ISOLATION AND CHARACTERIZATION OF RHIZOSPHERE BACTERIA WITH POTENTIAL TO IMPROVE THE PLANT GROWTH OF BANANA PLANTS IN JUJA, KENYA

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Isolation and characterization of rhizosphere bacteria with potential to improve the plant growth of banana plants in Juja, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to my family members and especially my parents Pr. and Mrs. Samuel Onchwari Okero. This is a token of my appreciation, gratitude, honor and respect for your constant inspiration. You have instilled in me the values of hard work, persistence, self-reliance and patience during my pursuit for excellence. You have been and will always be the best. What more can I ask for than what you have given? Thank you for always giving me a shoulder to lean on.

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ABBREVATIONS AND ACRONYMS

16S rDNA	Sixteen S ribosomal deoxyribonucleic acid
ANOVA	Analysis of variance
ARA	Acetylene Reduction Assay
BLAST	Basic local Alignment Search Tool
BNF	Biological Nitrogen Fixation
DNA	Deoxyribonucleic acid
dNTPs	Deoxy-nucleotide triphosphates
EDTA	Ethylene diamine tetra acetic acid
IAA	Indole acetic acid
IBR	Institute of Biotechnology Research
MR-VP	Methyl red-Voges-Proskauer test
NBIRP	National Botanical Research Institute's phosphate
NCBI	National Center for Biotechnology Information
NPK	Nitrogen Phosphorus Potassium
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
TAE	Tris-Acetic acid Ethylenediaminetetra acetic acid
TE	Tris- Ethylene diamine tetra acetic acid
UV	Ultraviolet
YEM	Yeast manitol agar

ABSTRACT

Banana (*Musa* spp.) is the world's third most important starchy food. It is widely grown in developing countries and requires large amounts of nitrogen fertilizers which are expensive to the poor resource farmers and may be hazardous to the environment. Phosphorus is another mineral nutrient which is essential for plant development and growth but it is usually unavailable to the plant due to its insolubility. Plant growth promoting rhizobacteria are found in the rhizosphere in association with roots and can enhance the growth of the plant. This study isolated rhizobacteria associated with bananas (*Musa* spp.) from rhizosphere soil samples collected from seven banana farms in Juja, Kenya and evaluated their potential as biofertilizers. Out of the 20 bacterial isolates obtained, nitrogenase activity was recorded in 19 isolates and 18 isolates solubilized phosphates. Phylogenetic analysis clustered the isolates into three phyla represented by six different genera; *Pseudomonas, Bacillus, Staphylobacterium, Chryseobacterium, Streptomyces* and *Paenibacillus.* The *Bacillus* and *Pseudomonas* spp. isolates recorded the highest phosphorus solubilization of 1.6 and 1.5 on the phosphate solubilization index. There was significant differences in the plantlet's mean height ($p<0.0001$) and mean mass in dry weight ($p<0.0006$) in all the five strains tested as compared to the control. *Bacillus megaterium* (KP797917) and *Streptomyces* sp.(KP797931) bacterial strains isolated from banana rhizosphere in Juja, Kenya had a significant difference in mean number of green leaves ($p=0.000$) as compared to the control. Plantlets treated with *Streptomyces* sp. registered a significantly higher growth in all parameters ($p<0.05$). The intake of zinc and iron was not influenced by inoculants (banana plantlets growth with the five bacterial isolates). The study showed that certain banana rhizosphere bacterial isolates such as *Streptomyces* sp. and *Bacillus megaterium* have plant growth promoting traits that could be utilized as biofertilizers in agriculture in the production of bananas.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background Information

Banana is a staple food in most parts of East Africa especially in Uganda, Tanzania, Rwanda, Burundi and part of Kenya. World economy review shows that banana is an important crop in the tropical countries. In rural areas, bananas are a source of income especially for the households that sell the banana as fruit in the local markets. In Kenya over 63,290 hectares area is under banana production giving an average yield of around 19 tonnes per hectare which opposes the potential average production of around 35-45 per hectare (Ngamau, Matiru, Tani, & Muthuri, 2014).

The banana in these areas is grown by the farmers mostly in small scale as food security for the homestead and as a source of income for the farmers. The areas where banana is grown include Central, Nyanza, Eastern and Western province. Despite the challenges facing the growth of banana in Kenya like lack of supportive policies and well-established institutions that optimize its growth, banana still plays a very important role in the economy and in establishing food security. The growth of banana production in Kenya is hindered by the infestation by various diseases and pests. Most farmers in Kenya use traditional exchange planting process. This practice aggravates the transmission of these diseases and discourages most farmers from investing in banana because the yields are greatly reduced (Karembu, 2007). It is important to establish a better agricultural practice, such as the use of plant growth promoting rhizobacteria as biofertilizers to ensure the threat created in employment, food security and in income are eliminated.

The rhizosphere is the thin layer of soil immediately surrounding the plant roots. It is extremely important and an active area for root activity and metabolism. There are a number of microorganisms that coexist in the rhizosphere such as bacteria, protozoa,

fungi and algae but bacteria are the most abundant. Usually, plants select those bacteria that contribute most to their growth and fitness by producing promotive organic compounds through exudates thereby creating an environment where diversity maybe low (Saharan & Nehra, 2011).

Plants are usually subjected to biotic and abiotic factors within the environment that influences their development and growth. From the economical point of view this is very important as the given factors may affect the root development and finally the production rate. The bacteria which provide benefits to the plant may either form symbiotic relationships with the plant, some may be free living in the soil and others may be found near or within the roots. The bacteria that may be beneficial and free living in the soil are usually called plant-growth-promoting rhizobacteria (PGPR). The rhizosphere soil has an abundance of nutritious elements, because of the continuous root activities of the plants. Rhizospheric zone is mixed with solid particles and an active community of bacteria (Haghighi, Alizadeh, & Firoozabadi, 2011).

There has been increasing interest in soil microorganisms due to a greater realization of their benefits and importance in maintaining soil fertility. The plant growth may actually be stimulated by phytohormones which are produced by bacteria, products that are vitamin-related or even the pathogen suppression activities (Mia, Shamsuddin, & Mahmood, 2010b).

Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties. Appropriate biophysical and biochemical environment should be provided in order to propagate any bacterium for any purpose. The nutritional or the biochemical environment can be made available through the culture medium provided. Depending on the bacteria's special needs, different types of culture media have been developed having different uses and purposes. Yeast manitol agar is a differential medium that supports the growth of rhizobium species while nutrient agar is the kind of medium which contains nutrients that supports the growth of all kinds of

microorganisms while nitrogen free media doesn't contain nitrogen and organisms which grow in this media are able to fix atmospheric nitrogen for their growth. Bacteria use the nutrients that have been released from the roots of the plants for their growth, as they in turn secrete metabolites to the rhizosphere. Many of these metabolites act as signalling compounds which the neighboring cells perceive in the same micro-colony or by cells of some other bacteria and also by cells of roots of the host plant within the rhizosphere (Van Loon, 2007).

The plant growth promoting rhizobacteria (PGPR) are a small portion of rhizosphere bacteria (2–5%) that promote growth. In order to improve the plant uptake process, phosphate solubilization, fixation of nitrogen and production of phytohormones like indole -3- acetic acid are some examples of the direct mechanisms that influence the plant growth. Biologically controlling plant pathogens and deleterious microoganisms by production of lytic enzyme, antibiotics, siderophore and hydrogen cyanide through competition for nutrient and space can significantly improve plant health and promote growth by increasing of seedling emergence, vigor and yield (Chaiharn, Chunhaleuchanon, Kozo, & Lumyong, 2008).

Biofertilization is the process of using soil microorganisms in increasing the availability and the mineral nutrient uptake for plants. Plant growth promoting rhizobacteria act as biofertilizers directly when they help to provide nutrients to the host plant, and indirectly by their positive influence to the growth of roots and morphology or in aiding in some other beneficial symbiotic relationships though not all PGPR are biofertilizers. A number of PGPR stimulate the plant growth by controlling pathogenic organisms (Vessey, 2003).

1.2 Rhizosphere Bacteria

The rhizosphere zone is the volume of soil which has direct influence on the presence of living plant roots or it can be the compartment of soil influenced by the roots. The rhizosphere supports active and large microbial populations which exert neutral,

beneficial and detrimental effects on the plants. Rhizobacteria (root colonizing bacteria) exerting the beneficial effects on the host plant growth via direct or indirect mechanisms are known as plant growth promoting rhizobacteria (Joseph, Ranjan, & Lawrence, 2007).

The interactions of plant microbes in the rhizosphere are responsible for improving the health of the plant and fertility of soil. PGPR strains use one or more indirect or direct mechanisms to promote the health and growth of plants. These mechanisms are active simultaneously or independently in different stages of plant growth. Studies show that PGPR directly enhance plant growth through a variety of mechanisms: by fixing atmospheric nitrogen, mineral solubilisation such as phosphorus, siderophores production, and plant growth hormones synthesis of Indole-3- acetic acid (IAA), cytokinins, gibberellic acid, and ethylene (Roesch, Triplett, de Quadros, & Camargo, 2007; Maiyappan, Amalraj, Santhosh, & Peter, 2010).

Indirect mechanisms may involve biologically controlling plant pathogens and deleterious microorganisms, through producing antibiotics, hydrogen cyanide, lytic enzymes, catalase and siderophore or by competing for space and nutrients which significantly improves plant health and promotes growth, as seen by increase in vigor, seedling emergence, and yield. After fixation of nitrogen, phosphate solubilization is also very important in plant growth promoting activity (Naik, Sahoo, Goswami, Ayyadurai, & Sakthivel, 2008). A large number of soluble inorganic phosphates that are added to the soil are actually fixed soon after the application as insoluble forms and become unavailable to the plants. A number of soil bacteria in particular those that belong to the genera *Bacillus* and *Pseudomonas*, do possess the ability of changing insoluble forms to soluble forms by secretion of organic acids such as propionic acid, formic acid, glycolic, lactic, succinic acid and fumaric acid. Microbial inoculants which are biofertilizers promote plant growth, productivity and also increase the nutrient content of the host plant. Biofertilizers are internationally accepted as another source of

chemical fertilizers in which significant increase has been reported in crop yields by applying PGPR microbial inoculants (Kumar *et al.*, 2012).

1.3 Banana Plant Rhizosphere Bacteria

The roots of banana (*Musa* spp.) are adventitious and horizontally proliferate in the topsoil and cannot get water and nutrients from the deeper profile of soil like other fruit crops. The undeveloped root system prevents the production of the bananas in large-scale under adverse tropical soil condition where these root systems are crucial for the support of the plant, water and nutrient acquisition and also in production of plant growth regulators by rhizosphere bacteria (Mia *et al.*, 2010b).

The banana fruit crop is cultivated widely in tropical areas where large amount of chemical fertilizers (NPK fertilizer) may commonly be applied. Commercial cultivation of banana needs substantial inorganic fertilizers such as urea. Banana plants are not able to store "N" so deficiency symptoms soon develop because the supply may not be sufficient. Therefore, "N" should be supplied at some short intervals during the growth which is difficult and costly to maintain. Moreover, excessive "N" which is beyond the critical level is not only uneconomical and wasteful but also pollutes water bodies as well as the soil environment. Bananas are affected from a mismatch of its nitrogen requirements because its nitrogen may be supplied as chemical fertilizer and as a result there is a 50-70% loss of the fertilizer applied. One approach in solving this problem is to regulate the time of nitrogen application as based on the nutrient requirement of banana plant. This increases the efficiency of the plant's use of nitrogen. The other approach is by increasing the efficiency in which the available soil nitrogen is used and meeting the additional nitrogen demand through making banana capable of its own nitrogen fixation either directly, or by a close interaction with diazotrophic bacteria (Roesch *et al.*, 2007).

The nitrogen-fixing activity that is detected in the rhizosphere of plants is actually not the outcome of some individual nitrogen-fixing strains, but it is the sum of interactions

taking place in the members existing in the rhizosphere community (Holguin, Guzman & Bashan, 1992). Inorganic phosphates are taken up by plants in two soluble forms which may be the monobasic ($H_2PO_4^-$) and also the dibasic (HPO_4^{2-}) ions. Some microorganisms of the soil solubilize these insoluble phosphorus forms through the production of organic acid process, ion exchange reactions and chelation and as a result they are available to plants (Banerjee, Palit, Sengupta, & Standing, 2010).

Biofertilizer, an alternative nitrogen fertilizer may be applied in increasing crop growth via biological fixation of nitrogen, availability of nutrients from the soil and uptake of water to ensure a sustainable banana production. Various studies have shown that PGPR strains successfully form colonies on the surface of banana roots, whereby a number of bacterial cells are seen in root hair proliferation zone. Application of PGPR alone can produce significant benefits which require minimal levels of fertilizer-N as a result producing a synergistic effect on root development and growth. The inoculation may also increase the "N" yield and fix nitrogen in association with banana roots, subsequently increasing the yield. This also improves the physical attributes of the fruit quality and may initiate early flowering (Mia, Shamsuddin, Zakaria, & Marziah, 2009).

1.4 Research on Rhizosphere Bacteria

Numerous investigations on PGPR have been done focusing on biotechnological applications in horticulture, agriculture, environmental protection and forestry (Zahir, Arshad & Frankenberger, 2004). In the early 1950's studies began with a focus on nitrogen fixing bacteria. Since then, PGPR in large numbers that belong to different classes of bacteria and genera with multifunctional traits have been described. PGPR strains are distributed broadly among many taxa which includes *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria*, to an extent that determination of the background population size and the PGPR activity in resident microbial communities has become difficult to assess based on the analysis of the structure of microbial community or abundance of some particular taxonomic group (Tilak *et al.*, 2005).

The main purpose of biotechnological development based on PGPR is to develop soil inoculants which contribute to sustainable agriculture and as a result, diminish the use of pesticides and chemical fertilizers. The associations between plants and bacteria can be grouped into three categories: negative, neutral or positive. Most rhizobacteria that are associated with plants are commensals, in which bacteria may establish an interaction which does not have any seen effect on the physiology and growth of the plant (Martínez-Viveros, Jorquera, Crowley, Gajardo, & Mora, 2010). The rhizosphere contains rhizobacteria which negatively influence the growth and physiology of the plants, including phytopathogens. In addition to disease causing and parasitic organisms, such bacteria may produce phytotoxic substances, such as ethylene or hydrogen cyanide that inhibits root growth. PGPR exert a positive effect on the plant growth by production of growth regulators. Indirect mechanisms are stimulation of mycorrhizae development, competitive pathogen exclusion or removal of phytotoxic substances which are produced by plant roots and deleterious bacteria under stress condition mechanisms (Haghighi *et al*., 2011).

1.5 Importance of Rhizosphere Bacteria in Plants

Plant growth promoting rhizobacteria influence plant health and their productivity through a variety of mechanisms; solubilization of mineral nutrients, root growth stimulation and root disease suppression. Since the first studies on PGPR, many candidate PGPR strains have been evaluated and screened in the laboratory, field studies and greenhouses across the world (Zehnder Murphy, Sikora, & Kloepper, 2001). Nevertheless, implementation of this biotechnology is hindered by lacking consistency and variation in responses obtained in field trials from one site to the other, year to year, or different crops. Establishment of the introduced bacteria successfully depends on properly selecting PGPR that must be inoculated to the soil and crop combination. Other problems that may be related to storage, inoculum production and delivery have mostly prevented the use of non-spore forming bacteria as soil inoculants. There has been considerable confusion over the definite effects of PGPR, which confuse scientific

studies aimed at adding their contribution to plant growth. This is to a large extent due to poor understanding of the associations between PGPR and their host plant and the microflora residing within them, as well as limited information on how environmental factors affect the processes that contributes to plant growth promotion (Martínez-Viveros *et al.*, 2010).

1.6 Effects of Microbial Inoculants

Microbial inoculants can help in promoting the health of the plant since they form symbiotic relationships with the plant. The inoculants provide a suitable microenvironment which combines with the physical protection for a long period of time preventing a sudden decline of the bacteria introduced. Inoculants act a source of bacteria which can survive in the soil and can be easily available to the plants. They can be applied to improve the nutrition of the plant and promote their growth by plant hormone stimulation. The tissue culture banana plantlets can mitigate the risks associated with pests and diseases. In addition, the inoculants are very beneficial in reducing the risks that are associated with the use of chemical pesticides and fertilizers (Trabelsi & Mhamdi, 2013). Therefore, the banana plantlets are protected from environmental stress that might hinder their growth, survival, and health. The use of inoculants is being adapted in various crops since it helps in increasing the growth and production. The inoculants are also less expensive as compared pesticides and fertilizers (Bashan, de-Bashan, Prabhu, & Hernandez, 2014).

The inoculants increase the development and the growth of the roots in the tissue cultured banana plantlets. This can be observed by the increased growth of root hairs resulting from the interaction of bacteria with the host plant. Similar results have been observed in other cereal crops like in wheat where inoculants enhance the growth by the division of cells in the root tips and in maize where they increase the diameter and the length and branching of lateral roots. Plant growth promoting rhizobacteria inoculation in plants can also increase the amount of nutrients available in the plant. It increases the concentration of Nitrogen in the leaves and roots by 28%-40% resulting in the overall

accumulation of Nitrogen in the dry matter (Mia, Shamsuddin, Zakaria, & Marziah, 2010a). The micro propagated "Grand Naine" banana plantlets inoculated with rhizobacteria in combinations or alone shows significantly higher growth in parameters such as aerial dry weight, leaf area, total fresh weight, and shoot length than in the control with non-treated bananas. The content in minerals i.e N, P and K increased significantly after the application. The process of inoculation stimulates the growth of roots as well as the development of bananas (Mia *et al.*, 2010b).

1.7 Biofertilizers

Biofertilizers can be defined as microbial inoculants that help in promoting productivity and plant growth. Internationally they have been accepted as another source of fertilizer since they can be environment friendly and ensure a sustainable production of bananas. In biofertilizer technology, new systems are being developed that may increase biological fixation of nitrogen with non-legumes and cereals leading to the development of bacteria that fix nitrogen within the roots (Cocking, 2000).

In order for a biofertilizer to be effective, bacteria that promote growth should be able to fix nitrogen as well. Inoculation of free living as well as associative N_2-fixing bacteria produces advantageous effects on the growth of the plant. Indicative increase in the yield of crops after application of PGPR has been reported under some diverse field conditions. Reports have shown that they fix atmospheric nitrogen with cereals, grasses and also help in improving nutrient uptake (Vessey, 2003). The way PGPR respond to different fruits is not similar to those that are found in gramineous crops. From recent studies, PGPR strain UPMB 10 (*Bacillus sphaericus*), an isolate from oil palm produced beneficial effects on some plantation crops like banana, coconut and oil palm (Mia *et al.*, 2009). For the past 40 years field inoculation and greenhouse studies with these PGPR have shown that rhizobacteria can promote the yield of important crops agriculturally that have been grown on different climatic conditions and soils (Mia *et al.*, 2010b).

1.8 Statement of the Problem

The banana plant requires high amounts of potassium and nitrogen followed by phosphorus, calcium and magnesium in order to maintain high yields. The physiological limitation in the storage capacity of nitrogen is also a constraint for the cultivation of this crop commercially. Deficiency symptoms quickly develop and extra nitrogen has to be frequently applied even on fertile soil. Increase in use of chemical fertilizer is undesirable because its production is energetically a costly process and most of the energy utilized is by the consumption of fossil fuels which are non-renewable (Mia *et al.*, 2009). As a result, considerable pollution is caused through the use and production of inorganic nitrogen fertilizers and this is compounded by relatively low absorption efficiency by plants due to their non-extensive root system. The resource poor farmers cannot afford the chemical fertilizers. In addition, chemical fertilizers may not be eco-friendly as they reduce microbial diversity (Mahdi *et al.*, 2010).

Better understanding of the rhizosphere bacteria, may help to elucidate their potential role and function more effectively. This can help develop a sustainable and affordable system of crop production which may include the development and use of biofertilizers. However, information on rhizobacteria and their role in growth promotion and production of bananas in Juja and Kenya at large is not yet available.

1.9 Justification

Banana, being an important fruit crop requires large amounts of chemical fertilizers for its commercial cultivation. This is a costly process and can be hazardous to the environment when used excessively. Plant growth promoting rhizobacteria (PGPR) could in turn be used for growth promotion, nutrient uptake and also as an alternative source of nitrogen fertilizer of non-leguminous crops (Mia *et al.*, 2010a). However, the use of this important technique in banana production system is limited. Information on PGPR, their application on bananas and even on different crop plants shows the potential of PGPR as a biofertilizer and bioenhancer for banana cultivation. Nitrogen

fixation, improved nutrient absorption and plant growth promotion are important criteria for the achievement of a sustainable banana production system. Unlike chemical fertilizers, biofertilizers are environment friendly, cleanse the plant from the precipitated chemical fertilizers and help maintain soil microbial diversity (Vessey, 2003). Therefore there is need of finding an alternative nutrient supply source such as biofertilizers. One possibility is by using rhizobacteria which have potential to improve plant growth. This study aimed at characterizing PGPR associated with banana roots and determining their potential use as bio-fertilizer in banana plants.

1.10 Hypothesis

There are no bacteria in the rhizosphere soil of banana plant in Juja that have the potential to improve the plant growth.

1.11 Objectives

1.11.1 General Objective

To isolate rhizosphere bacteria from banana plants within Juja farms and assess their potential as plant growth promoting rhizobacteria.

1.11.2 Specific Objectives

1. To isolate, characterize and identify bacteria from banana plant rhizosphere in Juja.
2. To determine if the isolates can fix nitrogen, solubilize phosphate or produce auxins.
3. To evaluate phenotypic effects of potential plant growth promoting rhizobacteria on banana plant.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study Site

Soil samples were collected from farmers' fields in Juja, Kenya (Figure 2.1).

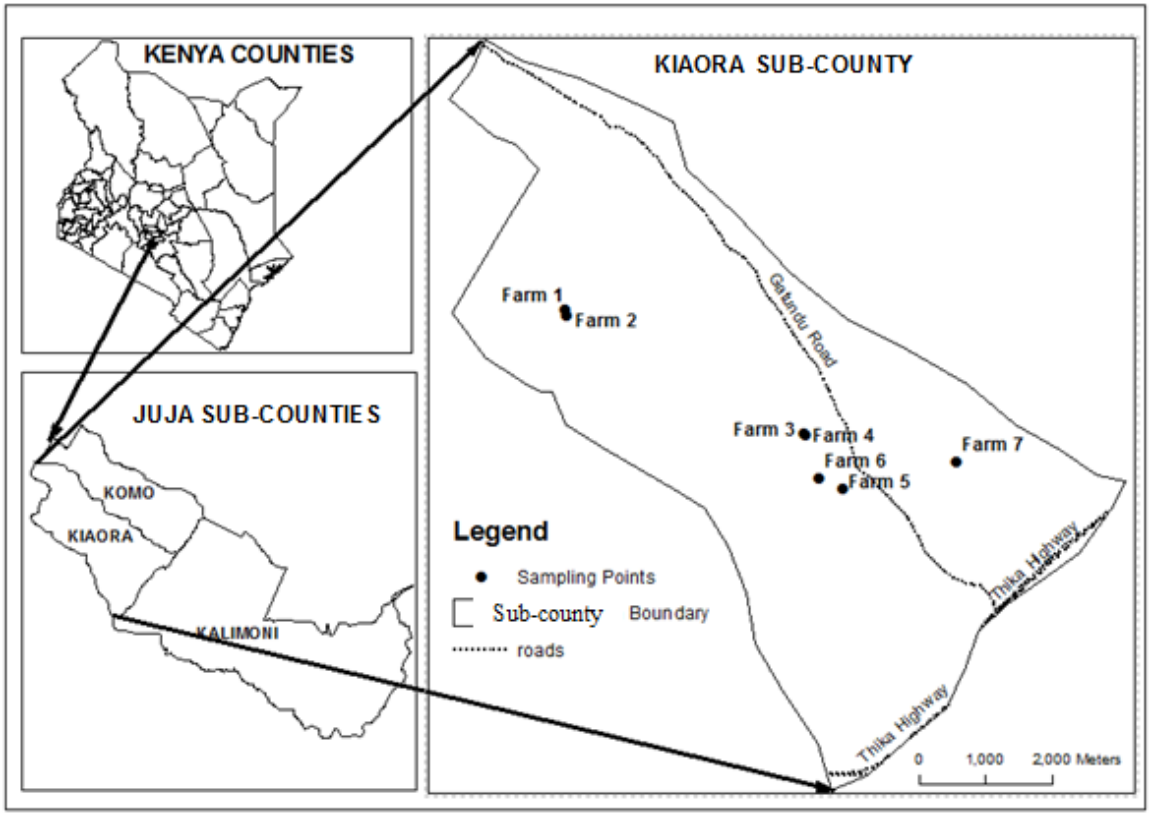

Figure 2.1: A map of Kenya and Juja showing the sampling points

2.2 Experimental Design

Twenty nine (29) soil samples of cooking and thirty eight (38) soil samples of ripening banana varieties were randomly collected from Juja experimental sites. The soil samples were plated on three different types of media and the isolates obtained were then

characterized using morphological, biochemical and molecular characterization processes.

2.3 Determination of Sample Size

A study was conducted on the experimental site, and averages of around 80 banana plants were located in each farm. This was then taken as the population size and was used in calculating the sample size as shown below. A sample size of 67 soils was used which was determined using the formula by Israel, (2009). Where: n = sample size, N = Population size, e = precision at 95% confidence level and P= 0.05

$$n = \frac{N}{1 + N(e^2)}$$

$$n = \frac{80}{1 + 80(0.05^2)}$$

$$n = 67$$

2.4 Collection of Soil Samples

Soil samples from cooking and ripening banana varieties were randomly collected (Table 2.1) from Juja farmer's fields in polythene bags and transported to the laboratory for rhizosphere bacteria isolation. The samples were collected from seven farms with the following GSP coordinates [Farm 1- S1˚4'0.4512", E36˚57'25.848" Farm 2- S1˚4'3.0468", E36˚57'26.8092" Farm 3- S1˚4'59.8116", E36˚59'21.5736" Farm 4- S1˚5'1.0176", E36˚59'22.3188" Farm 5- S1˚5'26.7324", E36˚59'40.6644" Farm 6- S1˚5'21.6528", E36˚59'29.22" Farm 7- S1˚5'14.1216", E37˚0'35.6328"]. The rhizosphere soil surrounding the banana plant roots at the depth of 20-40 cm was collected and kept as such in polythene bags and transported to the laboratory. Isolation, morphological and biochemical characterization were done at the microbiology laboratory of Food Science Department, while molecular characterization was carried out at the Institute of Biotechnology Research (IBR) in Jomo Kenyatta University of Agriculture and Technology, Juja.

Table 2.1: Soil samples from cooking and ripening banana varieties in Juja

Sample No.	Farm No.	Type of banana	Banana Variety
1	Farm 1	Ripening	Grand naine
2	Farm 1	Ripening	Giant Cavendish
3	Farm 1	Ripening	Williams
4	Farm 1	Ripening	Williams
5	Farm 1	Ripening	Vallary
6	Farm 1	Cooking	Matoke
7	Farm 1	Cooking	Matoke
8	Farm 1	Cooking	Matoke
9	Farm 1	Cooking	Matoke
10	Farm 1	Cooking	Matoke
11	Farm 2	Cooking	Githumo
12	Farm 2	Ripening	Nyoro
13	Farm 2	Cooking	Githumo
14	Farm 2	Ripening	Nyoro
15	Farm 2	Ripening	Njajiba
16	Farm 2	Ripening	Nyoro
17	Farm 2	Cooking	Githumo
18	Farm 2	Cooking	Githumo
19	Farm 2	Cooking	Githumo
20	Farm 2	Ripening	Nyoro long
21	Farm 2	Ripening	Nyoro
22	Farm 2	Cooking	Githumo
23	Farm 2	Cooking	Githumo
24	Farm 2	Cooking	Githumo
25	Farm 2	Cooking	Githumo
26	Farm 3	Ripening	Nyoro long
27	Farm 3	Ripening	Sweet bananas
28	Farm 3	Ripening	Nyoro long
29	Farm 3	Cooking	Githumo
30	Farm 3	Cooking	Githumo
31	Farm 3	Ripening	Kibutu
32	Farm 3	Ripening	Kibutu
33	Farm 3	Ripening	Nyoro
34	Farm 3	Cooking	Githumo
35	Farm 3	Cooking	Gichagara
36	Farm 4	Ripening	Kibuu

37	Farm 4	Ripening	Kibuu
38	Farm 4	Ripening	Kibuu
39	Farm 4	Cooking	Githumo
40	Farm 4	Cooking	Githumo
41	Farm 5	Ripening	Kampala
42	Farm 5	Ripening	Kampala
43	Farm 5	Ripening	Kampala
44	Farm 5	Ripening	Kampala
45	Farm 5	Ripening	Kampala
46	Farm 5	Ripening	Nyoro
47	Farm 5	Ripening	Nyoro
48	Farm 5	Ripening	Nyoro
49	Farm 5	Ripening	Mshirain
50	Farm 5	Ripening	Gichuru
51	Farm 6	Ripening	Kibutu
52	Farm 6	Cooking	Githumo
53	Farm 6	Cooking	Githumo
54	Farm 6	Cooking	Githumo
55	Farm 6	Cooking	Githumo
56	Farm 6	Ripening	Israel
57	Farm 6	Ripening	Israel
58	Farm 6	Ripening	Mboo
59	Farm 6	Cooking	Gichagara
60	Farm 6	Cooking	Gichagara
61	Farm 7	Ripening	Kibuu
62	Farm 7	Cooking	Githumo
63	Farm 7	Cooking	Githumo
64	Farm 7	Ripening	Israel
65	Farm 7	Ripening	Israel
66	Farm 7	Ripening	Mboo
67	Farm 7	Cooking	Gichagara

2.5 Preparation of Culture Media

Three different media were used; yeast manitol agar, nutrient agar and nitrogen free medium (Ngamau, Matiru, Tani, & Muthuri, 2012). The ingredients were weighed (Appendix 8) and then dissolved in distilled water as described by warming them on a hot plate. These were autoclaved under pressure for 15 min at 121°C. The media was poured in sterile petri dishes and allowed to cool.

2.6 Isolation by Serial Dilution Technique

The soil stuck around the roots was removed by washing the roots and serial dilutions of the rhizospheric soils was done up to 10^{-5} dilutions and then inoculated on yeast manitol agar (YEM), nitrogen free medium and nutrient agar. The cultures were then incubated both aerobically and anaerobically for 24-48 h at 30^{o}C to allow for rhizosphere bacteria to grow. Individual colonies were then picked and streaked on fresh media to purify them and generate pure cultures. The pure cultures were thereby used to perform morphological and biochemical tests. The potential plant growth promoting rhizosphere bacterial cultures were also used to perform molecular characterization.

2.7 Morphological Characterization of Bacterial Isolates

Morphological characterization was carried out to determine the shape of the bacterial cell. In this process, classical Gram staining method was used (Bathlomew, 1962). Smears from the bacterial isolates were made and heat fixed, subsequently they were flooded with crystal violet (Sigma Aldrich, Steinheim, Germany) and allowed to stand for one minute. Smears were then washed with tap water gently, and flooded with Grams iodine (Sigma Aldrich, Steinheim, Germany). After one minute they were rinsed with tap water. 95% ethanol was then used for decolourization (Scharlab S.L., Spain), and then counterstaining followed that took some forty five seconds with safranin. After that the smears were washed gently with tap water, and dried to be observed under oil immersion on a light microscope (Bathlomew, 1962).

2.8 Biochemical Characterization of Bacterial Isolates

The following biochemical tests were carried out:

2.8.1 Acetylene Reduction Assay (ARA)

The reduction of acetylene to ethylene (C_2H_4) is a method widely used to check nitrogenase activity in isolates, cell-free extracts and natural samples. The reduction of acetylene (C_2H_2) to ethylene is catalyzed by enzyme nitrogenase. This test measures nitrogenase activity, which is the total amount of nitrogen that an organism or the system has fixed (Eckert *et al.*, 2001). The biological nitrogen fixation process may be summarized as:

$$N_2 + 8\,H^+ + 6\,e^- \xrightarrow{Nitrogenase} 2\,NH_3 + H_2$$

Bacteria grown for three days in nitrogen free medium were placed in semisolid agar media that contains 2.3g of agar per liter. Media of 5ml was then placed in 10ml vials. To reach a concentration of 12% v/v, acetylene was added and ethylene produced was determined on a Shimadzu Gas Chromatograph (GC-9A, Japan) after 12 h as described by Eckert et al., (2001). 1ml of the gas in the reaction vials was injected into the GC machine using a syringe and a chromatograph that showed the retention time produced. Standard ethylene gas was used as the positive control while un-inoculated tube used as a negative control.

2.8.2 Urease Test

The urease test resolves an organism's ability to split urea by producing a urease enzyme (Harold, 2002).

Urease test summary:

$$(NH_2)_2CO + H_2O \xrightarrow{urease} CO_2 + 2NH_3(\text{ammonia})$$

$$\text{Ammonia} + \text{phenol red} \longrightarrow \text{deep pink color}$$

Ammonia formed was detected as a result of some alkaline conditions in the presence of an enzyme and increased pH by a pH indicator. Christensen's urea contains phenol red as the pH indicator which is yellow under acidic conditions (pH 6.8). In alkaline conditions (pH 8.4) the media is turned by the indicator to rose pink. The isolates' ability to attack carbon and nitrogen bonds in amide compounds was determined by using urea broth medium (Oxoid Ltd., Basingstoke, Hampshire, England) that contained phenol red as the pH indicator (Cappuccino & Sherman, 2002). The isolates were inoculated aseptically into sterile Christensen's urea broth by using a sterile wire loop and then incubated in a clean incubator (IN-81, Yamato, Japan) for 24 h at 30°C, thereafter observations were made depending on the color change (Cappuccino & Sherman, 2002).

2.8.3 Nitrate Reduction Test

In this test the enzyme nitrate reductase is produced which reduces nitrates to nitrites or water and free nitrogen gas. The nitrite produced combines with sulphanilic acid and alpha-naphthylamine to form a diazo red dye.Nitrate reduction was tested by inoculation of substrates into nitrate broth medium that contains 1% potassium nitrate and then incubating them for 72 hat 30ºC. After incubation, drops of sulphanilic acid and alpha-naphthylamine were added. Nitrate reduction was observed by adding 0.2 ml naphthylamine and 0.2 ml sulphanilic acid reagent to each sample tube. Positive test gave a red color while negative reactions remained yellow (Cappuccino & Sherman, 2002).

NO_3^- *nitrate reductase* $\rightarrow$ NO_2^- denitrifying bacteria $\rightarrow$ $N_{2(g)}$

2.8.4 Phosphate Solubilisation Test

Plate assay method was used to test for phosphate solubilizing microorganisms in phosphate solubilisation media. This media was prepared and poured on sterile petri plates and a sterile wire loop used for inoculating the isolates onto the media. The inoculated plates were placed in a clean incubator (IN-81, Yamato, Japan) at 30°C for

two weeks and observations done (Cappuccino & Sherman, 2002). If a halo was formed around the bacterial colonies this indicated a positive result for phosphate solubilisation.

2.8.5 Indole Acetic Acid Production Test

Indole acetic acid production was detected calorimetrically using the Salkowski reagent as specified by Glickmann & Dessaux (1995). The pure isolates of bacteria were inoculated aseptically into the sterile nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England), by using a sterile wire loop and then incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 72 h. In order to obtain cell free broth, centrifugation of the cultures was carried out (3000rpm, 5 min, 30°C). Then few drops (0.5 ml) of Salkowski's reagent was added to the cell free broth and thereafter incubated at room temperature for 30 min (Glickmann & Dessaux, 1995). Pink color formation indicated a positive test. Salkowski's reagent is a 35% $HClO_4$ solution that contains 10 mM $FeCl_3$, and when it is mixed with IAA, tris-(indole-3- acetate), iron (III) complex is actually formed that displays pink coloration (Rahman, Sitepu, Tang, & Hashidoko, 2010).

2.8.6 Citrate Utilization Test

In the determination of the capability of the bacterial isolates to use citrate as their energy carbon source, Simmons' Citrate agar slants were used (Harold, 2002). The isolates were inoculated under aseptic conditions into sterile Simmon's Citrate agar slants (Sigma Aldrich, Steinheim, Germany), by using a sterile wire loop and then incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 h, observations were made on color change (Cappuccino and Sherman, 2002). The isolates were inoculated in a medium having sodium citrate and a pH indicator bromothymol blue. Inorganic ammonium salts were also present in the medium, which were utilized as a sole nitrogen source. Citrase enzyme is involved in the utilization of citrate and breaks down citrate to acetate and oxaloacetate. Oxaloacetate is broken down again to pyruvate

plus CO_2. Utilization of sodium citrate produces Na_2CO_3 which results in alkaline pH that changes the color of the medium from green to blue.

Sodium Citrate $\xrightarrow{\textit{Citrate permiase}}$ Pyruvic acid + Oxaloacetic acid + CO_2 *Citrase*

Excess sodium from sodium citrate + $CO_2 + H_2O \rightarrow Na_2CO_3$ (Alkaline pH- blue color)

2.8.7 Catalase Test

This test detects the enzyme catalase that is present in the majority of cytochrome containing aerobic bacteria that forms hydrogen peroxide the oxidative end result of aerobic sugar breakdown. Decomposition of hydrogen peroxide by catalase produces water and oxygen.

$$2\ H_2O_2 \xrightarrow{\textit{Catalase}} 2\ H_2O + O_2$$

The isolates were inoculated aseptically into sterile Trypticase Soy agar (Sigma Aldrich, Steinheim, Germany) using a sterile wire loop and placed in a clean incubator for incubation (IN-81, Yamato, Japan) at 30°C for 24 h, the catalase activity was then determined by adding to the cultures 3% hydrogen peroxide. A positive result was indicated when bubbles formed (Cappuccino & Sherman, 2002).

2.8.8 Hydrogen Sulphide Production Test

Sulfur-Indole Motility agar (Oxoid Ltd., Basingstoke, Hampshire, England) was the media used in demonstrating cysteine desulfurase production by the isolates. Sulfur containing amino acids were broken down by cysteine desulfurase producing ammonia, pyruvate and hydrogen sulfide. Iron found in the medium reacted with hydrogen sulfide to produce the characteristic black precipitate giving a positive result for hydrogen sulfide production by the bacterial isolates (Cappuccino & Sherman, 2002). This was summarized as follows:

Cysteine $\xrightarrow{\textit{Cysteine desulfurase}}$ pyruvic acid + ammonia + hydrogen sulfide gas

$H_2S + Fe^{+2} \longrightarrow Fe(NH_4)SO_4$

(Black precipitate)

In this test a loopful of the isolate was stubbed through Sulfur-Indole Mortility agar (Oxoid Ltd., Basingstoke, Hampshire, England) and then incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 h. The blackening of the media showed that hydrogen sulfide had been produced (Cappuccino & Sherman, 2002).

2.8.9 Methyl Red-Voges-Proskauer Test (MR-VP)

The MR-VP test was carried out to determine if the isolates had the ability to oxidize glucose meanwhile stabilizing and producing high acid concentration end products. The isolates were inoculated aseptically into sterile MR-VP broth (Sigma Aldrich, Steinheim, Germany), using a sterile wire loop and incubated inside a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 h. 1ml of every culture were picked and mixed with either methyl red indicator (MR test) or Barritts reagent (VP test) and observations were done depending on the color change (Cappuccino & Sherman, 2002).

Summary of MR test:

Positive test: glucose $\longrightarrow$ pyruvic acid (1 day)

Pyruvic acid $\longrightarrow$ lactic, acetic, and formic acids

Many acids (pH 4.2) + added methyl red $\longrightarrow$ red color

Negative test: glucose $\longrightarrow$ pyruvic acid (1 day)

Pyruvic acid $\longrightarrow$ neutral end products

Neutral end products (pH 6.0) + methyl red $\longrightarrow$ yellow color

Summary of VP test:

Glucose + ½ O_2 $\longrightarrow$ 2 pyruvate $\longrightarrow$ α-acetolactate $\longrightarrow$ acetion $\longrightarrow$ 2,3-butanediol

Acetion + Barritt's reagent $\longrightarrow$ diacetyl + creatine (pink complex)

2.8.10 Gelatine Hydrolysis

Gelatine liquefaction detected the breakdown of gelatine to polypeptides and amino acids by enzyme gelatinase (Harold, 2002). Gelatine protein is produced by hydrolysis of a component of the connective tissues and tendons of animals known as collagen. Gelatine is solid at room temperature but above 25^{o}C it turns into liquid. When gelatinase hydrolyses this protein into amino acids, it remains liquefied even at the low temperatures of an ice bath (Cappuccino & Sherman, 2002). The bacterial isolates were inoculated onto nutrient broth supplemented with 12% gelatine and 1.5% agar, to demonstrate hydrolytic activity of gelatinase. One un-inoculated tube was used as control for each isolate (Cappuccino & Sherman, 2002). After incubation, cultures that remained liquefied when placed in refrigerator at 4^{o}C for 30 min were considered positive for gelatine hydrolysis (Cappuccino & Sherman, 2002).

2.8.11 Indole Production

The test identifies isolates with the ability to produce the enzymes tryptophanase that removes the amino group from tryptophan to form indole, pyruvic acid and ammonia, and cysteine desulfurase, that produces pyruvate, ammonia and hydrogen sulphide from sulfur containing amino acids. Indole reacts with Kovacs reagent (*p*-dimethylamino-benzaldehyde) (Harold, 2002), to form a deep red color. Kovac's reagent was added to each tube of the 48 h culture according to the protocol of Harold, (2002). The presence of a cherry red layer in the media indicated positive result for indole production while negative results were indicated by color remaining brown (Cappuccino & Sherman, 2002).

2.8.12 Starch Hydrolysis

Using starch agar plates the bacterium was inoculated using aseptic technique. Streaking was done with the respective bacteria on the plate in a straight line. Incubation of the plate for 24 to 48 h at 30^{o}C was done. Drops of Gram's iodine were

placed on each of the line streaked on the starch agar plate. If the area around the line of growth was clear, starch had been hydrolyzed, and the test was positive; if it was not clear or the entire medium turned blue, starch has not been hydrolyzed, and the test was negative (Cappuccino & Sherman, 2002).

2.8.13 Identification of the Banana Rhizosphere Bacteria

The isolates obtained were identified based on their morphological observation plus their biochemical characterization. Bergey's Manual of Determinative Bacteriology (7^{th} edition) was then used as a reference in order to identify the isolates (Mohite, 2013).

2.9 Determination of Nitrogen Fixation, Phosphate Solubilisation and Auxin Production

Qualitative screening of the bacterial isolates from the rhizosphere of bananas was done to determine whether they are able to fix nitrogen, solubilize phosphates and even produce auxins.

2.9.1 Screening for Nitrogen Fixation Ability

In order to determine if the isolates have the ability of fixing atmospheric nitrogen, there was qualitative screening of growth done on solid N-free medium(1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 1g $CaCO_3$, 0.2 g NaCl, 5 mg $FeSO_4.7H_2O$, 10 g glucose, 5 mg $NaMoO_4$ per litre and 1.5% agar at pH 7.0). The growth parameter that was used was growth on N-free medium and thereafter data taken four days after inoculation (Ngamau *et al.*, 2012).

2.9.2 Screening for Phosphate Solubilization

For the phosphate solubilizing isolates qualitative screening was carried out using the National Botanical Research Institute's phosphate (NBIRP) growth medium. The medium consisted of 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2$, 0.25 g $MgSO_4$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ per litre and 1.5% agar at pH 7. Phosphate solubilizers screening was

based on the formation of visible halo zones on the prepared agar plates. This is as a result of organic acids being produced into the surrounding medium. The halo size measures the relative efficiency of the isolates to solubilize phosphates. The diameters of both the halo and colony were measured 14 days after inoculation. Halo size was determined by subtracting from the total diameter the colony diameter (Ngamau *et al.*, 2012).

2.9.3 Screening for Auxin Production

Production of indole acetic acid was determined calorimetrically using the Salkowski reagent as explained by Glickmann & Dessaux (1995). The pure isolates were inoculated aseptically using a sterile wire loop into sterile nutrient broth and then incubated in a clean incubator at 30°C for 72 h. Centrifugation of the cultures was done (3000 rpm, 5min, 25°C) to obtain cell free broth. Some few drops (0.5 ml) of Salkowski's reagent was added to the cell free broth and then incubated for thirty minutes at room temperature. Pink color formation indicated a positive test. Salkowski's reagent is a 35% $HClO_4$ solution that contains 10 mM $FeCl_3$, and when mixed with IAA, tris-(indole-3- acetato) iron (III) complex is formed which displays pink coloration (Rahman *et al.*, 2010).

2.10 Molecular Characterizaton of the Rhizosphere Isolates

2.10.1 DNA Extraction

The genomic DNA from all the twenty bacteria isolates was extracted after 48 h growth in nutrient broth and incubation in a shaker at 37ºC. Before the extraction, cells from the broth were harvested in 1.5 ml Eppendorf tube by centrifugation of 1 ml of culture at 13,000 g for 10 min. Washing of the pellet was done by re-suspending the cells in an equal volume of TE buffer, followed by centrifugation at 13,000 g for 5 min and discarding the supernatant. The cells were again re-suspended in 200 µl of solution A [50 mM Tris (pH 8.5), 50 mM EDTA (pH 8.0) and 25% sucrose solution 5 µl of

lysozyme (20 mg/ml) and 5 µl of RNase A (20 mg/ml) and gently mixed (Sambrook, Maniatis, & Fritsch, 1989). The mixture was incubated at 37°C for 1 hr then 600 µl of solution B [10 mM Tris (pH 8.7), 5mM EDTA (pH 8.0), 1% sodium dodecyl sulphate] and 10 µl of proteinase K (20 mg/ml) were accordingly added, gently mixed and incubated at 55°C for 30 min (Sambrook *et al.*, 1989). Phenol-chloroform 400 µl (ratio 1:1) was added and then spun at 13,000 g for 10 min. The aqueous upper layer was transferred carefully into another 1.5 ml Eppendorf tube. This very step was repeated just before the addition of 500 µl of chloroform: Isoamyl alcohol (24:1) for washing off the phenol. The mixture was subjected to spinning at 13,000 g for 10 min and the supernatant discarded carefully (Sambrook *et al.*, 1989). The above method was repeated twice.

The DNA precipitation was done by the addition of 250 µl of absolute ethanol (ice cold) then 50µl sodium chloride and left overnight -20°C. The pellet was subjected to precipitation by centrifuging at 13,000 g for 10 min and the supernatant discarded. An addition of 500 µl of 70% ethanol was done and centrifuged for 10 min at 13,000 g. The supernatant was carefully discarded not to pour down the pellet. The above procedure was repeated twice before air-drying the pellet completely at room temperature (residual ethanol is eliminated in this step) as described by Sambrook *et al.*, (1989). The dry pellet was thereafter re-suspended in 100 µl of TE buffer (TE is advantageous because EDTA chelates magnesium ions that makes the residual DNases more difficult to degrade the DNA) and later kept at -20°C for future use (Magarvey, Keller, Bernan, Dworkin, & Sherman, 2004). The visualization of the DNA was done under UV on a 1% agarose gel in 1X TAE buffer after staining with ethidium bromide (Sambrook *et al.*, 1989). The quantification of the DNA was done by using a spectrophotometer and the purity of the DNA determined at an absorbance of 260 nm and 280 nm.

2.10.2 Polymerase Chain Reaction

For the amplification of the 16S rDNA, 1µl of DNA from each of the twenty (20) rhizopshere soil isolates were amplified using Taq polymerase and 10xbuffer according to manufacturer's (QIAGEN) instructions.

Nearly full-length 16S rDNA gene sequences were PCR-amplified using bacterial primer pair 27F forward 5'-GAGTTTGMTCCTGGCTCA-3' and 1492R reverse, 5'-TACGGYTACCTTACGACT-3' (Bioneer, USA) according to the position in relation to *Escherichia coli* gene sequence (Embley & Stackebrandt, 1994). Amplification was performed using an Eppendorf AG, model 22331 thermal cycler (Hamburg). Amplification of the DNA was done in a 50 µl mixture that constituted of 0.2 Units of Taq polymerase, 20pmol of 27F forward primer, 20pmol of 1492R reverse primer, 1.25mM dNTPs mix (QIAGEN), 10x PCR buffer (QIAGEN), 1 µl of template DNA and 29.8 µl of PCR water. The negative control includes all of the above except the DNA template. On the other hand, the positive control had all the above plus 1 µl of DNA template. The thermal program was initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, primer annealing at 43°C for 2 min, chain extension at 72°C for 1.5 min for 35 cycles, followed by final extension at 72°C for 5 min (Roux, 1995). The above denaturation, annealing and extension processes were repeated for 35 cycles. The amplified products (7.0 µl) were then separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1X TAE buffer and visualized by staining using ethidium bromide (Sambrook *et al.*, 1989).

2.10.3 Purification of the PCR Products

A 1.1 volume of the Binding buffer was added to the completed PCR mixture (for every 100µl of the reaction mixture, add 100µl of binding buffer) and mixed thoroughly (Sambrook *et al.*, 1989). The color of the solution was then checked, whereby yellow indicates an optimal pH for DNA binding. 800µl 0f the solution above was then transferred to the Gene JET Purification column. Centrifugation was done for 60 sec

and the flow-through was then discarded. 700µl of the wash buffer diluted with ethanol was then added to the Gene JET purification column (Thermo Scientific Gene JET Genomic DNA Purification Kit). Centrifugation for 60 sec was done, the flow-through discarded and purification column placed back into the collection tube (Thermo Scientific Gene JET Genomic DNA Purification Kit). The empty Gene JET Purification column was centrifuged for additional 1 min to completely remove any residual ethanol in the DNA sample that was to inhibit subsequent reactions (Werle, Schneider, Renner, Völker, & Fiehn, 1994). The Gene JET Purification column was transferred to a clean 1.5µl micro centrifuge tube and 50µl of elution buffer was the added to the center of the Gene JET Purification column membrane and centrifuged for 1 min. The Gene JET Purification column was then discarded and the Purified DNA store at -20^{o}C (Thermo Scientific Gene JET Genomic DNA Purification Kit). The amplified products (7.0 µl) were then separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1X TAE buffer and visualized by staining using ethidium bromide (Sambrook *et al.*, 1989).

2.10.4 Sequencing and Molecular Data Analysis

All the 20 rhizosphere bacteria isolates were taken for sequencing. Sequencing of the purified PCR products was carried out by a commercial service provider (Macrogen, South Korea). The sequences obtained were checked and then manually corrected using the chromas program. The gene sequences of the 16S rDNA were compared to the ones available in the public database by using a Basic Local Alignment Search Tool (BLASTn) on the National Center for biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) so as to determine the similarities as compared to sequences in the Gene bank database (Altschul, Gish, Miller, Myers, & Lipman, 1990; Shayne, Hugenholtz, Sangwan, Osborne, & Janssen, 2003). Alignment of the sequence was done by using the CLUSTAL Omega program (http://www.clustal.org) which was against the nearest neighbors. From the aligned sequences a neighbor-joining tree was then constructed (Saitou & Nei, 1987) using MEGA V5.10 (Tamura *et al.*, 2011). Using the

Maximum Composite Likelihood method, evolutionary distances were computed (Tamura, Nei, & Kumar, 2004). For obtaining statistical support values for the branches, bootstrapping (Felsenstein, 1985) was thereafter conducted with 1000 replicates. All sites, gaps included in the sequence alignment, were pairwise excluded in the phylogenetic analysis. By the use of resultant neighbor-joining tree, every isolate was assigned to its proper taxonomic group. The confirmation of taxonomic assignment was at a 95% confidence level by using the naïve Bayesian rRNA classifier on the RDP website (Cole, Chai, & Farris, 2005).

2.11 Assessment of Effect of Selected Isolates on Banana Plant Growth

Based on plant growth promoting traits screened previously like ARA, phosphate solubilisation and auxin production, five strains F1RWL, F5RNY, F1RWL, F6RMB and F5RNY were assessed for plant growth enhancement under controlled greenhouse conditions. Bacterial cultures grown to log phase [OD_{600}=0.8] at 30ºC for 48 h were washed by centrifuging at 1000g for 5min, re-suspended several times and rinsed with sterile distilled water (Mia *et al.*, 2009).

Infection of tissue cultured banana plantlets was done by dipping the 3 month old plantlets into the specific bacterial isolates treatment for 48 h at room temperature while distilled water was used for experimental control. The plantlets were grown in a sterile nutrient inert cocopeat media (Amiran, Kenya) supplemented with nitrogen phosphorus and potassium [NPK] slow release fertilizer at 5 levels [0gms, 2.5gms, 5gms, 7.5gms and 10gms] in a random block design in three replicates. Agronomical features of the plantlets: height, diameter and number of green and dry leaves were measured at 14 days intervals for 3 months (Mia *et al.*, 2009).

The plants were destructively sampled by gentle uprooting, washing off the media under running tap water and measuring the fresh weight. Dry weight was taken after drying the plantlets at 70ºC for 14 days until constant weight then nutrient levels of nitrogen,

phosphorus, potassium, calcium, iron and magnesium was assessed according to Plank (1992).

2.11.1 Nutrient Analysis

Two methods were used for the analysis of the nutrients in the banana tissues. The tissues analyzed were the roots, stem and leaves. The most preferred method was dry ashing. This was the method used for all the nutrients except nitrogen. Dry ashing is used commonly since it is simple, safer and even economical unlike wet ashing where some potentially explosive and carcinogenic substances are used (Ali, Zoltai, & Radford, 1988). Some elements volatilize when dry ashed since temperatures above 550ºC are used (Blanchar, Rehm, & Caldwell, 1965).

Wet ashing was used for the nitrogen analysis because it is volatile, may stick to the crucible if dry ashed and also forms some compounds which cannot be soluble in the acids used. In the dry ashing method, 1g of the sample containing the mixture of the roots, stem and leaves was weighed and placed in a crucible. It was then dry ashed at 550°C for about 8h. It was then cooled and the samples removed and diluted to 50ml in volumetric flasks (Ali *et al.*, 1988).

2.11.1.1 Phosphorus Analysis

For the analysis of phosphorus, the ashed (dry) stock, 1gm was weighed to 10 ml distilled water, 5ml was then taken in a flask and 3 drops of the indicator (0.5% P nitro phenol indicator) was added. After the addition of 6N ammonia solution drop by drop, the color changed to yellow. Decolorization was done by adding drop by drop 1N HNO_3, 5mls ammonium vanadate/molybdate was then added and made to the mark 50mls by using distilled water. The contents were allowed to stand for 30min. The yellow color of the sample was then run in the colorimeter or UV-Vis spectrophotometer 1240 SHIMADZU-JAPAN at 400nm. The concentrations of the P was obtained from the curve using the following standards: 0ppm P, 1ppm P, 2ppm P, 3ppm P, and 4ppm P which were obtained from the stock 50pm P (Konieczyński & Wesołowski, 2007).

2.11.1.2 Nitrogen Analysis

Nitrogen analysis was carried out by taking 1g of the dry tissue which was weighed and placed in the digestion tube and 5ml of the digestion mixture was added (mixture of 4ml conc. H_2SO_4 + 0.4 Selenium powder and 1g of Lithium sulphate). The contents were taken to the fumehood heater set at 300ºC for at least two hours. After two hours, the heater was put off in order to cool. The sample digest was then diluted to 50ml volumetric flask with distilled water, the distillation apparatus was then set up for distilling the already topped distillate. From the stock (50ml), 5ml was pippeted into a test tube and steam-distillation was done into a 100ml conical flask containing 1% Boric acid that had few drops of the mixed indicator, 10mls of 40% NaOH and 2mls of distilled water. Then distillation was continued drop by drop till the indicator made the boric acid solution green. To get the % N, the green color was back titrated to the original color of 1% Boric acid plus a mixed indicator which were red (Campbell, 1992).

$$\% N = \frac{a-b \times 0.2 \times V \times 100}{1000 \times W \times al}$$

Where a =Volume of the std 0.01N HCl of the sample

b= Volume of the std 0.01N HCl of the blank

V= Volume of the digestion 50mls stock

W= weight of the sample (1g)

Al= aliquot (5ml)

2.11.1.3 Other Minerals (K, Ca, Mg, Zn, Cu, Mn and Fe)

In the analysis of other minerals, 1gm was taken from the ashed sample and added to 10ml distilled water. 5ml was pipetted to a 50ml volumetric flask. It was then aspirated in a flame of atomic absorption spectrophotometer (AA-62000 SHIMADZU-JAPAN). Potassium was determined on a flame photometer (410 CORNING-JAPAN) (Tanaka, 1986). In the analysis of Ca and Mg, a leasing agent was added which was 2% strontium chloride to the mixture above though sometimes 1% lanthanum chloride can

also be used to stop interferences in the sample which may make that the exact Conc. of Mg^{2+} or Ca^{2+} not to be found. Only 1ml of the leasing agent was added to each sample (Konieczyński & Wesołowski, 2007).

2.12 Data Analysis

The procedures on biochemical characterization were carried out in replicates of three. Observations on the replicates were made in order to define the nature of each of the tests whether they are positive or negative. This information was also used during the phylogenetic trees generation to identify the banana rhizosphere bacteria. During the molecular characterization the 16S rDNA sequences obtained were analyzed using Chromas pro software (www.technelysium.com.au) and phylogenetic trees generated using MEGA V5.10 package (www.megasoftware.net). The plantlet diameter, height, number of green and dead leaves plus the dry weight of plant and finally nutrient analysis (for control and experimental) were taken. The agronomical data such as mean, ±SD from three experimental replicates were collected for analysis. Differences in agronomical parameters among the bacterial treatments were tested by analysis of variance procedure (ANOVA) using SPSS software. The student t-test was used to assess the variation between each of the treatments taken against the control experiment (Walpole, 1986). A probability of 5% was considered significant ($P \leq 0.05$) as described by Mia et al., (2009).

CHAPTER THREE

RESULTS

3.1 Isolation and Culturing of Rhizosphere Bacterial Isolates

Soil samples from the seven banana farms within Juja were found to be having the rhizospheric bacteria (Table 3.1). A total of 67 soil samples were obtained from the seven farms which included the ripening and the cooking varieties. Rhizosphere bacterial growth was observed on nutrient agar and yeast manitol agar after twenty four hours of incubation. Nutrient agar was the media that was supporting the growth of different types of organisms since it had all the nutrients that microorganisms needed for their growth. Yeast manitol agar supported the growth of rhizobium species. Microorganisms that were able to grow on this media did prove that they are able to fix atmospheric nitrogen and convert it to simple nitrogenous compounds in order to support their growth since this media had no nitrogen.

Bacterial colonies of different colors such as white, cream and orange were observed on the growth media. Diversity of isolates was found in the rhizosphere soil samples. After streaking and pure isolation processes, 20 pure isolates were obtained. The incubation of these isolates anaerobically using the anaerobic jar gave a positive growth for all the 20 isolates. The 20 pure isolates were then coded as follows: F(1-7) depending on which farm it was isolated, R (ripening) and D (cooking) depending on the variety and WL- Williams; NY-Nyoro; IS-Israel; MS-Mshirain; GC- Giant Cavendish; GN- Grand naine; KB- Kibuu; GG- Gichagara; VL- Vallary; MB- Mboo according to the type of bananas (Table 3.1).

3.2 Morphological Characterization of Rhizosphere Bacterial Isolates

Morphological characterization performed for the 20 isolates as based on Gram test showed that three of the bacterial isolates from the rhizosphere were Gram negative rods

while 14 of them were Gram positive rods and the rest remaining three were Gram positive cocci (Plate 3.1, Table 3.1).

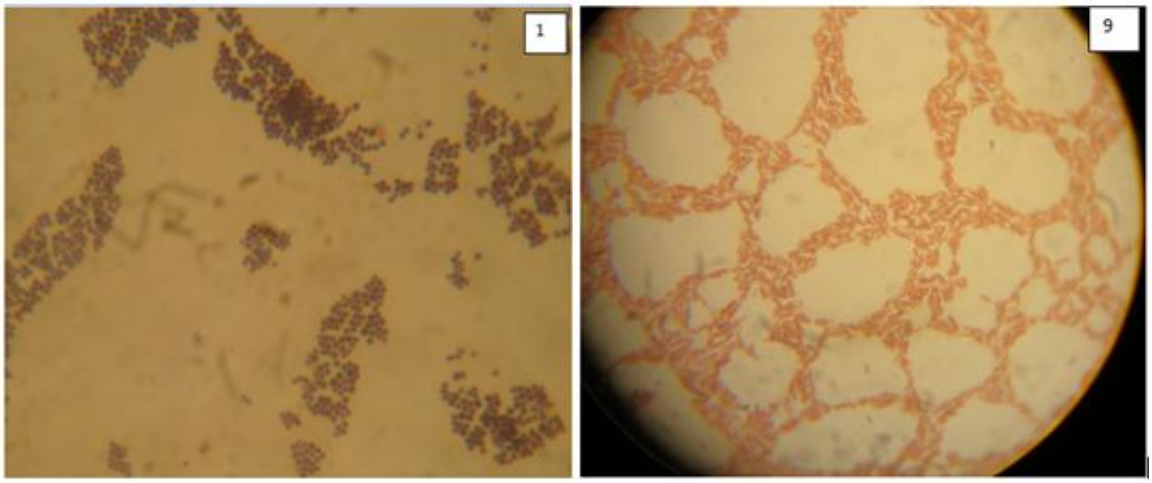

Plate 3.1: Gram test; Photographs showing Gram reaction of selected isolates from banana rhizosphere in Juja. Gram positive bacteria (1 - *Staphylococcus* sp.), Gram negative ones appear red/pink (9 - *Chryseobacterium* sp.)

The bacteria that were Gram positive retained crystal violet stain which is the primary stain during the decolourisation process and appeared purple when seen under a microscope while those bacteria that were Gram negative lost the primary stain during decolourisation and in turn took up safranin which is the secondary stain to appear red as seen under the microscope. The soil contained both the Gram negative and Gram positive bacteria (Table 3.1).

3.3 Biochemical Characterization of Bacterial rhizospheric Isolates

Various biochemical tests for all the 20 bacterial rhizospheric isolates were carried out, and the results are recorded as seen in (Table 3.1).

Table 3.1: Morphology, physiological and biochemical characterization of twenty rhizosphere bacteria isolated in 2012 from banana farms in Juja, Kenya

Serial no	Isolate code	Gram test	Morphology	Catalase test	Urease test	Nitrate reduction	Citrate utilization	Hydrogen sulfide production	Phosphate solubilisation	Methyl Red	Voges Proausker	Acetylene Reduction Assay	Indole Acetic Acid	Gelatin hydrolysis	Casein hydrolysis	Starch hydrolysis	Probable identity
1	F1RWL	+	Cocci	+	+	-	-	-	+	+	+	+	-	+	+	+	*Staphylococcus* sp.
2	F1RWL	-	Rods	+	-	-	-	-	-	-	-	+	-	+	+	+	*Chryseobacterium* sp.
3	F2RNY	+	Cocci	+	+	-	-	-	-	+	-	+	-	-	+	-	*Staphylococcus* sp.
4	F1RWL	+	Cocci	+	+	-	-	-	+	+	+	+	-	+	+	+	*Staphylococcus* sp.
5	F5RNY	+	Rods	+	-	-	-	-	+	+	-	+	-	+	+	+	*Bacillus* sp.
6	F6RIS	+	Rods	+	-	+	-	-	+	+	-	+	-	+	+	+	*Bacillus* sp.
7	F5RMS	+	Rods	+	-	-	+	-	+	+	-	+	-	+	+	-	*Bacillus* sp.
8	F4RKB	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
9	F5RMS	-	Rods	+	+	-	-	-	+	-	-	-	-	+	+	+	*Chryseobacterium* sp.
10	F1RGC	+	Rods	+	-	-	+	-	+	+	+	+	-	+	+	-	*Bacillus* sp.
11	F1RWL	+	Rods	+	+	-	+	+	+	-	-	+	-	+	+	+	*Streptomyces* sp.
12	F1RGN	+	Rods	+	-	+	+	-	+	+	+	+	-	+	-	-	*Paenibacillus* sp.
13	F1RGC	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
14	F4RNY	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
15	F6DGG	+	Rods	+	-	-	+	+	+	+	+	+	-	+	+	+	*Bacillus* sp.
16	F6RKB	+	Rods	+	-	-	+	-	+	+	+	+	-	+	+	-	*Bacillus* sp.
17	F1RVL	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
18	F1RWL	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
19	F6RMB	-	Rods	+	-	-	+	-	+	-	-	+	-	-	+	-	*Pseudomonas* sp.
20	F5RNY	+	Rods	+	+	-	+	+	+	-	-	+	-	+	+	+	*Streptomyces* sp.

Key: F1-Farm 1; F2-Farm 2; F3-Farm 3; F4-Farm 4; F5-Farm 5; F6-Farm 6; F7-Farm 7; R-ripening; D-Dessert; WL- Williams; NY-Nyoro; IS-Israel; MS-Mshirain; GC-Giant Cavendish; GN- Grand naine; KB- Kibuu; GG- Gichagara; VL- Vallary; MB-Mboo; + (positive); - (negative).

There were both the negative and positive outcomes observed for the citrate utilization test, urease test, phosphate solubilisation test, hydrogen sulfide production test, Methyl Red, Voges-Proskauer test, IAA production test, Acetylene reduction assay, Catalase test, denitrification test, casein hydrolysis, gelatin hydrolysis, and starch hydrolysis tests.

3.3.1 Assessment of Catalase Activity in Rhizosphere Isolates

In this study all the 20 isolates were seen to be catalase positive (Table 3.1) as observed by the formation of bubbles when hydrogen peroxide was added to the cultures. Hydrogen peroxide is decomposed to water and oxygen by catalase. Formation of bubbles indicates a positive result on addition of hydrogen peroxide to the cultures.

3.3.2 Assessment of Utilisation of Citrate by the Rhizosphere Bacteria

For citrate utilization test 13 isolates were shown to test positive and the other seven isolates gave a negative result (Table 3.1, Plate 3.2E). The media turns to Prussian blue from green indicating positive test for citrate utilization due to Bromothymol blue indicator which is incorporated. For a negative test there is no change in color. The positive isolates from the rhizosphere for citrate utilization were both Gram negative and Gram positive.

3.3.3 Assessment of the Production of Hydrogen Sulphide by the Rhizosphere Bacteria

Of all the isolates only three were positive for hydrogen sulphide production test the rest 17 were negative (Table 3.1). The blackening of the media indicates a positive result for the hydrogen sulfide production.

3.3.4 Nitrate Reduction Test

Seven isolates were positive for nitrate reduction test since the addition of sulphanilic acid and alpha- naphthylamine reacted with nitrite released from nitrate and turned red

in colour and 13 were negative for the test since they were yellow (Table 3.1, Plate 3.2A).

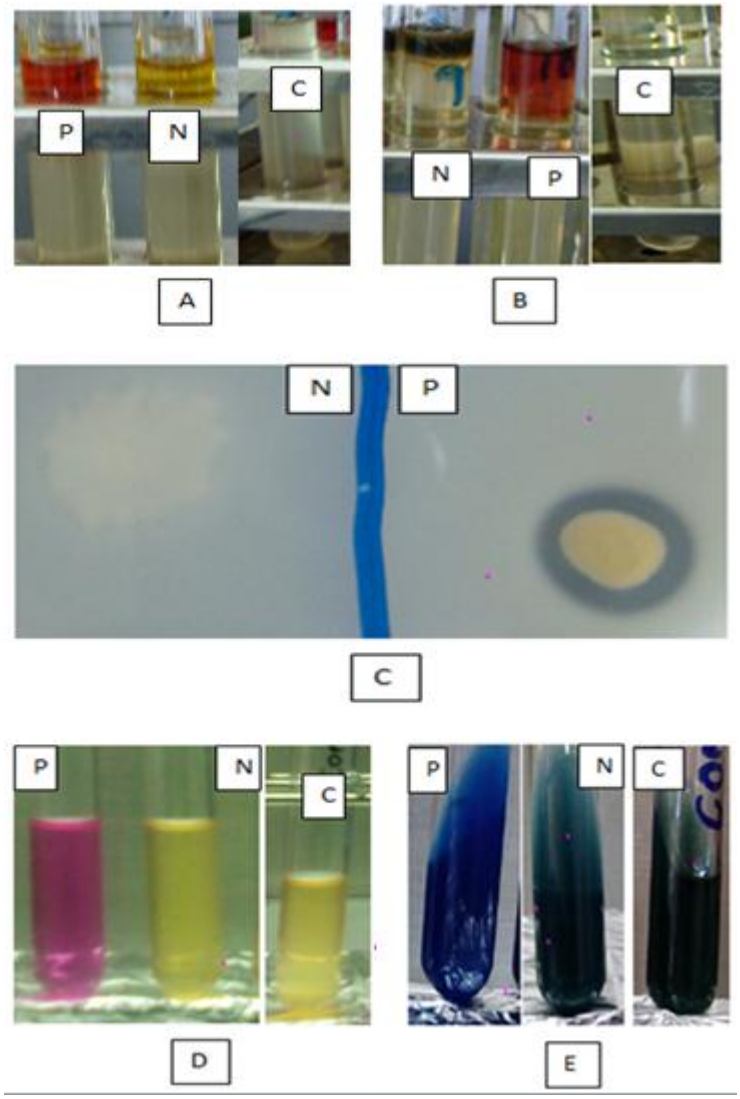

Plate 3.2: Photographs showing (A) Nitrate reduction test, (B) Voges- Proskauer test, (C) phosphate solubilisation test, (D) Urease test and (E) citrate utilization test as part of biochemical tests carried out

Legend: C-control, N- negative, P- positive

3.3.5 Methyl Red-Voges-Proskauer Test (MR-VP)

Fifteen of the isolates showed a positive result while five gave negative result for the methyl red test. Further, eleven of the isolates were positive, while the remaining nine tested negative for the Voges-Proskauer test (Table 3.1, Plate 3.2B). Formation of red color in methyl red test did indicate a positive result, while a negative test was indicated by the absence of red coloration. Upon addition of Barrit's reagent, formation of pink color indicated a positive result for Voges-Proskauer test while absence of red color formation was an indication of a negative test. The bacteria that were methyl red positive constituted both Gram negative and positive. The Voges-Proskauer positive isolate was a Gram positive bacterium.

3.3.6 Assessment of Indole Acetic Acid Production by Rhizosphere Bacterial Isolates

The results obtained showed that all the isolates were not indole acetic acid producers (Table 3.1). On addition of few drops of Salkowski's reagent into the cell free broth, a pink color is formed which is a positive test, but after incubation for thirty minutes at room temperature no color change was seen indicating a negative result.

3.3.7 Screening of Rhizosphere Bacteria for Ability to Solubilize Phosphorous

For phosphate solubilisation, 18 isolates tested positive while the remaining two gave a negative result (Plate 3.2C, Table 3.1). When there is a halo zone around the bacterial colonies after incubation of plates for fourteen days at 30°C it implies that it is a positive result for phosphate solubilisation.

3.3.8 Assessment of Activity of Urease on the Rhizosphere Bacterial Isolates

A rose pink color was observed for six isolates and a yellow color for the remaining 14 isolates when the isolates were inoculated on Christein's urea broth and incubated. These results obtained implied that the positive isolates for this test were six while those

that were negative for this test were fourteen (Table 3.1, Plate 3.2D). The pH indicator phenol red in Christensen's urea under alkaline conditions makes the media turn rose pink implying that it is a positive test. But under acidic conditions the indicator is yellow which implies that it is a negative test (Cappuccino and Sherman, 2002).

3.3.9 Screening of the Rhizospheric Bacterial Isolates for Nitrogen Fixation

Acetylene was reduced to ethylene through the following equation:

$C_2H_2 + 2H^+ + 2e^- \longrightarrow C_2H_4$

The time for retention of acetylene on gas chromatography was 1.5 min. It was also observed that the time for retention for ethylene gas (standard) on the gas chromatograph was 1.367 min while the one for the experimental isolate was 1.392 min. For Acetylene Reduction Assay, 19 isolates tested positive (Table 3.1) while one was negative. This was established when retention times on chromatographs of the standard (Plate 3.3) was compared with experimental sample. Both Gram negative and Gram positive isolates obtained in this study were positive for Acetylene Reduction Assay.

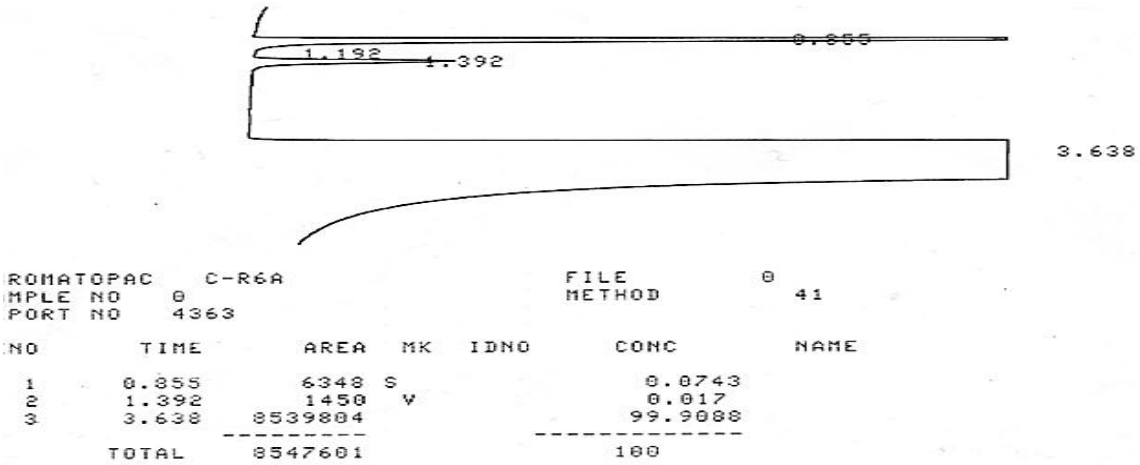

Plate 3.3: Standard chromatograph for showing the retention time (1.392) of ethylene.

3.4 The Potential of the Bacterial Isolates to Fix Nitrogen, Solubilize Phosphate or Produce Auxins

Nineteen bacterial isolates were observed to grow on the solid N-free medium (Table 3.1), thus they are capable of fixing atmospheric nitrogen. All *Bacillus* isolates were found to have nitrogenase activity and one of the *Bacillus* sp. showing the highest concentration of 0.7547 µl/ml followed by another with 0.1515 µl/ml indicating their potential in fixing nitrogen. *Chryseobacterium* spp. showed a nitrogenase activity of 0.069 µl/ml and 0.0145 µl/ml which could be a potential nitrogen fixer since members of these genus exhibit plant-growth promoting activities as they are universal symbionts of some higher plants. Eighteen bacterial isolates were positive for phosphate solubilization test. This was observed by the formation of a visible halo zone around the colonies on the agar plates containing NBRIP medium within 14 days after inoculation. The *Bacillu s*and *Pseudomonas* spp. isolates showed strong phosphorus solubilization activity than the rest of the isolates. *Pseudomonas* sp. had phosphate solubilization activity of 1.6 being the highest, followed by *Bacillus* spp. having 1.5 and 1.45 (Table 3.2). The bacterial isolates obtained were not able to produce auxins since there was no pink color observed after the addition of Salkowski's reagent.

Table 3.2: Phosphate solubilisation on agar and ethylene production by bacterial isolates from Juja, Kenya

Serial No.	Isolate code	Mean Phosphate solubilization index in agar assay	Mean Concentration of Ethylene (µl/ml)
1	F1RWL	1.33	0.0309
2	F1RWL	-	0.0145
3	F2RNY	-	0.0113
4	F1RWL	1.25	0.0474
5	F5RNY	1.33	0.0252
6	F6RIS	1.2	0.0635
7	F5RMS	1.25	0.0706
8	F4RKB	1.25	0.0510
9	F5RMS	1.33	-
10	F1RGC	1.33	0.0067
11	F1RWL	1.11	0.1459
12	F1RGN	1.2	0.0611
13	F1RGC	1.33	0.0339
14	F4RNY	1.2	0.0669
15	F6DGG	1.25	0.1515
16	F6RKB	1.5	0.7547
17	F1RVL	1.11	0.0466
18	F1RWL	1.42	0.0631
19	F6RMB	1.6	0.0508
20	F5RNY	1.2	0.0508

Key: F1-Farm 1; F2-Farm 2; F3-Farm 3; F4-Farm 4; F5-Farm 5; F6-Farm 6; F7-Farm 7; R-Ripening; D-Dessert; WL- Williams; NY-Nyoro; IS-Israel; MS-Mshirain; GC- Giant Cavendish; GN- Grand naine; KB- Kibuu; GG- Gichagara; VL- Vallary ; MB- Mboo.

Phosphate Solubilisation Index=Total diameter/Colony diameter.

3.5 Identification of Banana Rhizospheric Isolates

After characterization and identification process the isolates were grouped into; *Chryseobacterium*, *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Paenibacillus* and *Streptomyces* based on Bergey's manual (7^{th} Edition).

3.6 Molecular Characterization of the Rhizospheric Isolates

Based on the similarities in the biochemical and morphological characteristics, all the isolates were able to be classified into six groups. Twenty isolates were obtained from the samples collected from Juja. Only bacterial rhizosphere isolates which had the potential to enhance plant growth were selected for molecular characterization. The attributes considered to be most important included Acetylene reduction assay (nitrogen fixation), phosphate solubilisation, production of IAA, and activity of some enzymes like Catalase, nitrate reductase and urease. There were some other biochemical tests which were taken into consideration in order to identify any differences seen among the isolates. The genomic DNA for all the obtained twenty isolates was isolated and then amplified by using the primers 27F and 1492R. These primers targeted the 16S rDNA region of the genomic DNA. The expected band of 1500 bp for all the twenty isolates was amplified and representatively presented as seen in Plate 3.4.

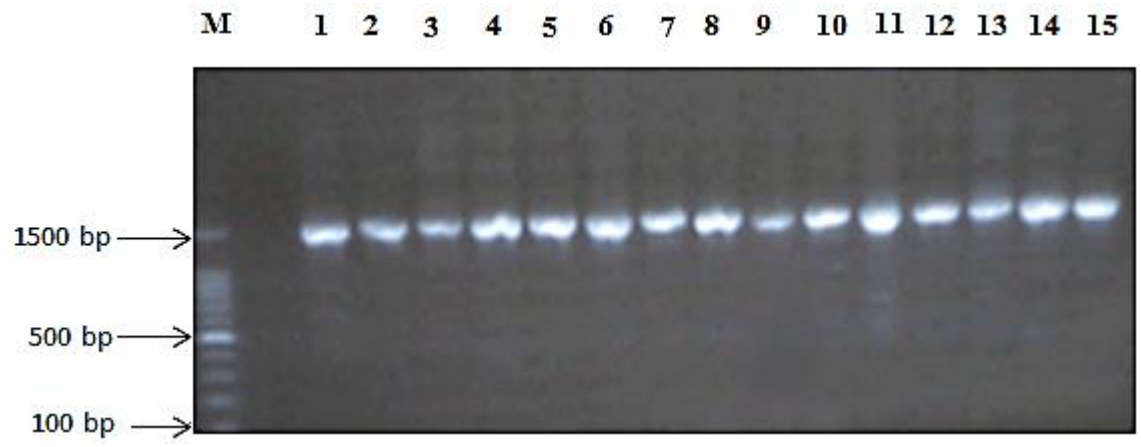

Plate 3.4: PCR products of rhizospheric isolates from Juja using universal primers bac 27F and bac 1492R on a 1.0% agarose gel. M is a 1kb DNA ladder (Biolabs) used as a molecular size marker. Lane 1-15 denotes some of the PCR products obtained after purification.

3.6.1 Sequencing of the PCR Products

The PCR products of all the twenty isolates were taken for sequencing which was done by a commercial service provider (Macrogen, South Korea). The morphological characteristics and the biochemical characteristics of the isolates were taken into consideration in order to identify if there is any notable differences between the isolates. The partial sequences for the 16S rRNA genes were deposited in the DNA DATA BANK of JAPAN and assigned accession numbers KP797914 to KP797931 (Table 3.3).

Table 3.3: Taxonomic affiliation and percentage sequence similarities of bacterial isolates with closest relatives from Genbank database

Sample ID.	Sample accession No.	Source	Closest relatives accession No.	Taxonomic affiliation	% ID.
Bacto1	KP797914	Banana rhizosphere	KF015518	***Staphylococcus gallinarum***	100
Bacto2	KP797915	Banana rhizosphere	KC853202	***Chryseobacterium* sp.**	100
Bacto4	KP797916	Banana rhizosphere	KF010812	***Staphylococcus saprophyticus***	98
Bacto5	KP797917	Banana rhizosphere	KM624612	***Bacillus flexus***	100
Bacto6	KP797918	Banana rhizosphere	KJ009393	***Bacillus firmus***	100
Bacto7	KP797919	Banana rhizosphere	KJ719344	***Bacillus marisflavi***	100
Bacto8	KP797920	Banana rhizosphere	JQ695929	***Bacillus licheniformis***	98
Bacto10	KP797921	Banana rhizosphere	KF923459	***Bacillus pumilus***	98
Bacto11	KP797922	Banana rhizosphere	FJ857946	***Streptomyces* sp.**	99
Bacto12	KP797923	Banana rhizosphere	KF554095	***Paenibacillus xylanilyticus***	100
Bacto13	KP797924	Banana rhizosphere	KJ920932	***Bacillus aquimaris***	100
Bacto14	KP797925	Banana rhizosphere	JX155762	***Bacillus cereus***	100
Bacto15	KP797926	Banana rhizosphere	KC741546	***Bacillus sonorensis***	100
Bacto16	KP797927	Banana rhizosphere	KF923459	***Bacillus pumilus***	98
Bacto17	KP797928	Banana rhizosphere	JX155762	***Bacillus cereus***	100
Bacto18	KP797929	Banana rhizosphere	HQ009875	***Bacillus megaterium***	100
Bacto19	KP797930	Banana rhizosphere	KF475842	***Pseudomonas monteilii***	100
Bacto20	KP797931	Banana rhizosphere	KJ152148	***Streptomyces* sp.**	99

3.6.2 Phylogenetic Analysis of the Sequences for the PCR Products

Out of 20 total isolates (prefixed in the Table 3.3 and phylogenetic tree as Bacto in Figure 3.1) sequenced, 18 of the isolates were included into the phylogenetic tree. The other two sequences had chimeras and were excluded in the subsequent analysis. Gene sequences that were obtained were analyzed by using MEGA V5.10 package (www.megasoftware.net) in order to generate the phylogenetic tree which was used to identify the isolates belonging to different genera. The 16S rDNA sequence analysis suggested that isolates 1 and 4 are phylogenetically related to *Staphylococcus* spp. with 100% sequence similarity (Figure 3.1).

Phylogenetic analysis of isolates 5, 6, 7, 8, 10, 13, 14, 15, 16, 17 and 18 suggested that they were related to members of the genus *Bacillus* (Figure 3.1) which tested positive in catalase test, phosphate solubilisation, ARA, gelatin and casein hydrolysis but negative for urease and IAA. Isolate 5 was closely related to *Bacillus flexus* with 100% sequence similarity, isolate 6 was closely related to *Bacillus firmus* with 100% sequence similarity, isolate 7 was a close relative to *Bacillus marisflavi* with 100% sequence similarity, isolate 8 was related to *Bacillus licheniformis* strain CRRI-HN-2 with 98% sequence similarity, isolate 10 and 16 were closely related to *Bacillus pumilus* with 98% sequence similarity, isolate 13 was *Bacillus aquimaris* with 100% sequence similarity, isolate 14 and 17 were related to *Bacillus cereus* with 100% sequence similarity, isolate 15 was *Bacillus sonorensis* strain MBCU2 with 100% sequence similarity, and finally isolate 18 was closely related to *Bacillus megaterium* with 100% sequence similarity (Figure 3.1).

All these isolates that were found to belong to the genus *Bacillus* were seen to be of Gram positive. Isolate 11 and 20 were phylogenetically related to *Streptomyces* sp., with 99% sequence similarity. Isolate 12 was a closely relative to *Paenibacillus xylanilyticus* with 100% sequence similarity. Positioning of some isolates phylogenetically showed that isolate 19 was related to *Pseudomonas monteilii* strain IHB B 2329at 100% sequence similarity (Figure 3.1).

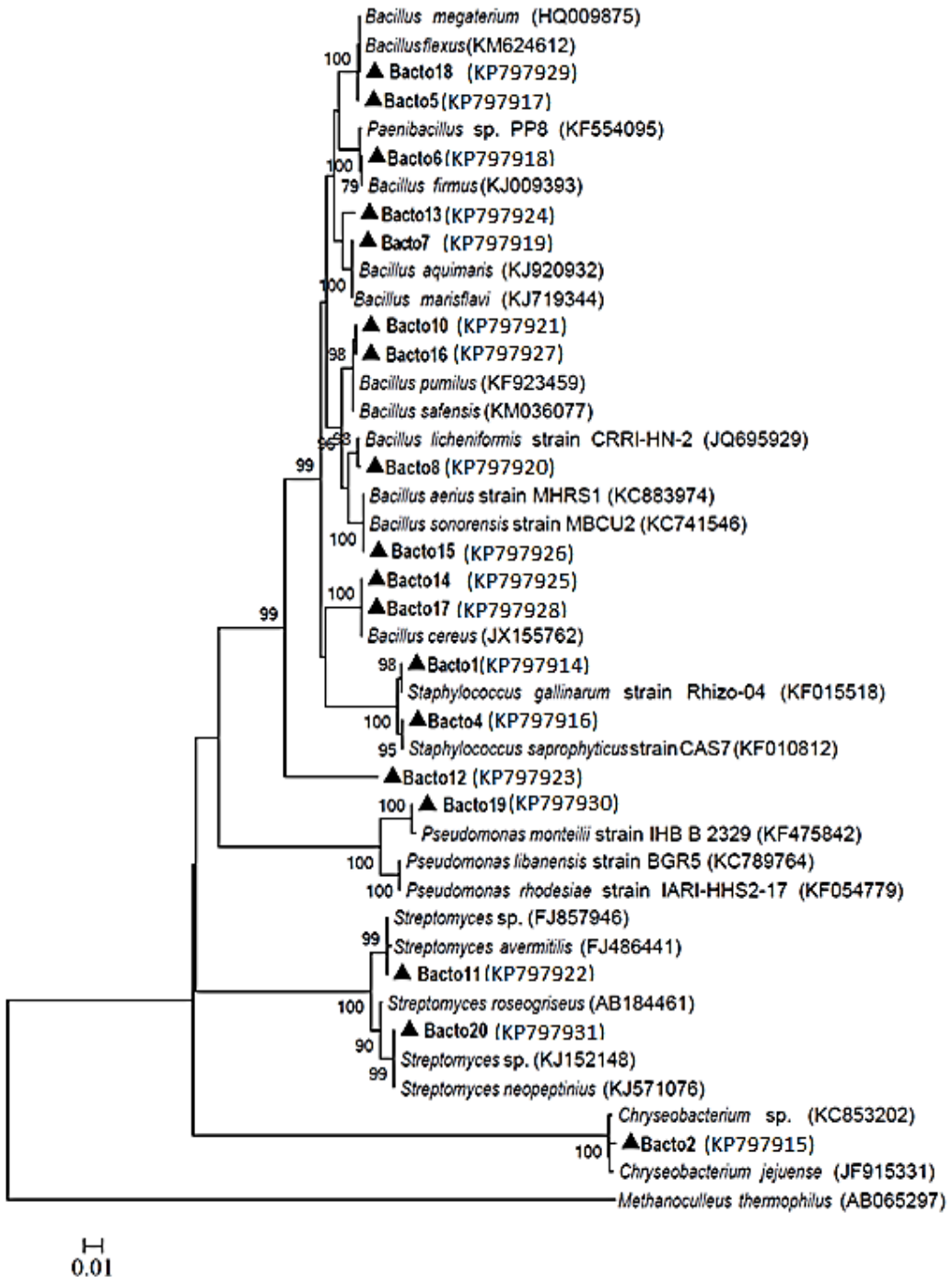

Figure 3.1: Neighbour joining phylogenetic tree showing the position of the banana rhizosphere isolates from Juja, Kenya. Eighteen strains from seven different farms in

Juja that were isolated in this study are indicated in bold font as Bacto. The accession numbers of the sequences are indicated in parantheses.

3.7 Assessment of Effect of Selected Isolates on Banana Plant Growth

Inoculation of the plants with five selected isolates had a positive growth influence on tissue cultured banana plantlets treated with 5gms, 7.5gms and 10gms levels of NPK fertilizer levels in height, diameter, number of green leaves and mass in dry weight compared with their respective controls. The mean plant diameter (cm) as seen from Figure 3.2 was higher in all plantlets as compared to the control, with the plantlet inoculated with *Bacillus flexus* recording the highest diameter of 3.34 cm when taking into consideration 10 gms fertilizer level. The plantlet inoculated with *Pseudomonas monteilli* had the highest mean plant height of 70.2 cm (Fig 3.3) and in the mean number of green leaves, plantlet inoculated with *Streptomyces* sp. showed the highest mean of 9.17 (Fig 3.4). For mean mass in dry weight, plantlet treated with *Bacillus megaterium* showed the highest mean of 54.8 gms at 10gms fertilizer level (Fig 3.5).

In the mean plant height, there was a notable increase in all the plantlets treated with different isolates as compared to the control in all the different levels of fertilizers used (Fig 3.3). As the levels of fertilizers were increased, there was also an increase in the mean diameter, height, number of leaves and even in mass in dry weight with few exceptions. For example in the mean mass in dry weight, there was significant increase in all the plantlets treated with different isolates at all levels of fertilizer used as compared to the control except in the plantlet treated with *Bacillus flexus* (Fig 3.5). There were significant differences in the plantlet's mean height ($p<0.0001$) and mean mass in dry weight ($p=0.0006$) in all the five tested plantlets treated with the different strains as compared to the control. The plantlet treated with *Bacillus megaterium* had a significant difference in mean number of green leaves ($p=0.000$). Plantlets treated with *Streptomyces* sp. registered a significantly higher growth in all parameters ($p<0.05$).

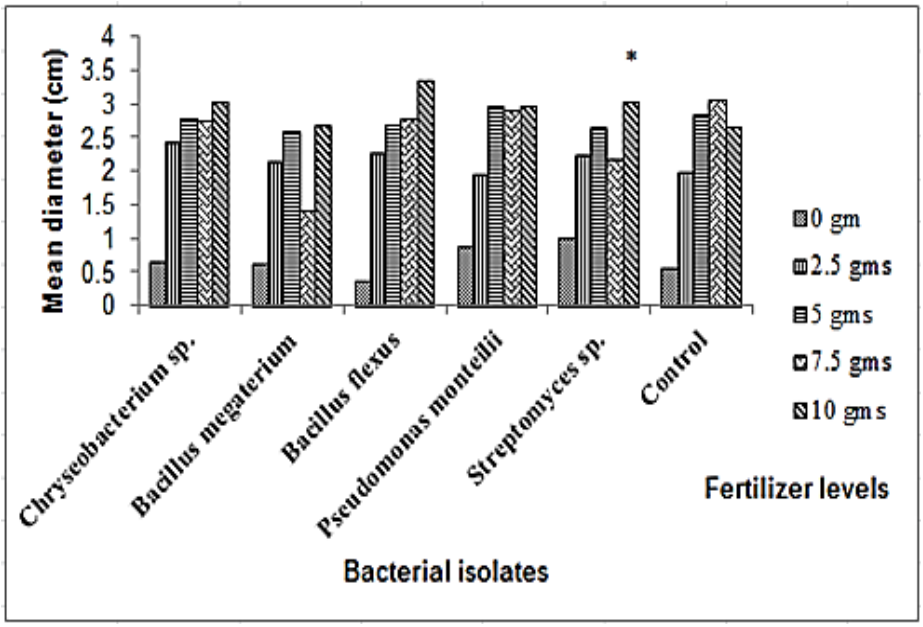

Figure 3.2: Effect of selected bacterial isolates from Juja, Kenya on banana plant diameter after 3 months of growth

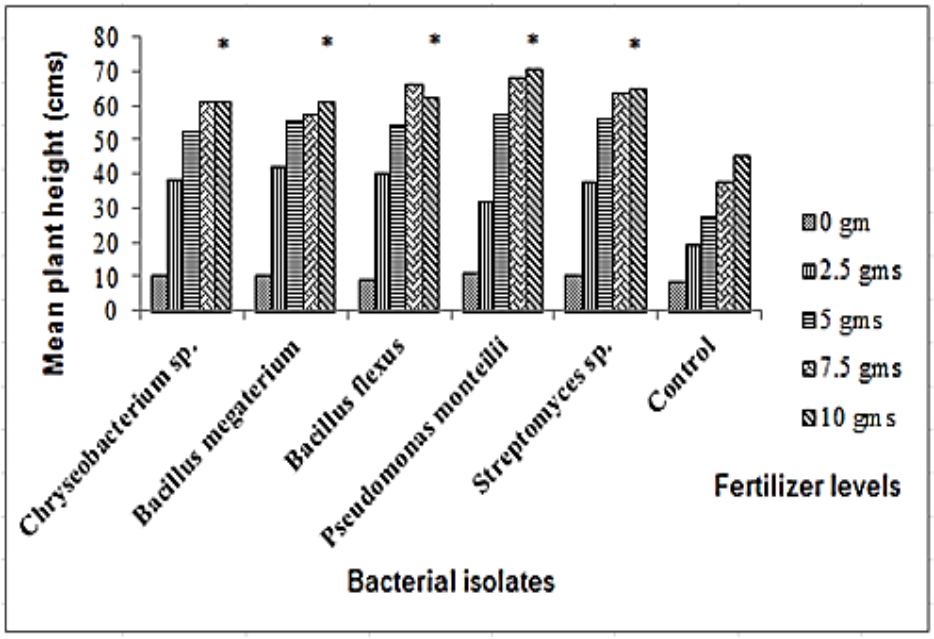

Figure 3.3: Effect of the selected bacterial isolates from Juja, Kenya on banana plant height after 3 months of growth

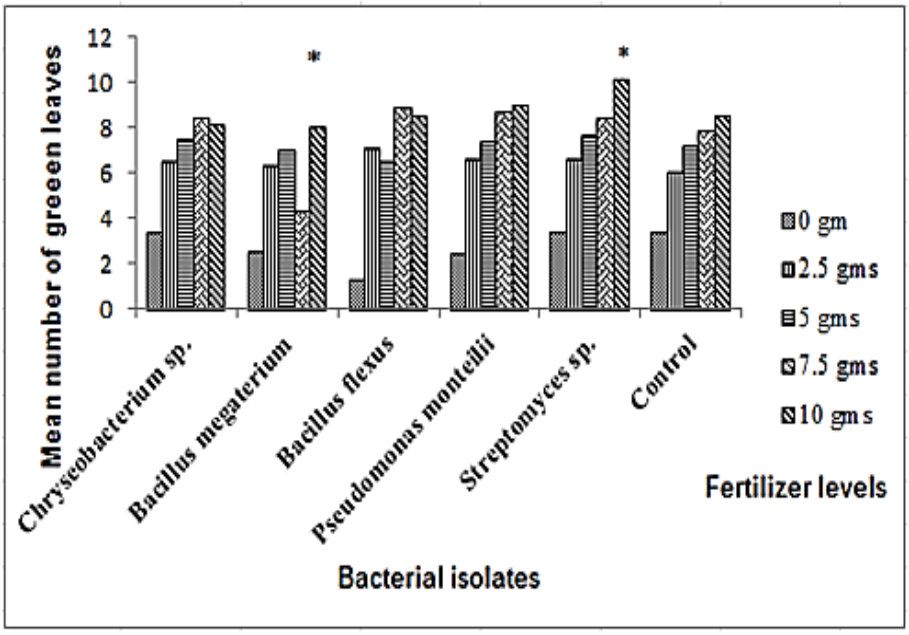

Figure 3.4: Effect of the selected bacterial isolates from Juja, Kenya on banana plant's number of green leaves after 3 months of growth

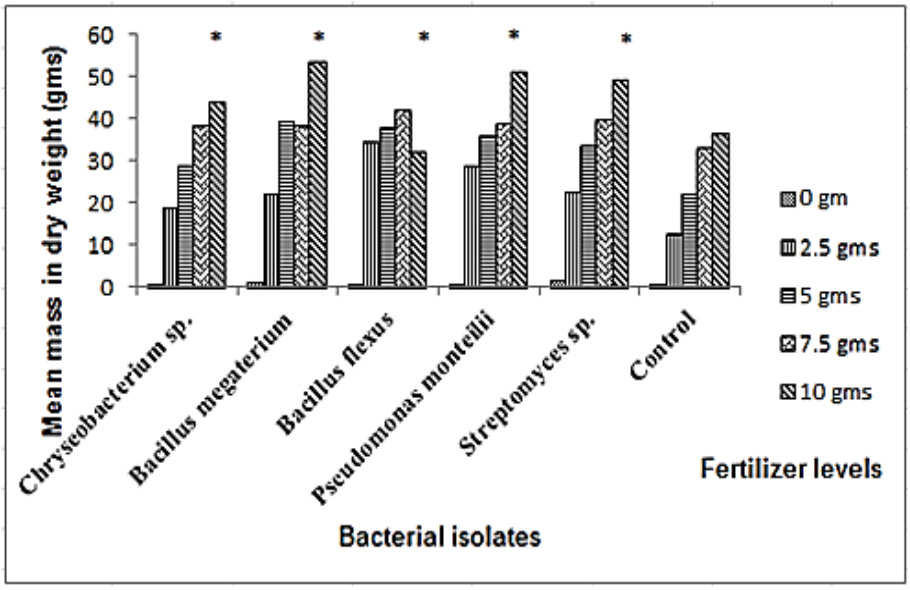

Figure 3.5: Effect of the selected bacterial isolates from Juja, Kenya on banana plant's mass in dry weight after 3 months of growth.

The levels of the nutrients in most of the banana plantlets used were relatively higher as compared to the controls. This was observed in all the analyzed samples. For example taking into consideration the fertilizer levels of 10gms, banana plantlet inoculated with *Chryseobacterium* sp. showed the highest growth in nitrogen and phosphorus which was 3.5ppm and 26.1ppm respectively. Banana plantlet inoculated with *Bacillus megaterium* had the highest potassium concentration of 10.1ppm with the least recording at 5.1ppm from the plantlet inoculated with *Pseudomonas monteilli*. Levels of potassium (5.1-10.1ppm), magnesium (2.43-5.97ppm), phosphorus (9.07-26.1ppm) and nitrogen (1.37-3.50ppm) were significantly different from each other at 10gms fertilizer level. Levels of iron (0.13-0.63ppm), copper (0.002-0.005ppm) and manganese (0.03-0.14ppm) were insignificantly different from one another. Levels of iron were significantly less as compared to their control (Table 3.4).

There were significant effects within the plantlets inoculated with the isolates in nutrient analysis in parameters such as nitrogen which had a p-value of 0.00000684, phosphorus had 0.000163, magnesium had 4.46E-09 and calcium 3.77E-05 which were significantly different (Appendix 1-7).An increase in the levels of fertilizers used, significantly influence the level of nutrients in the plantlets treated with the bacterial strains as compared to the control. The control plantlets showed a minimal increase in the levels of nutrients as the levels of fertilizers were gradually increased. For instance, the level of phosphorus for the control was not significantly different for all the fertilizer levels used. Whereas the levels of phosphorus in the plantlets treated with the isolates showed a significant difference in all the levels of fertilizers added. For example, the plantlet treated with *Chryseobacterium* spp. showed an increase of phosphorus nutrients from 10.5-26.1pmm, with the gradual increase of fertilizer from 0-10gms fertilizer levels (Table 3.4).

Table 3.4: Levels of mineral elements in banana plantlets (Variety- Grand-naine) cultivated using the selected bacterial isolates at JKUAT from Oct, 2013 - Jan 2014.

		Fertilizer levels				
Mineral elements (pmm)	**Samples**	**0 gms**	**2.5 gms**	**5 gms**	**7.5 gms**	**10 gms**
K	*Chryseobacterium* sp.	4.00±2.0	5.87±1.0	10.6±1.8	9.13±1.6	6.23±1.1
	Bacillus megaterium	6.86±2.8	7.60±4.1	6.27±0.9	7.00±0.9	10.1±3.2
	Bacillus flexus	8.20±5.8	7.30±2.9	5.33±2.7	10.8±1.9	8.73±4.1
	Pseudomonas monteilii	4.47±1.9	4.80±2.0	9.97±4.1	5.80±5.1	5.10±2.2
	Streptomyces sp.	3.17±2.8	4.90±0.6	11.5±10.5	10.2±3.1	6.03±1.2
	Control	6.80±1.2	6.93±2.3	6.86±1.2	5.43±1.1	7.50±0.6
Ca	*Chryseobacterium* sp.	0.36±0.1	1.79±0.1	1.48±0.6	1.82±0.6	1.49±0.9
	Bacillus megaterium	0.95±0.9	1.57±0.4	1.67±0.2	2.22±0.4	1.99±0.8
	Bacillus flexus	0.41±0.3	1.59±0.1	1.77±0.3	1.64±0.4	2.11±0.0
	Pseudomonas monteilii	0.92±0.8	1.76±0.3	1.31±0.2	2.95±2.2	1.74±0.8
	Streptomyces sp.	0.90±0.3	1.55±0.6	2.08±0.4	2.05±0.1	1.71±0.6
	Control	1.45±0.6	1.31±0.8	1.07±0.1	1.07±0.3	1.07±0.3
Mg	*Chryseobacterium* sp.	0.41±0.4	2.15±1.6	3.01±1.1	5.19±1.3	4.02±2.0
	Bacillus megaterium	2.01±2.8	4.29±0.4	4.34±1.5	5.40±0.9	3.78±0.7
	Bacillus flexus	0.61±0.3	4.56±0.7	3.31±1.6	4.15±2.3	4.19±0.4
	Pseudomonas monteilii	0.67±0.3	4.98±1.8	7.40±3.7	3.52±0.7	3.77±2.0
	Streptomyces sp.	0.38±0.1	5.08±1.2	4.63±0.4	4.58±0.8	5.97±1.2
	Control	2.00±0.9	2.78±0.3	2.52±0.5	2.61±0.6	2.43+0.7

		Fertilizer levels				
Mineral elements (pmm)	**Samples**	**0 gms**	**2.5 gms**	**5 gms**	**7.5 gms**	**10 gms**
Zn	*Chryseobacterium* sp.	0.19±0.2	0.07±0.0	0.09±0.0	0.16±0.0	0.06±0.1
	Bacillus megaterium	0.11±0.0	0.39±0.3	0.13±0.1	0.18±0.1	0.14±0.0
	Bacillus flexus	0.07±0.1	0.10±0.0	0.18±0.1	0.28±0.1	0.09±0.0
	Pseudomonas monteilii	0.10±0.1	0.08±0.0	0.17±0.1	0.26±0.2	0.27±0.1
	Streptomyces sp.	0.10±0.1	0.07±0.0	0.11±0.0	0.17±0.1	0.21±0.1
	Control	0.10±0.1	0.09±0.0	0.13±0.1	0.12±0.1	0.09±0.0
Mn	C*hryseobacterium* sp.	0.13±0.2	0.13±0.2	0.27±0.2	1.05±1.5	0.09±0.0
	Bacillus megaterium	0.05±0.0	0.16±0.0	0.21±0.1	0.40±0.5	0.06±0.0
	Bacillus flexus	0.004±0.0	0.20±0.1	0.16±0.1	0.33±0.4	0.07±0.1
	Pseudomonas monteilii	0.05±0.0	0.16±0.0	0.14±0.2	0.14±0.2	0.14±0.1
	Streptomyces sp.	0.24±0.2	0.17±0.1	0.20±0.1	0.20±0.1	0.08±0.1
	Control	0.06±0.0	0.05±0.0	0.04±0.0	0.05±0.0	0.03±0.0
Cu	*Chryseobacterium* sp.	0.003±0.0	0.003±0.0	0.005±0.0	0.004±0.0	0.005±0.0
	Bacillus megaterium	0.004±0.0	0.002±0.0	0.004±0.0	0.003±0.0	0.005±0.0
	Bacillus flexus	0.005±0.0	0.008±0.0	0.004±0.0	0.003±0.0	0.002±0.0
	Pseudomonas monteilii	0.001±0.0	0.005±0.0	0.001±0.0	0.006±0.0	0.002±0.0
	Streptomyces sp.	0.005±0.0	0.008±0.0	0.011±0.0	0.003±0.0	0.005±0.0
	Control	0.0004±0.0	0.003±0.0	0.004±0.0	0.0004±0.	0.005±0.0

		Fertilizer levels				
Mineral elements (pmm)	**Samples**	**0 gms**	**2.5 gms**	**5 gms**	**7.5 gms**	**10 gms**
P	*Chryseobacterium* sp.	10.5±8.5	12.8±1.5	14.6±2.7	11.3±7.7	26.1±17.5
	Bacillus megaterium	8.54±7.7	12.9±3.2	10.2±4.2	16.3±1.8	19.2±1.2
	Bacillus flexus	1.84±0.4	11.1±4.3	10.3±6.9	13.7±4.4	18.0±2.7
	Pseudomonasmonteilii	6.07±2.9	13.0±3.2	14.7±2.0	16.1±2.8	14.0±2.9
	Streptomyces sp.	4.84±3.9	11.5±3.0	12.7±4.3	20.6±14.5	14.1±2.5
	Control	6.93±1.7	9.43±3.1	6.67±2.1	6.50±6.7	9.07±1.6
Fe	*Chryseobacterium* sp.	0.12±0.1	0.28±0.1	0.36±0.1	0.39±0.2	0.13±0.1
	Bacillus megaterium	0.54±0.6	0.43±0.5	0.20±0.1	0.14±0.1	0.19±0.2
	Bacillus flexus	0.04±0.0	0.34±0.3	0.43±0.5	0.22±0.1	0.13±0.1
	Pseudomonas monteilii	0.07±0.0	0.63±0.8	0.24±0.2	0.32±0.1	0.41±0.5
	Streptomyces sp.	0.15±0.1	0.37±0.2	0.27±0.2	0.30±0.4	0.13±0.0
	Control	0.26±0.2	0.15±0.1	0.37±0.1	0.26±0.0	0.63±0.2
N	*Chryseobacterium* sp.	0±0	0.77±0.7	0.77±0.7	1.60±0.3	3.50±2.6
	Bacillus megaterium	0.5±0.8	1.1±0.1	0.87±0.4	1.10±1.0	1.37±0.4
	Bacillus flexus	0±0	0.97±0.2	1.80±0.4	1.87±0.3	1.57±0.1
	Pseudomonas monteilii	0±0	1.83±0.6	1.63±0.2	1.43±1.3	1.90±0.1
	Streptomyces sp.	0±0	0.77±0.3	1.30±0.1	1.57±0.4	1.43±0.2
	Control	1.43±1.2	1.80±0.5	1.53±0.4	4.16±4.2	1.77±0.4

Key: Values indicate means ± standard deviation

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

4.1.1 Isolation and Identification of Rhizosphere Bacteria

The results from this study indicate that the banana rhizospheric soil collected from Juja farmers' fields contains rhizobacteria. Twenty isolates were obtained after the isolation processes. Studies have also shown that plant growth promoting rhizobacteria can be isolated from rhizosphere soils of different plants and crops. Plant growth promoting rhizobacteria have been isolated from banana plants rhizosphere as indicated by Apastambh, Tanveer, & Baig, (2016). These bacteria colonize the roots of the banana plants and in return support in promoting their growth. They help in nitrogen fixation and also in increasing the supply of other nutrients and even in the production of plant hormones. Different media have been used to culture different microorganisms and in this study, three different media were used for the isolation, characterization and identification of the isolates. Growth on nitrogen free media indicated that the isolates were able to fix atmospheric nitrogen as also indicated by Ngamau et al., (2012).

From the Acetylene reduction Assay, 19 isolates tested positive which shows their ability in reducing acetylene to ethylene a method widely used to measure nitrogenase activity in isolates, natural samples, and even cell free extracts (Cappuccino & Sherman, 2002). The isolates were also able to grow on nitrogen free media indicating that they have that potential of fixing the atmospheric nitrogen. Studies show that nitrogen is among the nutrients commonly required by the plant for their optimal growth and productivity (Ahemad & Khan, 2011). This is a very important and crucial element for bananas as nitrogen may be a limiting factor in the production and growth of bananas. The use of nitrogen fixing bacteria can be able to reduce the application of chemical fertilizers and as a result can lower the cost of production (Saharan & Nehra, 2011).

In the phosphate solubilisation test, 18 bacterial strains were positive which is an important trait associated with plant P nutrition (Chen *et al*., 2006). The *Bacillus* and *Pseudomonas* spp. in this study were shown to have the potential of phosphorus solubilization. Phosphate solubilizing species have been shown to solubilize the fixed soil phosphorus and the applied phosphates resulting in better plant development and higher yields (Canbolat, Bilen, Cakmakci, Ahin, & Aydın, 2006). Studies show that soil or seed inoculations with phosphate solubilizing microbes (PSM) have to a large extent been used for the enhancement of crop growth and production (Banerjee *et al*., 2010). Phosphorus is the mineral nutrient second only to nitrogen needed by the plant. During phosphate solubilisation, organic acids are produced which play a great role in mineralization of soil organic phosphorus (Vessey, 2003).

Six bacterial isolates were able to break down urea and give simple forms such as nitrogen that can be readily available and absorbed by the plant and promote their growth. This can be a very important aspect in the development and growth of banana since bacteria have this potential of converting urea to simpler forms absorbed readily by plants. None of the bacterial strains was able to produce Indole Acetic Acid. The ability of the organism in the rhizosphere to produce Indole Acetic Acid is dependent on the precursors available and microbial Indole Acetic Acid uptake by the plant. Promotion of growth may depend on other mechanisms like production of hormones in the rhizosphere (Joseph *et al*., 2007).

The 16S ribosomal DNA sequence analysis showed that the isolates belong to the genera *Staphylococcus*, *Chryseobacterium*, *Bacillus*, *Streptomycetes*, *Paenibacillus* and *Pseudomonas*. This was shown by morphological and biochemical data as well. The phylogenetic position of isolate 19 did show that it is related to *Pseudomonas monteilii*. This was supported by the results obtained from the morphological and biochemical characterization. In this study, the isolate was not able to produce Indole Acetic Acid but was positive for Acetylene Reduction Assay and phosphate solubilisation test. *P. monteilii* is one of the species that is dominant in banana rhizosphere. Some studies

show that *P. monteilii* may be a sub group of *Pseudomonas putida* which are ecologically important in the rhizosphere and also can be utilized in sustainable agriculture. *P. putida* and *Pseudomonas fluorescens* have been isolated from bananas and rice roots in Sri Lanka ((Vlassak, Holm, Duchatea, Vanderleyden, & Mot, 1995). *P. fluorescens* and other *Pseudomonas* sp. have the ability to fix nitrogen as reported (Gowda & Watanabe, 1985; Chan, Barraquio, & Knowles, 1994). Species of *P. fluorescens* help to maintain soil health and are functionally and metabolically diverse. *P. fluorescens* isolated from shoots, roots, and the rhizosphere soil of sugarcane give significant increase in dry and fresh masses (Mehnaz *et al.*, 2009). Specific strains of putida group have been recently used on crop plants as seed inoculants in promoting growth and in yield increase. The *Pseudomonads* have been shown to rapidly colonize plant roots of potato, sugar beet and even radish as well as cause statistically significant increases in yield up to 144% in field tests (Kloepper & Schroth, 1978).

Morphological, biochemical and phylogenetic analyses of other isolates suggested that they are closely related to members of the genus *Bacillus*. All the 11 isolates that were identified as *Bacillus* during this research were found to have the potential to fix nitrogen since they tested positive in Acetylene Reduction Assay. *Bacillus* species that have been used as biofertilizers have direct effects on the growth of plant as they synthesize plant growth hormones, fix nitrogen and solubilize phosphates (Amer & Utkhede, 2000; Sahin, Cakmakci, & Kantar, 2004 and Cakmakci, Kantar, & Sahin, 2001). Phosphate-solubilizing *Bacillus* spp. stimulates plant growth by enhancing phosphate nutrition, increasing the uptake of nitrogen, potassium, phosphorous and iron (Whitelaw, Hardenand, & Bender, 1997; Biswas, Ladha, & Dazzo, 2000). In this study the 11 isolates belonging to genus *Bacillus* were able to solubilize phosphorous, which together with the effect of nitrogen fixation boost the potential to enhance plant growth. The most abundant genus in the plant rhizosphere is *Bacillus* and the activities of PGPR of some of the strains have been known for several years (Ngamau *et al.*, 2012; Probanza, Lucas García, Ruiz Palomino, Ramos, & Gutiérrez Mañero, 2002; Gutiérrez

Mañero, Probanza, Ramos, Lucas, & García, 2003). Metabolites released by *Bacillus* strains increase the availability of nutrients for the plants (Charest, Beauchamp, & Antoun, 2005).

Bacillus magaterium is required in the improvement of several root parameters such as root length, rooting performance and dry matter content of roots in mint (Kaymak, Yarali, Guvenc, & Donmez, 2008). *B. megaterium* var. *phosphaticum* and potassium solubilising bacteria (KSB) *Bacillusmucilaginosus* if inoculated in nutrient limited soil consistently increase mineral availability, plant growth and uptake of pepper and cucumber suggesting its potential use as a fertilizer (Han, Supanjani, & Lee, 2006). *Bacillus subtilis* is present naturally in the immediate plant root vicinity and maintains stable contact with higher plants thereby promoting their growth. In the inoculation of *Bacillus licheniforms* on tomato and pepper plants there is a considerable colonization seen that qualifies it as a biofertilizer without alteration of normal management in the greenhouses (García, Probanza, Ramos, Palomino, & Mañero, 2004). *Bacillus* was also found to be potentially helpful in increasing the yield, growth and nutrition of raspberry plant grown under organic conditions (Orhan, Esitken, Ercisli, Turan, & Sahin, 2006). The *Bacillus pumilus* 8N-4 has been used as a bio-inoculant for production of biofertilizer in order to increase the yield of wheat crop variety *Orkhon* in Mongolia (Hafeez *et al*., 2006).

Isolate 12 was closely related to members of genus *Paenibacillus xylanilyticus*. The nitrogen-fixing *Bacillus* strains such as *B. polymyxa, B. macerans, B. azotofixans* have been assigned to *Paenibacillus*. Studies show that they are spore forming. *P. xylanilyticus* in this study showed plant growth promoting activities such as acetylene reduction assay and phosphate solubilisation. Some species of *Paenibacillu*s have a great capacity of fixing atmospheric nitrogen consistently *in vitro* (Jin, Jing, & Chen, 2011).

Isolate 11 and 20 were phylogenetically related to *Streptomyces* sp. It has been shown that actinomycetes are important microorganisms present in the rhizosphere that

antagonize phytopathogenic fungi (Cao, Qiu, You, Tan, & Zhou, 2005). Previous studies on interactions of plant and microbe in the rhizosphere indicate that root and nodule colonization by *Streptomyces* have led to increase in the average size of nodules and also in improvement of the vigor of bacteroids due to enhancement of iron nodules assimilation and some other nutrients from the soil (Cao, *et al*., 2005).

Isolate 2 was phylogenetically related to members of genus *Chryseobacterium*. Isolate 2 was positive for acetylene reduction assay but negative for indole acetic acid production and phosphate solubilisation tests. Studies have shown that the members of the *Chryseobacterium* genus are an important group of bacteria that are associated with plants and currently there is enough evidence which shows that these strains exhibit plant-growth promoting activities (Montero-Calasanz, *et al*., 2013). Recent studies do suggest that *Chryseobacterium* sp. can as well be used as PGPR. They are the universal symbionts of some higher plants and enhance the adaptative potentiality of their hosts by a variety of mechanisms, like fixing molecular nitrogen, mobilizing recalcitrant nutrients of the soil, synthesis of phytohormones and controlling of phytopathogens (Van Peer & Schippers, 1989; Lugtenberg, De Weger, & Bennett, 1991; Weller & Thomashow, 1994).

Isolate 1 and 4 were phylogenetically related to *Staphylococcus* spp. From this study, the isolates were positive for urease test, phosphate solubilisation and Acetylene Reduction Assay tests which proved that they are able to promote plant growth but were not able to produce IAA. Previous studies show that *Staphylococcus* and bacteria from other genera can stimulate crop growth when a major pathogen is absent by affecting plant metabolism directly (Bashan, Puente, Luz, & Hernandez, 2008). *S. saprophyticus* is efficient in the formation of biofilm and can also colonize the roots of the plant and stimulate plant growth against factors of biotic stress; however this organism needs amino acids for successful survival, growth and formation of biofilm (Qurashi & Sabri, 2011). More studies are yet to be carried out on the potential of this isolate in promoting plant growth.

4.1.2 Functional Potentiality of Bacterial Isolates as Biofertilizers

The isolates were tested for their ability to fix nitrogen, solubilize phosphates and produce auxins, 19 isolates tested positive for acetylene reduction assay test, while 18 tested positive for phosphate solubilisation while none of the isolates was positive for auxin production. Studies have shown that nitrogen fixation capability and plant growth promotion are useful criteria for rhizobacteria to be used effectively as a biofertilizer. When bananas are inoculated with rhizobacteria, they contribute a considerable amount of nitrogen produced by biological nitrogen fixation system. This was the first proposed mechanism in explaining plant growth improvement after inoculation with rhizosphere bacteria because of the increase in the number of nitrogenous compounds plus activity of nitrogenase in the inoculated plants (Mia *et al*., 2010a).

Phosphate-solubilizing bacteria have been used in solubilization of applied phosphates and in fixing soil phosphorus as a result producing higher crop yields (Banerjee *et al*., 2010). There are a lot of phosphate solubilizing microbes which have been isolated routinely from rhizospheric soil of different plants including bananas (Apastambh *et al*., 2016). The main mechanism for solubilization of mineral phosphate is organic acids production where acid phosphatases play an important role in organic phosphorus mineralization in soil. Previous studies show that strains from *Bacillus, Pseudomonas*, and *Rhizobium* genera are actually some of the best phosphate solubilizers (Khan, Rawat, & Izhari, 2011). This is also true with the findings of this study as many of the isolates were from *Bacillus* and *Pseudomonas* genera. Several reports show that bacteria that enhance plant growth have the ability of solubilizing organic and/or inorganic soil phosphate after they have been inoculated in plant seeds or soil. This is evidence to show their importance in plant nutrition and as a result in plant growth improvement and performance. Several inoculations have been done using *Bacillus firmus, Bacillus cereus* and *Bacillus polymyxa* which show increase in phosphate uptake as well as the yield in crops (Rodríguez, & Fraga, 1999).

The most effective growth hormone in plants is known as auxin, and indole acetic acid is one of the most common among the different hormones. Studies have shown that indole acetic acid production in rhizobacteria play an important role in the growth of host plant (Banerjee *et al.*, 2010). In this research the isolates were not able to produce auxin, but it is evident that microorganisms not only play a role of nutrient uptake and synthesis but also help in exporting phytohormones which in turn helps to promote plant growth (Fischer, Fischer, Magris, & Mori, 2007). In many cases, phytohormones change or affect the growth patterns in roots resulting in many branched roots that have a greater surface area. Indole acetic acid assists in root initiation, division of cells, cell enlargement and greater surface area which helps in acquiring more soil nutrients. This hormone is produced by the plant growth promoting rhizobacteria. Indole acetic acid production has really helped in promoting growth in host plants (Vessey, 2003).

4.1.3 Effects of the Selected Isolates on Banana Plant Growth

The use of biofertilizers especially ones prepared from PGPR has received a much greater attention due to their easy production, low cost and lack of environmental hazards. Therefore by considering these factors, searching for microbiota which can be able to express the plant growth activities have increased alarmingly. Therefore this study aimed at testing the influence of the selected isolates on tissue culture banana plantlets (Ahmad, Zaidi, & Khan, 2016). Rhizosphere bacterial isolates were inoculated to the roots of the tissue culture banana plantlets of Grand-naine variety and evaluated within three months to see the results in growth considering the parameters such as height, diameter, number of leaves and also the dry weight content.

The greenhouse study showed that the strains that were used had significant effects on mean plant diameter, height, number of green leaves and even mass in dry weight as compared to the controls at 95% significant level. Plantlets treated with *Streptomyces* sp. registered a significantly higher growth in all parameters ($p<0.05$). This implies that this isolate enhance plant growth of bananas at the green house level. Use of PGPR has shown to increase the shoot, root length and even dry weight of the inoculated wheat

seedlings (Majeed, Abbasi, Hameed, Imran, & Rahim, 2015). Studies have shown that there is a positive growth influence after treatments with the microbial inoculants on the tissue culture plantlets (Mia *et al.*, 2010b). As the levels of fertilizers were increased, there was also an increase in the mean diameter, height, number of leaves and even in mass in dry weight with few exceptions. However, the direct effect of these isolates could not be determined because of the effect of the confounding fertilizer levels. But there were some significant differences comparing the plantlets with the bacterial isolates and the control, which shows that the plantlets with the treatments were higher than those without the isolates or the controls. This therefore shows that PGPR can be used as bifertilizers to assist in improving the growth and productivity of banana plants.

There were significant effects in terms of nutrient analysis taking into consideration parameters such as nitrogen with a p-value of 0.00000684, phosphorus 0.000163, magnesium 4.46E-09 and calcium 3.77E-05 which were significantly different. Previous studies have shown that when PGPR are inoculated into the bananas, there is increase in nitrogen concentration in leaves and roots as a result a greater accumulation is seen in dry weight (Mia *et al.*, 2010a, b). Data from this study suggests that these PGPR strains can be used as biofertilizers and for crop enhancement. The inoculation process stimulates root growth in all dimensions as reported by Mia *et al.*, (2010a) and also stimulates the reproductive growth as seen through early flowering.

PGPR not only fixes nitrogen but also increases nitrate assimilation. In order to produce high yields and quality fruits, bananas relatively require large amounts of nutrients since they extract some considerable nutrient quantities from the soil. Elements such as P and K have been suggested to play a very important role in plant-bacterium interaction (Mia *et al.*, 2010b). There is an increased K and P uptake amounts in banana when plants are inoculated with plant growth promoting rhizobacteria (Almaghrabi, Massoud, & Abdelmoneim, 2013). For example a combined inoculation of phosphate-solubilizing bacteria such as *Bacillus polymyxa* and *A. brasilense* on a field grown with sorghum significantly increased grain, dry matter yields and also the uptake of P and K as

compared with a single individual organism inoculation (Bashan *et al.*, 2008). The inoculation of PGPR changes many shoot and root parameters. The changes may directly be attributed to the positive bacterial effects on the uptake of minerals by the plant. PGPR inoculation improves mineral uptake in many cereal species, and its inoculation in banana nursery shows better growth, health seedling and consequently increases the seedling survival rates (Jaizme-Vega, Rodríguez-Romero, & Guerra, 2004).

Studies on PGPR have shown that they can be used as biofertilizers to promote sustainable agriculture. The bacteria colonize the roots of plants and promote the growth of plants through nitrogen fixation, solubilization of inorganic phosphates and excretion of growth regulators like indole acetic acid (Martinez-Viveros *et al.*, 2010; Park *et al.*, 2005; Ryu, Hu, Locy, & Kloepper, 2005).

4.2 Conclusions

1. Twenty rhizospheric bacterial isolates from rhizosphere soil of bananas within Juja were isolated and identified as *Pseudomonas*, *Bacillus*, *Staphylobacterium*, *Chryseobacterium*, *Streptomyces* and *Paenibacillus* spp.
2. The twenty rhizospheric bacterial isolates were able to enhance plant growth promotion through phosphorus solubilisation and nitrogen fixation.
3. The rhizospheric bacterial isolates from Juja were able to enhance the banana plant growth promotion in the greenhouse.

Based on these conclusions, the hypothesis that "there are no bacteria in the rhizosphere soil of banana plant in Juja that have the potential to improve the plant growth" was therefore rejected.

4.3 Recommendations

Therefore it can be recommended from the findings of this study that:

1. The isolates that support the growth can be used to develop a biofertilizer which can be used in enhancing growth of banana plant at the green house level since they have demonstrated so in this study.
2. Some further work on the isolates should be done that focuses on field trials in order to determine which individual isolates can support the growth promotion of the banana plant.
3. Further characterization should be further done on the isolates in order to establish if they are important in some other areas other than agriculture.

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APPENDICES

Appendix 1: ANOVA table for Nitrogen analysis

ANOVA: Nitrogen

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Bacteria Strains	31.75733	4	7.939333	7.360321	0.00000684	2.525215
Fertilizer Concentration	14.53867	5	2.907733	2.695674	0.029061	2.36827
Interaction	25.688	20	1.2844	1.190729	0.293726	1.747984
Within	64.72	60	1.078667			
Total	136.704	89				

Appendix 2: ANOVA table for Potassium analysis

ANOVA: Potassium

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Bacteria Strains	85.8021	4	21.45052	2.162534	0.084088	2.525215
Fertilizer Concentration	111.7707	5	22.35413	2.253631	0.060412	2.36827
Interaction	348.9264	20	17.44632	1.75885	0.048148	1.747984
Within	595.1497	60	9.919161			
Total	1141.649	89				

Appendix 3: ANOVA table for Phosphorus analysis

ANOVA: Phosphorus

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Bacteria Strains	877.4395	4	219.3599	6.679529	0.000163	2.525215
Fertilizer Concentration	321.3869	5	64.27737	1.957252	0.098143	2.36827
Interaction	875.8104	20	43.79052	1.333425	0.19445	1.747984
Within	1970.437	60	32.84062			
Total	4045.074	89				

Appendix 4: ANOVA table for Magnesium analysis

ANOVA: Magnesium

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Bacteria Strains	118.3796	4	29.5949	16.26926	4.46E-09	2.525215
Fertilizer Concentration	20.27146	5	4.054293	2.228774	0.062937	2.36827
Interaction	101.6176	20	5.08088	2.793121	0.00113	1.747984
Within	109.1441	60	1.819069			
Total	349.4128	89				

Appendix 5: ANOVA table for Zinc analysis

ANOVA: Zinc

Variation source	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Bacteria Strains	0.072707	4	0.018177	2.05283	0.098328	2.525215
Fertilizer Concentration	0.064543	5	0.012909	1.457874	0.217082	2.36827
Interaction	0.334373	20	0.016719	1.888167	0.030555	1.747984
Within	0.531267	60	0.008854			
Total	1.00289	89				

Appendix 6: ANOVA table for Calcium analysis

ANOVA: Calcium

Variation source	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Bacteria Strains	12.95337	4	3.238343	7.837228	3.77E-05	2.525215
Fertilizer Concentration	2.362179	5	0.472436	1.143359	0.347665	2.36827
Interaction	9.728482	20	0.486424	1.177212	0.3048	1.747984
Within	24.792	60	0.4132			
Total	49.83603	89				

Appendix 7: ANOVA table for Iron analysis

ANOVA: Iron

Variation source	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Bacteria Strains	0.328718	4	0.082179	1.009148	0.409968	2.525215
Fertilizer Concentration	0.216032	5	0.043206	0.530567	0.752243	2.36827
Interaction	1.466829	20	0.073341	0.900619	0.58745	1.747984
Within	4.886067	60	0.081434			
Total	6.897646	89				

Appendix 8: Components of the media used

Each media consisted of the following ingredients:

I. Yeast Manitol agar (10g//L manitol, 0.5g/L K_2PO_4, 0.2g/L NaCl, 0.8g/L MgSO4, 1g/L yeast extract, 0.01g/L $FeCl_3$, 15g/L agar) (Sigma Aldrich, Steinheim, Germany)

II. Nitrogen free media (0.5g/L K_2PO4, 0.2g/L NaCl, 0.2g/L $MgSO_4$, 6.6g/L $NaMoO_4(H_2O)$, 15mg/L $FeCl_3$, 15g/L agar) (Sigma Aldrich, Steinheim, Germany)

III. Nutrient Agar (28g/L) (Sigma Aldrich, Steinheim, Germany).