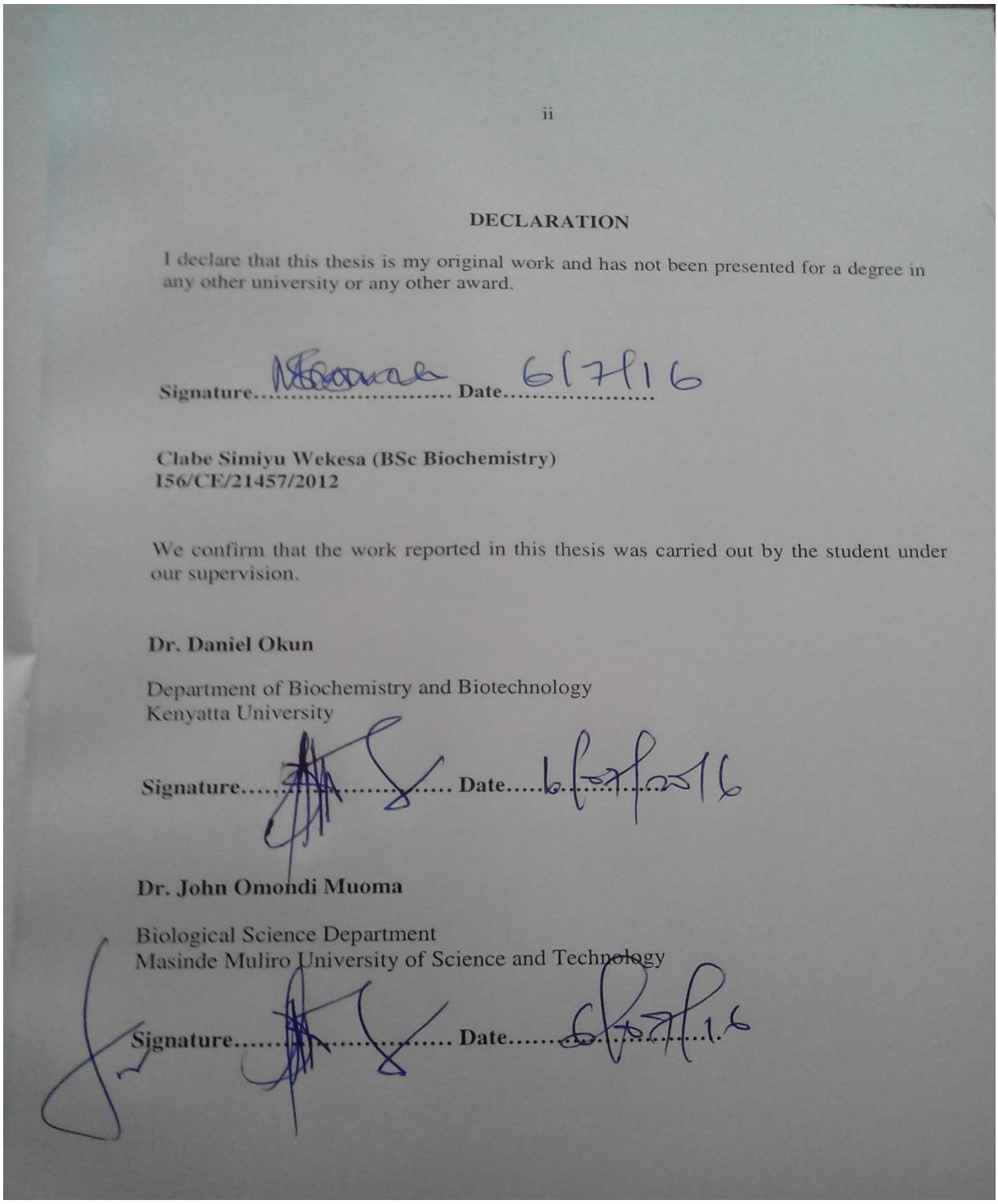
ABUNDANCE, GENETIC DIVERSITY AND SYMBIOTIC POTENTIAL OF COMMON BEAN (*Phaseolus vulgaris* L.) NODULE ASSOCIATED BACTERIA IN WESTERN KENYA SOILS

WEKESA CLABE SIMIYU
I56/CE/21457/2012

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

JULY 2016

DECLARATION



ii

DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

Signature..... **Date**.....6/7/16

Clabe Simiyu Wekesa (BSc Biochemistry)
I56/CE/21457/2012

We confirm that the work reported in this thesis was carried out by the student under our supervision.

Dr. Daniel Okun

Department of Biochemistry and Biotechnology
Kenyatta University

Signature..... **Date**.....6/7/2016

Dr. John Omondi Muoma

Biological Science Department
Masinde Muliro University of Science and Technology

Signature..... **Date**.....6/7/16

DEDICATION

To my father, Mr. Alex Wekesa, mother Evelyn Navangala. They have been my greatest support and strength throughout my study. Their moral and material support enabled me go through the difficulties experienced in the course of my studies.

ACKNOWLEDGEMENTS

I want to thank my supervisors, Dr. Daniel Okun and Dr. John Omondi Muoma for their continued. They worked hand in hand with me in my research work, read and corrected my thesis and provided moral and material support. I am grateful to Masinde Muliro University of science and Technology for allowing me to use their land for farming, laboratory and equipment for my work. My heartfelt gratitude also goes to Inter-University Council for East Africa for funding my studies; without this assistance I would not have managed to complete this work. I also thank laboratory technicians at Masinde Muliro University such as Mr. Peter Nyongesa, Mr. Nicholus Kitulungi and Mr. Willy Aganyanya. They made it possible for the laboratory equipments to be available for me during my research work. I want to thank Mr. Kelvin Juma of Kenyatta University for he assisted me in statistical data analysis and presentation; it added value to my work. I am grateful to my fellow students both at Masinde Muliro University of Science and Technology and Kenyatta University who assisted me financially, moral and sometimes working with me in the farm. More importantly I thank the Almighty God for providing me with health throughout the period of my studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

16SrRNA:	16S ribosomal ribonucleic acid
ACC:	1-aminocyclopropane-1-carboxylate
ANOVA:	analysis of variance
ATP:	Adenosine triphosphate
BLAST:	basic local alignment search tool
BLASTn:	nucleotide BLAST
DAPG:	2,4-diacetylphloroglucinol
DNA:	Deoxyribonucleic acid
HCN:	hydrogen cyanide
Hup:	hydrogen uptake
Hyp:	hydrogenase pleitropic
IAA:	Indole acetic acid
ISR:	Induction of systemic resistance
MPN:	Most probable number
NAB:	Nodule associated bacteria
NCBI:	national centre for biotechnology information
Nif:	nitrogen fixing gene
PCA:	phenazine-1-carboxylic acid
PCR:	polymerase chain reaction
PGPR:	Plant growth promoting rhizobacteria
PPP:	pods per plant
PSB:	Phosphate solubilizing bacteria
RAPD:	randomly amplified polymorphic DNA
RFLP:	Restriction fragment length polymorphism
RPGPR:	Rhizobia with plant growth promoting rhizobacteria
SAM:	S-adenosyl-L-methionine
SAR:	Systemic acquired response
SAR:	systemic acquired response
SPP:	Seeds per plant
TDM:	total shoot dry matter
VAM:	Vesicular-arbuscular mycorrhiza
WPP:	Weight of pods
YMA:	yeast extract mannitol agar

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are beneficial native soil bacteria that colonize plant roots and result in increased plant growth. Those that colonise the nodules of legumes are known as nodule associated bacteria (NAB). The aim of this study was to determine the distribution and genetic diversity of NAB that colonize *Phaseolus vulgaris*, their abundance, and symbiotic efficiency when coinoculated with *Phaseolus vulgaris* in Western Kenya soils. The soil samples were collected from cultivated lands in Kisumu near Lake Victoria, slopes of Mt. Elgon and Kakamega. In each of these regions, the soil samples were collected from four regions. 1ml of soil solution at 10 fold dilution for seven dilution steps (10^{-1} to 10^{-7}) and three replications for each dilution was used to inoculate common bean seedling in Leonard jars. They were harvested after four weeks to determine abundance of NAB using most probable number method. Common bean nodules were also collected directly from the farmers' farms in the above three regions. Harvested nodules and those collected from the field were cleaned and surface sterilized, crushed and exudates streaked on YEM agar growth media. Pure colonies were further cultured in YEM broth at 28^0C for three days and the genomic DNA isolated from the bacteria using Qiagen DNA extraction kit. 16SrRNA gene was amplified by 27F and 1492R primers and PCR products resolved by agarose gel electrophoresis and sequenced. 16SrRNA gene analysis revealed that NAB that nodulate with common beans are genetically diverse as they formed clusters on the phylogenetic tree and their distribution depends on chemical characteristics of the soil. BLASTn showered that isolated strains belonged to the genus *Pseudomonas, Providencia, Rhizobia, Klebsiella, Sphingobacterium, Enterobacter, Delfitia, Acinetobacter* and one strain did not have sequence homology at the GenBank. Mt. Elgon region had the highest population of NAB (120000 cells per gram of the soil), followed by Kisumu (1290 cells per gram of the soil) and Kakamega region had the lowest (17 cells per gram of the soil). The effect of PGPR on the yield of common beans was significantly higher ($p < 0.001$) when co-inoculated with *Rhizobia* compared to the yield of *Rhizobia* inoculated alone or control (not inoculated) ($p < 0.05$). This study therefore provides knowledge on the type of NAB that nodulates with common beans and factors that favour their distribution necessary for production of PGPR inoculants suitable to the soils of Western Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Recent census findings on the population of Western Kenya region shows that more than 80% of the population is involved in agricultural activity (Albinus *at al*., 2008). These farmers repeatedly cultivate their land causing a reduction in soil fertility and hence crop productivity. Chemical fertilizers currently being used in this region are very expensive and also cause soil and water pollution. The economic implications on the use of inorganic fertlizers together with their negative impacts to the environment have become a global concern therefore there is need to shift to sustainable farming practices (Osoro *et al*., 2014).

Some couple of decades in the past, the use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported (Kloepper *et al*.1980; Chen *et al*. 1995; Figueiredo *et al*. 2007; Acuña, *et al*., 2011). PGPR can affect plant growth by different direct and indirect mechanisms (Rajendran *et al*., 2011). Some examples of these mechanisms, which can probably be active simultaneously or synergistically at different stages of plant growth, are (1) increased mineral nutrient solubilization and nitrogen fixation, making nutrients available for the plant; (2) repression of soil borne pathogens (by the production of hydrogen cyanide,

siderophores, antibiotics, and/or competition for nutrients); (3) improving plant stress tolerance to drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA) (Gupta *et al*., 2000). Moreover, some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of ethylene in plants (Bashan and de-Bashan, 2010). By lowering ethylene concentration in seedlings and thus its inhibitory effect, these PGPR stimulate seedlings root length (Gamalero and Glick, 2011). In addition, biological control of plant pathogens and destructive microorganisms, through the production of antibiotics, lytic enzymes, hydrogen cyanide, and siderophores or through competition for nutrients and space can improve significantly the plant health and promote growth.

The leguminous plants are symbiotically associated with the rhizobia and this requires active nitrogen fixation and the interaction plays a key role in the agricultural crop production. Enhancement of legume nitrogen fixation by coinoculation of rhizobia with plant growth promoting rhizobacteria (PGPR) is a way to improve nitrogen availability in sustainable agriculture production systems. Many PGPRs are known to promote plant growth directly by the production of plant growth regulators and improvements in plant nutrient uptake (Hamaoui *et al*., 2001; Figueiredo *et al*., 2010; Pandey *et al*., 2005) or indirectly by the production of metabolites like antibiotics, siderophores, and so forth that decrease the growth of phytopathogens (Stajković *et al*., 2011; Beneduzi *et al.*, 2012). PGPR also have beneficial effects on legume growth and some strains enhance legume nodulation and nitrogen fixation by affecting interaction between plant and rhizobia (Parmar and Dadarwal, 1998).

Many studies have shown that simultaneous infection with rhizobia and rhizospheric bacteria increases nodulation and growth in a wide variety of legumes (Tahmasebpour *et al*., 2013; Mohamed *et al*., 2009). Such nodule assisting bacteria may be free-living rhizobacteria or endophytic. Endophytic bacteria reside intercellularly or intracellularly within host tissues and therefore are at advantage as compared to free-living counterparts by being protected from environmental stresses and microbial competition. Depending on their effect on the host plant, endophytic bacteria can be categorized into three groups: plant growth promoting, plant growth inhibiting, and plant growth neutral (Parmar and Dadarwal, 1998); however a major proportion of bacterial endophytes have plant growth promoting effect (Beneduzi *et al.,* 2012).

The presence of other bacteria other than Rhizobium in root nodules has been reported severally (Oliveira *et al*., 2012) in the Leguminosae. These studies demonstrated the isolation of bacteria of several genera from legume tissues, including *Aerobacter, Pseudomonas, Agrobacterium, Bacillus, Chryseomonas, Curtobacterium, Enterobacter, Erwinia, Flavimonas,* and *Sphingomonas*. Majority of the recent studies on legume-rhizobacterial interaction have been confined to soya bean (*Glycine max* L), cowpea (*Vigna unguiculata* L), chickpea (*Cicer arietinum* L), the common bean (*Phaseolus vulgaris* L), and red clover (*Trifolium pretense* L). Coinoculation of other PGPR with rhizobia is shown as an important practice in the development of sustainable agriculture. Available reports indicate improved plant yield and plant health under greenhouse conditions with respect to increase in root wet weight and nodulation when coinoculated with nodule endophytes, compared to inoculation with rhizobia alone

(Rajendran *et al*., 2011). PGPR that have been coinoculated with rhizobia include strains of the following well-known rhizobacteria: *Azospirillum* (Hamaoui, *et al*., 2001), *Azotobacter* (Tahmasebpour *et al*., 2013), *Bacillus* (Schwartz *et al*., 2013), and *Pseudomonas* (Tahmasebpour *et al*., 2013). Coinoculation of some *Pseudomonas* and *Bacillus* strains along with effective *Rhizobium sp*. is shown to stimulate chickpea growth, nodulation, and nitrogen fixation (Parmar and Dadarwal, 1998). Some *Serratia* strains, such as *S. proteamaculans* and *S. Liquefaciens*, have beneficial effects on legume plant growth (Mohamed *et al*., 2009). Bacilli are spore-forming, gram-positive, rod-shaped bacteria that comprise one of the most common soil bacterial groups and they are frequently isolated from the rhizosphere of plants. Bacillus species, common endophytes are readily adaptable to field applications due to their spore-forming capability. *Exiguobacterium sp*. fall in the class Bacilli, order Bacillales and family Bacillaceae. They are non-spore-forming motile gram-positive bacilli. The objectives of this study are to determine: the genetic diversity of bacteria associated with common bean nodules, their abundance and symbiotic relationship with common bean (*Phaseolus vulgaris*) plant to isolate the strains that are well adapted to Western Kenya soils.

1.2 Problem statement

Intensive farming practices that achieve high yield require continuous application of chemical fertilizers in our agroecosystems. However, the prices and availability of these chemical fertilizers become the limiting factor for crop production especially in developing countries around the world. Continuous application of N fertilizers may

result in negative impacts on agro-ecosystem such as leaching, pollution of water resources, gaseous emissions to atmosphere thus causing irreparable damage to the overall ecosystem and environment. Similarly, phosphorus (P) is one of the major essential macronutrients for biological growth and application of P fertilizers is indispensable component for crop production. The prices of P fertilizers jumped up several folds during recent years making P fertilizers not-affordable to a common resource poor farmer. During the last two decades, use of microbial techniques and introduction of rhizobacteria in agriculture has increased tremendously due to their potential for N_2-fixation and P solubilization thus increasing N and P uptake by the plants and therefore yields (Zahid *et al*., 2015). Studies have shown that PGPR strains vary widely and their growth promoting ability may be highly specific to certain plant species, cultivar, soil, and genotype (Hung *et al*., 2004). Under such conditions, knowledge of native bacterial population and their identification is important for understanding their distribution and diversity. It is important to explore and identify region specific microbial strains which can be used as potential plant growth promoters to achieve higher yields under specific ecological and environmental conditions. There are no published studies that determined diversity and distribution of NAB in the soils of Western Kenya associated with *Phaseolus vulgaris*. Therefore information concerning the diversity, distribution and symbiotic ability of these important microbes is not available, and for this reason, production of PGPR inoculants specific for this region is not possible.

1.3 Justification of the study

Common bean (*Phaseolus vulgaris*) is consumed widely in Kenya and more so Western Kenya, but its low yields due to limiting available nitrogen and phosphorus and infection by phytopathogens are a cause for worry. In order to protect this crop, inoculant companies have to get enough and satisfactory information concerning nitrogen fixing bacteria, phosphorus solubilising bacteria and those involved in the inhibition of phytopathogens that are efficient because development of inoculants requires selection of isolates that are symbiotically efficient and well adapted to local soil chemical factors (Zahid *et al*., 2015).

1.4 Research questions

1. Does the soil properties influence the abundance of NAB associated with *Phaseolus vulgaris* in Western Kenya soils?
2. Are NAB associated with *Phaseolus vulgaris* in Western Kenya soils genetically diverse?
3. What is the effect of NAB when inoculated with *Phaseolus vulgaris*?

1.5 Hypothesis

H_0: Soil properties have no influence on the abundance of NAB associated with *Phaseolus vulgaris* in Western Kenya soils.

H_1: NAB are not genetically diverse

H_2: NAB have no effect on the yield of common beans.

1.6 Research objectives

1.6.1 General objective

To determine genetic diversity of indigenous NAB nodulating *Phaseolus vulgaris* in Western Kenya soils

1.6.2 Specific objectives

The specific objectives of the study will be to:

i) Determine the effect of soil chemical properties (nitrogen, phosphorus, carbon, aluminium and potassium) on the abundance of indigenous NAB associated with *Phaseolus vulgaris* in Western Kenya soils.
ii) Determine the genetic diversity and distribution of indigenous NAB associated with *Phaseolus vulgaris* in Western Kenya soils.
iii) Determine effect of NAB on the yield of *Phaseolus vulgaris* in Western Kenya soils.

1.7 Expected output of the study

This study will provide the knowledge about the type, distribution, genetic diversity and symbiotic efficiency of indigenous PGPR in Western Kenya soils that are associated with *Phaseolus vulgaris* for inoculant companies to make PGPR inoculants that are well adapted for Western Kenya soils.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mechanisms Used by PGPR to improve plant growth

Mechanisms used are both direct and indirect. They are outlined in the subsequent sections below.

2.1.1 Provision of Nutrients

Plant growth promotion by bacteria can also occur as a consequence of the provision of nutrients that are not sufficiently available in the soil; these nutrients include phosphate, nitrogen, and iron. The main mechanisms involved, are the solubilisation of phosphate, nitrogen fixation, and iron chelation through siderophores.

2.1.1 Phosphate Solubilisation

Although the amount of phosphorus (P) usually in soil is between 400 and 1,200 mg kg^{-1} of soil, the concentration of soluble P in soil is typically ~1 mg kg^{-1} or less (Glick and Gamalero, 2011). P in soil is present in two main insoluble forms: mineral forms such as apatite, hydroxyapatite, and oxyapatite, and organic forms including inositol phosphate (soil phytate), phosphomonoesters, phosphodiesters, and phosphotriesters (Rodriguez and Fraga, 1999).

Since P is an essential macronutrient for plant growth and has only limited bioavailability, it is considered to be one of the elements that limit plant growth (Hinsinger, 2001). To satisfy plants' nutritional requirements, P is usually added to soils as fertilizers synthesized through high-energy-intensive processes. However, plants can

use only a small amount of this P since 75–90% of added P is precipitated by metal–cation complexes, and rapidly becomes fixed in soil. Thus, solubilization and mineralization of P by phosphate-solubilising bacteria (PSB) is one of the most important bacterial physiological traits in soil biogeochemical cycles (Rodriguez and Fraga, 1999). The major mechanism used by PSB for solubilization of inorganic P is based on the synthesis of low molecular weight organic acids such as gluconic and citric acid (Tahir *et al*., 2013). These organic acids bind phosphate with their hydroxyl and carboxyl groups thereby chelating cations and also inducing soil acidification, both resulting in the release of soluble phosphate (Mardad *et al*., 2013). Other mechanisms that have been proposed in solubilization of inorganic phosphate are the release of H^+, the production of chelating substances and inorganic acids (Bhalla *et al*., 2009). In addition, exopolysaccharides synthesized by PSB participate indirectly in the solubilization of tricalcium phosphates by binding free P in the medium, affecting the homeostasis of P solubilization (Khan *et al*., 2007). The mineralization of organic P occurs through the synthesis of phosphatases, including phosphomonoesterase, phosphodiesterase, and phosphotriesterase, catalyzing the hydrolysis of phosphoric esters (Rodriguez and Fraga, 1999). In addition, P solubilization and mineralization can coexist in the same bacterial strain.

Genera able to solubilize phosphate include *Pseudomonas* (Tahmasebpour *et al*., 2013), *Bacillus* (Toro *et al*., 1997), *Rhizobium* (Gupta *et al*., 2000), *Burkholderia*, *Enterobacter* (Toro *et al*., 1997), and *Streptomyces* (Karpagam and Nagalakshmi, 2014). Recently, the potential of *Streptomycete actinomycetes* to solubilize insoluble phosphates in soil

and to promote plant growth has been investigated (Khan *et al*., 2009). In particular, a highly rhizosphere competent isolate of *Micromonospora endolithica* able to solubilize considerable amounts of P, to produce acid and alkaline phosphatases as well as several organic acids, was found to be unable to synthesize any other stimulatory compounds (such as auxin, cytokinin, and gibberellin) and yet promoted the growth of beans.

The role of phosphate solubilization in plant growth promotion is often overshadowed by other plant beneficial activities expressed by the PSB. When Das *et al*. (2013) selected ten pseudomonads on the basis of their high phosphate solubilization activity on tricalcium phosphate and inoculated seeds with these strains, which also synthesize indole-3-acetic acid, ACC deaminase, and siderophores, the plants showed increased root elongation and biomass, however, under the conditions employed, P uptake was unaffected. Notwithstanding the difficulty that sometimes exists in pinpointing the contribution of phosphate solubilization activity to plant growth promotion, numerous reports demonstrate direct connections between phosphate solubilization activity and increased P in tissues of plants inoculated with PSB (Rodriguez and Fraga, 1999).

Besides the low rhizospheric competence of some PSB strains, specificity for the host plant or soil type could play a role. For example, solubilization of Ca–P complexes is quite prevalent among PSB, whereas the release of P by Fe–P or Al–P is very rare. Thus, release of soluble P is prevalent in calcareous soils and low in alfisols (Gupta *et al*., 2000). Frequently, the relatively high PSB density in soil does not correspond to the amount of soluble P present in soil. The efficiency of various PSB also depends upon

their physiological status, and the level of P released by phosphate solubilization is considered to be inadequate to induce a substantial increase of biomass. To obviate this problem, plants are often inoculated with PSB at concentrations that are higher than what is normally present in soil.

As a consequence of the heterogeneous results obtained by inoculating plants with PSB, the commercial application of PSB-based biofertilizers has been quite limited. Longer bacterial survival of PSB may be achieved by cell encapsulation inside nontoxic polymers such as alginate that increase the shelf life of the bacteria, protect them against many environmental stresses, and release them to the soil gradually (Sivakumar *et al*., 2014). This may be more effective than the application of cell suspensions with, improvement of growth promotion efficacy related to enhanced phosphate solubilization activity in lettuce inoculated with encapsulated but not free-living *Enterobacter sp*. cells (Jezequel *et al*., 2003).

The highest efficiency in stimulating plant growth was observed when PSB were co-inoculated with bacteria with other physiological capabilities such as N fixation (Rojas *et al*., 2001), or with mycorrhizal (Toro *et al*., 1997; Babana and Antoun, 2006) or nonmycorrhizal fungi (Babana and Antoun, 2006). Thus, the use of mixed inocula with different plant beneficial activities appears to be a promising strategy. In one set of experiments, increased amounts of both nitrogen fixation and phosphate solubilization were observed in mangrove seedlings treated with a mixture of the nitrogen-fixing *Phyllobacterium sp*. and the PSB *Bacillus licheniformis*, compared to plants inoculated

with individual cultures (Rojas *et al*., 2001). In fact, when the two bacterial species were cocultivated *in vitro*, they affected one another's metabolism: N fixation increased in *Phyllobacterium sp*., and phosphate solubilization increased in *B. licheniformis*. However, the growth of the coinoculated plants did not differ from that of plants treated with a single bacterium.

Finally, the genetic manipulation of PGPR to obtain expression or overexpression of genes involved in phosphate solubilization is an attractive strategy for improving the efficacy of some bacterial inoculants. With this approach, it may be possible to avoid competition among microorganisms that is often observed when mixed inoculants are employed. Unfortunately, largely for political rather than scientific reasons, the deliberate release of genetically modified organisms to the environment is still controversial in many jurisdictions (Rodriguez *et al*., 2006).

2.1.2 Iron Chelation and Siderophores

Iron is the fourth most abundant element on earth; however, in aerobic soils, iron is mostly precipitated as hydroxides, oxyhydroxides, and oxides so that the amount of iron available for assimilation by living organisms is very low, ranging from 10^{-7} to 10^{-23} M at pH 3.5 and 8.5, respectively. Both microbes and plants have a quite high iron requirement (i.e., 10^{-5} to 10^{-7} and 10^{-4} to 10^{-9}M, respectively), and this condition is more accentuated in the rhizosphere where plant, bacteria, and fungi compete for iron (Glick and Gamalero, 2011). To survive with a limited supply of iron, in bacteria, cellular iron deficiency induces the synthesis of low-molecular weight siderophores, molecules with

an extraordinarily high affinity for Fe^{3+} (K_a ranging from 10^{-23} to 10^{-52}) as well as membrane receptors able to bind the Fe–siderophore complex, thereby allowing iron uptake by microorganisms (Loper and Buyer, 1990).

Many *Pseudomonas spp*. and related genera produce yellow–green, water soluble, fluorescent pigments collectively called pyoverdines, composed of a quinoleinic chromophore bound together with a peptide and an acyl chain, conferring a characteristic fluorescence to the bacterial colonies (Meyer and Abdallah, 1978). About 100 different pyoverdines have been identified and represent about 20% of the microbial siderophores that have been characterized (Boukhalfa and Crumbliss, 2002). Pyoverdine-mediated iron uptake confers a competitive advantage on to fluorescent pseudomonads over other microorganisms. Regulation of pyoverdine synthesis is not only based on iron availability but also on quorum sensing whereby cell-to-cell communication mediated by N-acyl homoserines lactones occur activating siderophore synthesis.

In plants, active iron uptake occurs mainly through two strategies. Strategy I, exploited by dicotyledonous and nongraminaceous monocotyledonous plants, is based on acidification of the rhizosphere by H^+ excretion, leading to the reduction of Fe^{3+} to Fe^{2+} and its transport inside root cells (Bauer *et al*., 2002; Slatni *et al*., 2009). Strategy II, used in grasses and graminaceous plants including wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), and maize (*Z. mays*), relies on the synthesis of Fe^{3+} chelators called phytosiderophores and on the uptake of the Fe–phytosiderophore

complex in root cells mediated by specific transporter molecules. The iron dynamics in the rhizosphere are under the control of the combined effects of soil properties and plant and microbially produced compounds (Lemanceau *et al*., 2009). Plant iron nutrition can affect the structure of bacterial communities in the rhizosphere. For example, transgenic tobacco that overexpresses ferritin and accumulates more iron than nontransformed tobacco has less bioavailable iron in the rhizosphere. As a consequence, the composition of the rhizosphere bacterial community differed significantly when compared to nontransformed tobacco lines (Salisbury *et al*., 1988).

Siderophores are involved both in plant growth promotion and health protection (Lemanceau *et al*., 2009). The benefits of microbial siderophores have been demonstrated by supplying radiolabeled ferric-siderophores to plants as a sole source of iron (Bar-Ness *et al*., 1992). The role of siderophores in plant nutrition is further supported by the absence of iron-deficiency symptoms (i.e., chlorosis) and by the fairly high iron content in roots of plants grown in nonsterile soils compared to plants grown in sterile systems (Saharan and Nehra, 2011). Thus, mung bean (*Vigna radiate* L.) plants, inoculated with the siderophore producing *Pseudomonas* strain GRP3 and grown under iron-limiting conditions, showed reduced chlorotic symptoms and an enhanced chlorophyll level compared to uninoculated plants (Johri *et al*., 2003). In addition, the Fe–pyoverdine complex synthesized by *Pseudomonas fluorescens* C7 was efficiently taken up by the plant *Arabidopsis thaliana*, leading to an increase of iron content inside plant tissues and to improved plant growth (Vansuyt *et al*., 2006).

Plant iron nutrition improvement by soil bacteria is even more important when the plant is exposed to an environmental stress such as heavy metal pollution. Metal mobility in soil can be affected by microbial metabolites and especially by siderophores that can bind to magnesium, manganese, chromium (III), gallium (III), cadmium, copper, nickel, arsenic, lead, and zinc and radionuclides, such as plutonium (IV) as well as to iron (Guo *et al*., 2010; Sangthong *et al*., 2015). In addition, by supplying iron to the plants, siderophores may help to alleviate the stresses imposed on plants by high soil levels of heavy metals. *Kluyvera ascorbata*, a PGPR able to synthesize siderophores was able to protect canola, Indian mustard, canola, and tomato from heavy metal (nickel, lead, and zinc) toxicity (Burd *et al*., 1998). The siderophore overproducing mutant SUD165/26 of this bacterium provided even greater protection, as indicated by the enhanced biomass and chlorophyll content in plants cultivated in nickel contaminated soil (Burd *et al*., 1998).

When two mutants of strain *Pseudomonas putida* ARB86, one impaired in siderophore synthesis and the other overproducing siderophores were used to inoculate *A. thaliana* plants exposed to nickel, symptoms induced by the metal were relieved to the same extent in plants inoculated with both mutants and wild type suggesting that alleviation of Ni toxicity in this case is siderophore independent (Parmar and Dufresne, 2011). Similarly, two siderophore-producing bacterial strains reduced Zn uptake by willow (*Salix caprea*) suggesting that bacterial siderophores may bind to heavy metals from soil and inhibit their uptake by plants. On the other hand, enhancement of Zn and Cd uptake in willow inoculated with a Streptomyces strain unable to produce siderophores

highlights the importance of other physiological traits for heavy metal accumulation by *S. caprea* (Kuffner *et al*., 2008). The bottom line for these seemingly contradictory results is that the effect of siderophores in the presence of high concentrations of metals is quite complex, depending on soil composition, metal type and concentration, and the siderophore(s) and plant(s) utilized. Thus, the impact of siderophore in metal-contaminated soils needs to be assessed on a case by case basis.

2.1.3 Nitrogen Fixation

Agriculture has become increasingly dependent on chemical sources of nitrogen derived at the expense of petroleum. Besides being costly, the production of chemical nitrogen fertilizers depletes non-renewable resources and poses human and environmental hazards. To complement and eventually substitute mineral fertilizers with biological nitrogen fixation would represent an economically beneficial and ecologically sound alternative. However, despite nitrogen's abundance in the atmosphere, it must first be reduced to ammonia before it can be metabolized by plants to become an integral component of proteins, nucleic acids, and other biomolecules (Zahran, 1999). The most important microorganisms that are currently used agriculturally to improve the nitrogen content of plants include a range of *Rhizobia*, each specific for a limited number of plants. Other nitrogen-fixing bacteria, notably *Azospirillum spp*., are also employed as bacterial inoculants; however, it is generally thought that for free-living bacteria, the provision of fixed nitrogen is only a very small part of what the bacterium does for the plant (Glick and Gamalero, 2011).

Martyniuk *et al*. (2004) outlined the status of rhizobial taxonomy and enlisted 36 species distributed among seven genera (*Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Methylobacterium, Rhizobium and Sinorhizobium*) derived, based on the polyphasic taxonomic approach.

Nitrogen-fixing (diazotrophic) bacteria fix atmospheric nitrogen by means of the enzyme nitrogenase, a two component metalloenzyme composed of (a) dinitrogenase reductase, a dimer of two identical subunits that contains the sites for Mg-ATP binding and hydrolysis, and supplies the reducing power to the dinitrogenase, and (b) the dinitrogenase component that contains a metal cofactor (Swain and Abhijita, 2013). Overall, nitrogenase biosynthesis (nif) genes include structural genes, genes involved in the activation of the Fe protein, iron molybdenum cofactor biosynthesis, electron donation, several genes of unknown function, and the regulatory genes required for the synthesis and function of the nitrogenase. The nif genes may be carried on plasmids as in most *Rhizobium* species or, more commonly, in the chromosome of free-living (Desnoues *et al*., 2003) and associative nitrogen-fixing bacteria. The nif genes from many different diazotrophs are arranged in a single cluster of approximately 20–24 kb with seven separate operons that together encode 20 distinct proteins. All of the nif genes are transcribed and translated in a concerted fashion, under the control of the nifA and nifL genes. NifA protein is a positive regulatory factor which turns on the transcription of all of the nif operons (except its own). The DNA-bound NifA protein interacts with transcription initiation factor sigma 54 before transcription from the nif promoter is initiated. NifL protein is a negative regulatory factor which, in the presence

of either oxygen or high levels of fixed nitrogen, acts as an antagonist of the NifA protein. Because of the complexity of the nif system, genetic strategies to improve nitrogen fixation have been elusive (Glick and Gamalero, 2011).

Since nitrogen fixation requires a large amount of ATP, it would be advantageous if rhizobial carbon resources were directed toward oxidative phosphorylation, which results in the synthesis of ATP, rather than glycogen synthesis, which results in the storage of energy as glycogen. A strain of *R. tropici* with a deletion in the gene for glycogen synthase was constructed (Marroqui *et al*., 2001). Treatment of bean plants with this mutant strain resulted in a significant increase in both the number of nodules that formed and the plant dry weight in comparison with treatment with the wild-type rhizobial strain.

Oxygen is both inhibitory to nitrogenase and is a negative regulator of nif gene expression; however, it is required for *Rhizobia spp*. bacteroid respiration. This difficulty can be resolved by the introduction of leghemoglobin, which binds free oxygen tightly resulting in an increase in nitrogenase activity. Since the globin portion of leghemoglobin is produced by the plant, more efficient strains of *Rhizobium spp*. may be engineered by transforming strains with genes encoding bacterial hemoglobin (Ramirez *et al*., 1999). Following transformation of *Rhizobium etli* with a plasmid carrying the *Vitreoscilla sp*. (a gram negative bacterium) hemoglobin gene, at low levels of dissolved oxygen in the medium, the rhizobial cells had a two- to three fold higher respiratory rate than the nontransformed strain. In greenhouse experiments, when bean

plants were inoculated with either nontransformed or hemoglobin-containing *R. etli* the plants inoculated with the hemoglobin-containing strain had approximately 68% more nitrogenase activity. This difference in nitrogenase activity leads to a 25–30% increase in leaf nitrogen content and a 16% increase in the nitrogen content of the seeds that are produced (Ramirez *et al*., 1999).

The most common strain of *R. etli* encodes three copies of the nitrogenase reductase (nifH) gene, each under the control of a separate promoter. To increase the amount of nitrogenase, the strongest of the three nifH promoters (i.e.,PnifHc) was coupled to the nifHcDK operon, which encodes the nitrogenase structural genes (nifHc is one of the three nifH genes). When the PnifHc–nifHcDK construct was introduced into the wild-type strain, the net result was a significant increase in nitrogenase activity, plant dry weight, seed yield, and the nitrogen content of the seeds. This genetic manipulation worked as well or better when the PnifHc–nifHcDK construct was introduced into the Sym plasmid from *R. etli* that contains all of the nif genes (Peralta *et al*., 2004). In addition, expression of the PnifHc–nifHcDK construct in a poly-b-hydroxybutyrate negative strain of *R. etli* enhanced plant growth to an even greater extent than when this construct was expressed in a wild-type poly-b-hydroxybutyrate positive strain. This is probably because in the poly-b-hydroxybutyrate negative strain there is an increased flux of carbon through the citric acid cycle and hence an increase in the amount of ATP to power nitrogen fixation (Peralta *et al*., 2004).

An undesirable side reaction of nitrogen fixation is the reduction of H^+ to H_2 by nitrogenase. ATP is wasted on the production of hydrogen and only 40–60% of the electron flux through the nitrogenase system is transferred to N_2, lowering the overall efficiency of nitrogen fixation (Glick and Gamalero, 2011). Some diazotrophic strains contain hydrogenase that can take up H_2 from the atmosphere and convert it into H^+ and the presence of a hydrogen uptake system in a symbiotic diazotroph improves its ability to stimulate plant growth by binding and then recycling the hydrogen gas that is formed inside the nodule by the action of nitrogenase. Although it is clearly beneficial to the plant to obtain its nitrogen from a symbiotic diazotroph that has a hydrogen uptake system, this trait is not common in naturally occurring rhizobial strains.

In *Rhizobium leguminosarum*, 18 genes are associated with hydrogenase activity. There are 11 hup (hydrogen uptake) genes responsible for the structural components of the hydrogenase, the processing of the enzyme, and electron transport. There are also seven hyp (hydrogenase pleitropic) genes that are involved in processing the nickel that is part of the active center of the enzyme. The hup promoter is dependent on the NifA protein so that hup genes are only expressed within bacteroids. On the other hand, the hyp genes are transcriptionally regulated by an FnrN-dependent promoter that is turned on by low levels of oxygen so that the hyp genes are expressed both in bacteroids and microaerobically. By modifying the chromosomal DNA of *R. leguminosarum* and exchanging the hup promoter for an FnrN-dependent promoter, a derivative of the wild type with an increased level of hydrogenase was created (Ureta *et al*., 2005). The engineered strain displayed a two fold increase in hydrogenase activity compared to the

wild type and no discernible amount of hydrogen gas was produced as a byproduct of nitrogen fixation with the net result that the amount of fixed nitrogen and hence plant productivity was greater.

A small localized rise in plant ethylene that can inhibit subsequent rhizobial infection and nodulation is often produced following the initial stages of *Rhizobia* infection. Some Rhizobia strains increase the number of nodules that form on the roots of a host legume by limiting the rise in ethylene either by synthesizing a small molecule called rhizobitoxine (Yuhashi *et al*., 2000) that chemically inhibits ACC synthase, one of the ethylene biosynthetic enzymes, or by producing ACC deaminase and removing some of the ACC before it can be converted to ethylene (Okazaki *et al*., 2004). The result of lowering the level of ethylene is that both the number of nodules and the biomass of the plant are increased by 25–40% (Okazaki *et al*., 2004). In the field, approximately 1–10% of rhizobial strains possess ACC deaminase (Glick, 2004) thus it is possible to increase the nodulation efficiency of *Rhizobia* strains that lack ACC deaminase by engineering these strains with isolated rhizobia ACC deaminase genes (and regulatory regions). In fact, insertion of an ACC deaminase gene from *R. leguminosarum* bv. viciae into the chromosomal DNA of a strain of *Sinorhizobium meliloti* that lacked this enzyme dramatically increased both nodule number and biomass of host alfalfa plants (Okazaki *et al*., 2004). Because of political/regulatory considerations, genetically engineered strains of *Rhizobia* may not currently be acceptable for use in the field; however, several commercial inoculant producers are already screening their more recently isolated *Rhizobia* strains for active ACC deaminase.

2.1.5 Phytoalexins production

Phytoalexins are low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms. The concept of phytoalexin is expanded because many isoflavonoids (the most widely studied class of phytoalexins) were shown to serve as signal molecules during infection of plant roots by symbiotic microbes (Mert-Türk, 2002). Phytoalexin synthesis can be used as an indicator of enhanced defence mechanism in bacteria-treated plants. An increase in the production of three phytoalexins, for example, rishitin, lubimin, and solvetivon were observed in potato slices and an inhibition in mycelia growth of *Phytophthora infestans* by culture filtrate of *Streptomyces*. It has also been observed that like other plants, Graminae contain secondary plant metabolites that have been found to be toxic to plant pathogenic fungi and bacteria and are proposed to be responsible for resistance to microbial pathogens (Mazid *et al*., 2011).

Similarly, in another study induction and accumulation of phytoalexins in cow pea roots were observed when infected with myccorhizal fungus and also their resistance to *Fusarium* wilt disease. From the studies, it is apparent that the rhizosphere bacteria such as fluorescent *Pseudomonads* and *Bacillus sp*. produce certain signal molecules which probably enhance the flavonoid production by plant roots. The enhanced flavonoid production could be an additional factor in nodule promotion (Jeandet *et al*., 2014). In addition, production of phytoalexins was demonstrated to increase after prior inoculation of chick pea (*C. arietinum* L.) seedlings with non-pathogenic isolates of

Fusarium oxysporum (inducers) and this was correlated with a delay on the onset of symptom and reduction of *Fusarium* wilt development.

2.1.6 Soil-borne Disease Suppression

Plant disease suppression by soil microorganisms is a possible alternative means of reducing the chemical input in agriculture. Biocontrol of plant pathogenic microorganisms relies on different traits including competition for colonization site or nutrients, production of antibiotics and enzymes, and induction of systemic resistance (ISR) against the pathogens (Raaijmakers *et al*., 2008).

2.1.6.1 Stimulation of Host Defence

When a plant comes into contact with a pathogenic microorganism, it responds with a systemic acquired response (SAR) where the plant's immune system is primed to defend itself against disease. Many phytopathogenic fungi, for example, are known to induce systemic acquired responses in plants. The most common parasitic fungi belong to genera *Pythium sp*., *Rhizoctonia sp.* and *Fusarium* sp. (Tahir *et al*., 2013). Some *Fusarium sp*. cause root rots and wilts and some feed on dead plant tissues. The exact mechanisms for how the plant immune system primes itself are still unknown; however, certain molecules in the pathway such as salicylic acid, for example, appear to play a critical role as a plant messenger once the plant is exposed to a pathogen (Nürnberger and Scheel, 2001). Some PGPR can stimulate a plant's defence system without the presence of a pathogen by emitting molecules similar to those in the plant's SAR. This response is called induced systemic resistance (ISR). Some of these molecules include

methyl salicylate (MeSA), methyl jasmonate (MeJA) and ethylene. Thus far, evidence of PGPR eliciting ISR has been observed in carnations, the common bean, cucumber and grapevine (Van Loon and Bakker, 2005). In other experiments, the colonization of root systems with PGPR, such as *P. fluorescens, P. putida, Bacillus pumilus* and *Serratia marcescens* was protected against foliar diseases (Ryu *et al*., 2003).

2.1.6.2 Siderophores

Siderophores are small molecules excreted by rhizobacteria when deficient in iron. By complexing with available iron in the rhizosphere it becomes less available to competing phytopathogens which also require iron thus inhibiting competitor growth. Siderophore production by *P. fluorescens* F113 has been shown to play a role in biocontrol of potato soft rot under iron limiting conditions (Antoun and Prévost, 2005). In addition, the antifungal activity of test isolates was greatly enhanced when both HCN and siderophores were produced indicating that together these two plant growth-promoting activities work synergistically to inhibit pathogenic fungi and protect plant health (Deshwal *et al*., 2003).

2.1.6.3 Antibiotics

Many rhizobacteria have been shown to produce antibiotics that inhibit the growth of an antagonistic bacterium. *P. fluorescens*, for example, has been shown to control the soft rot potato pathogen *Erwinia carotovora* subspecies *atroseptica* by producing the antibiotic 2,4-diacetylphloroglucinol (DAPG) (Bankhead *et al*., 2004). Three glucanase-producing actinomycetes, when used separately or more effectively in combination,

could significantly promote plant growth and therefore inhibit the growth of *Pythium aphanidermatum* (Shahverdi *et al*., 2013). Other major antibiotics produced by *B. cereusare* phenazine-e-carboxylic acid and phenazine-1-carboxamide; 2, 4-diacetyl phloroglucinol (phl), pyoluteorin, gluconic acid, 2-hexyl-5-propyl resorcinol and kanosamine (Lugtenberg and Kamilova, 2009).

Bacteriocins are proteins that normally kill or inhibit the growth of closely related bacterial strains. Bacteriocin thuricin17 was isolated from the PGPR *Bacillus thuringiensis* NEB17 (Babalola and Odhiambo, 2008). Oddly enough, this novel bacteriocin was able to inhibit the growth of not only related gram positive bacterial strains, but also of a gram negative strain of *Escherichia coli* MM294.

2.1.6.4 Antifungal Metabolites

Many antifungal metabolites have been produced and shown to be effective in vitro. These antifungal metabolites are also suspected to have antifungal activity in vivo. These metabolites include ammonia, butyrolactones, 2-4-diacetylphloroglucinol, HCN, kanosamine, Oligomycin A, Oomycin A, phenazine-1-carboxylic acid (PCA), pyoluterin (Plt), pyrrolnitrin (pln), viscosinamide, xanthobaccin and zwittermycin A (Milner *et al*., 1996;). In addition, certain fungi have been shown to be sensitive to particular combinations of metabolites.

2.2 Interactions of Plant Growth Promoting Rhizosphere Microorganisms

These interaction plays very important role in plant promotion and survival of the microorganisms themselves and are as follows;

2.2.1 Interactions between plants and bacteria

Interactions may be associative, symbiotic, neutralistic, or parasitic depending upon plant nutrient status in soil, soil environment, plant defence mechanism, and the type of microorganism proliferating in the rhizosphere zone.

In legume–*Rhizobium* symbiosis, *Rhizobium* induces nitrogen-fixing nodules on the roots of leguminous plants. In this process, dinitrogen which is chemically inert and makes up approximately 80% of the volume present in the earth's atmosphere is reduced to ammonia by the bacterial enzyme nitrogenase. The plants provide a micro-aerobic environment for the effective functioning of the oxygen-sensitive nitrogenase and carbohydrates for bacterial endosymbionts to support their metabolism. In return, the bacteria fix atmospheric nitrogen used by the plant for the synthesis of organic nitrogenous compounds to meet its biological needs.

Indirectly, PGPR can act as biofertilizers via asymbiotic nitrogen fixation and the solubilization of mineral phosphates and other nutrients. Rhizobacteria can also act as biocontrol agents by producing siderophores that compete with pathogenic organisms for iron, by producing antibiotics and bacteriocins that suppress bacterial pathogens and by producing anti-fungal metabolites (Milner *et al*., 1996; Shahzaman, 2014).

2.2.2 Interaction of Rhizobium with Azotobacter/Azospirillum

Interactions of *Azotobacter/Azospirillum* with the *Rhizobium* as co-inoculants have been observed to be synergistic in a majority of studies conducted under laboratory, greenhouse or field conditions. *Azotobacter sp*. influence *Rhizobium* by significantly

increasing nodulation. Increasing N_2 content within roots and shoots of respiring/metabolizing plant cells improves conditions within the rhizosphere and enhances synergistic interactions between host and *Azotobacter sp*. In an open field conditions, *Azotobacter* and *Azospirillum* have both been shown to improve growth yields in various soil mineral compositions. This suggests that a mutualistic relationship exists between *Azotobacter* and *Azospirillum* where both interact with the *Rhizobium* to improve *Cicer arietinum* (chick pea) yields (Narula and Vasudeva, 2007; Dashadi *et al.*, 2011).

The beneficial effects of *Azotobacter* and *Azospirillum* on plants are mainly attributed to improvements in root development, an increase in the rate of water and mineral uptake by roots, the displacement of fungi and plant pathogenic bacteria, and to a lesser extent, biological nitrogen fixation (Mikhailouskaya and Bogdevitch, 2009). Associative effect of *Azospirillum lipoferum* and *Azotobacter chroococcum* with *Rhizobium sp*. improved the growth of chick pea grown on both loamy sand and sandy soils (Rokhzadi and Toashih, 2011). Associative effect of *A. chroococcum* on *Bradyrhizobium* strains (BM 42 and BM 43) specific to moong bean (*Vigna radiata*) was also observed. The effect was more pronounced when *A. chroococcum* was co-inoculated with both the strains of *Bradyrhizobium*.

Certain species of *Azospirillum* have been used to study the relationship between free-living nitrogen-fixing rhizobacteria and legumes. Abundant in the rhizosphere, *Azospirilla* possesses a versatile metabolic system where carbon and nitrogen are

metabolized readily. In unfavorable arid or nutrient-deficient conditions, *Azospirilla* can morphologically transform into what appears to be enlarged cysts and the development of an outer polysaccharide coat by accumulating poly-L-hydroxybutyrate granules which serve as carbon and energy sources. A phenotypic advantage, such as a flagellum, allows the highly motile *Azospirillum* genus to swim toward nutrients via chemotactic attraction thus enhancing growth and increased yields (Steenhoudt and Vanderleyden, 2000). The inoculation of legumes with *Azospirillum* prompts enlarged lateral roots and root hairs. This results in improved water uptake and retention with higher nutrient uptake.

Some of the studies have shown that a relationship exists between chemotactic behaviour and *Azotobacter's* influence on plant growth such as cotton (*Gossypium hirsutum* L.) and wheat (*Triticum aestivum* L.) (Kumar *et al*., 2014). In the areas of soil where plant root exudates such as sugars, glucose, amino acids and organic acids have been deposited, bacteria mobilize towards these exudates through chemotactic attraction. Increased yields and enhanced growth using *A. chroococcum* indicate a positive response attributed to nitrogen fixation, phosphorus mobilization, bacterial production and the release of phytohormones (Naher *et al*., 2009).

2.2.3 Interaction of Rhizobium sp. with Rhizobacteria

Rhizosphere bacteria, especially species of *Pseudomonas* and *Bacillus*, have been identified in the rhizosphere of various leguminous and non-leguminous crops that help in plant colonization and suppression of plant pathogens.

Vermar *et al*. (2010) studied co-inoculation of the rhizobacteria with effective *Rhizobium sp*. BHURC01 on chickpea and observed a significant increase in nodule weight, root and shoot biomass and total plant nitrogen when grown either in sterilized chillum jars or under pot culture conditions. The *Rhizobium* stimulatory *Pseudomonas sp*. "CRP55b" showed maximum increase in all the symbiotic parameters. On co-inoculation with *Rhizobium* strains "Ca181" and "Ca313", *Pseudomonas sp*. "CRP55b" and "CRS68" resulted in significant increases in nodule weight, root and shoot biomass and total plant nitrogen. The nodule stimulating rhizobacteria enhanced levels of flavonoid-like compounds in roots on seed bacterization.

Studies have shown that a combined inoculation of *Azospirillum spp*., *A. chroococcum* 5, *Mesorhizobium ciceri* SWR17 and *Pseudomonas fluorescens* P21 improved nodulation, increased dry matter accumulation in roots and shoots, grain yields, biomass and protein yield of chick-pea by a significant margin. This can be attributed to the cumulative effects of an enhanced supply of nutrients, mainly nitrogen and phosphorus and the production of growth promoting substances. In addition, *P. fluorescens* has been found to synergistically interact with additional rhizobacteria to form interactions within the rhizosphere, attributing to phytohormone production, the stimulation of nutrient uptake and the bio-control of deleterious soil bacteria and phyto-pathogenic fungi (Kumari and Khanna, 2014).

Synergistic effects of plant growth-promoting rhizobacteria and *Rhizobium* on nodulation and nitrogen fixation by pigeonpea (*Cajanus cajan*) were also observed

(Tilak and Ranganayaki 2006). Co-inoculation of a variety of PGPR such as *A. chroococcum* and *Pseudomonas putida* with *Rhizobium sp*. (AR-2-2 k) showed increased plant growth, nodulation and improved nitrogenase activity. The association of *Rhizobium sp.* with *P. putida*, *P. fluorescens* and *Bacillus cereus* seems to produce the best agronomical results.

Inoculation of *Rhizobium phaseoli* and PGPR such as *P. fluorescens* P-93 and *A. lipoferum* S-21on bean yield and plant growth parameters yielded promising results (Yadegari *et al*., 2008). In the dually inoculated plants, there were significant increases in quantity, weight, total dry matter, seed yield, and protein content. All treatment combinations resulted in higher yield; however, *Rhizobium* strain Rb-133 inoculated with *P. fluorescens* P-93 gave the highest number of seeds and pods per plant, seed protein yield, and overall seed quantity.

2.2.4 Interaction of Rhizobium with Actinomycetes

Actinomycete, a common antagonistic bacterium is often studied for its inhibitory effects on bacteria within the host rhizosphere. There are various studies in literature suggesting the antagonistic effect of *Actinomycetes* under *in vitro* and *in vivo* conditions. Out of 60 isolates of *Actinomycetes*, bacteria and fungi from pasture soil samples, where no nodulation was observed in clover and 25–70% isolates of *Actinomycetes* were antagonistic toward 12 strains of *Rhizobium trifolii* tested (Shugla and Dwivedi, 1979). Nine lysogenic *Streptomyces sp*. NSA4 were isolated from the nodule surface of black gram which was found to inhibit fast- and slow-growing strains

of cowpea and soybean *Rhizobia*. The fast-growing strain of *Rhizobium* (both cow pea miscellany and soybean) was more sensitive to antibiosis as compared to slow-growing stains. Another study observed that 90% of the *Actinomycete ssp*. isolated from soil obtained from field plots was antagonistic to *Rhizobium japonicum* (Bouizgarne, 2013). In addition, 70% of other *Actinomycete ssp*. isolated from soybean rhizosphere were antagonistic to its homologous *Rhizobia*. However, few isolates stimulated growth of *Bradyrhizobium japonicum*.

The isolates of *Streptomyces lydicus* WYEC108 from pea plants (*Pisum sativum*) were originally studied for its properties as an antifungal biocontrol agent. This strain is capable of mycoparasitic colonization of fungal root pathogens and the excretion of antifungal metabolites within plant rhizospheres. WYEC108 is a unique Streptomyces strain that has the ability to act as a PGPR. It was also hypothesized that root and nodule colonization is one of the several mechanisms by which *Streptomyces* acts as a naturally occurring plant growth-promoting bacterium in pea and possibly other leguminous plants. *Streptomyces* WYEC108 enhanced nodule growth, bacteroid differentiation and act as an aid in bacteroid assimilation of iron and other inorganic nutrients from soils, resulting in enhanced overall growth (Peck and Kende, 1998).

There are some specific interactions in plant rhizosphere among different genera of *Actinomycetes*. *Actinomycete mycelium* makes up to 20% of the total bacterial biomass in the rhizosphere. There is significant lytic activity within the rhizosphere. *Actinomycete mycelium* content within the rhizosphere is significantly higher in root

systems of healthy plants compared to those of plants suffering from root rot disease. Inoculating winter rye (*Secale cereal* L.) with *Actinomycetes* has beneficiary growth advantages; however, co-inoculation of *Actinomycetes* with the cow clover plants (*Trifolium pretense* L.) had no effect on growth (Sangmanee *et al*., 2006).

2.2.5 Interaction of Rhizobium with Mycorrhiza

Associative action of mycorrhizal fungi and *Rhizobia* in legumes has a great impact on root and shoots development and phosphorous uptake which results in the enhancement of nodulation and nitrogen fixation (Jia *et al*., 2004; Ardakani *et al*., 2014). Inoculation with an effective *Rhizobium* combination with VAM fungi had a variable effect on plant growth enhancement, nodulation, and N_2 fixation.

There are various studies in the literature describing many significant findings in the synergistic interaction between AMF and asymbiotic N_2-fixing bacteria such as *A. chroococcum, Azospirillum spp*. and *Acetobacter diazotrohicus* (Khan, 2006; Ordookhani *et al*., 2010). The role of AM fungi as P suppliers to legume root nodules is of great relevance when a specific AM fungus, *Rhizobacterium sp.* known for effective nodulation and N_2 fixation was found in a mycotrophic legume *Anthyllis cytisoidesin* a Mediterranean semi-arid ecosystem in Spain (Requena *et al*., 1996, 2001). The strain *Glomus intraaridices* was found to be more effective with *Rhizobium sp*. NR 4, whereas *Glomus coronatum* was effective when co-inoculated with *Rhizobium sp*. NR9 strain. Research has provided evidence that the genetic pathway of AM symbiosis is shared in part by other root–microbe symbioses such as N_2-fixing *Rhizobia* (Gherbi *et al*., 2008).

Such specific interactions between AM fungi, *Rhizobium*, and PGPR have provided an insight into specific functional compatibility relationships between AMF and PGPR and their management when used as biofertilizers or biocontrol agents.

2.3 Characterization of Plant Growth Promoting Rhizobacteria

Application of molecular techniques have led to more rapid and accurate strategies for examining microbial diversity including the discovery and identification of novel organisms and their catabolic genes involved in the biodegradation of organic contaminants in soil and water (Zahid *et al*., 2015). Microbial identification and diversity characterization has been enhanced by utilising the highly conserved gene, 16SrRNA which is ubiquitous in all microorganisms (Olsen *et al*., 1986). 16SrRNA gene sequences are conserved enough to enable the design of PCR primers which target different taxonomic groups (from kingdom to genus), but have enough variability to provide phylogenetic comparisons of microbial communities (Issar *et al*., 2012; Mahbouba *et al*., 2013).

Microbial community compositions can be analysed based on profiles generated from the physical separation of rRNA or DNA sequences on a gel (Sabir *et al*., 2013). In this regard, several techniques based on the amplification and comparisons of PCR amplified DNA sequences have been developed and used to characterize microbial communities from contaminated environments. These methods detect differences between DNA/RNA sequences, which often include PCR-amplified 16SrRNA gene fragments. The different genetic community profiling methods include amplified

ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE) and terminal-restriction length polymorphism (T-RFLP) (Reski, 2006).

2.3.1 Amplified ribosomal DNA restriction analysis (ARDRA)

For amplified ribosomal DNA restriction analysis, PCR amplified 16S rRNA fragments are digested or cut at specific sites with restriction enzymes and the resulting digest separated by gel electrophoresis. Different DNA sequences will be cut in different locations and will result in a profile unique to the community being analysed. Divergence of the community rRNA restriction pattern on a gel is highly influenced by the type of restriction enzyme used (Spaepen *et al*., 2007). Banding patterns in ARDRA can be used to screen clones or be used to measure bacterial community structure (Kadouri *et al*., 2003).

2.3.2 Ribosomal intergenic spacer analysis (RISA)

RISA is a PCR-based technique that amplifies the region between the 16S and 23S rRNA operons. The intergenic spacer region, depending on the species, has both sequence and length (50–1500 bp) variability (Chung and Tzeng, 2004) and this unique feature facilitates taxonomic identification of organisms (Stajkovic *et al*., 2009). RISA has been used to distinguish between different strains and closely related species of *Staphylococcus, Bacillus, Vibrio* and other medically important microorganisms.

RISA is a very rapid and simple rRNA fingerprinting method but its application in microbial community analysis from contaminated sources is limited partly due to the limited database for ribosomal intergenic spacer sequences is not as large or as comprehensive as the 16S sequence database (Sudadi, 2012). As a result, community analysis using RISA could reduce its effectiveness in the identification of unknown or nonculturable microbial species from contaminated sources. Furthermore, RISA sequence variability may be too great for environmental applications. Its level of taxonomic resolution is greater than 16S rRNA and hence may lead to very complex community profiles (Etesami *et al*., 2009).

2.3.3 Terminal-restriction fragment length polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism is a modification of ARDRA. The PCR primers used in T-RFLP analysis are fluorescently labelled and the resultant PCR products are visualised and quantified (Hussain *et al*., 2015). T-RFLP relies on variations in the positions of restriction sites among sequences and the determination of the length of fluorescently labelled terminal restriction fragments by high-resolution gel electrophoresis on an automated DNA sequences. The use of fluorescently tagged primers limits the analysis to only the terminal fragments of the digestion (Jangu and Sindhu, 2001). This simplifies the banding pattern, hence enabling the analysis of complex communities as well as providing information on diversity as each visible band represents a single operational taxonomic unit or ribotype (Boiero *et al*., 2007).

2.4 DNA sequencing

Recent advances in DNA sequencing have revolutionized the field of genomics, making it possible for even single research groups to generate large amounts of sequence data very rapidly and at a substantially lower cost (Benabdellah *et al*., 2011). These high throughput sequencing technologies make deep transcriptome sequencing and transcript quantification, whole genome sequencing and sequencing available to many more researchers and projects. However, while the cost and time have been greatly reduced, the error profiles and limitations of the new platforms differ significantly from those of previous sequencing technologies (Pandey *et al*., 2013). The selection of an appropriate sequencing platform for particular types of experiments is an important consideration, and requires a detailed understanding of the technologies available; including sources of error, error rate, as well as the speed and cost of sequencing (Leveau and Gerards, 2008).

2.4.1 Sanger capillary sequencing

Reverse strand synthesis is performed on these copies using a known priming sequence upstream of the sequence to be determined and a mixture of deoxy-nucleotides (dNTPs, the standard building blocks of DNA) and dideoxy-nucleotides (ddNTP, modified nucleotides missing a hydroxyl group at the third carbon atom of the sugar) (Hirose *et al*., 2008). The dNTP/ddNTP mixture causes random, non-reversible termination of the extension reaction, creating from the different copies molecules extended to different lengths (Nordström, 2004). Following denaturation and clean-up of free nucleotides, primers, and the enzyme, the resulting molecules are sorted by their molecular weight

(corresponding to the point of termination) and the label attached to the terminating ddNTPs is read out sequentially in the order created by the sorting step (Zazueta *et al*., 2013).

2.4.2 Roche/454 GS FLX Titanium sequencer

It is based on the pyrosequencing approach developed by Pal Nyren and Mostafa Ronaghi at the Royal Institute of Technology, Stockholm in 1996 (Ronaghi *et al*., 1996). In contrast to the Sanger technology, pyrosequencing is based on iteratively complementing single strands and simultaneously reading out the signal emitted from the nucleotide being incorporated (also called sequencing by synthesis, sequencing during extension). Electrophoresis is therefore no longer required to generate an ordered read out of the nucleotides, as the read out is now done simultaneously with the sequence extension (Fleet and Sun, 2005).

In the pyrosequencing process, one nucleotide at a time is washed over several copies of the sequence to be determined, causing polymerases to incorporate the nucleotide if it is complementary to the template strand (Porcel *et al*., 2014). The incorporation stops if the longest possible stretch of complementary nucleotides has been synthesized by the polymerase (Bottini *et al*., 2004). In the process of incorporation, one pyrophosphate per nucleotide is released and converted to ATP by an ATP sulfurylase. The ATP drives the light reaction of luciferases present and the emitted light signal is measured (Bianco and Defez, 2009).

2.4.3 Illumina Genome Analyzer

The reversible terminator technology used by the Illumina Genome Analyzer employs a sequencing-by-synthesis concept that is similar to that used in Sanger sequencing, i.e. the incorporation reaction is stopped after each base, the label of the base incorporated is read out with fluorescent dyes, and the sequencing reaction is then continued with the incorporation of the next base (Mazid *et al*., 2011). Two different adapters are added to the 5' and 3' ends of all molecules using ligation of so-called forked adapters. The library is then amplified using longer primer sequences, which extend and further diversify the adapters to create the final sequence (Mert-Türk, 2002).

2.4.4 Applied Biosystems SOLiD

In the sequencing-by-ligation process, a sequencing primer is hybridized to single-stranded copies of the library molecules to be sequenced. A mixture of 8-mer probes carrying four distinct fluorescent labels compete for ligation to the sequencing primer. The fluorophore encoding, which is based on the two 3'-most nucleotides of the probe, is read (Jeandet *et al*., 2014). Three bases including the dye are cleaved from the 5' end of the probe, leaving a free 5' phosphate on the extended (by five nucleotides) primer, which is then available for further ligation. After multiple ligations (typically up to 10 cycles), the synthesized strands are melted and the ligation product is washed away before a new sequencing primer (shifted by one nucleotide) is annealed (Raaijmakers *et al*., 2008). The same process is followed for three other primers, facilitating the read out of the dinucleotide encoding for each start position in the sequence. Using specific

fluorescent label encoding, the dye read outs (i.e. colors) can be converted to a sequence (Husen *et al*., 2009; Yim *et al*., 2010).

2.5 Abundance of nodule associated bacteria

One of the major strategies for enhancing and exploiting symbiotic nitrogen fixation by legumes in crop production system in through inoculation with NAB. Inoculation of legumes is especially critical when compatible NAB are absent. When population densities are low, or when native NAB are not effective (Cheminingw'a *et al*., 2011). Soils lacking in NAB may occur in areas where indigenous related legumes are absent or where levels of pH, osmotic stress, temperatures, and heavy metals are detrimental to PGPR populations (Shamseldin and Moawad, 2010) susceptibility to *Bacteriophage* and losing of symbiotic plasmids. From Kawaka *et al*., 2014 study, there is an apparent land use on abundance of *Rhizobia* with fallow system giving high abundance. Failure of inoculation to elicit response in legumes is a common phenomenon in Kenyan soils (Muthini *et al*., 2014). This could be due to the presence of effective indigenous NAB or highly competitive but inefficient indigenous strains that lock out the inoculant strains from occupying the nodules (Kawaka et al., 2014). Population size of effective indigenous soil NAB can be used as a reliable index of whether a legume would respond to inoculation or not (Cheminingw'a *et al*., 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Site of study

The soils were collected from farmers' fields in which beans had been grown frequently but for which there was no history of inoculation with rhizobia. The soils were collected from four sites of Lake Victoria shore, Kakamega and Mt. Elgon slopes. At Lake Victoria shores, soils and nodules were collected from farm A (S 00° 08.729'; E 034° 69.596'), Farm B (S 00° 08.828'; E 034° 69.654'), farm C (S 00° 08.852'; 034° 69.654') and farm D (S 00° 09.094; E 034° 69.715'), all in Korando B sub-location in Kisumu County. At Mt. Elgon region, soils were collected from Farm A (S 00° 79.209'; E 034° 63.688'), Farm B (S 00° 77.913'; E 034° 64.030'), farm C (S 00° 81.852'; 034° 61.654') and farm D (S 00° 82.094; E 034° 59.715'), all in Kapkateny sub-location in Bungoma County. At Kakamega, soils and nodules were collected from Farm A (S 00° 19.570'; E 034° 65.921'), Farm B (S 00° 20.779'; E 034° 65.663'), farm C (S 00° 18.982'; 034° 68.534') and farm D (S 00° 18.715; E 034° 68.607'), all in Kakamega south sub-county in Kakamega County. All the soils used in this experiment were obtained from the lands that had been previously used for common bean cultivation.

3.2 Soil sample collection

After clearing the surface debris, soils were sampled with a spade that was sterilized with 75% ethanol to a depth of 30 cm following a W pattern across the selected fields.

About 10g of the soils were taken at intervals of 6 metres along the W pattern running across the whole plot. Soil samples were passed through 2 mm sieve to have homogenous particles for further analysis. The soil samples from each farm were thoroughly mixed, bulked and sub-sampled. One sub-sample was used to determine the population of soil *rhizobacteria*, their diversity and their symbiotic efficiency while the other sub-sample was used for the chemical analyses of the soil. Soil sample for *rhizobacteria* analysis was loosely closed immediately in sterile khaki bags and stored at 4^0C while soil samples for chemical analysis was air-dried and stored at room temperature until it was used.

3.3 Soil chemical analysis

The bulked soil samples were analysed for soil pH, percent carbon, nitrogen, aluminium, potassium and Phosphorus at the University of Nairobi, College of agriculture and veterinary sciences using established protocols. Available nitrogen was determined by Cadmium reduction method (Margeson *et al*., 1980), soil pH by glass electrode method (Kalra, 1995), organic carbon by Walkley black method (Figueiredo *et al*., 2010), Phosphorus by Mehlich method (Watson, 2007), aluminium was extracted with KCl (Auxtero *et al*., 2012) and read with AAS and potassium was extracted with NH_4OAc (Hosseinpur and Zarenia, 2012) and read with flame photometer.

3.4 NAB count to determine the abundance

The numbers of NAB present in Western Kenya soils which can nodulate *P. vulgaris* was estimated by the most probable-number, plant infection technique, modified by Martyniuk and Oron (2008). Seeds of *P. vulgaris* cv. Roseccoco were surface sterilized by being rinsed in 95% ethanol and then submerged in 0.2% acidified mercuric chloride

for 3 min, and they were then germinated on sterile water-agar plates for 2 days. Single seedlings were transplanted into Leonard jars containing vermiculite and the jars were placed in a growth room at 24^0C for a few days. The seedlings were inoculated with 1 ml of 10-fold soil dilutions. In these tests, seven soil dilution steps (from $10^{-1}$to 10^{-7}) and three jars (seedlings) for each dilution, and the plants were watered as required with sterile N-free nutrient solution (Muthini *et al*., 2014). Nitrogen free nutrient solution contained in g/L: $CaCl_2$ 0.1, $MgSO_4.7H_2O$ 0.12, KH_2PO_4 0.1, $Na_2HPO_4.2H_2O$ 0.15, Ferric citrate 0.005, and 1.0 ml of trace elements stock solution. The trace elements stock solution contained: H_3BO_3 2.86, $MnSO_4.7H_2O$ 2.03, $ZnSO_4.7H_2O$ 0.22, $CuSO_4.5H_2O$ 0.08, and $NaMoO_2.2H_2O$ 0.14 in g/L. The pH of the nutrient solution was adjusted to 6.8 with 1M NaOH and 1M HCl. After 4 weeks of growth, the roots of the seedlings were gently washed in tap water and inspected for the presence of nodules in each dilution, and the total number of positive cases counted. Based on these scores, the most probable numbers (MPN) of NAB in the test soils and 95% confidence limits was calculated using MPNES program. Log-transformed numbers of rhizobia in the test soils was expressed per 1g of soil dry matter.

3.5 Determination of genetic diversity

3. 5.1 NAB isolation from the root nodules and culture

Nodules were collected from common beans obtained from the farmers' fields in Mt. Elgon, Kakamega and Kisumu. In addition, nodules were also obtained from common beans cultivated in the screen house; common beans were planted in the tins filled with

the soils obtained from the above regions. NAB were then isolated from surface-sterilized nodules according to the method described by Rincon *et al.* (2007).The surfaces of collected nodules were first sterilized with 75% ethanol and 0.1% $HgCl_2$ for 3 min each, and then extensively rinsed several times with sterile distilled water before being crushed with a flame-sterilized blunt-tipped pair of forceps. The exudates of the crushed nodules were cultured on yeast-mannitol agar (YMA) medium at 28°C for 3-5 days, and a single colony selected for further culture. The purity of the culture was validated by repeated streaking on YMA medium and microscopic examination and identification of cellular morphology. All isolates were stored in 20% glycerol at -70°C.

3. 5.2 DNA extraction, PCR of 16SrRNA gene, Extraction and Purification

Genomic DNA was isolated using Qiagen microbial DNA extraction kit following manufacturer's protocol.

Genomic DNA samples were amplified using the universal primers 27f (5'AGAGTTTGATCCTGGCTCAG 3') and 1492r (5' TACGGCTACCTTGTTACGACTT 3') which are complimentary to conserved regions of the bacterial 16S rRNA gene. Amplification was carried out in 25 µL reaction volumes containing the following: 2.5 µL 10X PCR reaction buffer, (100mM Tris-HCl, pH 8.3, 500 mM KCl) and 1.5 µL 25 mM $MgCl_2$ solution, 4.0 µL 1.25 mM, dNTPs, 0.5 µL of 27f primer (200ng/µL), 0.5 µL of 1492r primer (200ng/ µl), 0.1 µL AmpliTaq Gold DNA polymerase and 1 or 2 µL of DNA as template. Reaction volumes were made up to 25 µL with sterile ultrapure water. The thermal cycling profile consisted of

an initial denaturation step at 94^{o} C for 3 min, followed by 30 cycles of denaturation (1 min at 94^{o} C), annealing at 1 min at 57^{o}C, and extension of 2 min at 72^{o}C, followed by a final extension at 72^{o}C for 8 min. Negative controls were included to check for the presence of false positives due to reagent contamination. Amplified products were separated on 1.5% agarose gels in 1XTBE buffer at 10V cm^{-1} for 30 minutes. Amplification products were then stained with Ethidium bromide and observed with a BioRad UV transilluminator.

After the gel was photographed, the bands were located in by using UV lamp, cut out and placed in a 2mL eppendorf. The PCR fragments were then extracted from the gel using Qiagen Gel extraction kit using their protocol.

3. 5.3 Sequencing reactions of the 16SrRNA gene

Sequencing reactions were performed at Bioneer, South Korea using the BigDye Terminator v3.1 sequencing Kit (Applied Biosystems, USA) with the primers 27F, and 1492R. 12 µl of (4 µl ss DNA, 2 µg, 4 µl 0.8 µM primer, 2 µl 10x MOPS buffer and 2 µl 10x_Mn[2+] isocitrate buffer) was added in 1.5ml microcentrifuge tube, then incubated at 65-70^{0}C for 5 minutes to denature DNA and allow primers anneal. The reaction was allowed to cool at room temperature for 15 minutes, and then briefly centrifuged to reclaim condensation.

To each reaction, 22 µl (7 µl ABI terminator mix (401489), 2 µl diluted Sequenase [TM] (3.25 U/µl), and 1 µl 2 mM a-S dNTPs) was added and incubated for 10 minutes

at 37^0C before 20 µl 9.5 M ammonium acetate and 100 µl 95% ethanol was added and vortexed. It was then centrifuged again for 15 minutes, and carefully the supernatant decanted. DNA was then precipitated in ice-water bath for 10 minutes, centrifuged for 5 minutes at 12000 rpm in a microcentrifuge at 4^0C and supernatant carefully decanted and rinsed in 300 µl of 70-80% ethanol. DNA was then dried for 5-10 minutes in the Speedy-Vac.

Thermal-cycling Conditions included 60^0C for 30 min and holding at 4^0C. Sequenced products were analyzed in an automatic sequencer, ABI3730XL (Applied Biosystems).

3. 5.4 Phylogenetic analysis of the 16SrRNA gene

The 16SrRNA gene sequence reads from the sequencer were edited by ChromasLite (http://www.technelysium.com.au/chromas_lite.html) to remove unknown bases and consensus sequences generated from sequence fragments using BioEdit version (version 7) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and then nucleotide alignments of partial 16SrRNA gene was generated using the multiple MAFFT online alignment (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). As a first approach to identifying the isolates, a BLAST search on the GenBank database was performed.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa

clustered together in the bootstrap test (1000 replicates) are shown above the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 39 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 212 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2004).

In estimating evolutionary divergence between Sequences, the number of base substitutions per site from between sequences are shown. Analyses were conducted using the Jukes-Cantor model (Jukes and Cantor, 1969). The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 255 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2004).

3.6 Determining symbiotic efficiency of the NAB isolates

The plant inoculation studies with Common bean were carried out as mentioned in Rajendran *et al*. (2011). Some germinated Common bean seeds were inoculated with rhizobial strain *Rhizobium sp.* strain NUM466 obtained from the Nairobi Microbiological Resources Center (MIRCEN) collection, some with NAB isolates and the culture-coated seedlings were sown in autoclaved soil systems consisting of the following: autoclaved soil sown with seedlings without any bacterization as the

negative control (NC); seedlings with *Rhizobium sp.* strain NUM466 alone as a positive control (PC) and seedlings coinoculated with *Rhizobium sp.* strain NUM466 and NAB isolates as the experimental systems. All the pot experiments were performed in triplicates and plants were harvested after 56 days of inoculation and various parameters such as number of pods per plant (PPP), number of seeds per pod (SPP), weight of pods per plant (WPP), and total dry matter (TDM) of shoot per plant were monitored.

3.7 Data analysis

Comparison between population size of NAB and chemical soil concentrations was performed by Spearman's correlation implemented in Graphpad Prism (Version 5). Multiple comparisons of various parameters with plants inoculated with *Rhizobia* and other NAB, *Rhizobia* alone and uninoculated was done by one way ANOVA followed by Turkey's post hoc test implemented in Winks (Version 7.0.5).

CHAPTER FOUR

RESULTS

4.1 Soil Characteristics

The chemical and nodule associated bacteria in the soil are shown below.

4.1.1 Chemical characteristics of the soil

The sites from which the soils were collected varied in pH levels, Nitrogen, Phosphorus, Aluminium and potassium concentrations (Table 4.1). Kakamega site had lowest concentration of pH, followed by Kisumu and Mt. Elgon region had the highest. Mt. Elgon region had the lowest levels of aluminium followed by Kisumu and eventually Kakamega had the highest. Kakamega had the highest amount of nitrogen and inorganic carbon while Kisumu had the highest amount of phosphorus and potassium.

4.1.2 NAB population characteristics in the soils

All the soil samples collected from the three field sites contained common bean nodule associated bacteria (NAB). The population size of indigenous NAB in the field sites varied from 17 to 120000 bacterial cells per gram of dry soil. Mt. Elgon soils produced higher amount of bacteria cells per gram, followed by Kisumu and eventually Kakamega produced the least number. The population of NAB from Mt. Elgon soils was significantly higher than those of Kisumu and Kakamega ($p < 0.05$) but there was no significant difference between NAB population for Kakamega and Kisumu ($p > 0.05$) (Table 4.1; Appendix 1).

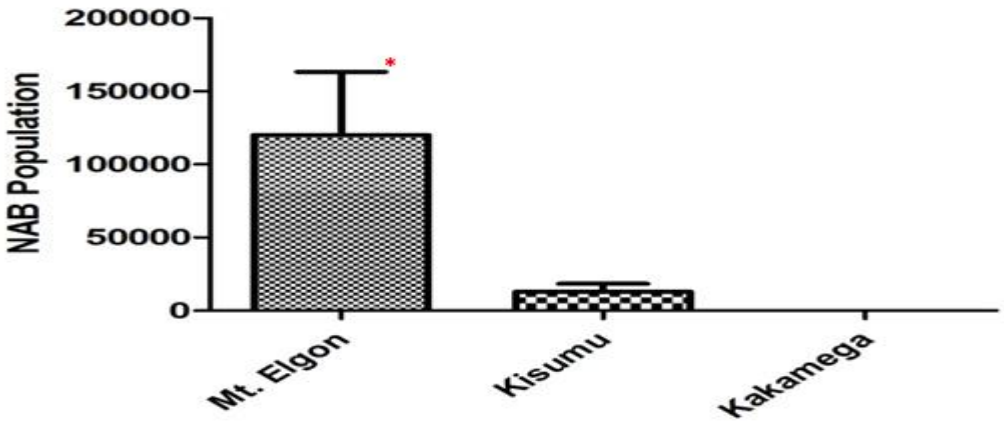


Figure 4.1: Rhizobia population in Kakamega, Kisumu and Mt. Elgon soils

4.2 Association between soil characteristics and NAB population

NAB population in Mt. Elgon region was positively correlated to the soil concentrations of pH, nitrogen, carbon, potassium and phosphorus but negatively correlated to the concentration of aluminium in the soil. However, only the correlation of NAB population with aluminium and phosphorus was significant ($p < 0.05$), but insignificant ($p > 0.05$) with all other factors considered (Table 4.2; Appendix 2).

In Kisumu region, organic carbon, pH and phosphorus were positively correlated to the NAB population though the correlation was not statistically significant ($p > 0.05$) while nitrogen, potassium and aluminium was insignificantly ($p > 0.05$) negatively correlated to the NAB population (Table 4.2; Appendix 3).

Population of NAB in Kakamega soils was positively correlated to the pH, nitrogen and phosphorus while the correlation with organic carbon, potassium and aluminium was negative (Table 4.2; Appendix 4). Only aluminium was found to have a statistically

significant correlation with the soil NAB ($p < 0.05$) while correlation of NAB population with other factors considered was found to be statistically insignificant ($p > 0.05$) in this study.

Table 4.1: Chemical and population characteristics of the selected study sites

	ELGON	KISUMU	KAKAMEGA
NAB Population	120000±86409	12920±10893	17±12.83
pH	6.09±0.44	5.48±0.22	4.61±0.46
% Nitrogen	0.2±0.03	0.15±0.03	0.27±0.07
% Carbon	1.89±0.22	1.085±0.07	2.27±0.3
Potassium (Cmol/Kg)	0.89±0.1	1.54±0.05	0.31±0.13
Phosphorus (PPM)	18.53±7.75*	118.71±94.17	19.75±7.91
Aluminium (Cmol/Kg)	3.7±0.78*	5.78±0.88	9.43±1.86*

* indicates significant difference in soil characteristics and NAB; NAB (nodule associated bacteria); pH (potential of hydrogen); N (nitrogen); C (carbon); K (potassium); P (phosphorus); Al (Aluminium); % (percent)

4.3 Growth characteristics of NAB

Root nodule associated bacteria were isolated from the nodules of common bean plants from different field areas on the YMA. Colonies showing different morphological characteristics on the plates were selected for further characterization. A total 30 strains were isolated with different morphological characteristics. Some of the isolates had pink colour, others had yellow colour, some were colourless, and others were first growing while others were slow growers. Below are some plates showing NAB growing on them.

4.4 Genetic diversity and distribution of Nodule associated bacteria

Genomic DNA of about 21226bp (base pairs) was extracted for all isolates. The PCR amplification resulted in about 1600bp (base pairs) bands of 16SrRNA gene for the thirty isolates (Plate 4. 1). This was reasonable as expected fragment size was about 1450-1580 base pairs.

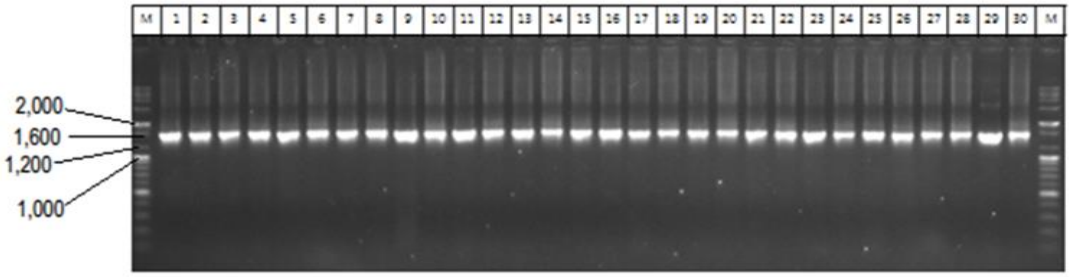


Plate 4.1: Gel electrophoresis of 16S rDNA observed on1% Agarose gel

M indicates marker ladder;
Numbers 1-10 indicates samples from Mt. Elgon region
Numbers 11-20 indicates samples from Kisumu shores
Numbers 21-30 indicates samples from Kakamega

4.4.1 Sequence identity

After sequence reads were cleaned and consensus sequences of between 1434 base pairs to 1557 (Appendix 9) generated, they were blasted in the GenBank database. This produced eight genera; *Enterobacter, Pseudomonas, Providencia, Klebsiella, Rhizobium, Delfitia, Sphingobacterium, Acinedobacter* and one was unknown as shown in the table 4.2 below.

Table 4.2: NCBI identity of NAB obtained from *Phaseolus vulgaris*

Genera Identity from NCBI	Sample name
Enterobacter	E1, E8, K1, K6 and K8
Pseudomonas	E2, E9, K4, K5 and S8
Providencia	E3, E4, E5, E6, K3 and K9
Klebsiella	E7 and K2
Rhizobium	E10, S2, S4, S6 and S9
Delfitia	K7, S3, S5 and S7
Sphingobacterium	S1
Acinedobacter	S10
Unknown	K10

The highest number of organisms belonged to *Providencia* (20%), followed by *Enterobacter*, *Pseudomonas* and *Rhizobia*, each with 16.7%, *Delfitia* with 13.3% , *Klebsiella* with 6.7% while *Sphingobacterium, Acinedobacter* and unknown strain had 3.3% each as shown in figure 4.2 below.

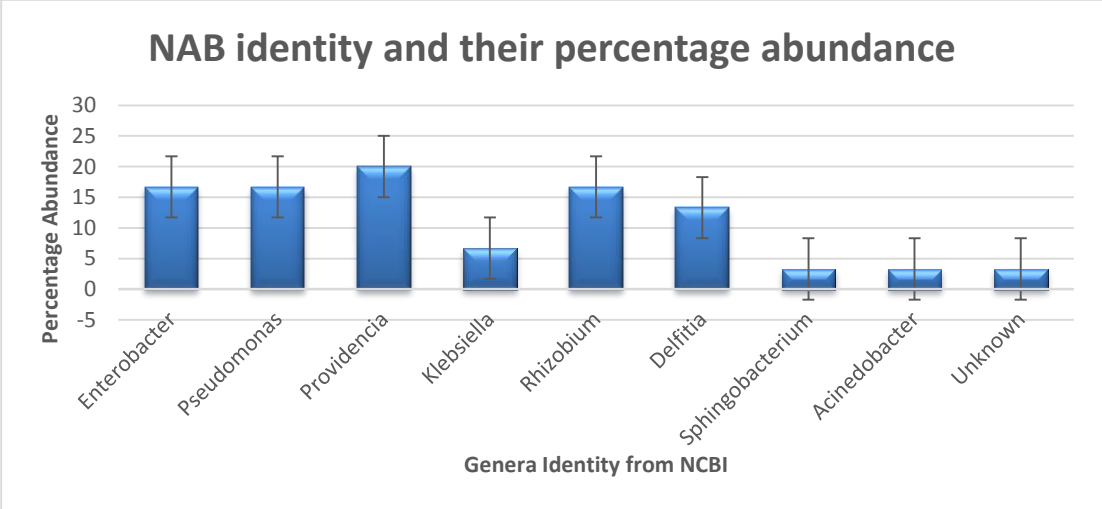


Figure 4.2: Population of NAB isolates in Western Kenya

It was found that Mt. Elgon soils contained 20% of *Enterobacter* and *Pseudomonas*, 40% of *Providencia* and 10% of *Klebsiella* and *Rhizobia*. Kisumu soils contained 10%

of *Pseudomonas*, *Acinetobacter* and *Sphingobacterium*, 30% *Delfitia* and 40% *Rhizobia*. Kakamega soils contained 10% of *Klebsiella*, *Delfitia* and Unknown bacteria strain, 20% *Pseudomonas* and *Providencia* and 30% *Enterobacter*. The graph on Figure 4.3 be below summarizes this distribution of NAB.

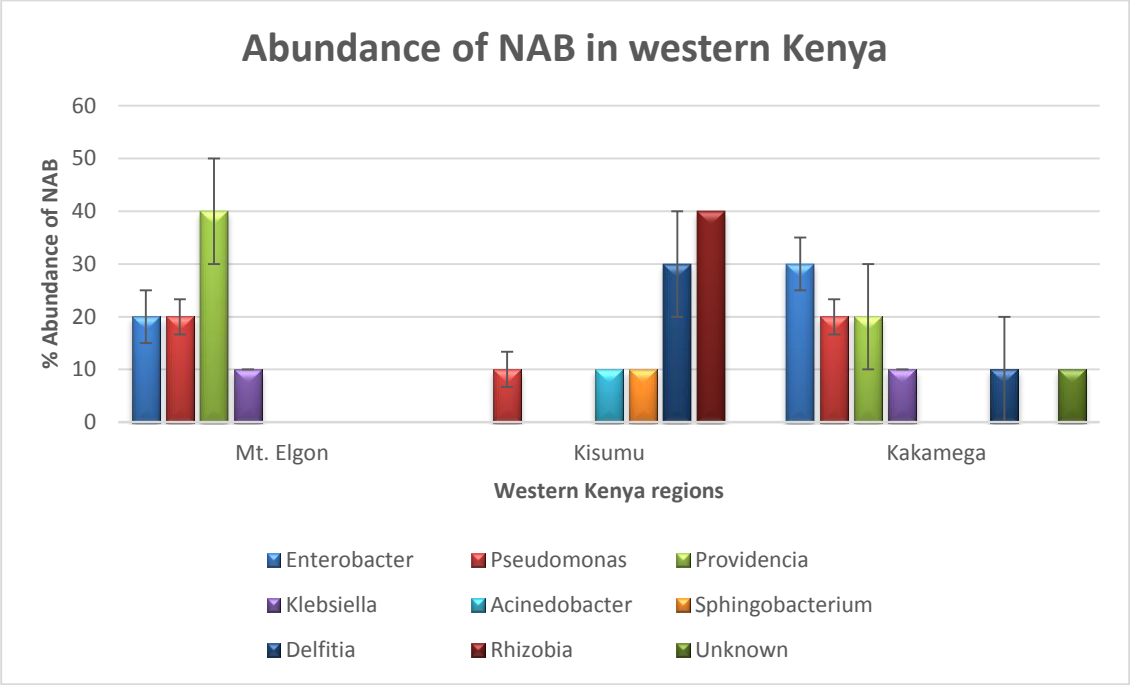


Figure 4.3: Distribution of NAB in three Western Kenya regions

4.4.2 16S rDNA phylogenetic analysis

MAFFT online alignment output is shown in the Plate 4.2 below.

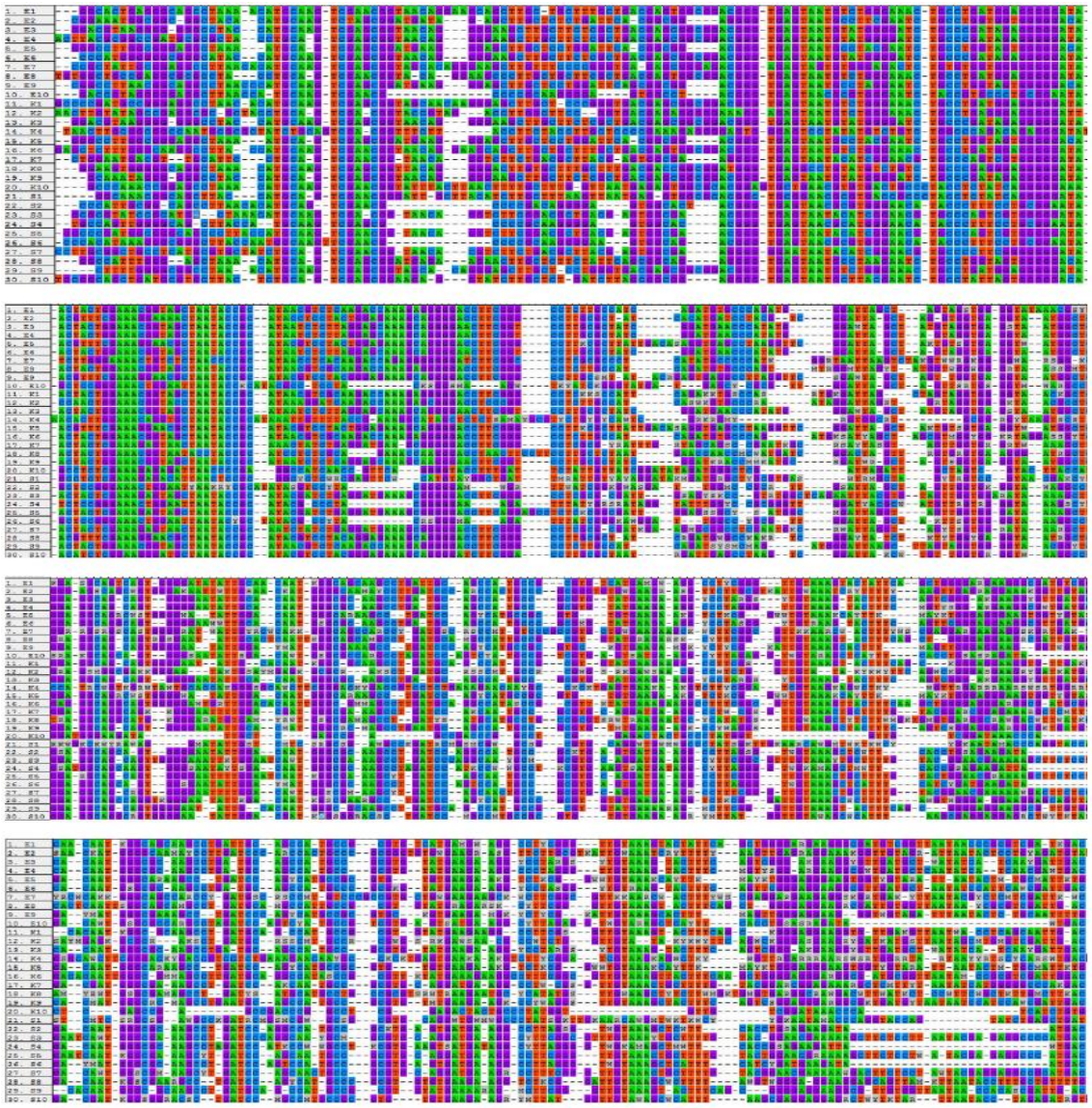


Plate 4.2: A section of 16SrRNA gene alignment produced by MAFFT online and viewed in MEGA: Similar colour indicates similar nucleotide and there the nucleotide conservation. Different colours down the column indicates different nucleotides thus diversity.

Partial 16SrRNA gene sequences from the isolates were used together with seven other 16SrRNA gene partial sequences downloaded from NCBI (http://www.ncbi.nlm.nih.gov/nucleotide/). Standard GenBank sequences *Providencia rattgeri* (NR042413), *Klebsiella variicola* (HQ407284), *Enterobacter sp.* (KF010358), *Acinetobacter calcoaceticus* (JF681282), *Pseudomonas fluoriscens* (NR102835), *Rhizobium sp.* (KF263563) and *Sphingobacterium multivorum* (EU240953) were used as comparisons for 16SrRNA genes from the isolates as shown in figure 4.4.

Phylogenetic tree of the sequences using Neighbour joining method and rooted with *Bacillus licheniformis* separated the root nodule bacteria into two main clusters. The first cluster contained isolates E10, S2, S4, and S6 together with the GenBank *Rhizobium sp.* (KF263563). The second cluster was subdivided into ten subclusters. The first one contained S1 and K10 clustered together with GenBank *Sphingobacterium multivorum* (EU240953).

The second contained K5, E5, E2, S8, S10 and E9 clustered together with the GenBank *Pseudomonas fluoriscens* (NR102835) and *Acinetobacter calcoaceticus* (JF681282). The third one contained K7, S7, S3 and S5 clustered together with the GenBank *Delfitia sp.* (KC702840). The fourth subcluster contained K1, and S9 clustered together with the GenBank isolate *Klebsiella variicola* (HQ407284) and *Enterobacter sp.* (KF0103558). The fifth one contained E6, K3, E3, E4 and K9 clustered together with the GenBank *Providencia rettgeri* (NR042413). K2, K4, K8, and E7 formed independent subclusters (Figure 4.4).

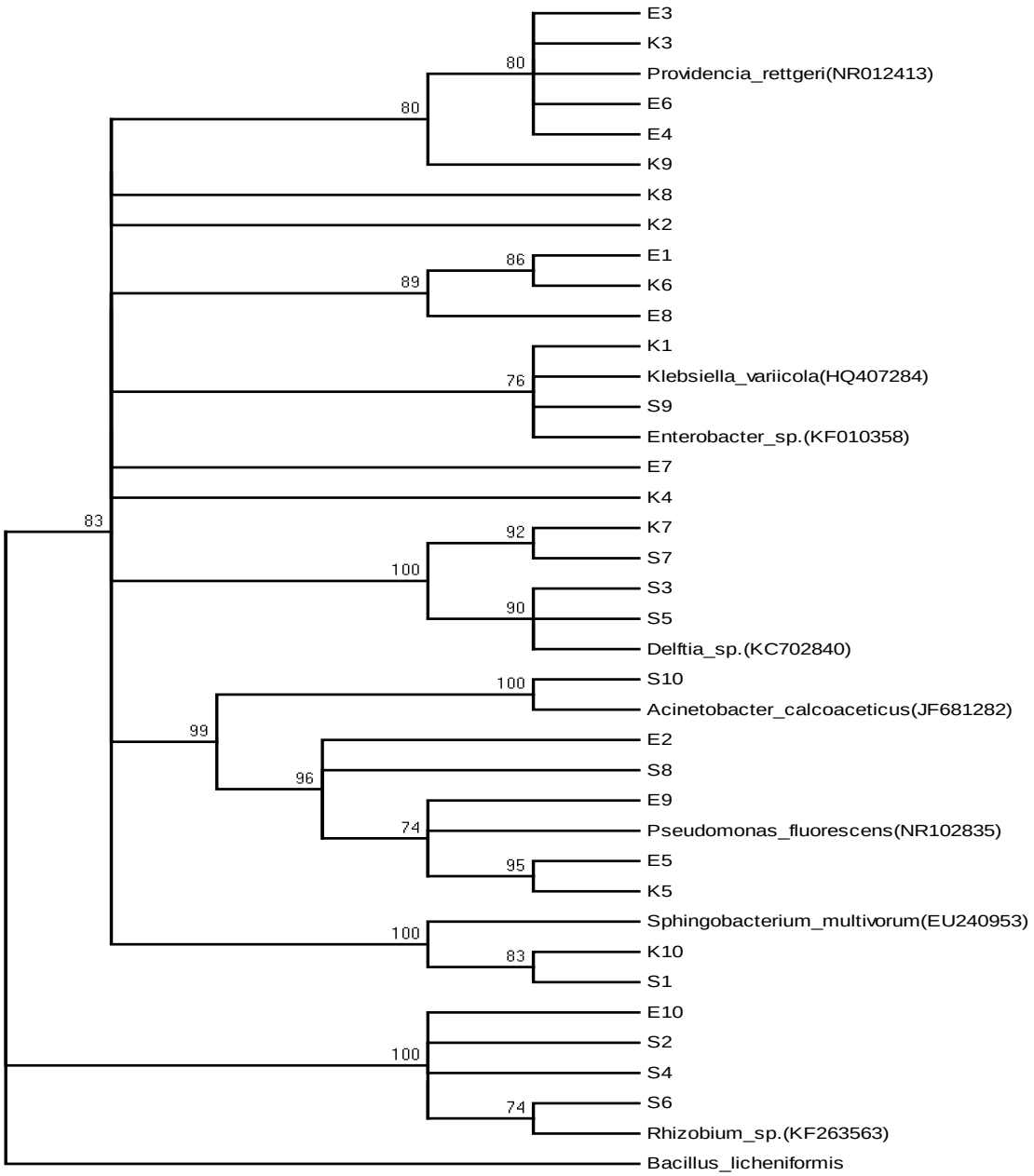


Figure 4.4: Phylogenetic tree rooted with *Bacillus licheniformis*: Organisms in the same cluster are genetically similar, while those in different clusters are genetically different.

The evolutionary divergence matrix is shown in figure 4.5 below

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1. E1																													
2. E2	0.233																												
3. E3	0.074	0.254																											
4. E4	0.070	0.249	0.028																										
5. E5	0.233	0.092	0.243	0.222																									
6. E6	0.061	0.243	0.012	0.016	0.227																								
7. E7	0.156	0.311	0.152	0.156	0.300	0.156																							
8. E8	0.028	0.243	0.078	0.070	0.227	0.066	0.161																						
9. E9	0.212	0.087	0.217	0.196	0.024	0.201	0.277	0.212																					
10. E10	0.386	0.380	0.373	0.386	0.420	0.380	0.406	0.380	0.400																				
11. K1	0.040	0.233	0.070	0.049	0.227	0.057	0.133	0.036	0.206	0.367																			
12. K2	0.147	0.373	0.171	0.161	0.354	0.156	0.243	0.142	0.348	0.492	0.133																		
13. K3	0.074	0.254	0.000	0.028	0.243	0.012	0.152	0.078	0.217	0.373	0.070	0.171																	
14. K4	0.330	0.406	0.342	0.330	0.400	0.336	0.373	0.311	0.373	0.539	0.306	0.373	0.342																
15. K5	0.233	0.092	0.243	0.222	0.000	0.227	0.300	0.227	0.024	0.420	0.227	0.354	0.243	0.400															
16. K6	0.028	0.233	0.061	0.074	0.243	0.066	0.147	0.028	0.217	0.380	0.040	0.142	0.061	0.336	0.243														
17. K7	0.265	0.294	0.249	0.243	0.282	0.249	0.348	0.233	0.277	0.393	0.254	0.342	0.249	0.484	0.282	0.243													
18. K8	0.380	0.555	0.367	0.354	0.531	0.348	0.500	0.380	0.523	0.720	0.367	0.434	0.367	0.680	0.531	0.386	0.547												
19. K9	0.070	0.238	0.024	0.024	0.227	0.012	0.166	0.078	0.201	0.380	0.066	0.161	0.024	0.317	0.227	0.078	0.254	0.342											
20. K10	0.484	0.589	0.523	0.507	0.606	0.515	0.572	0.470	0.580	0.633	0.500	0.580	0.523	0.633	0.606	0.484	0.563	0.680	0.523										
21. S1	0.563	0.589	0.606	0.589	0.563	0.606	0.670	0.555	0.547	0.680	0.597	0.670	0.606	0.651	0.563	0.580	0.606	0.818	0.606	0.249									
22. S2	0.406	0.427	0.400	0.400	0.462	0.406	0.427	0.393	0.427	0.110	0.386	0.492	0.400	0.563	0.462	0.406	0.380	0.762	0.400	0.633	0.661								
23. S3	0.217	0.271	0.206	0.196	0.249	0.201	0.311	0.186	0.233	0.348	0.206	0.300	0.206	0.434	0.249	0.201	0.044	0.515	0.206	0.531	0.563	0.342							
24. S4	0.367	0.380	0.354	0.348	0.406	0.361	0.393	0.354	0.367	0.078	0.348	0.470	0.354	0.507	0.406	0.354	0.354	0.680	0.354	0.606	0.642	0.061	0.311						
25. S5	0.206	0.249	0.212	0.191	0.238	0.201	0.306	0.191	0.222	0.336	0.201	0.311	0.212	0.441	0.238	0.201	0.061	0.515	0.201	0.539	0.563	0.354	0.020	0.317					
26. S6	0.361	0.380	0.354	0.361	0.420	0.361	0.386	0.354	0.386	0.083	0.348	0.462	0.354	0.500	0.420	0.367	0.354	0.690	0.354	0.580	0.615	0.070	0.317	0.049	0.323				
27. S7	0.265	0.306	0.254	0.254	0.311	0.260	0.336	0.243	0.294	0.380	0.260	0.354	0.254	0.448	0.311	0.254	0.066	0.531	0.254	0.507	0.572	0.348	0.074	0.317	0.083	0.323			
28. S8	0.243	0.074	0.243	0.243	0.057	0.233	0.294	0.249	0.053	0.406	0.233	0.367	0.243	0.386	0.057	0.249	0.306	0.555	0.227	0.563	0.563	0.448	0.271	0.393	0.260	0.393	0.317		
29. S9	0.036	0.238	0.066	0.057	0.238	0.053	0.133	0.044	0.212	0.361	0.016	0.133	0.066	0.323	0.238	0.036	0.271	0.373	0.061	0.515	0.606	0.386	0.222	0.348	0.206	0.348	0.265	0.243	
30. S10	0.271	0.196	0.254	0.249	0.152	0.249	0.348	0.249	0.137	0.413	0.271	0.367	0.254	0.427	0.152	0.265	0.254	0.580	0.254	0.547	0.563	0.413	0.222	0.386	0.233	0.373	0.277	0.156	0.277

Figure 4.5: Estimates of Evolutionary Divergence between Sequences: Numbers in the matrix indicate the level of genetic divergence among the isolates. The more the number, the higher the genetic difference among the isolates.

4.5 Determination of symbiotic efficiency

The means of number of pods per plant (PPP) for the plants inoculated with *Rhizobia* and other Plant growth promoting *Rhizobacteria* (RPGPR) was significantly higher (p<0.05) than those inoculated with *Rhizobia* alone and the uninoculated (control) plants while there was no significant difference between the means for the plants inoculated with *Rhizobia* alone and the uninoculated ones (p>0.05) (Table 4.3; Appendix 4).

The means of number of seeds per pod (SPP) for the RPGPR was significantly higher than the means of both from plants inoculated with *Rhizobia* alone and uninoculated ones ($p<0.05$) while the mean of SPP from plants inoculated by *Rhizobia* alone was also significantly higher than the uninoculated ones ($p<0.05$) (Table 4.2; Appendix 5).

The means of weight of pods per plant (WPP) for the plants inoculated with RPGPR was significantly higher than those of plants inoculated with *Rhizobia* alone and the uninoculated ones ($p<0.05$) and the mean of the WPP for the plants inoculated with *Rhizobia* alone and the means for the plants that were not inoculated were not inoculated were not significantly different ($p<0.05$) (Table 4.2; Appendix 6).

The means of total shoot dry matter of plants (TDM) of plants inoculated with RPGPR was significantly higher than the uninoculated and those inoculated with *Rhizobia* alone ($p<0.05$) while the means of TDM for plants inoculated with *Rhizobia* alone and the Uninoculated ones were not significantly different ($p<0.05$) (Table 4.2; Appendix 7).

Table 4.3: Effects of bacterial inoculation on plant growth and symbiotic characteristics

	Inoculated with NAB	**Inoculated with Rhizobia**	**Uninoculated**
Number of pods per plant	13.4±1.17*	10.3±1.00	10.1±3.07
Number of seeds per pod	4.8±1.32*	3.4±0.8	3.3±1.34
Weight of pods per plant	25.01±1.68*	23.32±0.85	22.53±1.94
Total dry matter per plant	26.19±0.62*	23.6±1.99	23.13±3.09

* indicates significant difference between plant yield parameters and various treatments

CHAPTER FIVE

DISCUSSION, CONCLUSION, RECOMMENDATION AND SUGGESTIONS FOR FURTHER RESEARCH

5.1 Discussion

Mt. Elgon region contained higher population of NAB and Kakamega soils had the least. NAB produced significantly higher yield than both positive and negative controls for all the parameters tested. Phylogenetic tree and Evolutionary distance matrix indicated that Common bean NAB that colonise Western Kenya soils are genetically diverse.

The indigenous populations of NAB nodulating common bean ranged from 17 bacteria cells per gram of dry soil to 120000 bacteria cells per gram of dry soil. These observations provide evidence that indigenous common bean nodulating bacteria are widespread in Western Kenya. The high population levels of common bean nodulating ingenious bacteria in the field sites could be attributed to the legumes' widespread integration in the cropping system in Kenya (Chemining'wa *et al*., 2011). This finding is in agreement with Zhang *et al*., (1996) who reported an increase in soil indigenous NAB population from 0.1% (in absence of legume) to 8-9% of the total aerobic bacteria when legumes were cultivated in the field.

Variation among the field sites in population sizes of root nodule bacteria (i.e. 17 to >120000 cells per gram of dry soil) observed in this study is a common phenomenon. This can be attributed to differences in levels of soil pH, plant nutrients, soil

type, soil moisture, temperature and crop/soil management, among other factors (Anyango *et al*., 1992). The results are in agreement with the findings of several authors. Bashan *et al*., (1993) reported inhibition of NAB by high aluminium concentration and low pH. The low population of NAB and legume nodulation noted with the Kakamega soil could be attributed to its low pH (4.56), high aluminium concentration (9.425 Cmol/kg), low organic carbon content (0.3125 Cmol/kg) that have been shown to adversely affect both survival of NAB and nodulation process in legumes (Egamberdieva, 2012).

This study has shown that the main factors that affect the abundance of soil *rhizobacteria* are aluminium and pH which just like in the other findings (Laurette *et al*., 2015). Kakamega soils with the highest concentration of aluminium and lowest pH had the lowest population of NAB (17cells/g) as compared to other regions. The fact that there was no significant difference between Kisumu and Kakamega soils can be attributed to the small difference between soil aluminium concentration and pH between these two regions. This indicates that farming practices which can reduce the concentration of soil aluminium and reduce acidification should be encouraged to improve the population of soil NAB, such as liming because even aluminium solubilization is due to increasing soil acidity (Soares *et al*., 2013).

High concentration of nitrogen has also shown to inhibit the growth of NAB though it was not significant in this study. This finding agrees with the other one (Shamseldin and Moawad, 2010; Chemining'wa *et al*., 2011). This therefore points to the fact that

inorganic fertilizers may not be the best option as far as farming is concerned because a part from being a soil pollutant (Verma *et al*., 2010), it also inhibits microbes which may be important in the solubilization of phosphorus (Khan *et al*., 2009) and production of antibiotics necessary in the destruction of phytopathogens (Jeandet *et al*., 2014). Inoculants containing NAB should therefore be produced to supplement the inorganic fertilizers.

The current study describes the isolation of nonrhizobial and *Rhizobia* bacteria that are closely associated with nodules of *Phaseolus vulgaris*. The isolation procedure adopted involved thorough surface sterilization of nodules and was thus specifically aimed to eliminate nodule epiphytes. Stajković *et al*., (2009) reported the isolation of 115 bacterial isolates from 15 root nodules, of which nearly 60% were rhizobia while the remainder was identified as belonging to several other genera of which eight species were exclusively found only in root nodules. According to the results reported by Rajendran *et al*. (2011) about 10% of the surface sterilized nodules tested showed presence of endophytic nonrhizobial flora and some nodules showed more than one morphologically distinct nonrhizobial colonies. In this study, similar results were obtained in which endophytes from nine genera were isolated from the nodules processed.

The phylogenetic analysis using the results of 16SrRNA gene sequence analysis showed a vast diversity in the microbial flora among the isolated nodule-associated organisms. There are reports stating the rhizospheric diversity (Antoun and Prévost, 2005) but this

study observed that diversity exists even amongst the organisms associated with the nodules. Probably all the organisms whose presence has a beneficial relation might get associated with the plant nodules.

Sequencing data of the 16SrRNA gene of the bacteria isolated in this study classified them in eight genera belonging to *Providencia spp*. being dominant, *Enterobacter, Pseudomonas, Rhizobium, Delfitia, Klebsiella, Sphingobacterium, Acinedobacter* and one of them had no sequence similarity in the GenBank, its phylogenetic relationship shows that it is closely related to *Spingobacterium sp.* This was in line with other findings, Kuklinsky-Sobral *et al*. (2004) reported the isolation of nodule endophytes belonged to the genera *Phyllobacterium, Sphingomonas, Rhodopseudomonas, Pseudomonas, Microbacterium, Mycobacterium,* and *Bacillus* from soya bean nodules. Costa *et al*. (2011) isolated the genera *Agromyces, Bacillus, Brevibacillus, Delftia, Dietzia, Enterobacter, Methylobacterium, Microbacterium, Micrococcus, Paenibacillus, Pseudomonas, Rhizobium, Rhodococcus, Sphingobacterium* and *Stenotrophomonas* from *Phaseolus vulgaris.*

Pseudomonas sp. is distributed in the whole of Western Kenya region because it was isolated in the samples from Kakamega, Kisumu and Mt. Elgon. Its population was high in Kakamega soils. This is an indication that it's the best adapted NAB in this region. Owing to its importance as plant growth promoting bacteria (Yadegari, 2014), more sensitive characterisation techniques are required to determine the type of species found in Western Kenya. *Rhizobia sp*. was isolated in the soils of Mt. Elgon and Kisumu, but

it was not isolated in Kakamega soils. Its population was high in Kisumu soils, in fact it was the most abundant species in Kisumu soils. *Enterobacter sp*. was isolated in Kakamega and Mt. Elgon soils but not Kisumu soils and its population was highest in Kakamega soils in fact it was the most abundant in that soil. *Providencia sp*. was isolated in Kakamega and Mt. Elgon soils and abundantly in Mt. Elgon soils. *Klebsiella sp*. was isolated in Mt. Elgon and Kakamega soil with similar abundance. *Delfitia sp*. was isolated from Kisumu and Kakamega soils and abundantly in Kisumu soils. *Sphingobacterium sp*. and *Acenetobacter sp*. was isolated only from Kisumu soils with similar abundance. This study also isolated uncharacterised strain of bacteria in Kakamega soils whose 16SrRNA gene is absent in the gene bank, this strain clustered with *Sphingobacterium sp*. on the phylogenetic tree.

Phylogenetic analysis on the basis of 16SrRNA gene sequences provided better understanding in evaluation of genetic diversity of NAB isolated in this study. The neighbour joining tree constructed and rooted with GenBank 16SrRNA gene of *Bacillus licheniformis* put the isolates into two main clusters. The second cluster was further subdivided into ten other subclusters. Even most isolates in the same subcluster differed in their genetic distances showing that most of the NAB in the soils of Western Kenya are genetically different. On the genetic distance table (Figure 4.6), only isolates there was no single group of isolates with zero genetic distance indicating that all NAB isolated in this study were genetically different.

The alignment of sequences shown in figure 4.4 shows that 16SrRNA gene of the isolates has very high conservation due to similar nucleotides in the same positions but also with variable regions which makes it a good marker in studying evolutionary diversity. This is in tandem with other studies which have shown that the 16SrRNA gene is efficient to define genera because it is conserved, but have variable regions, just enough to determine genetic diversity in organisms (Rosas *et al*., 2012). However, it has limitations to identify species, due to possible occurrence of genetic recombination and horizontal gene transfer resulting in sequence mosaicism (Mun *et al*., 2013; Ntushelo, 2013). Another disadvantage of bacterial identification based on the analysis of 16SrRNA genes is that, closely related species cannot always be differentiated because of high levels of sequence conservation (Martens *et al*., 2007). To surmount these difficulties, the use of other genes such as protein coding genes with greater sequence divergence than 16SrRNA genes, are recommended as supplementary genetic markers for identification of the NAB (Martens *et al*., 2007).

The effects of co-inoculation with PGPR strains on the symbiotic performance of common bean nodulating *Rhizobia* in the *P. vulgaris* were significant. Common bean is believed to be a poor nitrogen fixer due to the genetic characteristics of symbiotic partners as well as soil and environmental conditions (Cheminingw'a *et al*., 2011). However, selecting *Rhizobacteria* for increased survival in specific soil types, greater compatibility with crop species or cultivars, superior functioning under diverse climates, improved compatibility and competitiveness with other soil microorganisms and higher nitrogen-fixing efficiency have been shown that can

improve growth and yield components of inoculated legumes (Yadegari *et al*., 2010). Beneficial effects of rhizobia on common bean have been described in several studies with different climatic and soil conditions. Hamaoui *et al*. (2001), inoculation of chickpeas with *Azospirillum brasilense* and native *Rhizobia* resulted in a significant increase in nodulation, root and shoot growth, and crop yield as compared with non-inoculated controls and Abd-Alla *et al*. (2014) investigated the synergistic interaction of *Rhizobium* and arbuscular mycorrhizal fungi for improving growth of faba bean grown in alkaline soil. A significant increase over control in number and mass of nodules, nitrogenase activity, leghaemoglobin content of nodule, mycorrhizal colonization, dry mass of root and shoot was recorded in dual inoculated plants than plants with individual inoculation.

In this study, *Rhizobia* strains were able to increase only the number of seeds per pod but failed to significantly increase the number of pods per plant, weight of seeds per plant, and total dry matter of plants in seed filling stage when compared to uninoculated control plants. Co-inoculation of the common bean with *Rhizobium* and PGPR resulted in better nodulation which was translated into higher shoot dry matter and seed yield production. This is in agreement with previous reports demonstrating the beneficial effects of PGPR belonging to *Pseudomonas spp*. and *Azotobacter spp*. on symbiotic efficiency of *rhizobia* nodulating different legume crops (Verma *et al*., 2010; Dashadi *et al*., 2011). The results revealed that application of PGPR together with *Rhizobium* improved the growth and seed production by inoculated beans.

The mechanisms of growth and nitrogen fixation promotion by PGPR are not well understood (Yadegari, 2014); however, a wide range of possibilities including both direct and indirect effects have been suggested (Gupta *et al*., 2000; Jin *et al*., 2006; Martínez-Viveros *et al*., 2010; Gamalero and Glick, 2011; Glick, 2012). The regulation of root system development in plants depends on auxin activity. In legume root nodules, Indole Acid Acetic which is produced by most of PGPR, activates the enzyme H^+-ATPase, which is fundamental for energy production in the nodules (Figueiredo *et al*., 2007). It is also well known that plant root flavonoids are the inducers of nodulation gene (nod genes) expression in *Rhizobium* (Abd-Alla *et al*., 2014). *Rhizobacteria* have also been found to induce phytoalexins class of fluorescent compounds, closely related to flavonoids and isoflavonoids in roots of several crop plants. These phytoalexins have a direct bearing on plant protection mechanisms against pathogens, which help root development (Mert-Türk, 2001). Mobilization of insoluble nutrients such as phosphorus followed by enhancement of uptake by plants, and production of pathogen inhibiting substances (Loom and Bakker, 2005) can also positively affects the Rhizobium-legume symbiosis.

5.2 Conclusions

i. Rhizobacteria population in the soil is decreased by soil concentration of aluminium.

ii. Common bean nodule associated bacteria in Western Kenya soils are genetically diverse as shown by 16SrRNA phylogenic analysis.

iii. This study supports other findings performed in other regions that the yield in common beans increases when other NAB are coinoculated with *Rhizobia*.

5.3 Recommendations from the study

i. Farmers need to lime their soil if pH is low to reduce soil soluble aluminium because it is known that aluminium solubilisation increases with decreasing pH.

ii. Inoculant companies should utilize the information from this study to generate PGPR that are well suited in Western Kenya soils.

5.4 Suggestions for further research

i. A study should be carried out to determine the symbiotic efficiency for the respective isolates obtained in this study to find the most usefully indigenous PGPR.

ii. Now that NAB declined in the presence of high aluminium concentration, a study should be carried out to determine the strains that are tolerant to aluminium toxicity.

REFERENCES

Abd-Alla, M., El-Enany, A., Nafady, N., Khalaf, D. and Morsy, F. (2014). Synergistic interaction of *Rhizobium leguminosarum* bv. viciae and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (*Vicia faba* L.) in alkaline soil. *Microbiological Research*, **169:** 49–58.

Acuña, J., Jorquera, M., Martínez, O., Menezes−Blackburn, D., Fernández, M., Marschner, P., Greiner, R. and Mora, M. (2011). Indole acetic acid and phytase activity produced by rhizosphere bacilli as affected by pH and metals. *Journal of Soil Science and Plant Nutrition*, **11**: 1-12.

Albinus, M., Obando, J. and Bamutaze, J. (2008). Effects of land use practices on livelihoods in the transboundary sub-catchments of the Lake Victoria Basin. *African Journal of Environmental Science and Technology*, **2:**309–317.

Antoun, H. and Prévost, D. (2005). Ecology of plant growth promoting rhizobacteria. In Siddiqui Z. A. (ed.), PGPR: *Biocontrol and Biofertilization*, 1–38.

Anyango, B., Wilson, K., Beynon, J. and Giller, K. (1995). Diversity of *Rhizobia* nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Applied and Environmental Microbiology*, **61**: 4016–4021.

Ardakani, M., Pietsch, G., Friedel, J., Schweiger, P., Moghaddam, A. and Raza, A. (2009). Effect of co-inoculation with rhizobia and mycorrhiza on root parameters of lucerne (*Medicago sativa* L.) under dry organic farming conditions. *International Symposium "Root Research and Applications"* RootRAP, 2–4.

Auxtero, E., Madeira, M. and Parker, D. (2012). Extractable Al and Soil Solution Ionic Concentrations in Strongly Leached Soils from Northwest Iberia: Effects of Liming," *ISRN Soil Science.* **2012**: 1-15.

Babalola, O. and Odhiambo, G. (2008). Effect of Inoculation with *Klebsiella oxytoca*'10 mkr 7' on Striga Suicidal Germination in *Zea mays*. *World Applied Sciences Journal*, **3**: 57-62.

Babana, A. and Antoun, H. (2006). Effect of *Telemsi* phosphate rock solubilising microorganisms on phosphorus uptake and yield of field-grown wheat (*Triticum aestivum* L.) in Mali. *Plant Soil*, **287**: 51-58.

Bankhead, S., Landa. B., Lutton, E., Weller, D. and Gardener, B. (2004). Minimal changes in Rhizobacterial population structure following root colonization by wild type and transgenic biocontrol strains. *FEMS Microbiology Ecology*, **49**: 307–318.

Bar-Ness, E., Hadar, Y., Chen, Y., Shanzer, A. and Libman, J. (1992). Iron uptake by plants from microbial siderophores: A study with 7-nitrobenz-2 oxa-1,3-diazole-desferrioxamine as fluorescent ferrioxamine B analog. *Plant Physiology*, **99**: 1329-1335.

Bashan, Y. and De-Bashan, L. E. (2010). Chapter two-how the plant growth-promoting bacterium Azospirillum promotes plant growth—a critical assessment. *Advances in Agronomy*, **108:** 77-136.

Bashan, Y., Holguin, G. and Lifshitz, R. (1993). *Isolation and characterization of Plant Growth -Promoting Rhizobacteria*. In Methods in Plant Molecular Biology and Biotechnology. CRC Press.

Bay, Y., Souleimanov, A. and Smith, D. (2002). An inducible activator by a *Serratia proteamaculans* strain and its soybean growth-promoting activity under greenhouse conditions. *Journal of Experimental Botany*, **53**: 1495-1502.

Benabdellah, K., Abbas, Y., Abourouh, M., Aroca, R. and Azcón, R. (2011). Influence of two bacterial isolates from degraded and non-degraded soils and arbuscular mycorrhizae fungi isolated from semi-arid zone on the growth of *Trifolium repens* under drought conditions: Mechanisms related to bacterial effectiveness. *European Journal of Soil Biology*, **47**: 303-309.

Beneduzi, A., Ambrosini, A. and Passaglia, L. (2012). Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology*, **35**: 1044-1051.

Bhalla, K., Singh, S. B. and Agarwal, R. (2010). Quantitative determination of gibberellins by high performance liquid chromatography from various gibberellins producing Fusarium strains. *Environmental Monitoring and Assessment*, **167**: (1-4), 515-520.

Bianco, C. and Defez, R. (2009). *Medicago truncatula* improves salt tolerance when nodulated by an indole-3-acetic acid-overproducing *Sinorhizobium meliloti* strain. *Journal of Experimental Botany*, **60**: 3097–3107.

Boiero, L., Perrig, D., Masciarelli, O., Penna, C., Cassán, F. and Luna, V. (2007). Phytohormone production by three strains of Bradyrhizobium japonicum and possible physiological and technological implications. *Applied Microbiology and Biotechnology*, **74**: 874–880.

Bottini, R., Cassán, F. and Piccoli, P. (2004). Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. *Applied Microbiology and Biotechnology*, **65**: 497–503.

Bouizgarne, B. (2013). Bacteria for plant growth promotion and disease management. In *Bacteria in agrobiology: Disease management*. Springer Berlin Heidelberg.

Boukhalfa, H. and Crumbliss, A. (2002). Chemical aspect of siderophore mediated iron transport. *Biometals*, **15**: 325-339.

Burd, G., Dixon, G. and Glick, B. (1998). A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. *Applied and Environmental Microbiology*, **64**: 3663–3668.

Chemining'wa, G., Theuri, S. and Muthomi, J. (2011). Abundance of indigenous *Rhizobia* nodulating cowpea and common bean in central Kenyan soils. *African Journal of Horticultural Science*, **5**: 92-97.

Chen, C., Bauske, E., Musson, G., Rodriquez-Kabana, R. and Kloepper, J. (1995). Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. *Biological Control*, **5**: 83-91.

Chung, K. and Tzeng, D. (2004). Biosynthesis of indole-3-acetic acid by the gall-inducing fungus *Ustilago esculenta*. *Journal of Biological Sciences* **4**: 744-750.

Costa, L. E. D. O., Queiroz, M. V. D., Borges, A. C., Moraes, C. A. D. and Araújo, E. F. D. (2012). Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (Phaseolus vulgaris). *Brazilian Journal of Microbiology*, **43**: 1562-1575.

Das, A., Kumar, M. and Kumar, R. (2013). Plant growth promoting rhizobacteria (PGPR): an alternative of chemical fertilizer for sustainable, environment friendly agriculture. *Research Journal of Agriculture and Forestry Sciences*, **1**: 21-23.

Dashadi, M., Khosravi, H., Moezzi, A., Nadian, H., Heidari, M. and Radjabi, R. (2011). Co-inoculation of *Rhizobium* and *Azotobacter* on growth of faba bean under water deficit conditions. *American-Eurasian Journal of Agriculture & Environmental Sciences*, **11**: 314-319.

Deshwal, V., Pandey, P., Kang, C. and Mahashwari, D. (2003). Rhizobia as a biological agent against soil borne plant pathogenic fungi. *Indian Journal of Experimental biology*, **41**: 1160-1164.

Desnoues, N., Lin, M., Guo, X., Ma, L., Carreno-Lopez, R. and Elmerich, C. (2013). Nitrogen fixation genetics and regulation in a *Pseudomonas stutzeri* strain associated with rice. *Microbiology*, **149**: 2251–2262.

Egamberdieva, D. (2012). The management of soil quality and plant productivity in stressed environment with rhizobacteria. In *Bacteria in Agrobiology: Stress Management*. Germany: Springer-Verlag Berlin Heidelberg.

Etesami, H., Alikhani, H. and Akbari, A. (2009). Evaluation of plant growth hormones production (iaa) ability by iranian soils rhizobial strains and effects of superior strains application on wheat growth indexes. *World Applied Sciences Journal*, **6**: 1576-1584.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**: 783-791.

Figueiredo, M. V. B., Martinez, C. R., Burity, H. A. and Chanway, C. P. (2008). Plant growth-promoting rhizobacteria for improving nodulation and nitrogen fixation in the common bean (Phaseolus vulgaris L.). *World Journal of Microbiology and Biotechnology*, **24**: 1187-1193.

Figueiredo, M., Seldin, L., de Araujo, F. and Mariano, R. (2010). Plant growth promoting Rhizobacteria: Fundamentals and applications. In *Plant Growth and Health Promoting Bacteria*. D.K. Maheshwari (ed.), Berlin Heidelberg: Springer-Verlag.

Fleet, C. M. and Sun, T. P. (2005). A DELLAcate balance: the role of gibberellin in plant morphogenesis. *Current Opinion in Plant biology*, **8:** 77-85.

Gamalero, E. and Glick, B. (2011). Mechanisms used by plant growth-promoting bacteria. In *D.K. Maheshwari (ed.), Bacteria in Agrobiology: Plant Nutrient Management*. Berlin: Springer-Verlag Berlin Heidelberg.

Gherbi, H., Markmann, K., Svistoonoff, S., Estevan, J., Autran, D., Giczey, G., Auguy, F., Péret, B., Laplaze, L., Franche, C. and Parniske, M. (2008). SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and Frankiabacteria. *Proceedings of the National Academy of Sciences*, **105**: 4928-4932.

Glick, B. (2004). Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiology Letters*, **251:** 1–7.

Glick, B. (2012). Plant growth-promoting bacteria: Mechanisms and applications. *Hindawi Publishing Corporation Scientifica*, **2012**: 1-16.

Guo, H., Luo, S., Chen, L., Xiao, X., Xi, Q., Wei, W., Zeng, G., Liu, C., Wan, Y., Chen, J. and He, Y. (2010). Bioremediation of heavy metals by growing hyperaccumulaor endophytic bacterium *Bacillus sp*. L14. Bioresource Technology, **101**: 8599–8605.

Gupta, A., Gopal, M. and Tilak, K. (2000). Mechanisms of plant growth promotion by rhizobacteria. *Indian Journal of Experimental Biology*, **38**: 856-862.

Hamaoui, B., Abbadi, J., Burdman, S., Rashid, A., Sarig, S. and Okon, Y. (2001). Effects of inoculation with *Azospirillum brasilense* on chickpeas (*Cicer arietinum*) and faba beans (*Vicia faba*) under different growth conditions. *Agronomie*, **21**: 553–560.

Hinsinger, P. (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant and Soil*, **237**: 173–195.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H. and Sakakibara, H. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of Experimental Botany*, **59:** 75–83.

Hosseinpur, A. and Zarenia, M. (2012). Evaluating chemical extractants to estimate available potassium for pinto beans (Phaseolus vulgaris) in some calcareous soils. *Plant Soil and Environmen*t. **58**: 42–48.

Hung, P. and Annapurna, K. (2004). Isolation and characterization of endophytic bacteria in soybean (*Glycine sp*.). *Omonrice*, **12**: 92-101.

Husen, E., Wahyudi, A., Suwanto, A. and Saraswati, R. (2009). Soybean seedling root growth promotion by 1-aminocyclopropane-1-carboxylate deaminase-producing Pseudomonads. *Indonesian Journal of Agricultural Science*, **10**: 19-25.

Hussain, A., Shah, S., Rahman, H., Irshad, M. and Iqbal, A. (2015). Effect of IAA on in-vitro growth and colonization of Nostoc in plant roots. *Plant-Microbe Interaction*, **6**: 1-9.

Issar, S., Sharma, S., Choudhary, D., Gautam, H. and Gaur, R. (2012). Molecular characterization of pseudomonas spp. isolated from root nodules of various leguminous plants of Shekhawati region, Rajasthan, *India. American Journal of Plant Sciences*, **3**: 60-63.

Jangu, O. and Sindhu, S. (2001). Differential response of inoculation with indole acetic acid producing *Pseudomonas sp.* in green gram (*Vigna radiata* L.) and black gram (*Vigna mungo* L.). *Microbiology Journal*, **1**: 159-173.

Jeandet, P., Hébrard, C., Deville, M., Cordelier, S., Dorey, S., Aziz, A. and Crouzet, J. (2014). Deciphering the role of phytoalexins in plant-microorganism interactions and human health. *Molecules*, **19**: 18033-18056.

Jezequel, K., Perrin, J. and Lebeau, T. (2003). Bioaugmentation with a Bacillus sp. to reduce the phytoavailable Cd of an agricultural soil: comparison of free and immobilized microbial inocula. *Chemosphere*, **59**:1323–1331.

Jia, Y., Gray, V. and Straker, V. (2004). The Influence of Rhizobium and Arbuscular Mycorrhizal Fungi on Nitrogen and Phosphorus Accumulation by *Vicia faba*. *Annals of Botany*, **94**: 251–258.

Jin, C., He, Y., Tang, C., Wu, P. and Zheng, S. (2006). Mechanisms of microbially enhanced Fe acquisition in red clover (*Trifolium pratense* L.). *Plant, Cell and Environment*, **29**: 888–897.

Johri, B., Sharma, A. and Virdi, J. (2003). Rhizobacterial diversity in India and its influence on soil and plant health. *Advanced Biochemistry and Engineering/Biotechnology*, **84**: 49–89.

Jukes, T. and Cantor, C. (1969). Evolution of protein molecules. In Munro HN, editor, *Mammalian Protein Metabolism*, New York: Academic Press.

Kadouri, D., Jurkevitch, E. and Okon, Y. (2003). Involvement of the reserve material poly-hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. *Applied and Environmental Microbiology*, **69**: 3244–3250.

Kalra, Y. (1995). Determination of pH of Soils by Different Methods: Collaborative Study. *Journal of AOAC International*. **78**: 310-324.

Kawaka, K. Dida, M., Opala, P., Ombori, O., Maingi, J., *et al*, (2014). Symbiotic efficiency of native Rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in soils of Western Kenya," *International Scholarly Research Notices*, **2014**: 1-8.

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

Khan, G. (2006). Mycorrhizoremediation—an enhanced form of phytoremediation. *Journal of Zhejiang University SCIENCE B*, **7**: 503-514.

Kloepper, J., Schroth, M. and Miller, T. (1980). Effect of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant development and yield. *Phytopathology*, **70**: 1078-1082.

Kuffner, M., Puschenreiter, M., Wieshammer, G., Gorfer, M. and Sessitsch, A. (2008). Rhizosphere bacteria affect growth and metal uptake of heavy metal accumulating willows. *Plant Soil*, **304**: 35–44.

Kuklinsky-Sobral, J., Araujo, W., Mendes, R., Pizzirani-Kleiner, A., and Azevedo, J. (2004). Isolation and characterization of endophytic bacteria from soybean (Glycine max) grown in soil treated with glyphosate herbicide. *Plant and Soil*, **273**: 91–99.

Kumar, A., Singh, R., Giri, D., Singh, P. and Pandey, K. (2014). Effect of *Azotobacter chroococcum* CL13 inoculation on growth and curcumin content of turmeric (*Curcuma longa* L.). *International Journal of Current Microbiology and Applied Sciences*, **3**: 275-283.

Kumari, S. and Khanna, V. (2014). Effect of antagonistic rhizobacteria coinoculated with *Mesorhizobium ciceris* on control of fusarium wilt in chickpea (*Cicer arietinum* L.). *African Journal of Microbiology Research*, **8**: 1255-1265.

Laurette, N., Maxémilienne, N., Henri, F., Souleymanou, A., Kamdem, K., Albert, N., Dieudonné, N. and François-Xavier, E. (2015). Isolation and screening of indigenous bambara groundnut (V*igna subterranea*) nodulating bacteria for their tolerance to some environmental stresses. *American Journal of Microbiological Research*, **3:** 65-75.

Lemanceau, P., Bauer, P., Kraemer, S. and Briat, J. (2009). Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes. *Plant Soil*, **321**:513–535.

Leveau, J. and Gerards, S. (2008). Discovery of a bacterial gene cluster for catabolism of the plant hormone indole 3-acetic acid. *FEMS Microbiological Ecology* **l65:** 238–250.

Loon, L and Bakker, P. (2005). Induced systemic resistance as a mechanism of disease suppression by Rhizobacteria. In *PGPR: Biocontrol and Biofertilization*, Z. A. Siddiqui (ed.), Dordrecht: Springer, pp. 39-66.

Loper, J. and Buyer, J. (1990). Sidoropheres in microbial interactions on plant surfaces. *Molecular Plant Microbe Interactions*, **4**: 5-13.

Lugtenberg, B. and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, **63**:541–56.

Mahbouba, B., Christine, L., Nadir, B., Nadia, Y. and Abdelhamid, D. (2013). Phenotypic and molecular characterization of plant growth promoting Rhizobacteria isolated from the rhizosphere of wheat (*Triticum durum* Desf.) in Algeria. *African Journal of Microbiology Research*, **7:** 2893-2904.

Mardad, I., Serrano, A. and Soukri, A. (2013). Solubilization of inorganic phosphate and production of organic acids by bacteria isolated from a Moroccan mineral phosphate deposit. *African Journal of Microbiology Research*, **7:** 626-635.

Margeson, J. Suggs, J. and Midgett, R. (1980). Reduction of nitrate to nitrite with cadmium. *Journal of Analytical Chemistry.* **52**: 1955–1957.

Marroqui, S., Zorreguieta, A., Santamari´A, C., Temprano, F., Soberon, M., Megias, M. And Downie, A. (2001). Enhanced Symbiotic Performance by *Rhizobium tropici* Glycogen Synthase Mutants. *Journal of Bacteriology*, **183**: 854-864.

Martens, M., Delaere, M., Coopman, R., Vos, P., Gillis, M. and Willems, A. (2007). Multilocus sequence analysis of Ensifer and related taxa. *International Journal of Systematic and Evolutionary Microbiology*, **57:** 489–503.

Martínez-Viveros, O., Jorquera, M., Crowley, D. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of Soil Science and Plant Nutrition*, **10**: 293 – 319.

Martyniuk, S., Oron, J. and Martyniuk, M. (2004). Diversity and numbers of root-nodule bacteria (*Rhizobia*). *Acta Societatis Botanicorum Polionae*, **74**: 83-86.

Mazid, M., Khan T., and Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants. *Biology and Medicine*, **3**: 232-249.

Mert-Türk, F. (2002). Phytoalexins: Defense or just a response to stress? *Journal of Cell and Molecular Biology*, **1**: 1-6.

Meyer, J. (1978). The fluorescent pigment of *Pseudomonas fluorescens* : Biosynthesis, purification and physicochemical properties. *Journal of General Microbiology*, **107:** 319-328.

Mikhailouskaya, N. and Bogdevitch, I. (2009). Effect of biofertilizers on yield and quality of long-fibred flax and cereal grains. *Agronomy Research*, **7:** 412–418.

Milner, J., Silo-Suh, L., Lee, J., He, H., Clardy, J. and Handelsman, J. (1996). Production of Kanosamine by *Bacillus cereus* UW85. *Applied and Environmental Microbiology*, **62**: 3061–3065.

Mohamed, Z., El-Sayed, S., Radwan, T. and El-Wahab, G. (2009). Potency evaluation of *Serratia marcescens* and *Pseudomonas fluorescens* as biocontrol agents for root-knot nematodes in Egypt. *Journal of Applied Sciences Research*, **4**: 93-102.

Multiple alignment program for amino acid or nucleotide sequences: http://align.bmr.kyushu-u.ac.jp/mafft/online/server/

Mun, S., Lee, J., Lee, S., Han, K. and Ahn, T. (2013). Phylogeny of flavobacteria group isolated from fresh water using multilocus sequencing analysis. *Genomics & Informatics*, **11**: 272-276.

Muthini, M., Maingi, J., Muoma, J., Amoding, A., Mukaminega, D., Osoro, N., Mgutu, A. and Ombori, O. (2014). Morphological assessment and effectiveness of

indigenous rhizobia isolates that nodulate *p. vulgaris* in water hyacinth compost testing field in Lake Victoria basin. *British Journal of Applied Science & Technology*, **4**: 718-738.

Naher, U., Radziah, O., Halimi, M., Shamsuddin, Z.and Mohd Razi, I. (2009). Influence of root exudate carbon compounds of three rice genotypes on rhizosphere and endophytic diazotrophs. *Pertanika Journal of Tropical Agricultural Sciences*, **32**: 209 – 223.

Narula N. and Vasudeva, M. (2007). Terrestrial Environment. *Environmental Microbiology*: 1-28.

National Center for Biotechnology Information: Retrieved from the web address:

http://www.ncbi.nlm.nih.gov/nucleotide/.

Nordström, A. (2004). Cytokinins in Arabidopsis, tools, pathways and interaction with auxin. *Doctoral thesis: Swedish University of Agricultural Sciences*.

Ntushelo, K. (2013). Identifying bacteria and studying bacterial diversity using the 16S ribosomal RNA gene-based sequencing techniques: A review. *African Journal of Microbiology Research*, **7**: 5533-5540.

Nürnberger, T. and Scheel, D. (2001). Signal transmission in the plant immune response. *TRENDS in Plant Science*, **6**: 372-379.

Okazaki, S., Sugawara, M. and Minamisawa, K. (2004). Bradyrhizobium elkanii rtxC Gene Is Required for Expression of Symbiotic Phenotypes in the Final Step of Rhizobitoxine Biosynthesis. *Applied and Environmental Microbiology*. **70**: 535–541.

Olsen, G., Lane, D., Giovannoni, S., Pace, N., Stahl, D. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Annu Rev Microbiol*, **40**: 337–365.

Ordookhani, K., Khavazi, K., Moezzi, A. and Rejali, F. (2010). Influence of PGPR and AMF on antioxidant activity, lycopene and potassium contents in tomato. *African Journal of Agricultural Research*, **5**: 1108-1116.

Osoro, N.O., Kawaka, F., Naluyange, V., Ombori, O., Muoma, J.O., Amodingd, A., Mukaminegae, D., Muthini, M. and Maingi, J.M. (2014). Effects of water hyacinth (Eichhornia crassipes [mart.] solms) compost on growth and yield of common beans (Phaseolus vulgaris) in Lake Victoria Basin. *European International Journal of Science and Technology*, **3**: 173-186.

Pandey, P., Kang, S. and Maheshwari, D. (2005). Isolation of endophytic plant growth promoting *Burkholderia sp*. MSSP from root nodules of *Mimosa pudica*. *Current Science*, **89**: 177-180.

Pandey, R., Swammy, K. and Khetmalas, M. (2013). Indole: A novel signaling molecule and its implications. *Indian Journal of Biotechnology*, **12**: 297-310.

Parmar, N. and Dadarwal, K. (1998). Stimulation of nitrogen fixation and induction of flavonoid like compounds by rhizobacteria. *Journal of Applied Microbiology*, **86:** 36–44.

Parmar, N. and Dufresne, J. (2011). Beneficial interactions of plant growth promoting rhizosphere microorganisms. In *Bioaugmentation, Biostimulation and Biocontrol*. Berlin Heidelberg: Springer-Verlag.

Patten, C. and Glick, B. (2004). Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. *Applied and Environmental Microbiology*, **68**: 3795–3801.

Peck, S. and Kende, H. (1998). A gene encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase produces two transcripts: elucidation of a conserved response. *The Plant Journal*, **14**: 573-581.

Peralta, H., Mora, Y., Salazar, E., Encarnacion, S., Palacios, F. and Mora, J. (2004). Engineering the nifH promoter region and abolishing poly-hydroxybutyrate accumulation in *Rhizobium etl*i enhance nitrogen fixation in symbiosis with *Phaseolus vulgaris*. *Applied and Environmental Microbiology*, **70**: 3272–3281.

Porcel, R., Zamarreño, A., García-Mina, J. and Aroca, R. (2014). Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. *BMC Plant Biology*, **14**: 1471-2229.

Raaijmakers, J., Paulitz, T., Steinberg, C., Alabouvette, C. and Moënne-Loccoz, Y. (2008). The rhizosphere: a playground and battlefield for soil borne pathogens and beneficial microorganisms. *Plant Soil*, **321**: 341–361.

Rajendran, G., Patel, M. and Joshi, S. (2011). Isolation and characterization of nodule-associated *Exiguobacterium sp*. from the root nodules of fenugreek (*Trigonella foenum*-graecum) and their possible role in pant growth promotion. *International Journal of Microbiology*, **2012**: 1-8.

Ramírez, M., Valderrama, B., Arredondo-Peter, R., Soberón, M., Mora, J. and Hernández, G. (1999). *Rhizobium etli* genetically engineered for the heterologous expression of *Vitreoscilla sp*. hemoglobin: effects on free-living and symbiosis. Molecular Plant-Microbe Interactions, **12:** 1008–1015.

Requena, N., Jeffries, P. and Barea, J. (1996). Assessment of natural mycorrhizal potential in a desertified semiarid ecosystem. *Applied and Environmental Microbiology*, **62**: 842–847.

Requena, N., Perez-Solis, E., Azcon-Aguilar, G., Jeffries, P. and Barea, J. (2001). Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. *Applied and Environmental Microbiology*, **65**: 495-498.

Reski, R. (2006). Small molecules on the move: homeostasis, crosstalk, and molecular action of phytohormones. *Plant Biology*, **8**: 277–280.

Rodríguez, H. and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, **17**: 319–339.

Rojas, A., Holguin, G., Glick, B. and Bashan, Y. (2001). Synergism between *Phyllobacterium sp*. (N_2-fixer) and *Bacillus licheniformis* (P-solubilizer), both from a semiarid mangrove rhizosphere. *FEMS Microbiology Ecology*, **35**: 181-187.

Rokhzadi, A. and Toashih, V. (2011). Nutrient uptake and yield of chickpea (*Cicer arietinum* L.) inoculated with plant growth promoting rhizobacteria. *Australian Journal of Crop Science*, **5**: 44-48.

Ronaghi, M., Karamohamed, S., Pettersson, B., Uhlén, M. and Nyrén, P. (1996). Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry*, **242**: 84-89.

Rossman, A. and Palm-Hernández, M. (2008). Systematics of plant pathogenic fungi: Why it matters. *Plant Disease*, **92**: 1376-1386.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Pare, P. W. and Kloepper, J. W. (2003). Volatiles produced by PGPR elicit plant growth promotion and induced resistance in Arabidopsis. In *Proceedings of the Sixth International Workshop on Plant Growth Promoting Rhizobacteria* (pp. 436-443).

Sabir, S., Asghar, H., Kashif, S., Khan, M. and Akhtar, M. (2013). Synergistic effect of plant growth promoting rhizobacteria and kinetin on maize. *The Journal of Animal & Plant Sciences*, **23**: 1750-1755.

Saharan, B. and Nehra, V. (2011). Plant growth promoting rhizobacteria: A critical review. *Life Sciences and Medicine Research*, **2011**: 1-30.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**: 406-425.

Sangmanee, P., Bhromsiri, A.and Akarapisan, A. (2006). The potential of endophytic actinomycetes, (*Streptomyces sp*.) for the biocontrol of powdery mildew disease in sweet pea (*Pisum sativum*). *Asian Journal of Food and Agro-Industry*, Special issue: S93-S98.

Sangthong, C., Duangboobpha, S. and Prapagdee, B. (2015). Cadmium removal from water and soil by a cadmium-resistant *Rhizobacterium* and its effect on plant root elongation. *EnvironmentAsia*, **2**: 50-54.

Schwartz, A., Ortiz, I., Maymon, M., Herbold, C., Fujishige, N., Vijanderan, J., Villella, W., Hanamoto, K., Diener, A., Sanders, E., DeMason, D. and Hirsch, A. (2013). *Bacillus simplex*—A little known PGPR with anti-fungal activity—Alters pea legume root architecture and nodule morphology when coinoculated with *Rhizobium leguminosarum* bv. viciae. *Agronomy*, **3**: 2073-4395.

Shahverdi, M., Mirshekari, B., Rahmani, H., Rashidi, V. and Ardakani, M. (2013). Response of forage quality in Persian clover upon coinoculation with native *Rhizobium leguminosarum* symbiovar (sv.) trifoli RTB_3 and plant-growth promoting *Pseudomonas florescence* 11168 under different levels of chemical fertilizers. *African Journal of Microbiology Research*, **8**: 155-161.

Shahzaman, S. (2014). Preliminary screening for fungal root pathogen suppression by plant growth promoting rhizobacteria in Chickpea (*Cicer arietinum* L.). *Scholarly Journal of Agricultural Science*, **4**: 295-298.

Shamseldin, A. and Moawad, H. (2010). Inhibition of nitrogenase enzyme and completely suppression of nodulation in common bean (*Phaseolus vulgaris* L.) at high levels of available nitrogen. *American-Eurasian Journal of Agriculture and Environmental Science*, **7**: 75-79.

Shugla, S. and Dwivedi, R. (1979). The effect of microbial culture filtrates on the growth behaviour of different strains of rhizobia. *Proceedings of the National Academy of Sciences*, 45: 150-153.

Sivakumar, P., Parthasarthi, R. and Lakshmipriya, V. (2014). Encapsulation of plant growth promoting inoculant in bacterial alginate beads enriched with humic acid. *International Journal of Current Microbiology and Applied Sciences*, **3**: 415-422.

Slatni, T., Krouma, A., Gouia, H. and Abdelly, C. (2009). Importance of ferric chelate reductase activity and acidification capacity in root nodules of N_2-fixing common bean (*Phaseolus vulgaris* L.) subjected to iron deficiency. *Symbiosis*, **47**: 35–42.

Soares, B., Ferreira, P., de Oliveira-Longatti, S., Marra, L., Rufini, M., de Andrade, M. and Moreira, F. (2013). Cowpea symbiotic efficiency, pH and aluminum tolerance in nitrogen-fixing bacteria. *Science Agriculture*, **71**: 171-180.

Spaepen, S., Vanderleyden, J. and Remans, R. (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiology*, **31**: 425–448.

Stajković, O., Delić, D., Jošić, D., Kuzmanović, D., Rasulić, N. and Knežević-Vukčević, J. (2011). Improvement of common bean growth by co-inoculation with Rhizobium and plant growth-promoting bacteria. *Romanian Biotechnological Letters*, **16**: 5919-1526.

Stajkovic, O., Meyer, S., Milicic, B., Willems, A. and Delic, D. (2009). Isolation and characterization of endophytic non-rhizobial bacteria from root nodules of alfalfa (*Medicago sativa* L.). *Botanica Sarbica*, **33**: 107-114.

Steenhoudt, O. and Vanderleyden, J. (2000). Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: Genetic, biochemical and ecological aspects. *FEMS Microbiology Reviews*, **24**: 487-506.

Sudadi. (2012). Exogenous application of tryptophan and indole acetic acid (IAA) to induce root nodule formation and increase yield of soybean. *Agricultural Science Research Journal*, **2**:134 – 139.

Swain, H. and Abhijita, S. (2013). Nitrogen fixation and its improvement through genetic engineering. *Journal of Global Biosciences*, **2**: 98-112.

Tahir, M., Mirza, M., Zaheer, A., Dimitrov, M., Smidt, H. and Hameed, S. (2013). Isolation and identification of phosphate solubilizer Azospirillum, Bacillus and Enterobacter strains by 16SrRNA sequence analysis and their effect on growth of wheat (*Triticum aestivum* L.). *Australian Journal of Crop Science*, **7**: 1284-1292.

Tahmasebpour, B., Rezaei, H. and Aliasgharzadeh, N. (2013). The effects of helping bacteria (P*seudomonas spp.*) in nitrogen green beans fixation and nodulation with *Rhizobium leguminosarum* by phaseoli. *International Journal of Science Inventions Today*, **2**: 556-566.

Tak, H. I., Ahmad, F., & Babalola, O. O. (2013). Advances in the application of plant growth-promoting rhizobacteria in phytoremediation of heavy metals. In *Reviews of Environmental Contamination and Toxicology Volume*, Springer New York. **223**: 33-52.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular Evolutionary genetics analysis using Maximum Likelihood,

Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, **28**: 2731-2739.

Tilak, K., Ranganayaki, N. and Manoharachari, C. (2006). Synergistic effects of plant-growth promoting rhizobacteria and Rhizobium on nodulation and nitrogen fixation by pigeon pea (*Cajanus cajan*). *European Journal of Soil Science*, **57:** 67–71.

Toro, M., Azcon, R. and Barea, J. (1997). Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. *Applied and Environmental Microbiology*, **63**: 4408–4412.

Ureta, A., Imperial, J., Ruiz-Argueso, T. and Palacios, J. (2005). *Rhizobium leguminosarum* biovar viciae symbiotic hydrogenase activity and processing are limited by the level of nickel in agricultural soils. *Applied and Environmental Microbiology*, **71**: 7603–7606.

Vansuyt, G., Robin, A., Briat, J., Curie, C. and Lemanceau, P. (2006). Iron Acquisition from Fe-Pyoverdine by *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions*, **20**: 441–447.

Verma, J., Yadav, J. and Tiwari, K. (2010). Application of *Rhizobium sp.* BHURC01 and plant growth promoting *Rhizobacteria* on nodulation, plant biomass and yield of chick pea (*Cicer arietinum* L.). *International Journal of Agricultural Research*, **5**: 1816-4897.

Watson, M. and Mullen, R. (2007). Understanding soil tests for plant-available phosphorus. School Environmental and Natural Resources, The Ohio State University, Columbus-OH, Fact Sheet.

Yadegari, M. (2014). Inoculation of bean (*Phaseolus vulgaris*) Seeds with *Rhizobium phaseoli* and plant growth promoting rhizobacteria. *Advances in Environmental Biology*, **8**: 419-424.

Yadegari, M., Rahman, H., Noormohammadi, G. and Ayneband, A. (2008). Evaluation of bean (*Phaseolus vulgaris*) seeds inoculated with *Rhizobium phaseoli* and plant growth promoting *Rhizobacteria* on yield and yield component. *Pakistan Journal of Biological Sciences*, **11**: 1935-1939.

Yadegari, M., Rahmani, H., Noormohammadi, G. and Ayneband, A. (2010). Plant growth promoting rhizobacteria increase growth, yield and nitrogen fixation in *Phaseolus vulgaris*. *Journal of Plant Nutrition*, **33**: 1733-1743.

Yim, W., Chauhan, P., Madhaiyan, M., Tipayno, S. and Sa, T. (2010). Plant growth promontory attributes by 1-aminocyclopropane-1-carboxylate (ACC) deaminase

producing *Methylobacterium oryzae* strains isolated from rice. *19th World Congress of Soil Science, Soil Solutions for a Changing World*, 1 – 6.

Yuhashi, K., Ichikawa, N., Ezura, H., Akao, S., Minakawa, Y., Nukui, N., Yasuta, T. and Minamisawa, K. (2000). Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. *Applied and Environmental Microbiology*, **66**: 2658–2663.

Zahid, M., Abbasi, M., Hameed, S. and Rahim, N. (2015). Isolation and identification of indigenous plant growth promoting rhizobacteria from Himalayan region of Kashmir and their effect on improving growth and nutrient contents of maize (*Zea mays* L.). *Frontiers in Microbiology*, **6**: 1-10.

Zahran, H. (1999). Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews*, **63**: 968–989.

Zazueta, N., Acosta, O., Herrera, L., Vázquez, R., López, E., Zúñiga, A. and Dorantes, A. (2013). Effect of inoculation with three phytohormone producers phytobacteria with ACC deaminase activity on root length of *Lens esculenta* Seedlings. *American Journal of Plant Sciences*, **4**: 2199-2205.

Zhang, F., Dashti, N., Hynes, R. and Smith, D. (1996). Plant growth promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.] nodulation and nitrogen fixation at suboptimal root zone temperatures. *Annals of Botany*, **77**: 453-459.

APPENDICES

Appendix 1: Comparison of NAB population in three regions of Western Kenya by one way ANOVA followed by Turkeys test.

One-way analysis of variance

P value	0.0155
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	6.86
R square	0.604

ANOVA Table	SS	df	MS
Treatment (between columns)	3.47e+010	2	1.74e+010
Residual (within columns)	2.28e+010	9	2.53e+009
Total	5.75e+010	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	95% CI of diff
Summary				
Mt. Elgon vs Kisumu	107080	4.26	Yes *	7795 to 206365
Mt. Elgon vs Kakamega	119983	4.77	Yes *	20698 to 219268
Kisumu vs Kakamega	12903	0.513	No ns	-86382 to 112188

Appendix 2: Correlation between population of NAB and chemical soil factors in Mt. Elgon region.

Parameter	pH	Nitrogen	Carbon	Potasium	Phosphorus	Aluminium
Number of XY Pairs	4	4	4	4	4	4
Pearson r	0.7895	0.4107	0.9108	0.8982	0.9721	-0.9707
95% confidence interval		-0.7116 to 0.9953		-0.9094 to 0.9836		-0.4037 to 0.9982
	-0.4603 to 0.9979		0.1667 to 0.9994		-0.9994 to -0.1433	
P value (two-tailed)	0.2105	0.5893	0.0892	0.1018	0.0279	0.0293
P value summary	ns	ns	ns	ns	*	*
Is the correlation significant? (alpha=0.05)	No	No	No	No	Yes	Yes
R square	0.6232	0.1687	0.8296	0.8067	0.9449	0.9423

Appendix 3: Correlation between population of NAB and chemical soil factors in Kisumu region.

Parameter	pH	Nitrogen	Carbon	Potasium	Phosphorus	Aluminium
Number of XY Pairs	4	4	4	4	4	4
Pearson r	0.1918	-0.3379	0.06242	-0.1464	0.6556	-0.3765

95% confidence interval	-0.9432 to 0.9735	-0.9806 to 0.9230	-0.9561 to 0.9656	-0.9709 to 0.9481	-0.8260 to 0.9918	-0.9822 to 0.9162
P value (two-tailed)	0.8082	0.6621	0.9376	0.8536	0.3444	0.6235
P value summary	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	No
R square	0.03680	0.1141	0.003896	0.02143	0.4297	0.1417

Appendix 4: Correlation between population of NAB and chemical soil factors in Kakamega region.

Parameter	pH	Nitrogen	Carbon	Potasium	Phosphorus	Aluminium
Number of XY Pairs	4	4	4	4	4	
Pearson r	0.8191	0.5399	-0.3962	-0.5037	0.8881	-0.9865
95% confidence interval	-0.6676 to 0.9961	-0.8756 to 0.9882	-0.9830 to 0.9123	-0.9870 to 0.8867	-0.4987 to 0.9977	-0.9997 to -0.4899
P value (two-tailed)	0.1809	0.4601	0.6038	0.4963	0.1119	0.0135
P value summary	ns	ns	ns	ns	ns	*
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	Yes
R square	0.6709	0.2915	0.1570	0.2537	0.7887	0.9732

Appendix 5: Effect of plant growth promoting rhizobacteria on the number of pods per plant.

One-way analysis of variance

P value	0.0010
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	9.058
R square	0.4015

Bartlett's test for equal variances

Bartlett's statistic (corrected)	13.78
P value	0.0010
P value summary	**
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	73.40	2	36.70
Residual (within columns)	109.4	27	4.052
Total	182.8	29	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff

Uninoculated vs Rhizobia alone	0.4000	0.6284	No	ns	-1.834 to 2.634
Uninoculated vs RPGPR	-3.100	4.870	Yes	**	-5.334 to -0.8664
Rhizobia alone vs RPGPR	-3.500	5.498	Yes	**	-5.734 to -1.266

Appendix 6: Effect of plant growth promoting rhizobacteria on the number of seeds per plant.

One-way analysis of variance

P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	587.4
R square	0.9775

Bartlett's test for equal variances

Bartlett's statistic (corrected)	13.94
P value	0.0009
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS	
Treatment (between columns)	1806	2	903.1	
Residual (within columns)	41.51	27	1.537	
Total	1848	29		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05? Summary	95% CI of diff
Uninoculated vs Rhizobia alone	-18.90	48.20	Yes	***	-20.28 to -17.52
Uninoculated vs RPGPR	-11.19	28.54	Yes	***	-12.57 to -9.814
Rhizobia alone vs RPGPR	7.710	19.66	Yes	***	6.334 to 9.086

Appendix 7: Effect of plant growth promoting rhizobacteria on the weight of pods per plant.

One-way analysis of variance

P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	146.0
R square	0.9154

Bartlett's test for equal variances

Bartlett's statistic (corrected)	14.60
P value	0.0007
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS	
Treatment (between columns)	491.3	2	245.6	
Residual (within columns)	45.41	27	1.682	
Total	536.7	29		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05? Summary	95% CI of diff
Uninoculated vs Rhizobia alone	1.540	3.755	Yes *	0.1009 to 2.979
Uninoculated vs RPGPR	9.250	22.55	Yes ***	7.811 to 10.69
Rhizobia alone vs RPGPR	7.710	18.80	Yes ***	6.271 to 9.149

Appendix 8: Effect of plant growth promoting rhizobacteria on the total dry matter of the shoot per plant.

One-way analysis of variance

P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	22.08
R square	0.6205

Bartlett's test for equal variances

Bartlett's statistic (corrected)	35.54
P value	< 0.0001
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS	
Treatment (between columns)	114.5	2	57.26	
Residual (within columns)	70.03	27	2.594	
Total	184.6	29		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05? Summary	95% CI of diff
Uninoculated vs Rhizobia alone	2.810	5.517	Yes **	1.023 to 4.597
Uninoculated vs RPGPR	-1.950	3.829	Yes *	-3.737 to -0.1629
Rhizobia alone vs RPGPR	-4.760	9.346	Yes ***	-6.547 to -2.973

Appendix 9: 16SrRNA gene Partial Sequences from isolates