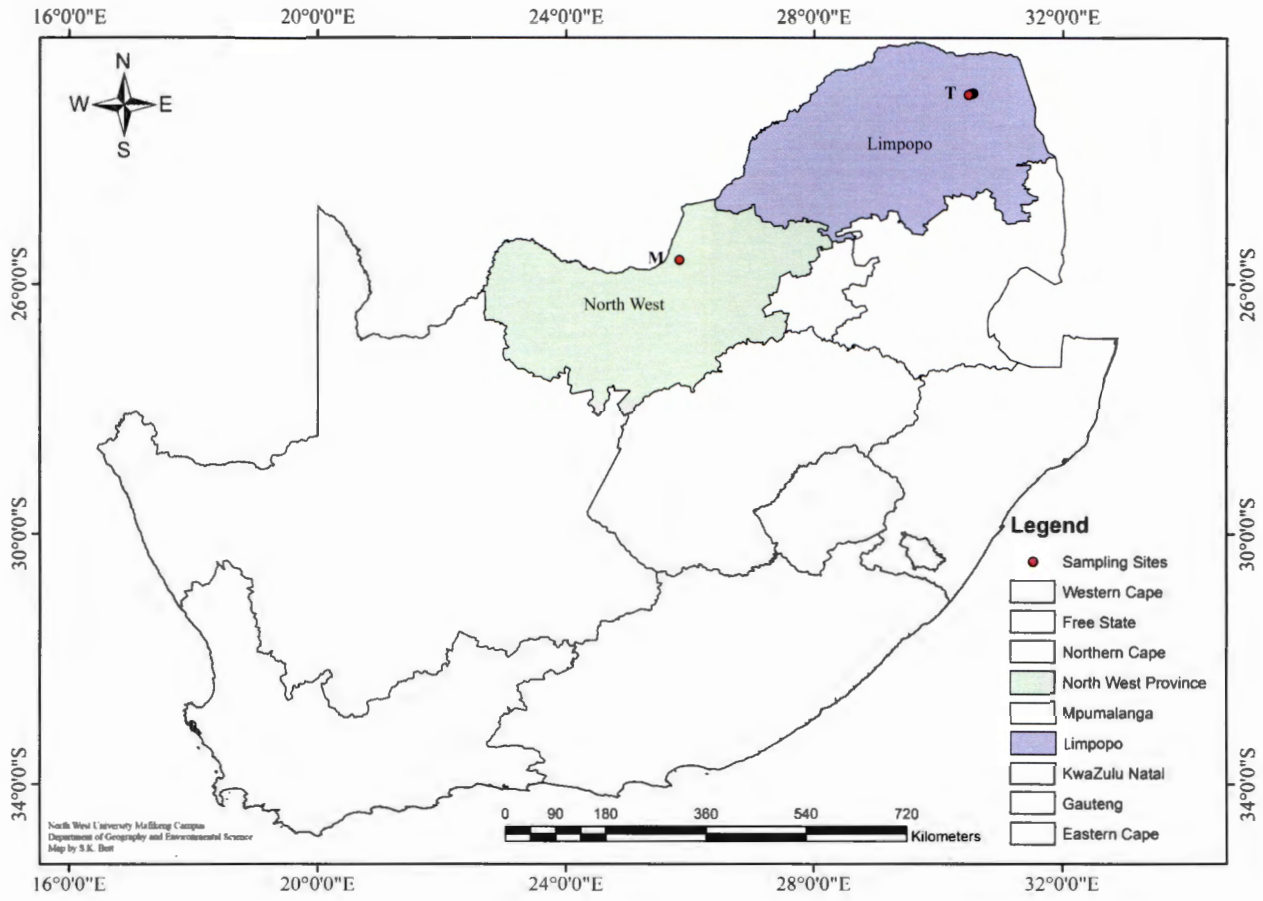


Figure 3.1 Geographic map of sampling sites, Ngaka Modiri Molema district, Mafikeng (M) North West province, and Vhembe district, Thohoyandou (T) Limpopo province, South Africa

Table 3.1 Soil sampling location, rhizosphere and rhizoplane sample codes

Location	Sample code	
	Rhizosphere	Rhizoplane
Mafikeng		
	MS1	MP1
	MS2	MP2
	MS3	MP3
	MS4	Mp4
	MS5	MP5
	MS6	MP6
	MS7	MP7
	MS8	MP8
Thohoyandou		
	TS1	TP1
	TS2	TP2
	TS3	TP3
	TS4	TP4
	TS5	TP5
	TS6	TP6

3.2 Sampling of cultivated soils

Soil samples that were distinct with respect to soil and site characteristics were collected from a wide array of cultivated sites. They were collected between November 2015 and April 2016. From each crop about 50 g of sample was collected at 5 to 10 cm depth from each of the surfaces using a hand trowel. Using a spatula, they were transferred into sterile paper bags. Sterile techniques were used during each collection. The soils were labeled properly and placed on a dry cool place to avoid moisture accumulation or extensive drying. The samples were immediately transported to the North-West University's Microbial Biotechnology research laboratory for analysis.

3.3 Phenotypic Characterization.

Isolation of bacteria

Rhizosphere samples were tested for plant growth promoting traits. To do this, 1 g of dry soil was weighed and was dissolved in 9 ml of distilled water and stirred using a magnetic stirrer. The purpose of stirring was to loosen bacteria that might have attached themselves to soil particles; thus making it difficult for detection. Ten-fold serial dilutions were prepared from each soil sample using distilled water and aliquots of 10µl were spread plated onto Tryptone Soy Agar. As for the rhizoplane, 10µl of the cell suspension, obtained from the pellet of 10 ml buffer used in roots shaking at 5,000 ×*g* for 10 minutes were spread plated unto Tryptone Soy Agar. The plates were incubated at 37°C for 24 h. To obtain pure cultures the colonies were streaked on fresh agar plates and were incubated at the same conditions as the original colonies.

3.3.1 Ammonium production test

Nitrogen is generally considered one of the major limiting nutrients in plant growth. Available genome sequences of rhizosphere and endophyte bacteria reveal in most cases a

versatile carbon and nitrogen metabolism. One specific conversion in the nitrogen cycle has been studied comprehensively: dissimilatory nitrate reduction or denitrification, by which nitrate (NO_3^-) is reduced to nitrite (NO_2^-) as an alternative respiratory pathway. Nitrite can further be converted to nitrogen oxides (N_2O and NO) or ammonia. Its role in plant growth promotion is related mainly to the latter compounds. NO is a potent signaling molecule in plants, altering root growth and proliferation in an auxin-dependent manner (Bulgarelli et al., 2013). The ammonium production test aims to determine whether the bacteria can fix nitrogen and be in the category of plant growth-promoting rhizobacteria. To prepare for the ammonium production test, 10 g of peptone reagent was suspended in 1000 ml distilled water. The mixture was allowed to dissolve by boiling and then it was distributed in 5 ml test tubes. The tubes were then autoclaved for 15 min at 121°C. Freshly grown cultures were inoculated in peptone solution, and it was incubated for 48-72 h at 37°C. About 0.5 ml Nessler's reagent was added to each test tube. Color change from brown to yellow indicated a positive test for ammonia production. No color change means a negative test for ammonium production (Cappuccino and Sherman, 1999).

3.3.2 Determination of indole acetic acid production

Plant growth is not stimulated only by indole acetic acid (IAA) production. In particular cases, IAA degradation by microorganisms can also stimulate root elongation, as illustrated by the interaction of *Pseudomonas putida* 1290 with radish plants. The source of IAA could be the plants themselves or other IAA-producing microorganisms. Thus, IAA degraders can have a function in the rhizosphere in auxin homeostasis by elevating or reducing local auxin concentrations (Leveau and Lindow, 2005). To determine this, a total of 8 g nutrient broth (Merck) was suspended in 500 ml; fresh grown cultures were inoculated into 10 ml nutrient broth in each test tube and incubated at 30°C for 48 h. A 4 ml culture was removed from each test tube and centrifuged at 10,000 rpm for 15 min. An aliquot of 1 ml supernatant was

transferred into a fresh tube to which 50µl of 10 Mm orthophosphoric acid and a 2 ml of reagent comprising of (1 ml of 0 .5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated at room temperature for 25 min. The development of a pink color indicated the presence of indole acetic acid (Ahmad et al., 2008).

3.3.3 Assessment of *in-vitro* antifungal susceptibility

Rhizobacteria can suppress the growth of various phytopathogens in a variety of ways like competing for nutrients and space, limiting available Fe supply through producing siderophores and producing lytic enzymes and antibiosis. Among PGPR, fluorescent pseudomonads are widely reported for their broad-spectrum antagonistic activity against a number of phytopathogens (Reddy, 2014). To assess this, 42 g of Potato dextrose Agar (Liofilchem) was suspended in 1000 ml distilled water. Selected fungi (*Fusarium*) and each test culture were spread on the prepared potato dextrose agar. Antibiosis of the test strain was assessed on the basis of the inhibition zone size after 4 days of incubation at 30°C (Mehnaz and Lazarovits, 2006).

3.3.4 Detection of Hydrogen cyanide (HCN) activity

Many plants have the ability to produce HCN; the mechanism for the production of HCN, in most species, is the degradation of cyanogenic glycosides. The HCN potential is a reflection of the concentration of cyanogenic glycosides in the plant which, upon degradation, leads to the release of HCN (Ahmad et al., 2008). Production of HCN was observed according to the method of Lorck (1948). Freshly grown cultures were spread on a Tryptone soy agar (30 g) containing glycine 4.5 g/l and a sterilized filter paper saturated with 1% solution of picric acid and 2% sodium carbonate was placed in the upper lid of the petri dish, the petri dish was then sealed with parafilm and incubated at 30°C for 4 days, a change in color of the filter paper from yellow to reddish brown was an index of cyanogenic activity.

3.3.5 Phosphate solubilzation

Strategies to improve phosphorus availability/uptake can contribute significantly to plant growth, because less than 5% of the phosphorus content of soils is bioavailable to plants. Microorganisms with the capacity to solubilize mineral phosphorus are abundant in most soils (up to 40% of the culturable population). Phosphate solubilzation is a complex phenomenon for selectively screening the bacteria which have the ability to release inorganic phosphate from tricalcium phosphate (Bulgarelli et al., 2013). We prepared a standard agar medium (pH 6-7) containing 5 g of tricalcium phosphate (TCP) as a sole source of phosphorus. The medium was poured on petri plates following autoclaving at 121°C for 15 min. Isolates were streaked on the plates and incubated for 3 days at 27°C. Phosphate solubilizing bacteria developed clear zones around colonies which indicated phosphate solubilzation capacity of the isolate (Pikovskaya, 1948).

3.3.6 Siderophore production

Similar to phosphorus, iron is abundant in soil, but it is not very available to plants owing to the low solubility of Fe^{3+}oxides. Plants have developed different strategies to counteract this low availability. In the first strategy (the reduction strategy, found mainly in dicots and non graminaceous monocots), protons and organic acids are released to decrease the soil pH, thereby increasing iron availability. In the second strategy (the chelation strategy, found mainly in grasses), plant roots release low-molecular-weight iron-chelating molecules (e.g., mugineic acid). These siderophores can efficiently bind iron and are then taken up by root cells (Conte and Walker, 2011). Isolates were grown in McCartney bottles with 20 ml production medium consisting of 25 g/l Sucrose, 4 g/l $(NH4)_2SO_4$, 3 g/l K_2HPO_4, 1 g/l citric acid, 0.08 g/l $MgSO_4$, and 0.0002 g/l $ZnSO_4$ at a pH of 6.8. The isolates were incubated for 48 h in a rotary shaker at 200 rpm. The siderophore assay is based on the color change that occurs as a result of ferric iron transfer from the reagent complex to the siderophore present

in the sample. The dye is initially blue, but on removal of iron or dye, the color changes to purple or pink (Milagres et al., 1999).

3.3.7 Chitinolytic and Cellulase activity

The degradation of cellulose and chitin by enzyme activity is an essential research topic due to its potential for efficient use of the energy and carbon content of these polymers. Chitin and cellulose are highly abundant and natural polymers of 1, 4-β-linked sugar units. They share similarities in both the structure and the enzymatic degradation mechanism. In general, different groups of enzymes interact in the polymer degradation process. For instance, exoenzymes, are active on both ends of the polymer chain; the endoenzymes, attack easily accessible glycosidic bonds or amorphous regions in the polymer chain; then there are dimer hydrolases, i.e., chitobiosidase, which hydrolyze oligosaccharides; and lastly the lytic polysaccharide monooxygenases, which break the crystalline region of the polymer chain and activate polymer unpacking (Howard et al., 2003, Vaaje-Kolstad et al., 2013). Chitinolytic and cellulase activity was done according to Cattelan et al. (1999). Bacterial isolates were plated on chitin agar and carboxymethyl modified cellulose (CMC) agar respectively. Plates were incubated for 5 days at 30°C. Development of halo zone around the colony was considered as positive for cell wall degrading enzyme production.

3.3.8 ACC Deaminase assay

The enzyme ACCD is an immediate precursor of the plant hormone ethylene, it converts ACC to α–ketobutyrate and ammonium (Saleh and Glick, 2001). The bacteria utilize the NH_3 evolved from ACC as a source of nitrogen and thereby decrease ACC within the plant (Siddikee et al., 2010). The enzyme has been found in soil bacteria and has been proposed to play a major role in plant growth and health (Glick et al., 2007). The possibility of a close mutualistic relationship between the plants and soil bacteria has been suggested. The more ACC that is utilized by soil bacteria, the lesser is that which is converted by ACC oxidase

into ethylene, resulting in reduced negative effect of stress ethylene and better plant growth (Penrose and Glick, 2003). The PGPR strains containing ACC deaminase activity are better effective for improving the growth of plants (Khantsi et al., 2013).

Bacteria were grown at 30°C in 10 ml of nutrient broth (supplemented with 15µg/ml of tetracycline for transconjugants) to late log phase, after which the cells were harvested by centrifugation at 9000 g for 10 min at room temperature. The cells were washed twice with 5 ml of 0.1M Tris-HCl buffer (pH 7.5). To induce ACC deaminase activity, the cells were suspended in 5 ml of M9 medium containing 3 mM ACC and then incubated for 18 h at 30°C in a rotary shaker. The bacteria were harvested by centrifugation, washed twice with 0.1M Tris-HCl buffer (pH 8.0), and resuspended in the similar solution. A 200 µl aliquot of bacterial suspension was removed and 10 µl of toluene was added. The cells were vortexed vigorously to enable the membrane to take up, and then 200 µl of 3 mM ACC was added to 50 µl of bacterial lysate. Some 0.1 M Tris-HCl buffer (pH 8.5) was added to the reaction mixture and incubated for 30 min at 30°C, 0.5 ml of 0.56 M HCl was added, and the mixtures were centrifuged at 9 000 rpm for 5 min. Then 200µl of 0.56 M and 75µl of 0.2% 2, 4-dinitrophenylhydrazine in 2 M NaOH was added to 500µl of the supernatant. Mixtures containing no cell suspension or no ACC were used as controls. ACC deaminase assay was quantified by monitoring the amount of α-ketobutyrate that was produced by the deamination of ACC via determination of the optical density at 540 nm (Honma and Shimomura, 1978), (Saleh and Glick, 2001).

3.3.9 Optical characterization of isolates

To obtain pure cultures, the colonies were streaked on fresh Tryptone Soy agar plates and were incubated at the same condition as the original colonies that is, they were incubated at 37 °C for 24 hours. Their cultural characteristics were recorded (Goodfellow et al., 2012).

3.4 Genotypic identification

Genomic DNA of all the isolates, both from the rhizosphere and rhizoplane was extracted using the Power Soil® isolation kit (MO Bio, BioCom Biotech USA), and purified using UltraClean® GelSpin® DNA Extraction kit, (MO Bio BioCom Biotech USA) following the manufacturer's specifications. For the rhizosphere genomic DNA extraction, 0.25g of soil samples were added to the PowerBead tubes, the mixture was then gently vortexed. This helps to disperse the soil particles, to dissolve humic acids and protect the nucleic acids from degradation. 60 µl of Solution C1 was added to the mixture and vortexed for 10 minutes. The step was critical for complete homogenization and cell lysis. Following that, the mixture was centrifuged at 10,000 ×g (Universal Z300K model centrifuge; HERMLE Labortechnik, Germany) for 1 min at room temperature. The supernatant (500µl) was transferred to a clean 2 ml collection tube. Some 250 µl of Solution C2 was added to the tube and vortexed briefly then incubated at 4°C for 5 min. The tubes were centrifuged at room temperature for 1 min at 10,000 ×g and 600µl of the supernatant was transferred to another new 2 ml collection tube. Some 200 µl of Solution C3 was added to the tubes and vortexed briefly. The mixture was incubated at 4°C for 5 min. The tubes were then centrifuged at room temperature for 1 min at 10,000 ×g. A volume of 750µl of the supernatant was transferred to the 2 ml collection tube and mixed with 1.2 ml of Solution C4 and vortexed for 5 sec. This allows the DNA to bind to the solution since the silica has high salt concentrations. Volumes of 675µl of the mixture were run three times into the Spin Filter then centrifuged at 10,000 ×g for 1 min at room temperature was processed, each time discarding the flow through. Contaminants passed through the filter membrane leaving only the DNA bound to the membrane. Some 500 µl of Solution C5 which is an ethanol based wash solution was added to further clean the DNA that is bound to the silica filter membrane while allowing the DNA to stay bound to the silica

membrane. The flow through was discarded. The tube was centrifuged at 10,000 ×g for 1 min. The Spin filter was placed in a clean 2 ml collection tube. A 100 µl of Solution C6 (10 mM Tris) which is the sterile buffer was added to the center of the white filter membrane. This resulted in the complete release of the DNA from the silica Spin Filter membrane. The tubes were centrifuged for 30 sec at 10,000 ×g at room temperature. The Spin filter was discarded and DNA in the tubes was subjected to downstream applications.

As for the rhizoplane, DNA was extracted from 0.5 ml of rhizoplane cell suspension, obtained from the pellet of 50 ml buffer used in roots shaking at 5,000 ×g for 10 minutes. The Mo Bio UltraClean™ soil DNA kit (Mo Bio Laboratories, USA) was also used and the same protocol described for the rhizosphere was used with no modifications. DNA concentrations were determined using NanoDrop 1000 (NanoDrop Technologies USA).

3.4 1 PCR Amplification of 16S rRNA

The 16S rRNA gene was amplified from genomic DNA obtained from extraction of both the rhizosphere and rhizoplane samples by polymerase chain reaction (PCR) with previously described primer 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'- TGA CTG ACT GAG GCT ACC TTG TTA CGA-3') (Lane, 1991). PCR was performed in a total volume of 25 µl containing 30-50 ng DNA, 100 mM each primer, 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. The thermal cycling conditions were: 10 min at 94°C for initial denaturation, 30 cycles of 30 s at 94°C for unealing, 1 min at 60°C, 1 min at 72°C for elongation, and final extension for 10 min at 72°C. The amplification reaction was performed with a Bio-Rad C100 Thermo Cycler (Bio-Rad, USA). The PCR amplicons were analyzed by electrophoresis in 0.8% (w/v) agarose gel. The gel containing ethidium bromide (10µg/ml) was viewed under ChemiDoc™MP System (Bio-Rad Laboratories, Hercules, CA, USA) to confirm the expected size of the PCR products. The

Sequencing of the PCR products was conducted at facilities of Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa.

Table 3.2 lists PCR primers used in this study. PCR conditions for 341f and 907r were described previously by Smalla et al. (2007) with some optimization. A volume of 1.25µl of Taq DNA polymerase AmpliTaq Gold, Perkin-Elmer, 15 mM Tris–HCl pH 8.0 , 50 mM of KCl, 2.5 mM $MgCl_2$, each deoxynucleoside triphos- 2 phate at a concentration of 200 mM, 2. 5 pmol of each primer and 10-29 ng/µl of the extracted DNA, The thermal cycle involved 10-min activation of the polymerase at 94°C before 2 cycles consisting of 1 min at 94°C, 1 min at 52°C and 2 min at 72°C. The annealing temperature was subsequently decreased by 5°C for every second cycle until it reached 47°C, at which point, 30 additional cycles were carried out; finally, a 10 min extension at 72°C was performed. Amplification of PCR products of the proper size was confirmed by electrophoresis through a 1.8% agarose gel stained with ethidium bromide.

PCR was performed in a total volume of 25 µl containing 5 µl -29 µl DNA, 0.8 mM of each primer, 0.05 U/µl Taq DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP contained, 0.3 mM of each deoxynucleotide (dNTP), 5 ml of 10Xbuffer (Promega, Madison, USA), 0.03 unit mlK1 REDTaq DNA polymerase (Sigma, USA), 3.75 mM $MgCl_2$, 2 ml of 10mg/mlK1 Bovine serum albumin (BSA) and double distilled, sterilized water to complete the mixture volume.

Table 3.2 PCR primers used in this study

Target organism	PCR	Sequence (5′→3′)
Bacteria	27F (F)	AGAGTTTGATCCTGGCTCAG
	1492 (R)	TGA CTG ACT GAG GCT ACC TTG TTA CGA
	515 (F)	GTGCCAGCMGCCGCGGTAA
	806 (R)	GGACTACHVGGGTWTCTAAT

3.4.2 PCR-RFLP

Reproducibility of the restriction fragment length polymorphism (RFLP) method was tested by duplicate PCRs of a sample DNA extract (Schütte et al., 2008). Restriction enzyme Hhai (GCG'C) (ThermoFisher Scientific, Biolabs, USA), was used in two single reactions. The enzymes was heat inactivated by incubating the samples at 65°C for 20 min. positive and negative controls were also subjected to PCR and restriction, to determine if restriction was complete and there was no bacterial DNA contamination. A 25µl PCR mixture of 10 µl of genomic DNA, 5 µl of restriction buffer provided in the kit , 1 µl of the restriction enzyme and 9 µl of free digest water was prepared and was incubated for 3 h at 37°C. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

3.4.3 Next generation sequencing using Illumina MiSeq

The 16S rRNA gene V4 variable region PCR primers 515 forward and 806 reverse were used in a 28 cycle PCR amplification (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (QIAGEN, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Sequences were joined, depleted of barcodes then sequences <150bp removed, sequences with ambiguous base calls removed. Sequences were deionized, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLAST against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov,http://rdp.cme.msu.edu).

3.5 Analysis

DNA fragments subjected to 16S rDNA Next Generation sequence (NGS) at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq, analysed by MR DNA analysis pipeline revealed the strains based on the percentages and names of classes, orders, families, genera, operational taxonomic unit (OTU), phyla and species. Taxa above the rank of class are not covered by the Rules of the *Bacteriological Code* (1990 Revision). Such names

cannot be validly published and they are cited below in quotes. Our interest is the kingdom bacteria covering their divisions of phyla with the highest sequence identity when the 16S rDNA sequence was searched in the NCBI database.

3.5.1 Molecular taxonomy determined by sequences and phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using a variety of software where a sequence was carried to process raw data into high quality sequences (Pappas et al., 2005). Nucleotide sequences were analyzed and edited using the BioEdit software (Hall, 1999). After this initial analysis, sequences were compared to the basic local alignment search tool database of sequences deposited at the National Centre for Biotechnology Information (NCBI) using the BLASTN website http://www.ncbi.nlm.nih.gov (Altschul et al., 1990). The partial 16S rDNA gene sequences were used to search the GenBank database with the BlastN algorithm to determine relative phylogenetic positions (Altschul et al., 1990). Multiple alignments of the sequences were carried out by the Mafft program 6.8.6 4 (Katoh and Toh, 2010) against corresponding nucleotide sequences retrieved from GenBank.

Chapter 4

4. Results

4.1 Phenotypic characterization of isolates

The bacterial isolates were assayed for plant growth promoting traits (Table 4.1 and 4.2). All isolates from both the rhizosphere and rhizoplane were able to produce ammonia. A total of 93% were able to solubilize phosphate on both the rhizosphere and rhizoplane. Comparatively, 64% of rhizosphere isolates were capable of producing siderophore compared to 57% of the rhizoplane, which are low molecular weight iron-binding molecules. Similarly 64% of isolates of the rhizosphere could synthesize indole acetic acid; compared to a high of 79% of the rhizoplane isolates. Interestingly, 64% of isolates form rhizosphere and rhizoplane showed ACC deaminase activity, chitinolytic activity and could produce hydrogen cyanide. However over 70% of isolates showed antifungal susceptibility, which proves to be able to suppress the growth of pathogens that could inhibit the plant growth and development. Rhizobacteria that promote plant growth are considered an alternative to the use of chemicals in agriculture.

Table 4.1 Plant growth promoting rhizobacteria traits from rhizosphere

Isolate name	NH_3 production	Phosphate	Antifungal	IAA	Siderophore	Chitinolytic	HCN	ACCD
MS1	+	+	+	+	-	+	+	+
MS2	+	+	+	+	+	+	+	+
MS3	+	+	-	+	+	-	-	+
MS4	+	+	+	-	+	+	+	-
MS5	+	+	+	-	-	+	-	-
MS6	+	+	-	-	+	+	+	-
MS7	+	+	+	+	+	+	+	+
MS8	+	+	+	+	+	+	+	+
TS1	+	+	+	+	+	+	-	+
TS2	+	+	-	+	+	-	+	+
TS3	+	-	+	-	_	-	-	-
TS4	+	+	+	-	-	-	-	-
TS5	+	+	+	+	-	+	+	-
TS6	+	+	+	+	+	-	+	+

PGPR traits of isolates from the rhizosphere samples included indole acetic acid production (IAA), hydrogen cyanide production (HCN), 1-aminocyclopropane-1-Carboxylic acid deaminase (ACCD) activity, antifungal susceptibility test, ammonium production, chitinolytic activity, siderophore production and phosphate production.

Table 4.2 Plant growth promoting rhizobacteria from rhizoplane samples

Isolate name	NH_3 production	Phosphate	Antifungal	IAA	Siderophore	Chitinolytic	HCN	ACCD
MP1	+	+	+	+	-	+	+	+
MP2	+	+	+	+	-	+	+	+
MP3	+	+	-	+	-	+	-	+
Mp4	+	+	-	-	+	-	-	-
MP5	+	+	-	-	+	+	+	-
MP6	+	+	+	+	-	+	-	-
MP7	+	+	+	+	+	+	+	+
MP8	+	+	+	+	+	+	+	+
TP1	+	+	+	+	+	-	+	+
TP2	+	+	+	+	+	-	-	+
TP3	+	-	-	+	-	-	-	-
TP4	+	+	+	+	-	-	-	-
TP5	+	+	+	-	+	+	+	-
TP6	+	+	+	+	+	+	+	+

PGPR traits of isolates from the rhizoplane samples included indole acetic acid production (IAA), hydrogen cyanide production (HCN), 1-aminocyclopropane-1-Carboxylic acid deaminase (ACCD) activity, antifungal susceptibility test, ammonium production, chitinolytic activity, siderophore and phosphate production.

Table 4.3 Comparison of PGPR traits production of rhizosphere and rhizoplane samples based on Tables 4.1 and 4.2

PGPR trait	**% production rate**	
	Rhizosphere	Rhizoplane
Ammonium Production	100	100
Phosphate	93	93
Antifungal	74	71
IAA	64	79
Siderophore	64	57
Chitinolytic	64	64
HCN	64	64
ACCD	64	64

4.2 Cultural characterization of isolates

The cultural characteristics of the isolates were given in table 4.4. The isolates showed different sizes which ranged from punctiform, small, moderate to large. The isolates varied in shape; among them are circular, irregular and also filamentous. They have different margins which were curly, others undulate and some were lobate. The table also revealed a distinctive elevation, of which others were raised, some were flat; while some of them were convex, or umbonate. The bacterial isolates showed very unique textures; others had a rough distinctive feature and the rest appeared to be smooth. The colour of isolates varied from pink, cream, tan, white, to yellow; they range from opaque to translucent.

Table 4.4 Cultural characteristics of rhizosphere isolates

Isolates number	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical density
MS1	Irregular	Curled	Umbonate	Small	Rough	Dull	White	Opaque
MS2	Irregular	Undulate	Flat	Small	Rough	Dull	White	Opaque
MS3	Irregular	Undulate	Flat	Large	Smooth	Dull	White	Opaque
MS4	Circular	Entire	Raised	Punctiform	Smooth	Partly shinning	White	Opaque
MS5	Irregular	Curled	Flat	Small	Rough	Dull	Cream	Translucent
MS6	Irregular	Undulate	Umbonate	Small	Rough	Dull	Tan	Opaque
MS7	Irregular	Undulate	Raised	Moderate	Smooth	Shinny	White	Opaque
MS8	Circular	Entire	Convex	Punctiform	Smooth	Shinny	Red	Opaque
TS1	Irregular	Undulate	Flat	Large	Smooth	Dull	White	Opaque
TS2	Irregular	Undulate	Flat	Large	Rough	Dull	Yellow	Opaque
TS3	Circular	Undulate	Flat	Large	Rough	Dull	White	Opaque
TS4	Irregular	Undulate	Raised	Small	Smooth	Partly dull	Cream	Translucent
TS5	Irregular	Undulate	Flat	Punctiform	Smooth	Shinning	Pink	Opaque
TS6	Irregular	Undulate	Flat	Large	Rough	Dull	White	Opaque

Cultural characteristics for the rhizosphere isolates showing unique optical properties

Table 4.5 Cultural characteristics of rhizoplane isolates

Isolates number	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical density
MP1	Irregular	Curled	Umbonate	Small	Rough	Dull	White	Opaque
MP2	Irregular	Undulate	Flat	Small	Rough	Dull	White	Opaque
MP3	Irregular	Undulate	Flat	Large	Smooth	Dull	White	Opaque
Mp4	Circular	Entire	Raised	Punctiform	Smooth	Partly shinning	White	Opaque
MP5	Irregular	Curled	Flat	Small	Rough	Dull	Cream	Translucent
MP6	Irregular	Undulate	Umbonate	Small	Rough	Dull	Tan	Opaque
MP7	Irregular	Undulate	Raised	Moderate	Smooth	Shinny	White	Opaque
MP8	Circular	Entire	Convex	Punctiform	Smooth	Shinny	Red	Opaque
TP1	Irregular	Undulate	Flat	Large	Smooth	Dull	White	Opaque
TP2	Irregular	Undulate	Flat	Large	Rough	Dull	Yellow	Opaque
TP3	Circular	Undulate	Flat	Large	Rough	Dull	White	Opaque
TP4	Irregular	Undulate	Raised	Small	Smooth	Partly dull	Cream	Translucent
TP5	Irregular	Undulate	Flat	Punctiform	Smooth	Shinning	Pink	Opaque
TP6	Irregular	Undulate	Flat	Large	Rough	Dull	White	Opaque

Cultural characteristics for the rhizoplane isolates showing unique optical properties

4.3 Genomic DNA results

Figure 4.1 shows the DNA concentrations extracted from rhizosphere and rhizoplane samples. The DNA concentrations obtained from rhizosphere samples varied from 5 to 30 ng µ/L, while DNA from rhizoplane samples ranges from 1 to 9 ng µ/L. The amplicons of the DNA determined by loading 1% (w/v) agarose gel in TBE buffer with the DNA extracts, revealed the amplicons size range of 20–1000 base pairs (Figure 4.2).

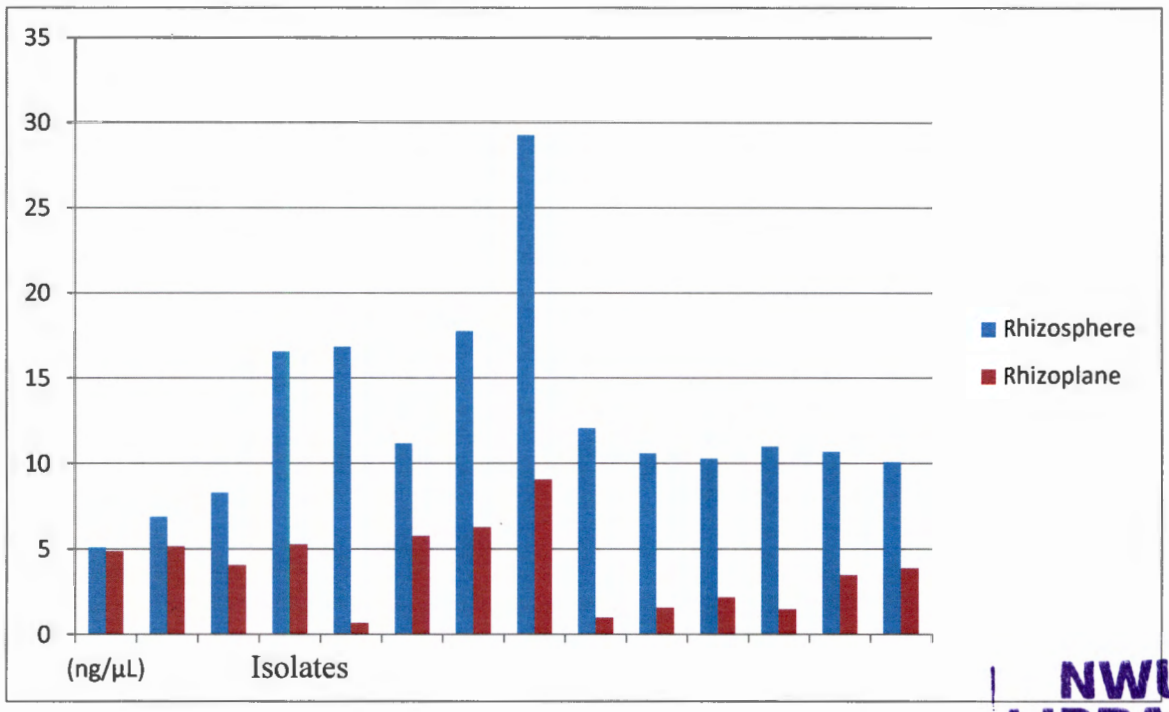


Figure 4.1 shows the NanoDrop readings of DNA concentrations of the rhizosphere higher compared to that of the rhizoplane of the bambaranut samples.

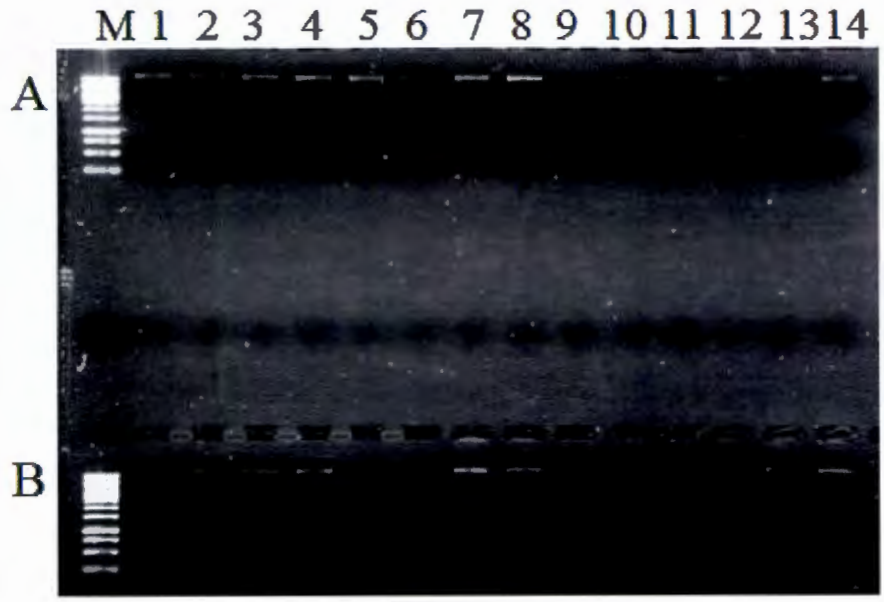


Figure 4.2 Agarose gel electrophoresis of the DNA of isolated strains M: 1 Kb DNA Ladder and numbers above are (A) strains from rhizosphere {1-8 from Mafikeng, 9-14 from Thohoyandou} and (B) from rhizoplane samples {1-8 from Mafikeng, 9-14 from Thohoyandou}.

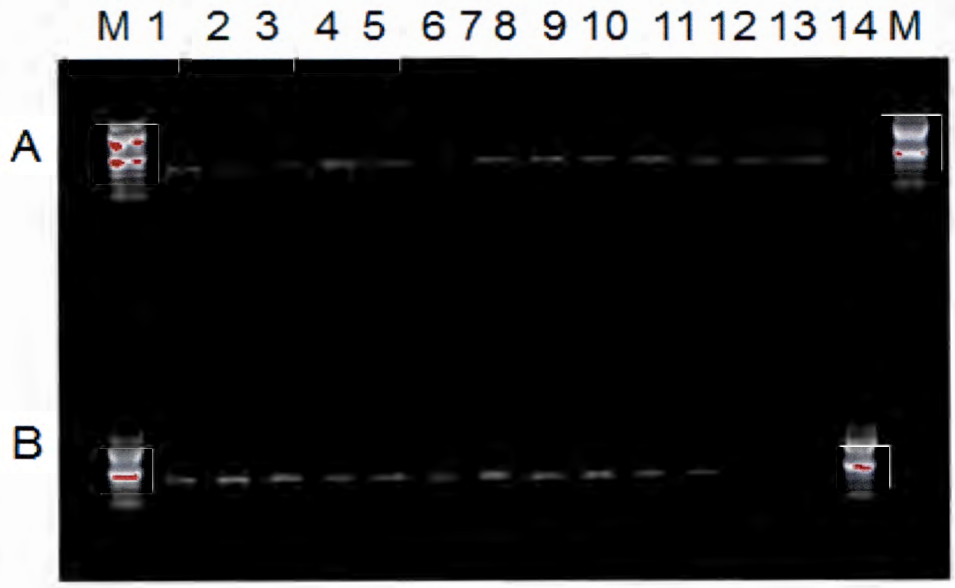


Figure 4.3 Agarose gel electrophoresis showing amplicons of the 27F and 1492R universal primers on the isolated strains M: 1 kb DNA ladder and numbers above are rhizobacteria strains (A) represents the rhizosphere isolates and (B) representing the rhizoplane isolates

4.3 PCR-RFLP identification of bacterial isolates

Figure 4.4 shows PCR-RFLP amplification of the genomic DNA with a Hhai restriction enzyme resulted in 1500 bp amplicons. The rhizosphere revealed 7 distinct groups while rhizoplane showed 5 distinct groups of communities.

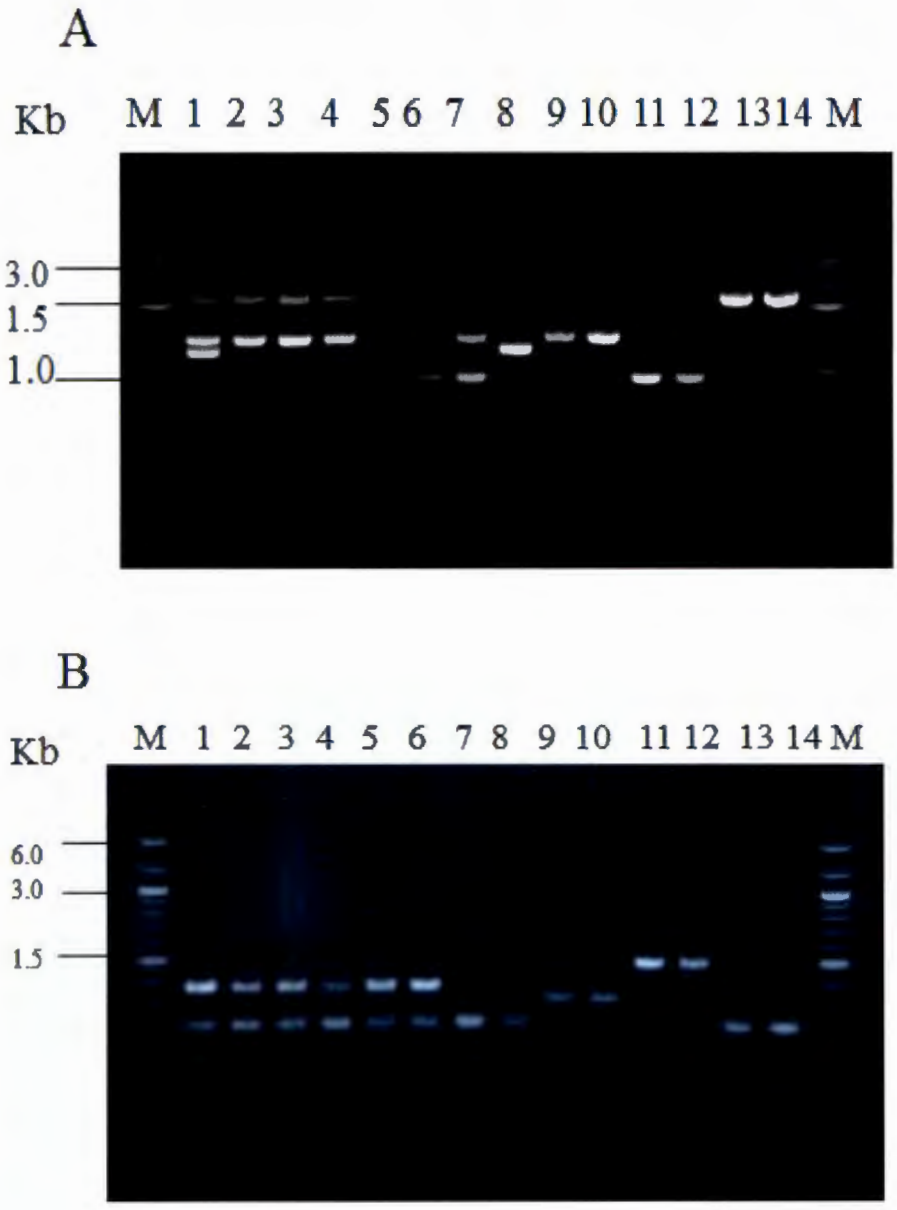


Figure 4.4 PCR-RFLP of Hhai showing the digestion of isolates (A) the rhizosphere samples and (B) rhizoplane samples indicating the cluster of microbial communities present in different samples.

4.4 Next Generation Sequencing (NGS) MiSeq

The resultant sequences of the 16S rRNA gene after NGS analysis showed that were similarities of phyla in both the rhizoplane and rhizosphere soil of the bambaranut. This is depicted in the Table 4.6 based on the operational taxonomic unit (OTU) of the phylum present in the rhizoplane and the rhizosphere of the leguminous soil. The most abundant bacteria phyla found in the entire samples are Actinobacteria, Proteobacteria, Acidobacteria, Cyanobacteria and Firmicutes (highlighted in Table 4.6). The bar graph in Figure 4.5 also depicts the relative abundance of these phyla. The molecular taxonomy determined by sequences and phylogenetic analysis of the five most abundant microbial groups revealed that these phyla are more prevalent in the rhizosphere than rhizoplane (Figure 4.6). The percentages of the relative abundance of bacterial phyla discovered within the selected cultivated locations are depicted in Figure 4.7.

Table 4.6 Details of 16S rRNA gene sequences retrieved in this study and the highlighted being more abundant and used for phylogenetic analysis

Phylum	MS8	TS6	MP7	TP5
Fibrobacteres	7	16	12	13
Thermodesulfobacteria	5	0	1	4
Elusimicrobia	2	40	2	2
Gemmatimonadetes	1925	1544	1275	1167
Candidatus Saccharibacteria	2	0	1	1
Actinobacteria	12386	7362	10112	10000
Nitrospinae	2	8	2	1
Armatimonadetes	102	22	98	77
Planctomycetes	2220	3828	1203	1458
Streptophyta	28	114	1081	91
Chlamydiae	5	14	3	1

Euryarchaeota	490	184	121	176
Basidiomycota	12	9	5	1
Cyanobacteria	304	689	454	184
Chlorophyta	6	52	12	2
Deinococcus thermos	21	67	30	23
Tenericutes	9	149	3	3
Nitrospirae	605	1105	278	363
Arthropoda	349	567	385	250
Firmicutes	3890	6465	3418	3313
Bacteroidetes	3435	6108	3682	2793
Eukaryota	124	3655	81	85
Bacillariophyta	10	571	38	4
Nematoda	21	21	155	17
Thaumarchaeota	10929	1810	3803	4075
Acidobacteria	5220	3688	2979	3663
Ignavibacteriae	9	64	10	5
Crenarchaeota	6	0	0	0
Eustigmatophyceae	0	35	1	0
Ascomycota	2	2	8	3
Apicomplexa	92	72	3	14
Fusobacteria	5	55	12	7
Spirochaetes	15	337	7	14
Chlorobi	1	5	5	1
Chloroflexi	3509	2723	1778	2575
Proteobacteria	26926	32419	19399	21519
Verrucomicrobia	203	426	233	199
Entomophthoromycota	0	0	0	3

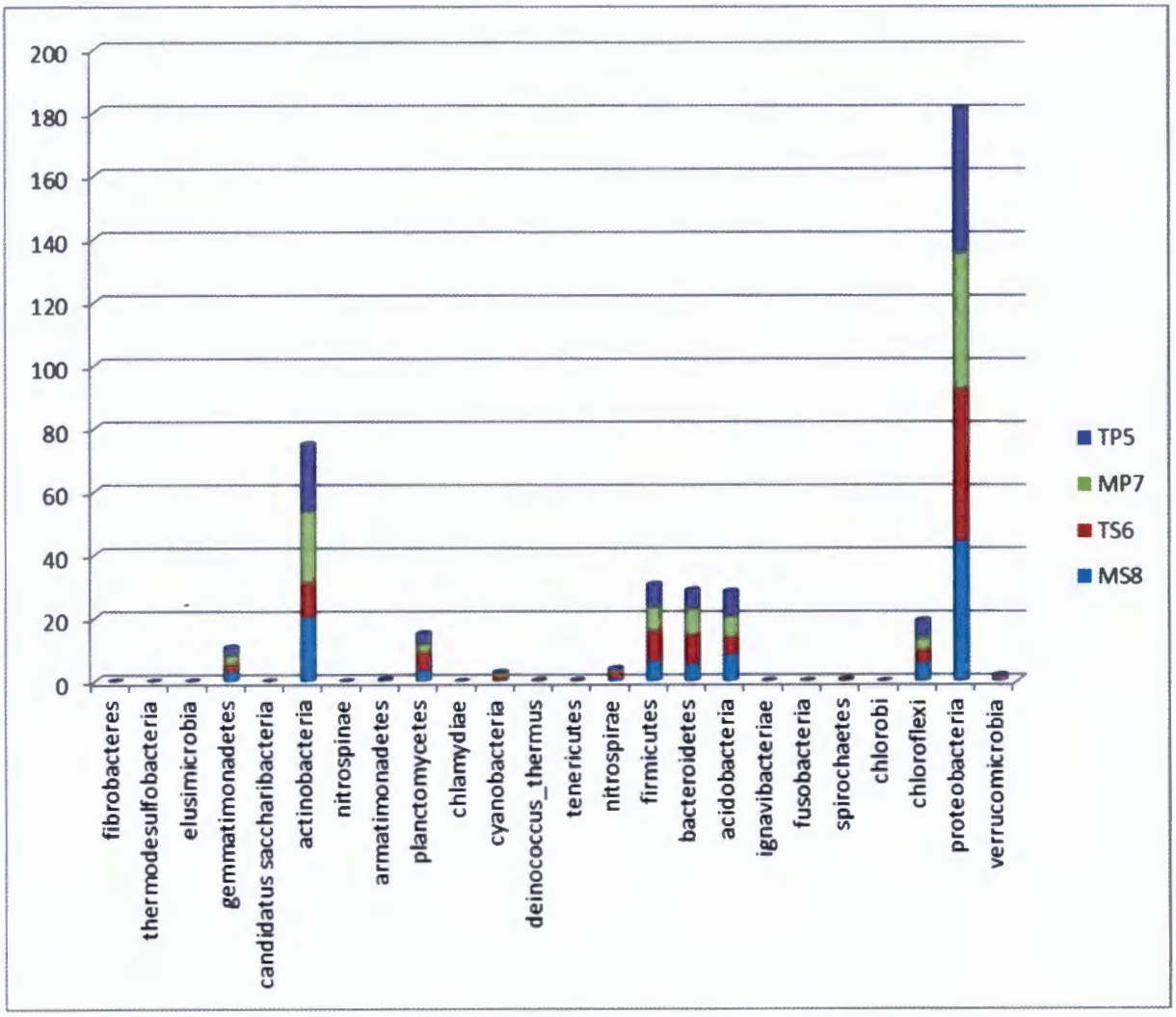
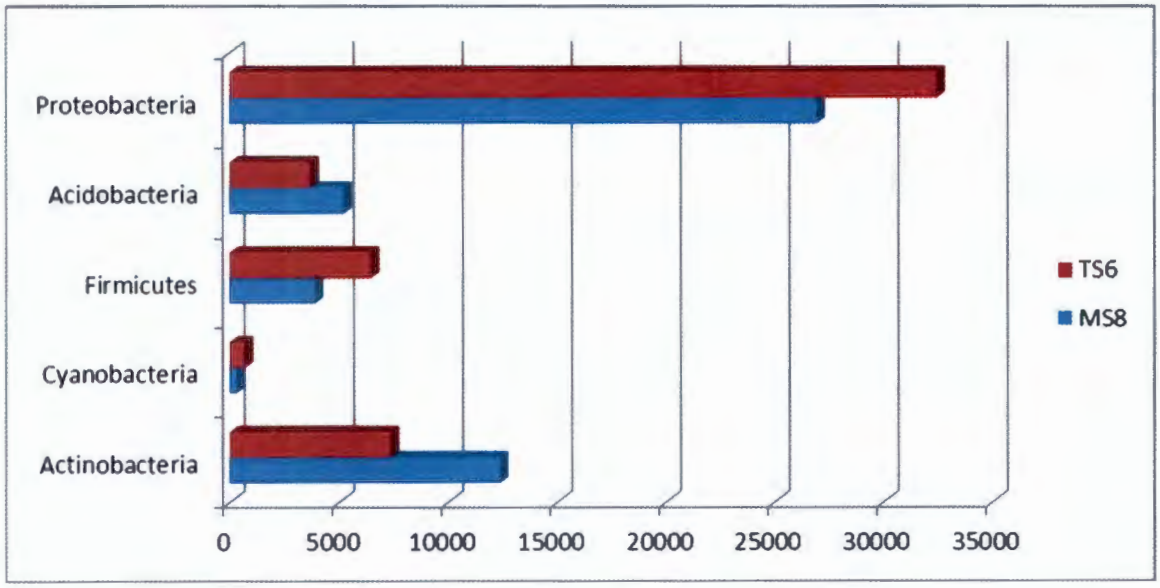


Figure 4.5 Relative abundance of bacterial phyla detected from the samples

A



B

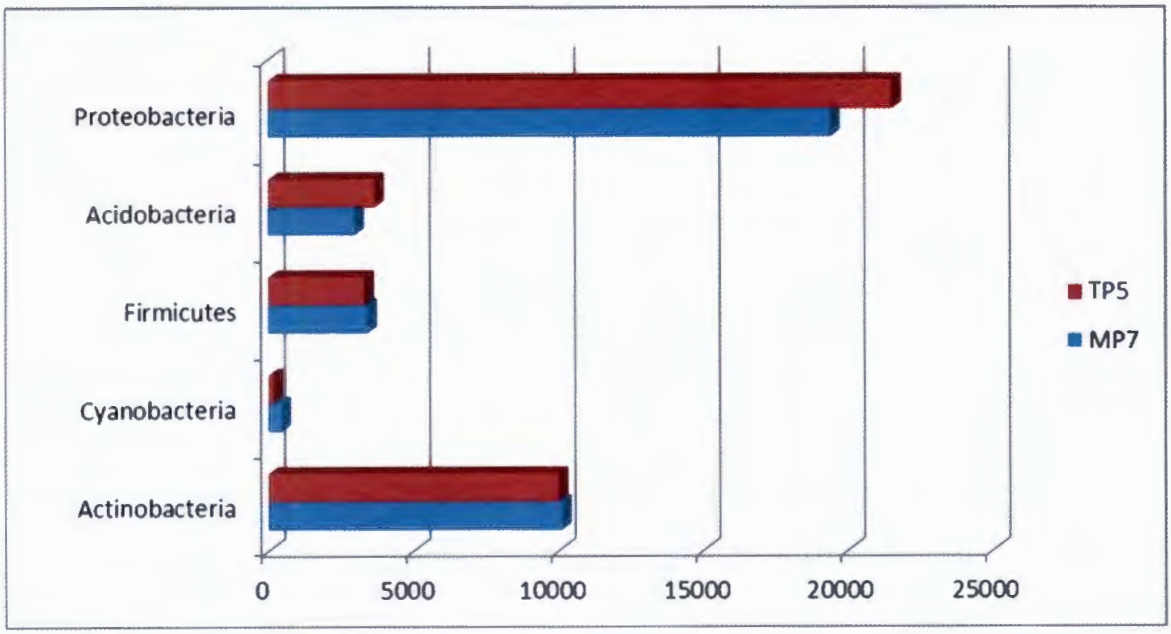


Figure 4.6 16S rRNA NGS analysis resulted in variation indices with five most abundant and studied rhizobia phyla including Actinobacteria, Cyanobacteria, Firmicutes, Acidobacteria, and Proteobacteria in the (A) rhizosphere and the (B) rhizoplane

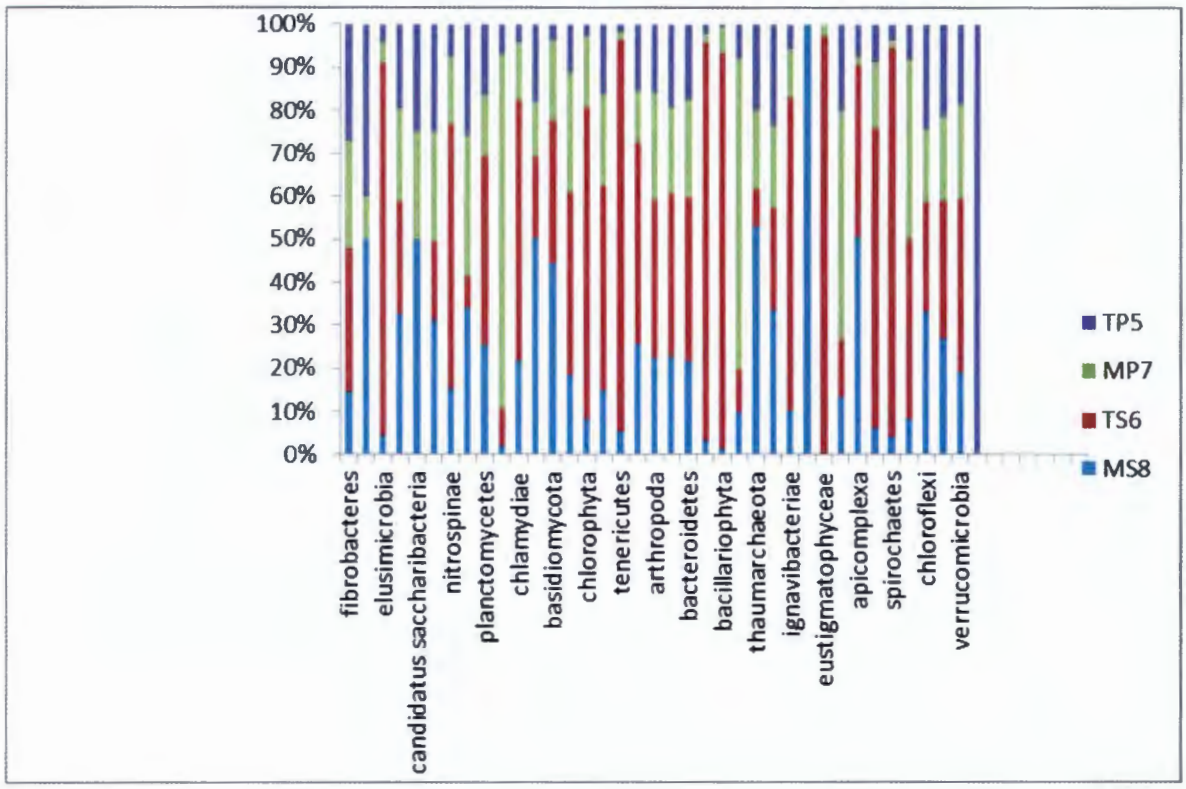


Figure 4.7 Relative abundance of bacterial phyla detected within the selected cultivated locations

Moreover, the NGS data set revealed a total of number 220,325 bacteria population in the entire samples analyzed. In all the samples, the total groups of bacteria are greater in the rhizosphere soil collected from the two locations. This is expressed in the Figure 4.8. Among the most abundant species found in the samples were: *Bacillus Spp., Arthrobacter Spp., Streptomyces Sp., Pelobacter Spp., Steroidobacter Spp., Acidobacterium Spp., Burkholderia Spp., Rhodopseudomonas Spp.* This is depicted in Figure 4.9.

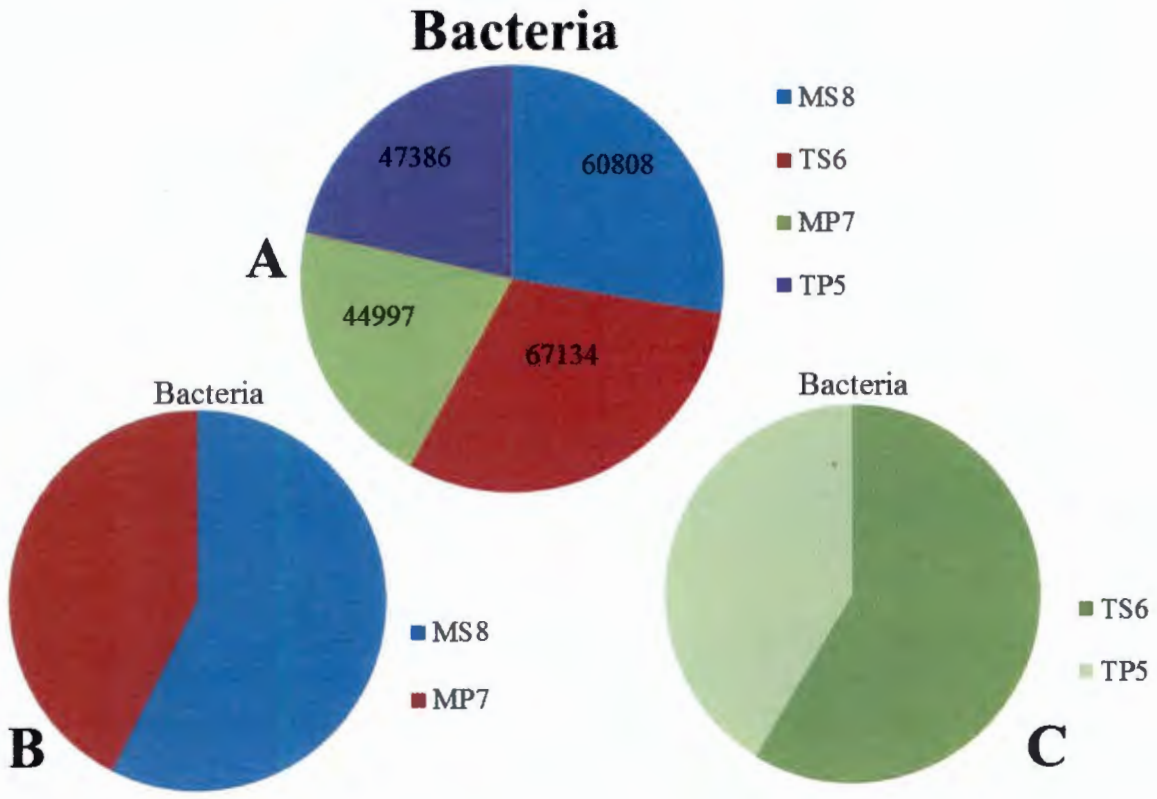


Figure 4.8 Diversity of bacterial groups within selected samples (A), diagrams of rhizosphere and rhizoplane bacteria diversity of samples from (B) Mafikeng and (C) Thohoyandou

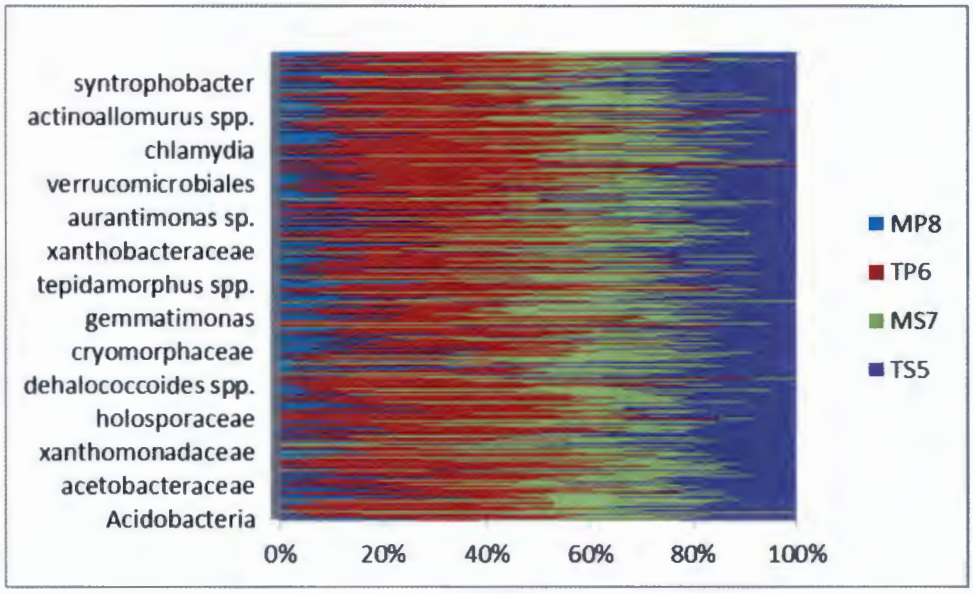


Figure 4.9 Species identity in the rhizosphere and rhizoplane

4.5 Discussion

Bambaranut is a nutritional food though it is underutilized. It has both immediate and indirect benefits to agriculture through a symbiotic relationship with bacteria around and on the plant, yet little attention has been paid from a research perspective. Molecular microbial biotechnology has improved ways towards describing, evaluating and categorizing bacteria for sustainable agriculture. The potential uses of biofertilizers in agriculture play an essential role of providing an economically viable level for achieving the goal to enhance food productivity. The use of chemicals to enhance plant growth, have varying degrees of side effects to the environment and are costly. Therefore, this work aimed at the determination and comparison of the intraspecific variation in the bacterial strains resident on the rhizoplane and rhizosphere of bambaranut. While comparing the nature of those bacterial strains living on and in the bambaranut roots as determined by their characteristics, we also compared the role played by the strains according to the next generation sequencing results.

Based on table 4.4 and 4.5, the isolates showed unique optical properties, however both the rhizosphere and rhizoplane isolates did not show any variation from their properties. Interestingly, 94 % of the isolates from both the rhizosphere and rhizoplane could solubilize phosphate to its availability state to plants. Since phosphorus is an essential macronutrient for plants and it is applied to soil as phosphate fertilizers; the chemical nourishment is speedily mobilized and becomes available to plants, resulting in an increase of nutrients to plants and eventually an increase in plant development and growth. It has been hypothesized that the rhizosphere effect has the greatest influence on microorganisms since this population can respond quickly to increased nutrients. They stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots. The species of bacteria present on rhizosphere and rhizoplane grown in the two soils were unique to each soil and only in a few

cases did the same species occur in both soils. However, the same genera were often present in both soils. This was proved when 100% of both rhizosphere and rhizoplane isolates possessed the ability to produce ammonium. Taxonomic partitioning between rhizosphere and rhizoplane did not occur among the cultured organisms in this study; no obvious population shifts in cultured organisms was observed between the rhizosphere and rhizoplane. The results of our work agree with result of another study which suggested that a great amount of field-to-field variation existed in the rhizosphere community and potentially have unpredictable effects governing the roots (Turnbull et al., 2012).

With reference to table 4.3, hormone activity of the siderophore and IAA, were less prevalent in the rhizosphere and rhizoplane samples with the microbial communities differing by soil type. Siderophore production serves as a measure of protecting plants from heavy metal toxicity whereas the production of IAA helps the bacterium to evade plant regulatory signals, and it induces uncontrolled growth in plant tissues. The results are in conformity with the findings of Flores-Félix et al. (2015). Equally important, the strains were able to produce hydrogen cyanide in equal percentages of both the rhizosphere and rhizoplane. Hydrogen cyanide is a pathogen depressant that inhibits pathogen growth, indirectly promoting plant growth. In addition 64% of the strains from both the rhizosphere and rhizoplane had the ability to degrade the cell wall when assayed for chitinolytic activity. Cell wall degradation allows easy nutrient uptake for plants and indirectly promoting plant growth (Gagne-Bourgue et al., 2013). Furthermore 64% of the strains on both the rhizosphere and rhizoplane expressed ACC deaminase activity; they proved to utilize ACC as a sole source of nitrogen, whereby it will lower the impact of different environmental stresses on plants resulting in plant growth (Khantsi et al., 2013). According to Glick (2014), a detailed understanding of the competitiveness of ACC deaminase containing bacteria is likely considerably more

complex than this study, nevertheless it becomes a mainstay of plant agriculture. These results are in conformity with von der Weid et al. (2000). This fact should be taken into account in the selection of strains for use as inoculants of bambaranut in field studies and for commercial use. Additional data are necessary to determine whether the same results would be obtained using different soils in different climates.

Microbial diversity is important for exploiting the potential of microbial resources from the environment. It is necessary to understand the overall survival microorganisms in our environment. Bacteria and Achaea have been widely studied with respect to their biodiversity in natural and constructed environment (Screen house studies). Studies employed traditional culture-dependent methods and resulted in the discovery of plenty of new bacterial taxa. Employing molecular biology methods, increasing evidence has suggested that the structures of microbial communities are related to soil processes, when determined by the use of terminal restriction fragment length polymorphism (RFLP) and sequencing of 16S rRNA genes (Rastogi and Sani, 2011) Although RFLP has been considered the default method to study microbial diversity in complex environments, sequencing is the next best method to understand the whole genome of a single gene (Rastogi and Sani, 2011, Powell et al., 2015). Evolutionary or taxonomic relationships among rhizobacteria are usually estimated through 16S rDNA sequence comparison. After PCR-RFLP restriction digestion, twenty eight strains each were clustered in groups of five for the rhizosphere and seven groups from the rhizoplane. This PCR-RFLP result did not show any sequence information, only a "fingerprint" of a specific community was expressed. These amplicons sequence analysis, based on the restriction enzyme site is not reliable, because more than one microorganism may share the same restriction enzyme site, which did not express the exact reflection of the entire microbial community. Metagenomics gives considerable information about the true

diversity of microbial community in the sample. This called for the introduction of next-generation sequencing in this work to revitalized research in environmental DNA. However, NGS allowed us to classify the bacterial populations for a valuable reduction in costs and a meaningful and reasonable increase in production and precision in more depth than the data obtained with RFLP and target potential bacterial species responsible for metabolic shifts. Several web based tools such as phylogenetic assignment tool (PAT), are methods of gathering sequence data, however, they require optimization and benchmarking before being used on samples of unknown nature to avoid false negatives and biased results. The excitement of using new platforms has generated momentum among researchers to apply NGS tools in various applications involving environmental DNA. Rapid progress has provided optimism for a bright future for the field of next-generation environmental DNA analysis. Several studies have analyzed soil bacterial diversity by examining 16S rDNA amplicons (Roesch et al., 2007, Rousk et al., 2010, Nacke et al., 2011). Four (4) genes (MS8, TS6, MP7 and TP5) of different geographical locations, and different climates were sequenced through the NGS and a comparison of their DNA sequences indicated their genes' similarities and variations (i.e., an average of 97% identities at the gene level). Compositions of bacterial communities within the samples were ascertained by analyzing 16S rRNA gene sequences from the dataset (Figures 4.7 A, B and C). Our analysis revealed that samples were mostly represented by the predominance of phyla *Proteobacteria*, *Actinobacteria, Cyanobacteria, Firmicutes* and *Acidobacteria* which were observed on both spheres with varying degrees of concentration. Sequences assigned to *Actinobacteria* were more abundant in the rhizosphere associated communities compared to the rhizoplane communities, which is a pattern that has also been noted in previous studies (Jin et al., 2014).

In comparison, a high abundance of the *Proteobacteria* was the largest and most diverse phylum in our dataset, followed by the *Actinobacteria*. Lineages belonging to *Proteobacteria*

are well known to survive in oligotrophic environments, capable of metal reduction and resistance and play very important roles in uranium immobilization in contaminated groundwater. The *Firmicutes* showed dominancy over the *Acidobacteria* and the *Cyanobacteria* were the least present in both samples.

Actinobacteria is a phylum whose members are physiologically diverse and ubiquitous in soils, but are underrepresented in culture at present. Phylum *Firmicutes* showed a strong phylogenetic affiliation in the rhizosphere as to the rhizoplane. This phylum is well-known for the presence of species such as *Bacillus* and *Clostridium* (Jin et al., 2014, Rosselli and Squartini, 2015). The actual role that the *Bacillus* species plays in soil wellness and turf health is becoming better understood. *Bacillus* species have the ability to withstand harsh environmental conditions, high temperatures, acidic environments, high and low water concentrations, hence the development of spores around the cell. They have developed a high resistance to abiotic and biotic stresses and as a result, are more dominant compared to other species. Naturally-selected *Bacillus* species have been utilized successfully for many industrial and agricultural purposes.

Bacillus species are naturally found in the soil at high levels and have a broad spectrum of activities. Bacillus species found in the areas of plant root system and/or in contact with the roots have been shown to improve turf grass performance. The species commonly found in soil have been recognized as bio control agents. *Bacillus* species can produce a group of bio surfactants (surface active agents of microbial origin), which act to improve the permeability of the cellular membranes for enhanced nutrient transfer. Different *Bacillus* strains produce a wide array of different antibacterial and antifungal compounds and no single *Bacillus* strain will produce all the beneficial lipo-peptides that have been identified to date.

Unfortunately, there are still areas which need to be addressed for greater clarity. One particular limitation of laboratory conditions is that they cannot mimic natural turf conditions that are consistently changing over time. However, the strength of laboratory research is the ability to control the environment to determine if there is or is not an impact based on the elimination of diverse soil or atmospheric conditions. Soil microbial behavior patterns are never constant and can vary greatly. *Cyanobacteria* were the ubiquitous group present more in the rhizosphere than in the rhizoplane soil habitats and possessing high tolerance to environmental stress. Little as they are they also can act as a biosorbent of heavy metals in sewerage soils (Wang et al., 2010, Haynes, 2014).

Based on this study, there is a slight intraspecific variation in the bacterial strains resident in the rhizoplane and rhizosphere of bambaranut. The isolates possess PGPR traits and therefore are considered possible alternatives to the use of chemicals in agriculture. PGPR-containing bacteria in agriculture might prove beneficial towards crop production and conservation and the isolates are likely to be potential candidates for biofertilizers, biocontrol and biotechnological application of commercial value.

4.6 Conclusion and future prospects

Food security is not just about the availability of food but the consistent access to safe, healthy and nutritious food. It is this holistic view of food security that I hope to carry forward in my future research, work and life. The rhizosphere is an ecosystem occupied by a variety of organisms involved in plant health and environmental sustainability. Abiotic factors influence microorganism–plant interactions, but the microbial community is also affected by expression of heterologous genes from host plant. Bacteria are characterized by extensive intraspecific variation of species. There is not only the sequence variation found in all species, but also the presence or absence of variation of resident strains. Intraspecific variation can facilitate coexistence, but this may be relatively unimportant in maintaining diversity in most real communities. This study revealed high levels of homogeneity within resident bambaranut rhizobium populations in the soils of both the rhizosphere and rhizosphere locations. The intraspecific variation observed in this study may be important to provide the plant with a variety of strains capable of coping with the different stresses caused by environmental conditions and consequently promoting growth and development. Future efforts may be focused on studying these bacteria in defined communities in soil with plants recombinant based on next generation sequencing results to understand the impact of one species on the whole community, their metabolite production and impact on the plant itself. Also, considering sustainable agriculture, the study will look into commercial production of the plant growth promoting isolates since they are environmentally friendly and could prove to be cost effective. Field inoculation trials and tests on competition for nodule occupancy are also needed to evaluate the potential of this abundant genetic resource for sustainable production of the under-utilized bambaranut which can supply alternative sources of nutrient rich diets to resource poor farmers.

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APPENDIX

Sampling co-ordinates

Sample sites (Group 1) Tshiulungoma village, Vhukati ha Manini, Dididi Ha-Mphengo na Thohoyandou (Group 2) North-West University Mafikeng, agricultural farm

Site number	Elevation (m)	South	East
Group 1			
A177	542	22.94774	30.56022
B179	542	22.94772	30.56032
C180	548	22.94974	30.5614
D181	562	22.9574	30.53573
E183	566	22.95887	30.52901
F184	570	22.96055	30.5238
G185	573	22.96394	30.51328
H186	600	22.96951	30.49717
I189	589	22.97355	30.48543
Group 2			
J190	1208	25.79956	25.23609

NANODROP readings of DNA concentrations extracted from samples

Rhizosphere		Rhizoplane	
Isolate no.	(ng/μL)	Isolate no.	(ng/μL)
MS1	5.1	MP1	4.9
MS2	6.9	MP2	5.2
MS3	8.3	MP3	4.1
MS4	16.6	Mp4	5.3
MS5	16.9	MP5	0.7
MS6	11.2	MP6	5.8
MS7	17.8	MP7	6.3
MS8	29.3	MP8	9.1
TS1	12.1	TP1	1.0
TS2	10.6	TP2	1.6
TS3	10.3	TP3	2.2
TS4	11.0	TP4	1.5
TS5	10.7	TP5	3.5
TS6	10.1	TP6	3.9

16S rDNA NGS Phylogenetic Microbial groups of the samples indicating the Bacteria Empire (kingdom) most dominant

Sample code	Bacteria	Fungi	Archaea	Eukaryota	Metazoa	Viridiplantae
MS8	60808	14	11425	226	370	34
TS6	67134	11	1994	4333	588	166
MP7	44997	13	3924	123	540	1093
TP5	47386	7	4251	103	267	93

16S rRNA Next Generation sequences analysis resulted in variation indices with five most abundant rhizobia phyla including Actinobacteria, Cyanobacteria, Firmicutes, Acidobacteria, and Proteobacteria

Phylum	Rhizosphere		Rhizoplane	
	MS8	TS6	MP7	TP5
Actinobacteria	12386	7362	10112	10000
Cyanobacteria	304	689	454	184
Firmicutes	3890	6465	3418	3313
Acidobacteria	5220	3688	2979	3663
Proteobacteria	26926	32419	19399	21519

16S rRNA NGS variation of species within the samples

Species	MP8	TP6	MS7	TS5
Acidobacteria	41	19	61	35
Sporichthya	192	27	160	255
Bosea Spp.	5	26	9	8
Adhaeribacter Spp.	22	8	16	10
Opitutus Spp.	103	286	146	89
Nitrobacter	8	4	4	4
Desulfotalea Spp.	190	44	72	119
Pedobacter glucosidilyticus	0	4	0	0
Devosia	1	4	2	2
Nannocystaceae	5	77	3	5
Brachybacterium Spp.	41	40	34	23
Ralstonia	1	7	1	1
Ohtaekwangia	175	162	310	152
Motilibacter Peucedani	2	1	1	3
Flavobacterium Urocaniciphilum	1	25	1	0
Microcoleus Sp.	2	0	11	0
Brevibacterium Picturae	0	10	1	0
Phaeospirillum Fulvum	4	2	2	4
Zavarzinella Spp.	23	70	13	12
Actinopolymorpha Spp.	0	0	4	1
Flavisolibacter	56	18	35	46

Arenimonas daechungensis	1	33	1	0
Pontibacter Sp.	25	24	17	25
Rhodopila globiformis	6	12	8	3
Elusimicrobium	0	2	1	0
Chloroflexia	82	185	51	79
Bradyrhizobiaceae	20	19	7	28
Thiobacter	2	183	4	2
Sideroxydans	3	172	5	2
Corynebacterium Spp.	3	6	4	7
Lysobacter brunescens	15	55	29	14
Cytophagaceae	62	127	67	55
Pelomonas saccharophila	2	26	37	7
Emticicia Sp.	0	3	0	0
Blastopirellula	1	14	1	0
Pirellula	140	214	69	75
Syntrophus	69	5	22	43
Cupriavidus Taiwanensis	6	42	31	12
Bacteriovoracaceae	4	26	4	7
Ochrobactrum Intermedium	0	0	3	0
Hyphomonas Spp.	21	22	10	10
Hydrogenophilaceae	22	84	10	8
Xanthomonas Spp.	8	47	15	4
Trichocoleus Spp.	10	11	44	8
Chloroflexus	0	3	0	0
Azorhizobium Spp.	8	12	3	5

Polaromonas Spp.	32	147	23	23
Flavitalea Spp.	39	11	24	22
Conexibacteraceae	215	74	126	160
Skermanella aerolata	257	166	172	112
Verrucomicrobia Subdivision 3	12	6	3	11
Thermobifida	24	1	14	8
Opitutus Sp.	22	22	32	20
Blastocatella	2	31	5	5
Agromyces humatus	6	36	40	14
Flavobacteriaceae	39	82	28	17
Desulfuromonas	0	9	0	0
Syntrophobacter aceae	242	28	69	189
Cytophaga hutchinsonii	1	2	0	0
Leptospira	0	5	0	0
Spirochaetia	15	41	6	10
Rhizobium sp.	3	38	3	2
Isosphaera spp	17	20	8	17
Microlunatus spp.	237	45	162	87
Anaerolineae	55	165	26	67
Flavisolibacter Sp.	192	58	93	71
Modestobacter Sp.	22	25	17	7
Pseudonocardia zijingensis	92	23	155	98
Rhodoplanes	9	56	6	7
Streptomyces violorubens	113	397	407	110
Chlamydiaceae	0	3	0	0

Mollicutes	0	22	0	0
Acetobacteraceae	50	112	33	45
Chondromyces apiculatus	2	2	3	2
Segetibacter	41	11	42	21
Chlorobium	1	1	5	1
Chryseobacterium formosense	0	10	2	0
Hymenobacter Spp.	1	14	28	0
Candidatus Saccharibacteria	2	0	1	1
Rhodovulum	2	2	1	0
Chitinophaga Sp.	1	5	1	6
Thermomonas	161	947	200	184
Geodermatophilus Sp.	31	17	16	10
Planctomyces	67	151	35	59
Rheinheimera Sp.	0	3	0	0
Nonomuraea Sp.	8	3	33	14
Pedosphaera	2	2	1	2
Nitrospiraceae	1	24	2	3
Caldilinea	12	17	3	8
Catenuloplanes Spp.	10	1	5	6
Cryptosporangium japonicum	28	4	5	4
Bacillus longiquaesitum	220	507	185	160
Rhodomicrobium Spp.	107	70	79	78
Micromonospora halophytica	42	45	26	31
Ammoniphilus Spp.	7	10	2	1
Comamonas	0	4	0	0

Candidatus kuenenia	5	40	7	6
Devosia Sp.	42	204	75	60
Haliea Spp.	1	7	1	0
Geodermatophilus Obscurus	96	72	97	51