GENETIC VARIATION IN GROUNDNUT (*Arachis hypogaea* L) NODULATING RHIZOBIA NATIVE TO PHOSPHORUS DEFICIENT SOILS OF WESTERN KENYA

BY

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A THESIS SUBMITTED TO THE SCHOOL OF SCIENCE IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BOTANY (GENETICS), BIOLOGICAL SCIENCES, UNIVERSITY OF ELDORET, KENYA

MAY 2017

DECLARATION

DECLARATION BY THE CANDIDATE

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This thesis is dedicated to my family for their moral support.

ABSTRACT

Soils in Western Kenya are characterized by high acidity with phosphorus (P) deficiency, affecting nodulation and nitrogen fixation of groundnut (*Arachis hypogaea* L.). The objectives of the study were to determine (1) the diversity of indigenous rhizobia strains nodulating groundnut using morphological and biochemical markers (2) identify indigenous groundnut rhizobia capable of nodulating groundnut under P deficiency, and (3) genotype the rhizobia isolates using polymerase chain-reaction-restriction fragment length polymorphism (PCR-RFLP) targeting 16S-23S ribosomal DNA (rDNA) intergenic spacer (IGS) and 16S rRNA gene. Phenotypic diversity among the isolates was assessed by morphological and biochemical attributes. Sixty four isolates out of the 68 were confirmed to be rhizobia due to their ability to nodulate groundnut. Ninety six percent of the isolates exhibited semi-globose to globose colony shape on yeast extract mannitol agar (YEMA). Groundnut was nodulated by both fast and slow growing rhizobia isolates with 81% being fast growers. Fifty one isolates representing 75% of the bacteria isolates showed ability to produce acid on YEMA medium supplemented with bromothymol blue (BTB). The isolates varied in response to low pH with 39 and 61 growing at pH 4.0 and 5.5, respectively. All the isolates grew at pH 7.0 and 8.5. YEMA medium containing glucose, sucrose, starch and citrate supported growth of 64, 61, 56 and 5 isolates, respectively. Sixty four isolates showed a clear zone of solubilization on medium containing insoluble inorganic phosphate. Solubilization index (SI) varied from 1.1 to 6.8. Fast-growing rhizobia isolates N01, B02, I06, Q01, F05, C02, E01, Q03, I01 and B01 recorded the highest solubilization index of 3.8, 4.5, 4.6, 4.6, 4.7, 5.0, 5.1, 6.1, 6.1 and 6.8, respectively. Genetic diversity among the isolates was assessed by sequence analysis of 16S-23S ribosomal. Amplification of IGS and 16S rRNA for 54 isolates produced a single fragment of 750 and 1,500 bp, respectively. Restriction of amplified 16S-23S rDNA IGS region with four endonucleases *Hinf* l, *Msp*1, *Taq*1 and *Csp*61 produced 1-10 restriction fragments in all the isolates. These fragments varied in length from 50 to 500 bp, 130 to 600 bp, 130 to 610 bp and 30 to 600 bp, respectively. Ten different combinations of patterns representing 10 different IGS genotypes (A-J) were detected in the 54 isolates. *Hinf* I was found to be the most discriminative detecting 10 genotypes (A–J) among the 54 rhizobia isolates analyzed as compared to *Msp*1 (8), *Taq*1 (8) and *Csp*61 (9). Digestion of the 16S rRNA gene amplicons with endonucleases *Hinf* 1, *Msp*1, *Taq*1 and *Csp6*1 each produced multiple fragments ranging between 1 and 8 depending on the isolate. Fragments ranged from 50 to 500 bp, 50 to 700, 150 to 1050 and 150 to 710 base pair in size, respectively. The number of genotypes per restriction enzyme varied (A-M) with *Hinf* 1, *Msp*1 and *Taq*1 delineating the largest at thirteen (13), and *Csp*61 representing the least (9) number of genotypes (A-I). Dendrogram from each cluster analysis with different enzymes and combined enzymes separated isolates from different sites of origin into two main groups. Both phenotypic and genotypic characterization showed great diversity among the 54 isolates with potential to solubilize inorganic phosphate and improve nitrogen fixation. The most promising rhizobia isolates from this study would be used as bio-fertilizer upon further validation in the greenhouse. The findings reported here if adopted will contribute to enhancing groundnut production on acid soils of western Kenya in particular and Africa in general.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	analysis of variance
BNF	biological nitrogen fixation
bp	base pairs
BTB	Bromothymol blue
cm	centimeter
DCP	dicalcium phosphate
DNA	deoxyribonucleic acid
dNTP	dinucleotide tri-phosphate
g	gram
GPA	glucose peptone agar
HCI	hydrochloric acid
IGS	intergenic spacer
kg	kilograms
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
N	Nitrogen
NaCl	Sodium chloride
NBRIP	National Botanical Research Institute Phosphate
P	Phosphorus
PCR	Polymerase chain reaction
PSB	Phosphate Solubilizing Bacteria
RNA	ribonucleic acid
RAPD	random amplified polymorphic DNA
µl	microlitre
µM	micromolar
W/V	weight by volume
YEMA	yeast extract mannitol agar

ACKNOWLEDGEMENT

I thank God almighty who has brought me this far and provided me with the strength and grace that has enabled me to finally come up with this thesis. I acknowledge with joy the splendid guidance and patience in my work by my supervisors Dr. Beatrice A. Were and Dr. Benson O. Nyongesa. I would like to thank National Council of Science, Technology and Innovation (NACOSTI) for the research grant (Ref No. NACOSTI/RCD/ST & 1/7TH CALL/Msc/060). I am grateful to Kent Hedman for his support and encouragement throughout my studies. Many thanks to management of the Biotechnology laboratory at the University of Eldoret for providing research space, facilities and technical support. Tribute to all the farmers who toil on soil, keep the glow on. Surely, "soil is mother". Let us not abandon her health. There are honest researchers and devotees to your welfare. Only that it takes long.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Rhizobia are gram negative, aerobic chemo-organotrophs, rod shaped, motile bacteria which are capable of nodulating legumes (Rajasundari, Ilamurugu & Logeshwaran, 2014). Rhizobia are capable of enhancing legume performance through N-fixation and phosphorus (P) solubilizing substance found within their cell wall (Sharma, Srivastava & Sharma, 2014). However soils deficient in P affect the survival and effective functioning of rhizobia isolates (Guiñazú, Andrés, Del Papa, Pistorio & Rosas, 2010). Inoculation of legumes with selected effective rhizobia strains has contributed to high symbiotic efficiency and enhanced legume grain yield and quality (Mulas, Seco, Casquero, Velazquez & Gonzalez-Andres, 2016; Tena, Wolde-Meskel, & Walley, 2016). In spite of the existing evidence that legume inoculation could increase crop performance and soil fertility, rhizobia inoculants are rarely used in most agricultural systems in Kenya. This is because legume nodulation by indigenous bacteria is usually assumed to be adequate.

The benefits, production and use of rhizobia inoculants for legume improvement has been a success for a very long time in developed countries, while it is still developing in sub-Saharan African countries, including Kenya. Australian agriculture, for instance, has demonstrated substantial increases in legume nodulation, grain and biomass yield, nitrogen fixation and post-crop soil nitrate levels for more than 100 years (Owen, Williams, Griffith & Withers, 2015). Similarly, commercial production and use of bio-inoculants started as early as 1895 in the U.S.A. and the U.K (Naveed,

Mehboob, Shaker, Hussain & Farooq, 2015). Nevertheless, efforts to establish local inoculants production in Africa started only in the 1980s and 1990s by Nitrogen Fixation in Tropical Agricultural Legumes (NifTAL), Food and Agriculture Organization (FAO) and the Regional Microbiological Resources Centres (MIRCENs). These organizations developed small scale inoculants production industries in many countries throughout Africa (Mutuma, Okello, Karanja & Woomer, 2014). Rhizobial inoculants are rarely used by farmers in Africa, except in experiments that are limited to research farms (Bala, Abaidoo & Woomer, 2010).

Groundnut (*Arachis hypogaea* L) is important as a source of food for direct human consumption and a good nitrogen fixer that significantly improves soil fertility (Pasupuleti, Nigam, Pandey, Nagesh & Varshney 2013; Ajeigbe *et al.,* 2015). In Western Kenya, groundnut is popularly sold to earn income and is a source of dietary protein hence the need to grow the crop so as to enhance economy and wellbeing of the rural communities in those areas.

The soils of western Kenya where groundnut is mainly cultivated are acidic, posing limitation to the potential production of this valuable oil crop. In acidic soils, nitrogen fixation is hindered as most rhizobia are sensitive to acidity. Inoculation with rhizobia especially in areas cultivated with groundnut for the first time has increased yields (El-Akhal *et al*., 2013). Therefore, productivity of the crop could be sustainably improved in areas with nutrient depleted soils through the use of rhizobia technology. To harness this technology, there is need to identify suitable strains that can establish effective symbiosis with groundnut under the prevailing field conditions in its production areas. Several indigenous rhizobia that have the potential to fix nitrogen have been isolated from cowpea, green gram and bambara groundnut in various parts

of Kenya (Mathu, Herrmann, Pypers, Matiru, Mwirichia & Lesueur, 2012; Benson, Beatrice, Regina, Koech, Skilton & Francesca, 2015). Such diversity of rhizobia presents opportunity for strain selection from among indigenous rhizobia in various groundnut growing agro-ecologies.

Groundnut nodulates freely and effectively with rhizobia of the cowpea group belonging to the genus *Bradyrhizobium* (Pule-Meulenberg *et al.*, 2010). Fast-growing groundnut rhizobia that acidify the medium and are closely related to *Rhizobium giardini* and *Rhizobium tropici* has been obtained in the soils of Central Argentina and Northeastern Brazil (Taurian, Ibañez, Fabra & Aguilar, 2006; de Freitas & Silva, 2013). Such diversity of groundnut nodulating rhizobia presents opportunity for strain selection from among indigenous rhizobia present in various groundnut growing agro-ecologies. Using phenotypic and molecular methods, genetic diversity of rhizobia has been observed in tropical soils and strains with valuable adaptations to stressful environments purified for commercial use (Mathu *et al.*, 2012). Although the assessment of diversity within natural populations of rhizobia in various regions of the world has received much attention, such studies are not well documented in Kenya.

Western Kenya is characterized by acid soils with phosphorus deficiency (Owino *et al.*, 2015). Phosphorus is the most important macro- element in the nutrition of plants, after nitrogen (N). It plays vital role in all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Khan, Zaidi, Ahemad, Oves & Wani, 2010). Phosphorus exists in two diverse forms; insoluble inorganic phosphorus and insoluble organic phosphorus in the soil solution (Kumar, Choudhary, Paswan, Kumar & Arun, 2014). However, due to low solubility and fixation in soils, only a small fraction of

phosphorus in soil solution is readily available to plants (Yang, Post, Thornton & Jain, 2013). The fully oxidized form of P, orthophosphate (Pi) is extremely unavailable in most soils of western Kenya because it forms a complex with Aluminium and iron oxides and it is fixed into organic forms that render the phosphate (Pi) largely inaccessible to plants (Kisinyo *et al.,* 2014). Due to their high P fixation capacity, addition of P fertilizers is needed to achieve adequate nodule establishment and function in order to enhance nitrogen fixation and legume production (Wasike *et al.,* 2009).

1.2 Statement of the Problem

In Kenya, groundnuts are popularly sold to earn income and are a source of dietary protein. Despite this, grain yield as low as 200-400 kg ha^{-1} has been observed in P deficient soils of western Kenya (Nekesa, Maritim, Okalebo & Woomer, 1999; Ojiem, Vanlauwe, de Ridder & Giller, 2007). Although it is well known fact that groundnut is a good source of fixed nitrogen, effort has not been made to study the indigenous rhizobia colonizing roots of this plant. Nodulation of groundnut by indigenous bacteria is presumed to be sufficient, and inoculation is hardly practiced by farmers in most growing areas in western Kenya. However, survival and effective functioning of rhizobia populations are hindered by phosphorus (P) deficiency (Al-Falih, 2002). N-fixation and overall growth performance of groundnut and other grain legumes are severely limited by low available P in the soils resulting in poor crop yield (Sanginga, 2003).

The increase of whole plant growth and plant nitrogen concentration in response to increased soil P supply have been noted for several leguminous species including

soybean (West, HilleRisLambers, Lee, Hobbie & Reich, 2005; Tsvetkova & Georgiev, 2003). Decreased specific- nitrogenase activity in nodules of P- deficient soybean plants was associated with decreased energy status of host plant cells of nodules. These latter observations imply specific involvement of phosphorus in symbiotic nitrogen fixation. Most groundnut growing regions in western Kenya are characterized by acid soils with P deficiency (Kisinyo *et al.,* 2013). In soils with high P fixation capacity, addition of chemical phosphate fertilizers is needed to achieve adequate nodule establishment and function in order to enhance nitrogen fixation and legume production (Sulieman & Tran, 2015).

Unfortunately, most of the phosphatic fertilizers applied to soils precipitate into insoluble forms and become unavailable to plants hence increasing the P requirements (Onwonga, Lelei & Macharia, 2013). Owing to continuous application of phosphatic fertilizers, most agricultural soils contain significant reserves of accumulated P that can be mobilized for plant use (Chen, 2006). One strategy to mobilize such soil P reserves is through inoculation with phosphate solubilizing microorganisms (Richardson, 2001). It is evident that certain strains of rhizobia are capable of solubilizing both organic and inorganic phosphates (Sharma, Sayyed, Trivedi & Gobi, 2013). Several indigenous rhizobia that have the potential to fix nitrogen have been isolated from cowpea, green gram and bambara groundnut in various parts of Kenya (Mathu *et al.,* 2012; Benson *et al*., 2015). Although indigenous groundnut rhizobia exist in Western Kenya soils, their genetic diversity and capacity to solubilize P is not well documented. In order to achieve desired performance and for expanded use, there is need to identify suitable strains that can establish effective symbiosis with groundnut.

1.3 Justification

Due to the importance of N and P for crop productivity, economic and environmental problems associated with the use of chemical fertilizers, nitrogen-fixing microorganisms have attracted significant attention, especially the ones capable of forming symbiosis with legumes generically known as rhizobia (Zahran, 2001). The potential for improving symbiotic nitrogen fixation in groundnut through rhizobial inoculation requires knowledge of the genetic diversity, adaptation and effectiveness of the indigenous rhizobia population in the soils.

Inoculation of legumes with rhizobial strains selected for high N_2-fixing capacity can improve nitrogen fixation in agriculture, particularly when local rhizobial strains are ineffective or absent from soils (Giller, Herridge & Sprent, 2016). Enhanced competitive ability of inoculants is a key requirement for successful colonization of plant roots, nodule formation, and subsequent N_2-fixation (Kyei-Boahen, Savala, Chikoye & Abaidoo, 2017). Groundnut is among the leguminous crops that can produce root nodules and fix atmospheric nitrogen by symbiotic relationship with cowpea- type rhizobia which predominate in tropical soils (Saharan & Nehra, 2011). The nitrogen fixing ability in this crop varies widely depending on groundnut genotypes and rhizobia strains (Giller, 2001). Rhizobia comprise a diverse group of bacteria that acts as the primary symbiotic fixer of nitrogen (Franche, Lindström & Elmerich, 2009). Groundnut is usually nodulated with the slow growing *Bradyhrizobium spp.* and in some cases fast growing rhizobia. These strains are tolerant to varying stress effect and are able to form effective (N_2-fixing) symbioses with their host legumes under salt, heat, and acid stresses.

Seed inoculation with Phosphate solubilizing bacteria (PSB) is known to improve the solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yield (Mehta & Nautiyal, 2001; Mohammadi & Sohrab, 2012). In fact, PSB render more phosphates into the soluble form than required for their growth and metabolism by secreting organic acids (Vessey, 2003). The interest in phosphate solubilizing bacteria has increased due to the prospective use of efficient strains as bio-inoculant components in organic agriculture (Rani, 2014). Specifically, the better potential of *Rhizobium leguminosarum* bv. viciae in solubilizing inorganic phosphate sources were reported by Alikhani, Saleh-Rastin & Antoun, (2007). Therefore, identification of effective groundnut nodulating rhizobia strains for development of bio-inoculants would boost nitrogen fixation and legume production in P deficient soils of Western Kenya.

1.4 Objectives

1.4.1 General Objective

The general objective of this study was to identify indigenous rhizobia that could be used to enhance groundnut production in P deficient soils of western Kenya.

1.4.2 Specific Objectives

The specific objectives of the research will be as follows:-

1. To determine the diversity of indigenous rhizobia strains nodulating groundnut using morphological and biochemical markers.

2. To determine the nodulation ability of phosphate solubilizing indigenous groundnut rhizobia

3. To genotype the rhizobia isolates using variation in ribosomal DNA

1.5 Research Hypothesis

The study was based on the following hypotheses:-

$H_{A1:}$ Indigenous rhizobia strains nodulating groundnut in P deficient soils show broad diversity in phenotypic characteristics.

H_{A2}: There is variation in nodulation ability of phosphate solubilizing indigenous groundnut rhizobia

$H_{A3:}$ Groundnut nodulating rhizobia isolates can exist in different genotypes that can be separated into distinct groups based on variation in ribosomal DNA.

CHAPTER TWO

LITERATURE REVIEW

2.1 Groundnut description, production and uses

Groundnut (*Arachis hypogaea L.*) is one of the most important plants cultivated all over the world and particularly in tropical and subtropical areas (Cuc, Mace, Crouch, Quang, Long & Varshney, 2008). It is an annual herb belonging to the family Leguminosae. The cultivated groundnut was first domesticated in the valleys of Peru (http://www.lanra.uga.edu); which might have taken place in Paraguay or Bolivia, where the wildest strains grow today. The plant grows 30 to 50 cm tall (Grichar, Dotray & Woodward, 2013). The leaves are opposite with two opposite pairs, each leaflet 1 to 7 cm long and 1 to 3 cm broad (Chenault, Ozias Akins, Gallo & Srivastava, 2008).

The flowers are a typical pea flower in shape, 2 to 4 cm across, yellowish orange with reddish veining (Cuc *et al.*, 2008). After pollination, the fruit develops into a pod 3 to 7 cm long, containing 1 to 4 seeds (Duke, 2012). Groundnuts grow best in light, sandy loam soil (Eze, Maduka, Ogbonna & Eze, 2013). They require five months of warm weather, and 500 to 1,000 mm of water (Grichar *et al.*, 2013). The pods ripen 120 to 150 days after the seeds are planted (Chenault *et al.*, 2008). If the crop is harvested too early, the pods will be unripe (Cuc *et al.*, 2008). If they are harvested late, the pods will snap off at the stalk, and will remain in the soil (Oladipupo, 2010). They prefer non acidic soil of preferably with pH of 5.9–7.0 (Duke, 2012).

Groundnuts provide over 30 essential nutrients and phytochemicals (Chen *et al.*, 2008). They are good source of niacin (Arya, Salve & Chauhan, 2016) which plays a

role in brain and blood flow, folate, fiber, magnesium and phosphorus. Plumpy Nut, a ready- to – use therapeutic food made from groundnuts, is used by UNESCO to cure acute malnourished children in Africa (Mallikarjuna, Shilpa, Pandey, Janila & Varshney, 2014). It contains about 25 percent protein, a higher proportion than any true nuts (Arya *et al.,* 2016). Groundnuts contain high concentrations of antioxide polyphenols (Craft, Kosińska, Amarowicz & Pegg, 2010) source of resveratrol, which is equivalent to that present in red grapes (Bråkenhielm, Cao & Cao, 2001). Resveratrol is a chemical associated with reduction in the risk of cardiovascular disease (Yang *et al.,* 2009), cancer and has anti-aging properties and therefore has major uses in both health and cosmetic industries.

Groundnuts can be used in recipes, eaten raw, oil making, textile materials. Boiled Groundnut is eaten as snack in Kenya, China, India and West Africa (Ezeonu & Ezeonu, 2016). Groundnut oil is used in cooking, making of cosmetics, plastics, dyes and paints. The flour is used as gluten free meal and also to make lactose free milk (Yao *et al.,* 2004). Husks or shell can be used as a source of fuel, roughage and oil or absorbent in livestock feeds, animal litter, mulch, soil conditioner and manure (Chenault *et al.,* 2008). Popular confectioneries from groundnuts include salted peanuts, peanut butter, peanut brittle, and shelled nuts (Hathorn, 2013).

Groundnut is grown in all continents with a total area of 24.6 million hectares, and a production of 41.3 million tons in 2012 (Garba *et al*., 2015). Africa had 11.7 million hectares of land used for groundnut production and 10.9 million tons of annual production in 2012, and thus was second only to the American continent (Akibode & Maredia, 2012). Despite the second position in terms of groundnut production, Africa has the lowest average yield per hectare (1 ton ha $^{-1}$) compared to Asia (1.8 tons ha $^{-1}$)

and America (3 tons ha $^{-1}$) (Garba *et al*., 2015). The low yields are related not only to the rain fed production systems with very low input but also to the use of traditional varieties that despite their genetic diversity are low yielding (Godfray & Garnett, 2010).

According to Asekenye, (2012), average yields in Western Kenya, a major groundnut growing region, varied depending on the farming system and type of seed farmers used. Higher yields were observed in farms that used improved varieties as compared to those that used local varieties. Similarly, groundnuts grown in pure stands gave better yields than those in mixed stands. Farmers obtained 30% to 50% lower yields than their potential (Kidula, Okoko, Bravo-Ureta, Thuo & Wasilwa, 2010).

2.2 Nitrogen Fixation

Nitrogen fixation is a synthetic method of converting atmospheric nitrogen to nitrogen oxides or ammonium ions that plants and other organisms are able to use (Burns & Hardy, 2012). Fixed nitrogen remains to be a limiting nutrient in most soils, with the main reserve of nitrogen in the biosphere being molecular dinitrogen from the atmosphere (De Bruijn, 2015). Dinitrogen is an inert gas with a triple bond that is energetically unfavorable to break (Kästner & Blöchl, 2007). Nitrogen availability is limiting for plant growth and has long been overcome through the application of synthetic nitrogen rich fertilizer (Beatty & Good, 2011). The use of large amounts of fertilizers boosts yields in plants such as cereals but has a high economic and environmental cost (De Bruijn, 2015; Fageria, 2016). The industrial production of nitrogen fertilizer costs more than US$ 100 billion because the energetically difficult reduction of the triple bond carried out at high temperature and pressure requires the use of large amounts of fossil fuel, a limited resource (De Bruijn, 2015). Therefore,

fertilizer costs are high and unaffordable to small scale farmers worldwide (Scialabba, 2000).

In addition, the use of fertilizer has a severe environmental impact, due to run-off of excess non-assimilated nitrate, and concomitant eutrophication of rivers, lakes and oceans, as well as contamination of the drinking water (De Bruijn, 2015). Moreover, carbon dioxide is produced during fossil fuel combustion which occurs during production of chemical fertilizer leading to the greenhouse effect, as does the decomposition of nitrogen fertilizer releasing nitrous oxide (Erisman *et al.,* 2015). Inoculation of legumes with rhizobial strains selected for high N_2-fixing capacity can improve nitrogen fixation in agriculture, particularly when local rhizobial strains are ineffective or absent from soils (Giller et al., 2016).

2.2.1 Biological Nitrogen Fixation

Biological Nitrogen Fixation (BNF) is the reduction of atmospheric dinitrogen to ammonia, which is carried out by a large and diverse group of free-living and symbiotic microorganisms (Wagner, 2012). BNF presents an inexpensive and environmentally sound, sustainable approach to crop production and constitute one of the most vital Plant Growth Promotion (PGP) scenarios (De Bruijn, 2015). BNF occurs when atmospheric di-nitrogen is converted to ammonia by an enzyme called nitrogenase (Ban & Iqbal, 2016)). The reaction for BNF is:

$$N_2 + 8\ H^+ + 8e^-\ 16\ Mg\ ATP \rightarrow 2NH_3\text{-}, + H_2 + 16Mg\ ADP + 16Pi$$

This process is coupled to the hydrolysis of 16 equivalents of ATP and is accompanied by the formation of one molecule of H_2. In free living diazotrophs, the nitrogenase released ammonium, is assimilated into glutamate through the glutamine

synthetase/glutamate synthase pathway (Patriarca, Tatè & Iaccarino, 2002). Organisms obtain from ATP by oxidizing organic molecules. Associative and symbiotic nitrogen fixing microorganisms obtain these compounds from plants' rhizospheres (Figueiredo *et al.*, 2013).

2.2.2 Symbiotic Nitrogen Fixation

Many microorganisms fix nitrogen symbiotically with a host plant. The plant provides sugars from photosynthesis that are utilized by the nitrogen fixing microorganism for the energy it needs for nitrogen fixation (Wagner, 2011). In exchange for these carbon sources, the microbe provides fixed nitrogen to the host plant for its growth. Nitrogen fixation by legumes begins with the formation of a nodule on the plant root or stem. Rhizobia invade the root and multiply within the cortex cells (Fournier *et al*., 2015). The plant provides all the necessary nutrients and energy for the bacteria. After infection, small nodules become visible with the naked eye within the plant fine roots (Cabeza *et al.,* 2015). These can be seen 2-3 weeks after planting, depending on legume species and germination conditions (Somasegaran & Hoben, 2012). When nodules are young and non-nitrogen fixing, they are normally white or grey inside (Cabeza *et al.,* 2015). As nodules grow in size they gradually turn pink or reddish in colour, showing nitrogen fixation has started. The pink or red colour is due to activity of leghemoglobin that controls oxygen flow to the bacteria (Kouchi, 2011).

Legume nodules that are no longer fixing nitrogen usually turn green, and may end up being discarded by the plant (Fournier *et al*., 2015). Pink or red nodules should predominate on a legume in the middle of the growing season; if white, grey, or green nodules predominate, little nitrogen fixation occurs as a result of an inefficient

rhizobial strain, poor plant nutrition, pod filling, or other plant stresses (Tairo & Ndakidemi, 2013).

N-fixation and overall growth performance of groundnut and other grain legumes are severely limited by low available P in the soils resulting in poor crop yield (Vadez *et al.*, 2012). Plants lack the genes encoding the nitrogenase enzymes which are used to reduce atmospheric dinitrogen into biologically utilizable form (Shin *et al.*, 2016). Hence, some plants form symbiosis with nitrogen-fixing microorganisms.

2.2.3 Acid soils of Western Kenya

It has been estimated that over 50% of the world potentially arable land are acidic (Zheng, 2010) posing a worldwide limitation to crop production. In developing countries like Kenya where food production is critical, acid soils occur up to 13% (Obura, 2008). High soil acidity limits nodulation, N_2 fixation and plant growth. Mugwe, Mugendi, Odee & Otieno, (2007) attributed poor nodulation of legumes to soil acidity, low soil P and lack of adequate indigenous rhizobia in Chuka, Eastern Kenya.

The low pH of the soil results in immobilization of phosphorous by aluminum, and iron ions. As a result, phosphorous availability as plant nutrient is limited, which in turn strap up energy acquisition for nitrogen fixation (Kisinyo *et al.*, 2014). However, there are legume nodulating bacteria such as *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium* strains possessing properties of solubilizing immobilized organic and inorganic phosphate sources (Kenasa, Jida & Assefa, 2014). Thus, besides the fixation of atmospheric nitrogen to its utilizable form, these bacteria also contribute to the growth of plants through solubilizing inorganic phosphates of low solubility.

High soil acidity found in Western Kenya is associated with aluminium (Al), hydrogen (H), iron (Fe) and manganese (Mn) toxicities and corresponding deficiencies of phosphorus (P), molybdenum (Mo), calcium (Ca), magnesium (Mg) and potassium (K) Kisinyo *et al.*, (2013). Phosphorus deficiency is due to inherent low soil P, high P fixation by Al and Fe oxides and insufficient fertilizer use to replace soil P removed through crop harvests (Smithson & Giller, 2002; Margenot, Singh, Rao & Sommer, 2016). The problem of P deficiency is discussed below.

2.3 Phosphorus in plant nutrition and nitrogen fixation

2.3.1 Role of Phosphorus

Phosphorus is one of the most essential macro-elements needed for growth and development of plants (Walpola & Yoon, 2012) and it is second only to nitrogen in importance to plant nutrition. Phosphorus nutrition is associated with development of roots, formation of flowers and seeds, strengthening the stalks and stems crop maturity and quality of the production, nitrogen fixation in legumes and strengthening the plant against diseases (Khan *et al.*, 2010). Phosphorus is required for metabolic processes such as energy transfer, signal transduction, macro-molecular biosynthesis, photosynthesis and respiration (Gyaneshwar, Kumar, Parekh & Poole, 2002). Inadequate Phosphorus restricts root growth, the process of photosynthesis, translocation of sugars, and other such functions which directly or indirectly influence N fixation by legume plants (Muhati *et al.*, 2011).

In groundnut, phosphorus is a vital ingredient for rhizobia to convert atmospheric N (N_2) into an ammonium NH_4 form useable by plants (Toro, 2007). Rhizobia are able to synthesize the enzyme nitrogenase which catalyzes the conversion of N to two

molecules of ammonia $(NH3)_{2.}$ Phosphorus becomes involved as an energy source when 16 molecules of adenosine triphosphate (ATP) are converted to adenosine diphosphate (ADP) as each molecule of N_2 is reduced to (NH_3).

Rhizobia have a high solubilization potential for various organic and inorganic phosphates and are hence preferred by their role in nitrogen fixation and P-solubilization (Alikhani *et al.,* 2007). Moreover, groundnuts associate with other phosphate solubilizing bacteria which convert the insoluble phosphates into soluble forms by acidification, chelation, exchange reactions and production of gluconic acid (Chen *et al*., 2006). This renders this crop potentially more adapted to soils with low P (Muhati *et al.*, 2011).

The efficiency of N-fixation in leguminous plants depends on root nodule development. P has specific roles in nodule formation, development, and functioning (Alikhani *et al.*, 2007) and nitrogen fixation as an energy source in form of adenosine triphosphate hydrolysed during the conversion of atmospheric N to ammonia (Patriarca *et al.*, 2002). Moreover, P is a major macronutrient for proper growth and survival of rhizobia in the P deficient soils that slows rhizobial growth survival, population density and infection capacity of rhizobia (McDonald, 2014; Gupta *et al.*, 2015). For these reasons, its deficiency can impair nodulation and N- fixation with deleterious effect on legume performance.

2.3.2 Phosphorus forms, availability and fixation

Phosphorus occurs in diverse forms in the soil solution that can be broadly categorized as insoluble inorganic phosphorus and insoluble organic phosphorus (Walpola & Yoon, 2012). Even in phosphorus rich soils, most of this element is in

insoluble form and only a small proportion (~0.1%) is available to plants (Kumar & Ram, 2014). Additionally, a large proportion of the phosphate fertilizers applied to soils precipitate into insoluble forms hence increasing the P requirement of the crop (Podile & Kishore, 2007). As a consequence of continuous application of phosphatic fertilizers at high doses, most agricultural soils generally contain large reserves of accumulated phosphorus (Richardson, 2001). Soon after application, a large amount of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized and becomes unavailable to plants (Kisinyo *et al*., 2014), when the fertilizer or manure phosphate comes into contact with the soil, a series of reactions begins which make the phosphate less soluble and less available. However, the degree of fixation and precipitation of phosphorus in soil is highly depended upon the soil conditions such as pH, moisture content, temperature and the minerals already present in the soil (Wani, Khan & Zaidi, 2007). In the case of acidic oils, free oxides and hydroxides of aluminium and iron play a key role in fixing phosphorus, while in alkaline soils, it is fixed by calcium (Walpola & Yoon, 2012). Therefore, phosphorus is often regarded as a limiting nutrient in agricultural soils (Guiñazú *et al.*, 2010).

Phosphorus deficiency is a common setback to agricultural productivity, particularly in developing countries where access to P fertilizers is limited (Alkama, Bolou, Vailhe, Roger, Ounane & Drevon, 2009; Godfray & Garnett, 2014). This constraint can be overcome by various mechanisms. One mechanism employed is root foraging strategies that improve acquisition of soil P, support higher yields in low P soil and thus lower the critical P requirement for plant growth. This enables agriculture to be operated at lower plant available P concentration and in turn, can slow the rate at which P accumulates in moderate to high P- soils (Guiñazú *et al.*, 2010). Secondary strategy is soil P mining that enhances the desorption, solubilisation or mineralization

of P from sparingly available pools and slowly mineralizing of resistant organic P pools (Delgado & Scalenghe, 2008). Soil mining P from agricultural soils is not, in itself, sustainable (Jeffries, Gianinazzi, Perotto, Turnau & Barea, 2003). However, the objective of this strategy is to increase the turnover of P in sparingly available P pools and thus slow the net accumulation of P that occur when moderate to high P soils are fertilized (Guiñazú *et al.*, 2010). Thirdly, plants with improved internal P-utilization efficiency could directly reduce the amount of P fertilizer required for agricultural production (Delgado & Scalenghe, 2008). Internal P efficiency is employed to extreme levels in slow growing species adapted to low landscapes but is also found in some of the plant species used in agriculture (Rose & Wissuwa, 2012).

Under diverse soil and agro-climatic conditions, microbial organisms with phosphate solubilizing strength have been shown to offer an economically sound alternative to the more expensive application of superphosphates and have a greater agronomic utility (Xiao, Chi, He, Qiu, Wang & Zhang, 2009); Walpola & Yoon, 2012). Accordingly, these microbial communities when used separately (Chen *et al.,* 2008) or together with other rhizosphere microbes produce measurable effects on crop plant performance (Wani *et al.*, 2007) .

As reported in several investigations, phosphate solubilizing bacteria could increase growth and production in several crops including maize (Hameeda, Harini, Rupela, Wani, & Reddy, 2008) chickpea, (Akhtar & Siddiqui, 2009), sugar beet (Walpola & Yoon, 2012) and bambara groundnut (Benson *et al.*, 2015). Focus is therefore, being placed onto the possibility of greater utilization of unavailable phosphorus forms wherein the phosphate solubilizing microbes could play a key role in making soluble phosphorus available to plants (Khan *et al.*, 2010). Inorganic forms of phosphorus are

solubilized by a group of heterotrophic microorganisms excreting organic acids that dissolve phosphatic materials and/or chelate cationic partners of the phosphate ion, PO_4^{3-} directly, releasing phosphorus into solution (Turan, Ataoğlu & Şahın, 2006).

2.3.3 P- Solubilization of living organisms

Microorganisms that take part in the solubilization of insoluble phosphorus include bacteria, fungi, actinomycetes and arbuscular mycorrhizal (AM) fungi (Sharma *et al.*, 2013; Kumar *et al.*, 2014). Apart from providing phosphorus to the plants, the phosphate solubilizing microorganisms also ensure the growth of plants by stimulating the efficiency of nitrogen fixation, accelerating the accessibility of other trace elements and by synthesizing essential growth promoting substances (Anzuay, Ludueña, Angelini, Fabra & Taurian, 2015) including siderophores (Wani *et al.*, 2007) and antibiotics (Yang *et al.*, 2009). They also provide plants protection from soil borne pathogens (Hamdali, Hafidi, Virolle & Ouhdouch, 2008).

The solubilization of insoluble phosphates in the rhizosphere is one of the most known mode of action of plant growth promoting bacteria (PGPB) that ensures nutrient availability to plants (Sharma *et al.*, 2013; Zahid, 2015). Phosphate solubilizing bacteria (PSB) secrete organic acids and phosphatases to convert the insoluble phosphates into soluble monobasic and dibasic ions, a process known as mineral phosphate solubilization (Taurian *et al.*, 2010). Phosphate solubilizing bacteria improve plant nutrition through an increase in phosphorus uptake by plants (Kumar & Ram, 2014) and their use as plant growth enhancing bacteria may contribute to fertilization of agricultural crops.

However, Plant growth promoting bacteria have inadequate numbers to compete with other bacterial strains established in the rhizosphere (Bloemberg & Lugtenberg, 2001). Therefore, for agronomic utility, inoculation of plants by target microorganisms to increase the levels found in soil is necessary to take advantage of their beneficial features for plant yield enhancement (Igual, Valverde, Cervantes & Velázquez, 2001). This requires isolation and characterization of native bacteria to be used as potential inoculants in the same area where they were obtained. The advantage of using natural soil isolates over the genetically manipulated or those isolated from a different environmental set up is the easier adaptation and success achieved when inoculated into the plant rhizosphere (Chen *et al.*, 2008).

Microorganisms play a key role in ensuring the availability of P to plant roots, and increasing P mobilization in soil, although the development of effective microbial inoculants remains a major scientific challenge (Kumar & Ram, 2014). Inoculation of PSB when used alone or as mixtures in soils, have demonstrated the capacity to enhanced overall performance of many crop plants including legumes around the world (Wani *et al.,* 2007; Shaharoona, Naveed, Arshad & Zahir, 2008). Therefore, PSB could be developed as suitable inoculants for increasing the productivity of agronomic crops in different agro-ecosystems. PSB can also enhance plant growth by improving the efficiency of BNF, promoting the availability of certain trace elements and by production of phyto-hormones in addition to providing P to plants (Ahemad & Mulugeta, 2014). Accordingly, increase in yield of several legumes has been demonstrated in seed or soil inoculation with N_2 ‗fixating organisms and PSB (Walpola & Yoon, 2012) or PSB when used with nodule bacteria (Zaidi, Ahemad, Oves, Ahmad & Khan, 2010). Several experiments on the co-inoculation effects of P-solubilizing and N_2 fixing bacteria, has shown that about 50% of P fertilizer

requirement could be saved by inoculation of N_2-fixers. An example is *Rhizobium* with P-solubilizers in legumes as reported in groundnuts (Taurian *et al*., 2010). This suggests that the approach holds greater potential for sustaining crop production with optimized P fertilization. A better alternative to PSB/rhizobia coinoculation is the use of monoinoculants from effective rhizobia with high phosphate solubilizing potential, which are preferred due to their role in nitrogen fixation and P-solubilization (Alikhani *et al.*, 2007). Moreover, the rhizobia with P-solubilising ability experience minimal competition from indigenous rhizosphere microflora and function better than free living soil PSB (Kumar & Ram, 2014). Therefore, ability to solubilse P should be considered when selecting rhizobia strains for use as inoculants.

2.4 Tolerance of rhizobia to Phosphorus deficiency in the soil

P deficiency occur when there is a low available P in the soil and rhizosphere, especially under acidic conditions, in which dissolved phosphorus salts may be precipitated in the presence of aluminum (Lazo, Dyer & Alorro, 2017). Slow growing strains of rhizobia appear more tolerant to low P levels than the fast growing rhizobia (Somasegaran & Hoben, 2012). Studies on effects of P on rhizobia have shown positive response to soil Phosphorus supplementation (Wang *et al*., 2013; Kumar and Ram, 2014). Somasegaran and Hoben (2012) observed that the ability of rhizobia strains to nodulate P - deficient soya beans was related to their tolerance of low P supply under in vitro growth. In this sense, rhizobia has a dual role to play in enhancing both P and N in nutrient deficient soils.

2.5 Genetic diversity of rhizobia

In ecological and agronomic studies, survival of released strains in the soil is of great interest. However, rhizobial identification is generally difficult, given that strains having different physiological properties may often be morphologically indistinguishable in culture and under a microscope. The standard classification of the genus *Bradyrhizobium* as presented in Bergey's manual of systematic Bacteriology considers only phenotypic features (Jordan, 1984; Berrada, Nouioui, Houssaini, Gtari & Benbrahim, 2012). There are different methods used for the assessment of genetic diversity in closely or distantly related bacterial species.

Traditionally, diversity has been demonstrated using features such as growth rate and colony morphology (size, shape, color, texture and general appearance) and antibiotic resistance methods (Somasegaran & Hoben, 1994). However, these methods are not discriminative enough to account for all the variation shown in the target species. They cannot depict sources of observed phenotypic diversity to its components that may be as a result of environmental factors or underlying genetic factors. Currently, DNA techniques are used to detect sequence polymorphisms within and between strains of bacteria (Wang *et al*., 2013) .

Over the past years, genotyping has employed several methods such as DNA (rRNA) nucleotide sequence analysis, amino acid sequence analysis, DNA: DNA hybridization, DNA: rRNA hybridization (Iteman, Rippka, de Marsac & Herdman, 2000). In the past, molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD) fingerprinting for repetitive sequences in the genome RNA oligonucleotide cataloguing and guanine plus cytosine (G+C%) of total DNA have been used (Janssen

et al., 1996). Amplified fragment length Polymorphism (AFLP) has also been used in fingerprinting (Vos *et al*., 1995). It is more reproducible and reliable than the other methods (Terefework, Kaijalainen & Lindström, 2001). However, for less complex bacterial DNA, 16S – 23S rDNA amplification and sequence analysis is used (Janssen *et al*., 1996). This is because the ribosomal intergenic spacer region (IGS) located between the 16S rRNA and 23S rRNA genes shows a high degree of length and sequence variation and holds potential for intra-species discrimination (Sadeghifard, Gürtler, Beer & Seviour, 2006).

2.5.1 PCR-RFLP analysis of the 16S- 23 rDNA intergenic region

PCR-RFLP analysis of the 16S- 23 rDNA intergenic region and sequence analysis of the 16S RNA gene are important tools in genetically clustering related rhizobia. They have been widely used in microbial taxonomy to determine inter and intra specific relationships (Abaidoo, Keyser, Singleton & Borthakur, 2000; Sarr, Neyra, Houeibib, Ndoye, Oihabi & Lesueur, 2005; Mallikarjuna *et al.*, 2014). In these methods, the generated PCR fingerprints are unique to each isolate and are used to classify them at strain level. Studies on the variation of bradyrhizobia from soybean used the PCR-RFLP analysis, a high-resolution genotypic fingerprinting technique based on the restriction of amplified fragments from total genomic DNA (Wang *et al*., 2013). The PCR-RFLP technique also demonstrated an insight into the extent of genetic diversity of indigenous *Bradyrhizobium* isolates nodulating cowpea (Mathu *et al.*, 2012) and groundnut (de Freitas & Silva, 2013).

2.5.2 Sequence analysis of 16S rDNA

Nitrogen-fixing rhizobia species have been effectively characterized basing on the sequence homology of 16S rDNA (Hameed *et al*., 2004). The 16S rDNA stretches of sequence are conserved to varying degrees and their positions are mostly known. Sequence information from the conserved region is of use for studying phylogenetic relationships (Mallikarjuna *et al.*, 2014) as well as for creating universal oligonucleotide probes and primers to be used for identification and amplification, respectively (Hameed *et al*., 2004). Isolation and characterization of bacterial strains from the perennial *Lespedeza species* have been studied showing diversity of bacterial strains enhancing its growth and nodulation (Sanginga, Danso & Bowen, 1989).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and collection of groundnut root nodules

Groundnut nodules were sampled in Busia (0^o and 0^o 45' North, 34º 25' East) and Siaya Counties (0° 33' 36" North, 34° 17' 10" East) in western Kenya (Figure 3.1). The counties cover a wide range of climatic conditions, with agro-ecological zones (AEZ) ranging from the Upper Midland Coffee and Maize Zone (UM4), the Lower Midland (LM_1) Sugarcane Zone and the drier Cotton/Sorghum/Sunflower Lower Midlands (LM_3 to LM_4) (Muhati *et al.*, 2011). Busia County receives an annual rainfall of between 760 and 2000 mm with average temperature of 22°C. The county experiences two rainy seasons, the long rainy season which is at its peak between late March and late May and the short rains occurring between August and October (Busia County Portal, 2015). The soils are sandy loams and generally poor with nitrogen and phosphorus as the main limiting macronutrients. The study sites were Alupe, Butula, Lukolis, Matayos, Bumala, Chakol, Nangina, Amagoro, Siginga and Namasali.

Siaya County has an annual rainfall ranging from 1,170 to 1,450 mm and an average temperature of 22.5ºC. The long rains fall between March and June, with a peak in April and May. Short rains are from late September to November (Siaya County Portal, 2015). The main soil type is ferrasol and generally poor in nitrogen and phosphorus as the main limiting macronutrients. The nodule sampling sites in Siaya County were located at Sega, Ugunja, Got nanga, Akala, Siaya town, Muhanda and

Nyamninia. Sites from both counties were chosen because they are located in different agro-ecological zones, have P deficient soils and form major groundnut growing areas in western Kenya

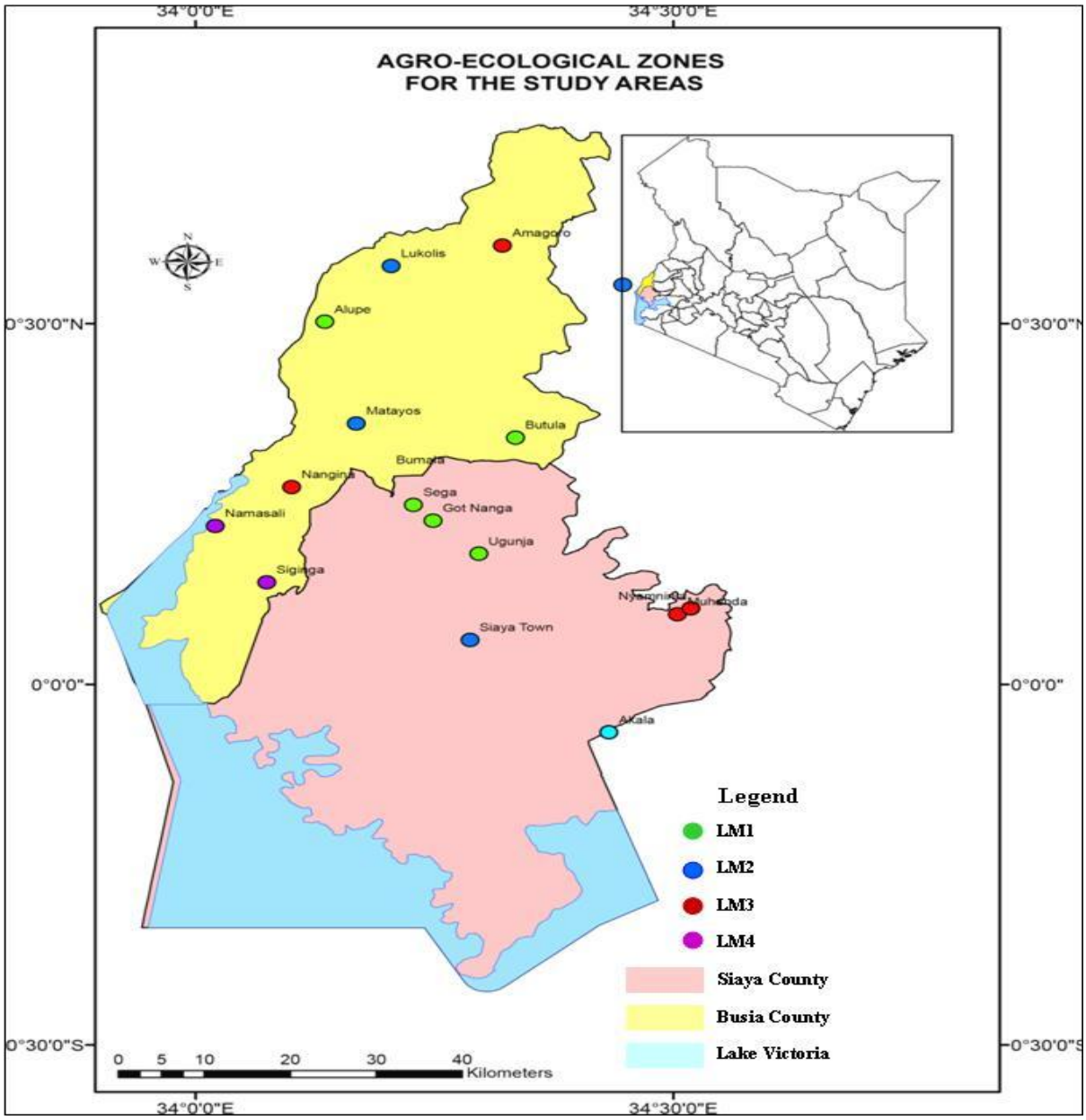

Figure 3. 1: Agro-ecological zones for the study areas
(Source: Author, 2016 modified from google map)

3.2 Soil Sampling and analysis

A random sample of farms was taken from groundnut growers in two Counties spanning agro-ecological zones (AEZ) LM_1, LM_2, LM_3, and LM_4. A total of three points per farm were selected from sites that had no previous history of legume nodulating bacteria inoculation. Individual farms in a given locality were on average 3 km apart. Soil sampling was conducted using a zigzag scheme on each groundnut field (Okalebo, Gathua & Woomer, 2002). From each sampling point, a soil sample was extracted using an Edelman soil auger at 0 to 20 cm depth from which the un-decomposed plant material were removed by hand. Three soil samples from each field were analyzed for soil pH and available P. Detailed procedures for soil analysis are presented in appendix II. Soil pH was measured using a glass electrode in a suspension of 5.0 g soil in 10ml water after equilibrating for 60 minutes. Soil extraction for available P was done using bicarbonate solution (0.5 M $NaHCO_3$ at pH 8.5) method (Hue, Uchida & Ho, 2000).

3.3 Isolation of rhizobia from nodules

Nodules were surface sterilized by immersion in 96% ethanol for 3 seconds followed by immersion in 2% sodium hypochlorite for 3 minutes and finally rinsed several times in sterile water. They were crushed in three drops of sterile water using a sterile glass rod and streaked on Yeast Extract Mannitol Agar (YEMA) media with 1% Congo red (CR). This was repeated on YEMA with 0.5% Bromothymol blue (BTB) (Vincent, 1970). The plates were sealed and incubated at 28°C and observed daily for isolated colonies. Single colonies were picked and checked for purity by repeated streaking. Viability of the isolates was maintained by sub-culturing on yeast extract

Mannitol agar (YEMA medium) after every one month to ensure the isolates remain active for future use (Gupta *et al.*, 2013).

3.4 Phenotypic characterization of bacterial isolates

Bacterial isolates were morphologically characterized based on size, shape, consistency, transparency, elevation, and acid or alkaline reaction (Vincent, 1970). Gram staining reaction was performed as per the standard gram's procedure (Huang, Lü, and Wu & Fan, 2007) to visualize cell morphology, size and motility under a light microscope.

3.5 Biochemical characterization of bacterial isolates

3.5.1 Congo Red Test

An aliquot of 2.5 ml of 1% solution of Congo red dye was added to one litre of YEMA (Singh *et al.,* 2008). Rhizobia isolates was streaked on YEMA plates and incubated for 5 days at 28°C to allow for growth of slow and fast growing isolates. Rhizobia stand out as white, translucent, glistering, elevated and comparatively smaller colonies with entire margins in contrast to red stained colonies of agrobacteria (Singh *et al.*, 2008).

3.5.2 Hofer's alkaline broth Test

Hofer's alkaline broth test at pH of 11 was used to differentiate rhizobia isolates (Singh *et al.,* 2008) from agrobacteria. Rhizobia do not grow at the high pH whereas agrobacteria grow (Huang *et al.*, 2007). The medium consisted of: 0.5 g K_2HPO_4; 0.2 g $MgSO_4$; 0.1 g NaCl; 0.05 g $CaCO_3$; 0.1 g yeast extract; 10 g mannitol, and 1 litre

water. The pH was adjusted to 11 by adding 28 ml of NaOH and 1 ml of 0.6% thymol blue.

3.5.3 Lactose agar Test

Agrobacteria utilize lactose to form reduced product 3-ketolactose, through the activity of the enzyme ketolactase while rhizobia is unable to utilize lactose. Culture of nodulated bacteria was streaked on lactose agar plate and incubated for 5 days as described by Hunter, Kuykendall & Manter, (2007). Benedict's reagent was poured over agar medium containing lactose (10 g/ litre) on which the isolated nodule bacteria were growing. The formation of yellow coloration due to copper II oxide (Cu_2O) indicates the presence of agrobacteria in the culture plate.

3.6 Confirmation of rhizobia isolates through nodulation test

The ability of each isolate to nodulate groundnut as host plant was tested in a growth bag containing sterile cotton wool (Laurette *et al.,* 2015). Healthy groundnut seeds were washed with detergent then sterilized using 1% hypochlorite solution for 10 minutes, rinsed thoroughly with sterile distilled water to remove traces of hypochlorite. Seeds were left to imbibe in sterile distilled water for 30 minutes and plated aseptically onto 0.8% (w/v) water agar in Petri dishes. Seeds were germinated in an incubator at 28ºC for two days. The pre-germinated seeds were transplanted into growth bags, after attaining a radical length of 1-2 cm and inoculated with individual rhizobia. Broughton and Dilworth N-free nutrient solution prepared according to (Somasegaran & Hoben, 1994) was dispensed into the growth bag through a small hole. Pregerminated groundnut seeds were inoculated with individual rhizobia isolates and transferred to the growth bags. The plants were maintained in the growth chamber

at 12h photoperiod; 28ºC day/night temperature and nodulation observed after six weeks (Laurette *et al.*, 2015).

3.7 Metabolic tests

3.7.1 Determination of nitrate reduction

Nitrate reduction test was performed to determine the isolates ability to reduce nitrate to nitrite (Gachande & Khansole, 2011). Nitrate broth prepared by mixing 1g KNO_3, 5g Peptone, 3g beef extract was inoculated with rhizobia culture, incubated at 28°C for 48 hours. Alpha napthylamine reagent containing 2g zinc chloride, 4g starch, 2g Potassium iodide, distilled water 1 litre, and 1 drop of dilute sulphuric acid (1 acid:3H_2O) was dispensed into a porcelain plate. One drop of nitrate broth culture was transferred to it and observation made. Blue colour change indicated a positive nitrate reduction test. In case there was no colour, powdered zinc metal was added, stirred using a magnetic stirrer to form a homogeneous mixture. The pH was adjusted to 6.8 by either adding 1M HCl or 1M NaOH. Red color change indicated a negative nitrate reduction test.

3.7.2 Urea hydrolysis

Urease production test was performed to determine the ability of isolates to reduce urea into ammonia (Gachande & Khansole, 2011). Urea broth medium containing 20g urea, 0.1g yeast extract, 9g KH_2PO_4, 9.5g K_2HPO_4, 0.01g phenol red, 1 liter distilled water and pH 6.8 was inoculated with rhizobial culture, incubated at 28°C for 48 hours. Urea reduction into ammonia was detected by a variety of bacteria producing enzyme urease that degrade urea into ammonia and carbon dioxide. Colour change

from pale red to pale brown was taken to be positive for the test and where the pale red colour was retained was taken to be negative result.

3.8 Carbon sources utilization

Utilization of broad range of carbon sources by isolates was tested on glucose, sucrose, starch, and citrate. The medium used for this test was a carbohydrate-free medium (Somasegaran & Hoben, 1994), yeast extract was reduced to 0.05 g. l^{-1}. Separately autoclaved sugars were added to the modified YEMA basal medium. After inoculation, plates were incubated at 28°C for 5 days and visual growth was recorded. All the plates were streaked with the freshly prepared liquid culture of each of the rhizobial isolates, and then incubated at 28°C for 5 days. Growth response of different isolates was recorded positive (visible growth) or negative (no growth**)**. Simmon's citrate agar medium containing 2 g sodium citrate, 0.2 g $MgSO_4$, 1g (NH_4) H_2PO_4,1g K_2HPO_4, 5 g NaCI, 0.08 g BTB,15 g agar, distilled water 1 litre, pH 6.8 was used to prepare slants which were then inoculated by stabbing to the base of the slants and tubes incubated at 28ºC for 48 hours. Colour change from green to blue indicated citrate utilization as carbon source.

3.9 Temperature assay

Agrobacteria can grow up to a temperature of 30ºC whereas rhizobia can tolerate higher temperatures of up to 44ºC (Huang *et al.*, 2007). To analyze the effect of temperature variation on the growth of isolates, YEMA medium was prepared with pH 6.8. After inoculation, the plates were incubated at 28ºC or 37ºC. Growth of the isolates at 37ºC was used to rule out the presence of agrobacterium in the culture.

3.10 pH assay

All isolates were tested for their tolerance to pH. The ability of the isolates to grow in acid and alkaline media was tested by inoculating them into YEMA medium adjusted to 4.0, 5.5, 7.0 and 8.5 with either NaOH or HCI before being autoclaved (Alshaharani & Shetta, 2015).

3.11 Phosphate solubilization

Phosphate solubilizing ability of each isolate was tested by plate assay using National Botanical Research Institute Phosphate (NBRIP) growth medium with pH adjusted to 7.0 (Mehta & Nautiyal, 2001). The growth medium was supplemented with 10% w/v insoluble $CaHPO_4$ as a sole source of P. The $CaHPO_4$ was generated by adding 50 mL of a 10 % K_2HPO_4 solution and 100 mL of a 10 % $CaCl_2$ solution (autoclaved separately) to complete 1,000 mL culture medium to precipitate the insoluble inorganic phosphate (Marra, Oliveira, Soares & Moreira, 2011). An aliquot of by 10 µl of fresh bacterial culture containing 10^8cfu/ml was spotted onto the plates and incubated at 28ºC for 7 days. Formation of a halo around the colonies indicated solubilizing ability. Solubilization index (SI) of each isolate was determined according to the procedure described by (Alikhani *et al.*, 2007) as follows:

$$Solubilization\ index = \frac{Halo\ zone\ diameter}{Colony\ diameter}$$

3.12 Molecular diversity studies

3.12.1 DNA extraction

Bacterial cells were grown in YEM broth for 48 hours at 28°C. Cells were rinsed with TES buffer (50 mMTris pH 8.0, 0.5 mM EDTA, 50 mM NaCl), and resuspended in saline EDTA (150 mM NaCl, 10 mM EDTA pH 8.0). Cells were lysed in 20% sodium dodecyl sulfate (SDS) warmed at 55 (°C). DNA was extracted using chloroform/isoamyl alcohol (24:1) and precipitated with ethanol (Maheswari, Chitra & Rani, 2013).

3.12.2 PCR amplification of the 16S-23S rDNA spacer region

Two primers FGPS 1,490-72 (5'-TGCGGCTGGATCCCCTCCTT-3') and FGPL 132-38 (5'-CCGGGTTTCCCCATTCGG-3') as described by Mathu *et al.* (2012) were used for PCR amplification. PCR amplification was carried out in a 50 µl reaction volume containing 10-20 ng pure total DNA extract, 1.5 U of Taq DNA polymerase, 20 mM Tris-HCL (pH 8.0), 100 mM KCl, 2.0 mM $MgCl_2$, 200 µM of each dNTP and 1.0 µM of each primer. PCR amplification was performed in Eppendorf thermal cycler using the following temperature profile: initial denaturation for 5 min at 95°C, 35 cycles of denaturation (30 s at 95°C), annealing at 57°C for 20 seconds and extension at 72°C for 30 seconds followed with a final extension at 72°C for 7 minutes. PCR amplified DNA was visualized by electrophoresis of 10 µl of the product on 1% (w/v) horizontal agarose gel in 1× TAE buffer (10 mMTris, 3M acetic acid, 1mM EDTA and pH 8.0). The gel was stained for 30 minutes in an aqueous solution of ethidium bromide (1 µg/ml) and photographed under UV illumination on Gel documentation (Beijing Junyi Dongfang Electrophoresis Equipment Co., Ltd).

3.12.3 PCR amplification of the 16S rRNA gene

For each sample, the 16S rRNA gene was amplified using the primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') corresponding to positions 27–46 of the *E. coli* 16S rRNA gene and 1492R (5-GGTT TAC CTT GTT ACG ACT T -3') corresponding to positions 1,525–1,506 of *E. coli* rRNA gene (Wasike *et al*., 2009). PCR amplification was carried out in a 50ul reaction volume. DNA was amplified by mixing template DNA, i.e., pure DNA (10-20 ng) with the polymerase reaction buffer 1.5 U of Taq DNA polymerase (Thermo Scientific), 20 mM Tris-HCL (pH 8.0), 100 mM KCl, 2.0 mM $MgCl_2$, 200 µM of each dNTP and 1.0 µM of each primer. PCR amplification was performed in Eppendorf thermal cycler adjusted to the following program:: initial denaturation for 5 min at 95°C, 35 cycles of denaturation (30 s at 95°C), annealing at 20 seconds at 55°C) and extension (30 seconds at 72°C) followed with a final extension (7 minutes at 72°C). PCR amplified DNA was visualized by electrophoresis of 10 µl of the producton 1% (w/v) horizontal agarose gel in 1× TAE buffer (10 mM Tris, 3M acetic acid and 1mM EDTA, pH 8.0). Electrophoresis was carried out at 80 V for 3 hours in 12 by 12 cm gels. Gels were stained and photographed as described in section 3.12.3 above.

3.12.4 Restriction fragment analysis of 16S-23S rDNA intergenic spacer region and 16S rRNA gene

Ten micro liters of PCR products were digested with the restriction endonucleases *Hinf*1, *Msp*1, *Taq*1, and *Csp6*1 according to manufacturer's instructions (Thermo Scientific) with 10 U of enzyme per reaction in a total volume of 50 µL. The restricted DNA fragments were separated by horizontal gel electrophoresis in 2.5% (w/v)

standard agarose (Thermo Scientific). Electrophoresis was carried out at 80 V for 3 hours in 12 by 12 cm gels. Gels were stained and photographed as described in section 3.12.3 above.

3.13 Statistical analysis

Nodulation data was log transformed using base ten to stabilize variance. Phosphate solubilization data was converted to SI (Alikhani *et al.*, 2007). Solubilization index and nodulation transformed data were subjected to analysis of variance and means were separated using Turkey's range test at $p < 0.05$ using statistical package R 3.2.5 (Team, 2016). Restriction fragment data was analyzed by hierarchical cluster analysis using Jaccard similarity co-efficient. A distance matrix based on the unweighted pair group method with averages (UPGMA) was used to construct a dendrogram (Sneath & Sokal, 1973). The stability of the groupings was estimated by bootstrap analysis on 1000 permutation in the same package. The trees were displayed using Tree View version 1.6.6, as described by Zhang *et al*., (1999).

CHAPTER FOUR

RESULTS

4.1 Collection site characteristics and isolates

The pH and available P values of the soils from nodule sampling sites in this study are presented in Table 4.1. Generally, soil pH ranged from 4.9-8.0 whereas available P was between 0.9-12.3 mg/kg^{-1} of soil. Ugunja in LM1 had the lowest pH of 4.9- 5.1 while Siginga in LM4 recorded the highest pH of 7.0- 7.9. Butula and Siaya town in LM1 and LM2 had 4.3 - 6.1 and 0.9 - 2.6 mg P kg^{-1} of soil respectively.

Table 4.1: Agro-ecological zones, rhizobia collection site and identity of isolates in this study

Agro-ecological zone	Collection Site	Soil Ph	Available soil P (mg/kg^{-1})	No. of Isolates	Isolate Identity
Low midland 1	Alupe	5.6-6.1	1.2-2.3	5	A01, A02, A03, A04, A05
	Butula	4.9-5.6	4.3-6.1	4	B01, B02, BO3, B05
	Ugunja	4.9-5.1	2.3-12.3	3	L01, L03, L05
	Got-Nanga	5.1-5.6	2.6-4.7	5	Q01, Q02, Q03, Q05, Q06
	Sega	4.9-6.6	1.2-4.9	4	K01, K03, K04, K05
Low midland 2	Lukolis	5.0-6.5	2.3-5.9	5	C01, C02, C03, C05, C06
	Matayos	5.0-5.7	2.5-5.0	3	D01, D03, D05
	Bumala	5.4-6.3	2.7-3.9	5	E01, E02, E03, E05, E06
	Chakol	5.8-6.7	2.6-7.4	4	F01, F03, F04, F05
	Akala	5.2-6.4	2.1-5.0	2	M01,M05
	Siaya town	4.9-6.2	0.9-2.6	3	N01, N03, N06
Low midland 3	Nangina	4.9-6.3	3.2-4.9	4	H01, H03, H04, H05
	Amagoro	5.8-6.5	3.8-5.9	5	G01,G02, G03, G05, G06
	Muhanda	5.8-6.8	2.3-5.0	3	O01, O03,O05
	Nyamninia	5.4-6.5	3.6-4.8	4	P01, P03, P05, P06
Low midland 4	Siginga	7.1-8.0	2.8-4.5	5	I01, I02, I03, I05, I06
	Namasali	5.6-5.9	2.0-5.0	4	J01, J02, J03, J05

Note: Soil pH and P values are range values of different farms in the collection site

4.2 Morphological and biochemical characterization of bacterial isolates

In this study, 68 bacteria isolates were obtained from root nodules of groundnut (Table 4.1). Bacteria colonies appeared on YEMA medium after 1 to 5 days of incubation at 28ºC. The diameter of the colonies ranged from 2 mm to 4 mm. Fifty five of the isolates representing 81% were fast-growing with colonies appearing within 1 to 2 days (Table 4.2). On the other hand, 19% of the isolates were slow growing with colonies emerging after 3 to 5 days of incubation. Sixty five isolates exhibited semi-globose to globose colonies on YEMA medium at 28ºC. The remaining three isolates D05, I01 and J05 had dome shaped colonies. All colonies had a smooth entire margin and raised surface with exception of D05, E02 and J05 that were pulvinate. The bacterial isolates had white, creamy, translucent and glistering colonies (Plate 4.1). Forty nine isolates representing 72% produced mucoid colonies while the rest were viscous.

Microscopic observation showed that 63 of the isolates were gram negative rod shaped cells occurring in chains (Table 4.2). Two isolates coded as B03 and LO1 were gram positive with single cells while one gram positive isolate, P06, had paired cells.

Majority of the isolates showed capacity to acidify the culture medium after 1 to 2 days of incubation at 28ºC. Fifty one isolates altered the colour of YEMA medium supplemented with bromothymol blue (BTB) from blue to yellow within 24 hours of incubation while 17 isolates retained the blue colour (Table 4.2, Plate 4.2).

Table 4.2: Morphological - biochemical characteristics and nodulation ability of bacterial isolates from groundnut nodules

Isolate	Diameter (mm)	Growth	Colony shape	Elevation	Consistency	Cell shape	Gram stain	BTB	C.R	HAB	LA	Nodules per Plant
A01	3	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
A02	4	Fast	Globose	Raised	Mucoid	Rod	-	Blue	-	-	-	39 abcd
A03	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	37 a-f
A04	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	37 abcd
A05	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	43 abc
B01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	+	-	29 f
B02	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
B03	2	Slow	Semi globose	Raised	Mucoid	Rod	+	Blue	-	-	-	00 g
B05	3	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	40 abcd
C01	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	37 a-f
C02	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	42 abc
C03	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	33 b-f
C05	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
C06	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	43 ab
D01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	+	-	40 abcd
D03	2	Slow	Semi globose	Raised	Viscous	Rod	-	Yellow	-	-	-	37 a-f
D05	3	Fast	Domed	Pulvinate	Viscous	Rod	-	Yellow	-	-	-	37 a-f
E01	4	Fast	Globose	Raised	Mucoid	Rod	-	Blue	-	-	-	37 a-f
E02	4	Fast	Globose	Pulvinate	Mucoid	Rod	-	Yellow	-	-	-	40 abcd
E03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	35 a-f
E05	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	33 c-d
E06	3	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	40 abcd
F01	3	Fast	Globose	Pulvinate	Mucoid	Rod	-	Yellow	-	-	-	40 abcd
F03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	39 abcd
F04	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	40 abcd
F05	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	38 abcd
G01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
G02	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	39 abcd
G03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
G05	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	44 a
G06	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
H01	2	Slow	Semi globose	Raised	Mucoid	Rod	-	Blue	-	-	-	39 abcd
H03	2	Slow	Semi globose	Raised	Viscous	Rod	-	Blue	-	-	-	37 a-f
H04	2	Slow	Semi globose	Raised	Viscous	Rod	-	Blue	-	-	-	39 abcd

BTB = Bromothymol blue, C. R = Congo red, HAB = Hofer's Alkaline broth reaction, LA = Lactose agar, - = negative, + = positive. Means with the same letter in a column are not significantly different at $P < 0.05$

Table 4.2 continued

Isolate	Diameter (mm)	Growth Rate	Colony Shape	Elevation	Consistency	Cell Shape	Gram stain	BTB	C.R	HAB	LA	Nodules per plant
H05	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	40 abcd
I01	4	Fast	Domed	Raised	Viscous	Rod	-	Yellow	-	-	-	40 abcd
I02	4	Fast	Globose	Raised	Viscous	Rod	-	Blue	-	-	-	38 a-f
I03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	38 a-f
I05	2	Slow	Semi globose	Raised	Viscous	Rod	-	Blue	-	-	-	38 abcd
I06	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	36 a-f
J01	4	Fast	Globose	Raised	Mucoid	Rod	-	Blue	-	-	-	35 a-f
J02	2	Slow	Semi globose	Raised	Mucoid	Rod	-	Blue	-	-	-	37 a-f
J03	2	Slow	Semi globose	Raised	Viscous	Rod	-	Blue	-	-	-	35 a-f
J05	4	Fast	Domed	Pulvinate	Mucoid	Rod	-	Yellow	+	-	-	00 g
K01	2	Slow	Semi globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	38 a-f
K03	2	Slow	Semi globose	Raised	Mucoid	Rod	-	Blue	-	-	-	38 a-e
K04	2	Slow	Semi globose	Raised	Mucoid	Rod	-	Blue	-	-	-	40 abcd
K05	4	Fast	Globose	Raised	Viscous	Rod	-	Yellow	-	-	-	37 a-f
L01	3	Fast	Globose	Raised	Mucoid	Rod	+	Yellow	+	-	-	00 g
L03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	40 abcd
L05	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
M01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	37 a-f
M05	3	Fast	Globose	Raised	Viscous	Rod	-	Blue	-	-	-	37 a-f
N01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	+	-	37 a-f
N03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	38 a-f
N06	2	Slow	Semi globose	Raised	Mucoid	Rod	-	Blue	+	-	-	29 ef
O01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	32 def
O03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	33 c-d
O05	4	Fast	Globose	Raised	Mucoid	Rod	-	Blue	-	-	-	33 b-f
P01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	35 a-f
P03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	40 abcd
P05	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	40 abcd
P06	2	Slow	Semi globose	Raised	Mucoid	Rod	+	Blue	+	-	-	00 g
Q01	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	38 a-e
Q02	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	42 abcd
Q03	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	35 a-f
Q05	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	39 abcd
Q06	4	Fast	Globose	Raised	Mucoid	Rod	-	Yellow	-	-	-	36 a-f

BTB = Bromothymol blue, C. R = Congo red, HAB = Hofer's Alkaline broth reaction, LA = Lactose agar, = negative, + = positive, - = negative, + = positive. Means with the same letter in a column are not significantly different at $P < 0.05$

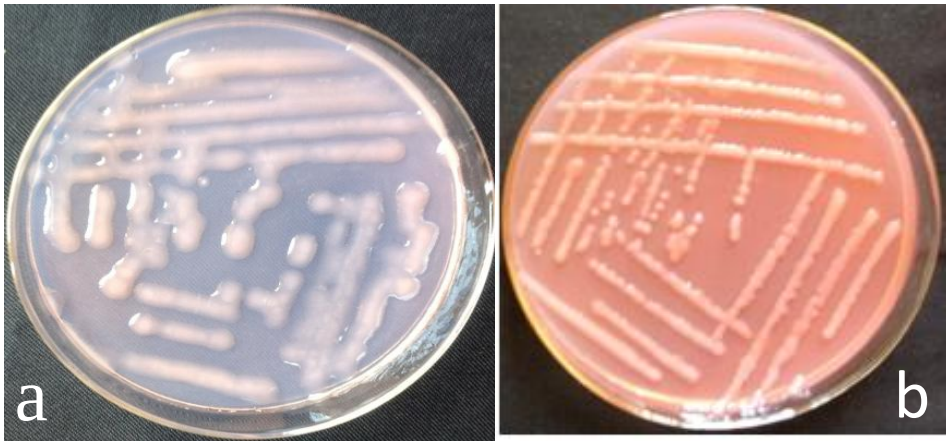

Plate 4.1: Growth characteristics of rhizobia on YEMA with Congo red Globose colonies with mucoid and milky texture (a) and (b) white, creamy, translucent, gummy and glistering appearance (Source: Author, 2016)

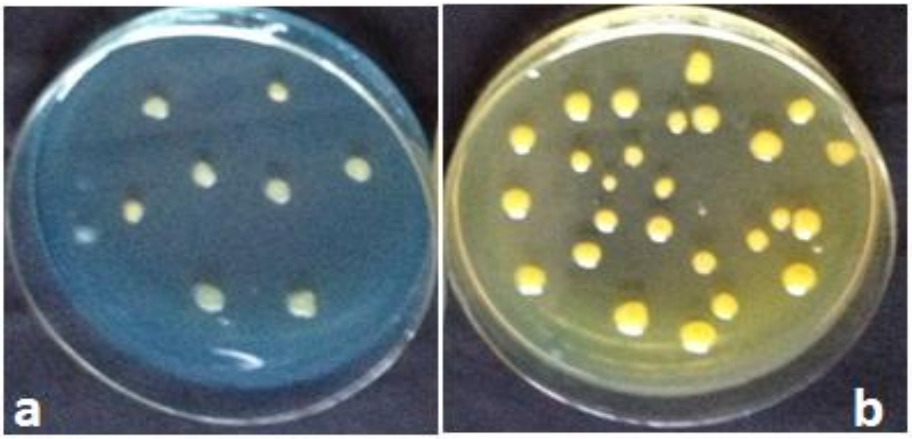

Plate 4.2: Growth of bacteria culture on YEMA bromothymol blue
Alkaline (a) and acid (b) producing isolates grown on YEMA supplemented with bromothymol blue (Source: Author, 2016*)*

4.3 Biochemical characterization of bacterial isolates

4.3.1 Congo Red Test

Rhizobia stood out as white, translucent, glistering, elevated and comparatively smaller colonies with entire margins in contrast to red stained colonies suspected to be agrobacteria (Plate 4.3). Sixty four isolates did not absorb the dye while isolates J05, L01, N06 and P06 absorbed the Congo red colour (Table 4.2).

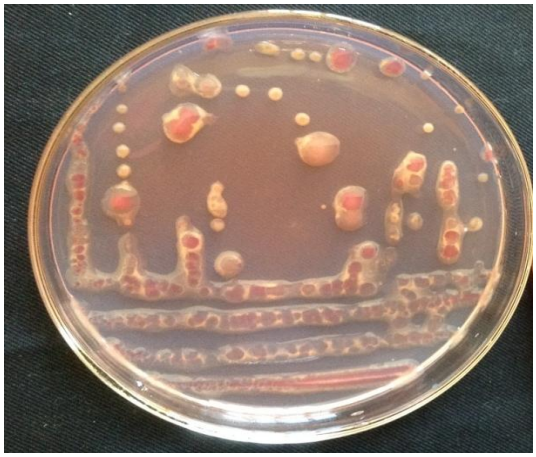

Plate 4. 3: Mixture of agrobacteria and rhizobia colonies (Source: Author, 2016)

4.3.2 Hofer's alkaline broth Test

All the isolates except B01, D01 and N01 did not exhibit detectable growth in Hofer's alkaline broth. This was depicted by colour change from blue to pale beige (Table 4.2; Plate 4.4).

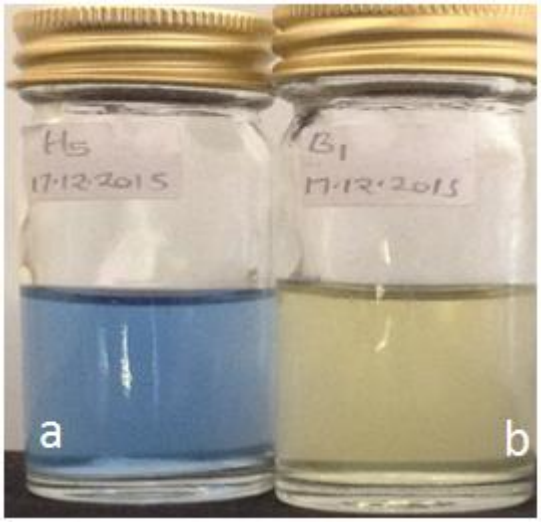

Plate 4.4: Growth of bacteria culture tested in Hofer's broth. No growth on Hofer's broth (a) and growth on broth (b) (Source: Author, 2016)

4.3.3 Lactose agar Test

All the sixty eight isolates grew on lactose agar. When Benedict's solution was poured over the lactose agar cultures, all the bacterial isolates showed negative results for 3-ketolactose production by failing to produce a yellow colouration (Table 4.2).

4.4 Confirmation of rhizobia isolates through nodulation test

Nodulation ability of groundnut nodule bacteria isolates differed significantly at $P < 0.05$ (Table 4.2). Sixty four isolates representing 94% were confirmed to be rhizobia due to their ability to nodulate groundnut (Table 4.2). Four isolates B03, L01, P06 and J05 failed to nodulate the test plant. Five isolates; G05, A05, C.06, C02 and Q02 that recorded the highest number of nodules; 44, 43, 43, 42 and 42, respectively were fast growing (Table 4.2). In contrast, isolate N06, a slow grower recorded the lowest number of nodules (29). Symbiotically active nodules showing pink colouration (Plate 4.5) occurred on both tap roots and lateral roots of the groundnut plants upon inoculation with rhizobia isolates. The leaves of the nodulated plants were dark-green while uninoculated unfertilized control plants showed yellowing typical of nitrogen deficiency.

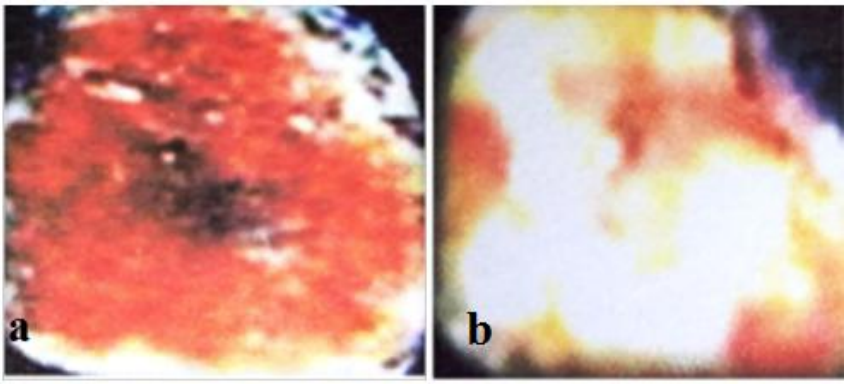

Plate 4.5: Cross section of nitrogen fixing nodules sampled from rhizobia inoculated groundnut seedlings after six weeks of growth. (a) Pink-dark red colouration (b) White-pink colouration (Source: Author, 2016)

4.5 Metabolic Identification of Rhizobia

4.5.1 Nitrate reduction test

Nitrate reduction test was positive for all the sixty eight isolates. Sixteen isolates readily reduced nitrate to nitrite, which was indicated by colour of the reaction mixture changing from brown to blue then colourless (Plate 4.6) within 48 hours after incubation. However, the remaining fifty two isolates changed the reaction mixture to blue but required addition of zinc dust for the complete reaction to occur.

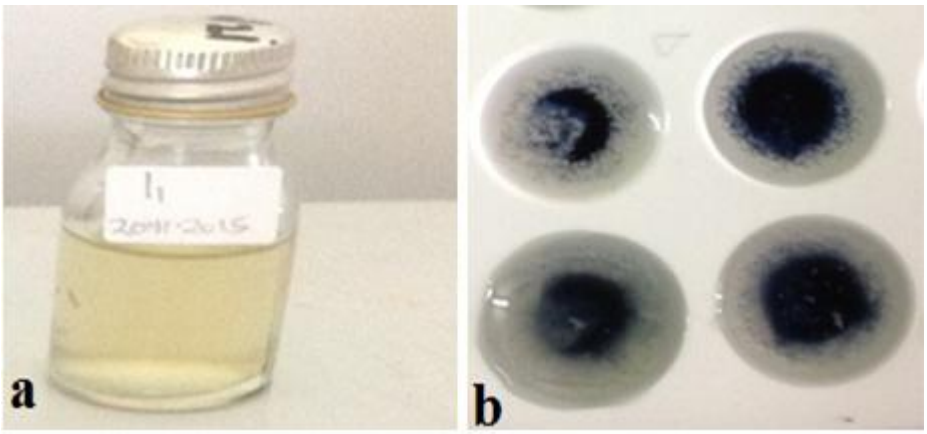

Plate 4.6: Nitrate reduction to nitrite by rhizobia isolates tested in broth. Nitrate broth before inoculation (a) and Porcelain plates showing colour change to blue (b) (Source: Author, 2016)

4.5.2 Urea hydrolysis test

Isolates A03, A04, K01, Q06 and G05 reduced urea into ammonia. This was deduced from the change in colour of the medium from pale red to pale brown (plate 4.7) after 48 hours of culture incubation.

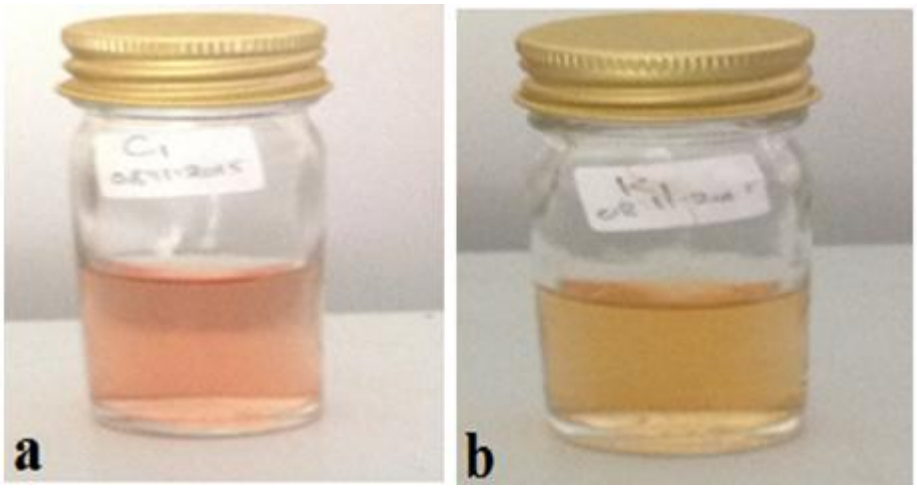

Plate 4.7: Urea reduction by bacteria culture tested in broth. Change in colour of the medium from brown (a) to pale brown (b) (Source: Author, 2016)

4.6 Carbon source utilization

Medium containing glucose (monosaccharide), sucrose (disaccharides), starch (polysaccharides) and citrate had 64, 61, 56 and 5 isolates growing, respectively (Figure 4. 1). Fifty two isolates out of 64 that utilized glucose were fast-growing while 80% of the isolates that utilized sucrose and starch were fast growing. Five isolates Q06, F04, C05, C01 and B02 that used citrate as carbon source were fast growers that could also utilize the other carbohydrates sources. In contrast, among the slow growers 19 % utilized glucose, 20% sucrose and starch respectively while none of them could utilize citrate.

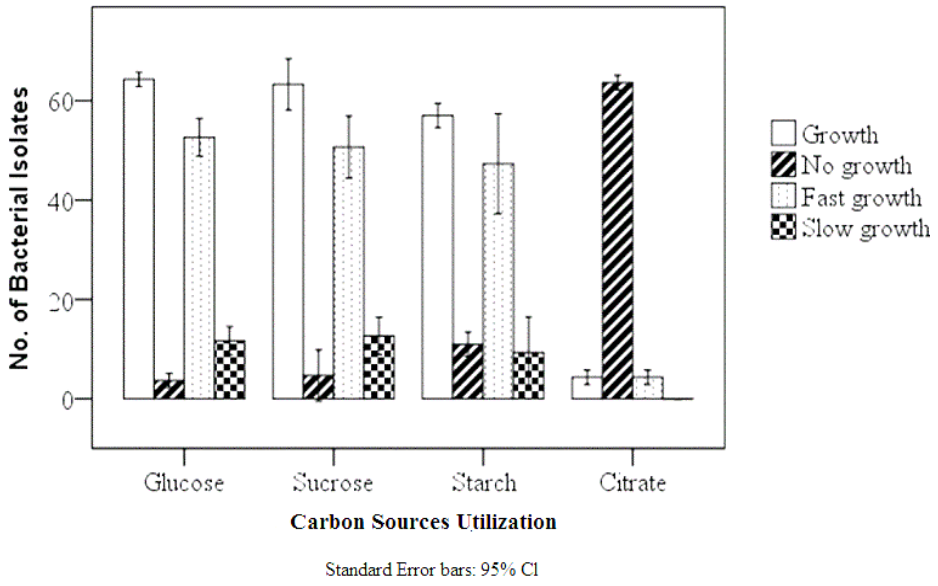

Figure 4.1: Growth of groundnut nodulating bacteria isolates on culture medium enriched with different carbon sources. Data presented are from three replicates

4.7 Temperature assay

All the isolates showed growth upon incubation at 28^{o}C on YEMA. Out of the sixty eight isolates incubated at 37 ^{o} C, isolate LO1 showed no growth.

4.8 pH assay

The isolates exhibited variation in their ability to grow on the medium adjusted to pH 4.0, 5.5, 7.0 and 8.5 (Figure 4.2). Thirty nine and 61 of the isolates grew at pH 4.0 and 5.5, respectively, indicating that they are acid tolerant. All the isolates grew at pH 7.0 and 8.5. Thirty four of the isolates that grew at pH 4.0 were fast growers.

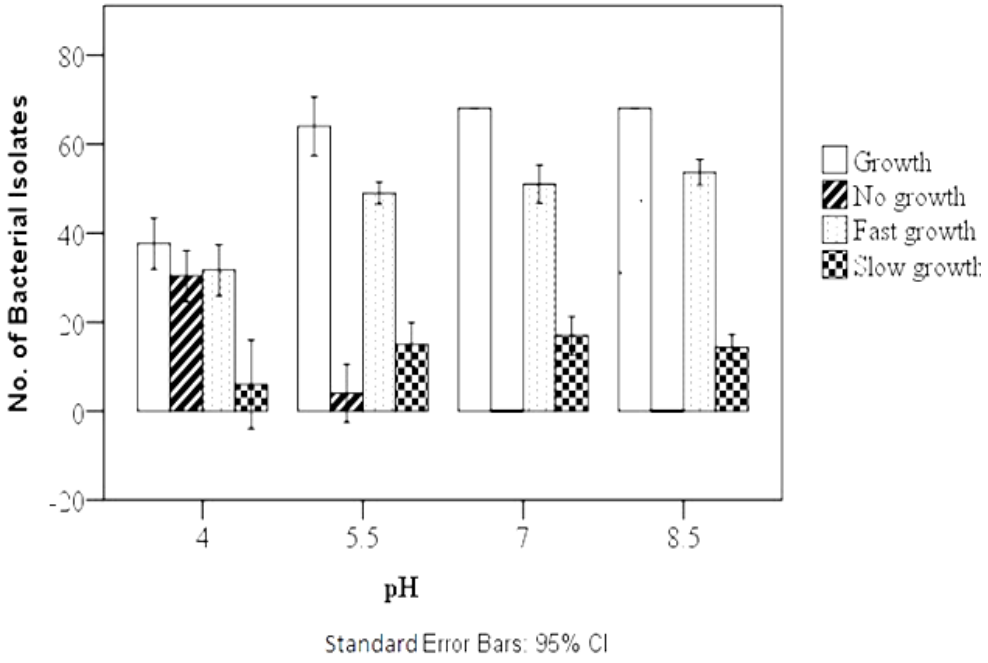

Figure 4.2: Tolerance of groundnut nodulating bacteria isolates to different pH Data presented are from three replicates

4.9 Phosphate solubilization

The phosphate solubilization index (SI) of groundnut nodule bacteria varied significantly at $P < 0.05$ (Table 4.3). A total of sixty four isolates representing 94% showed a clear zone of solubilisation around the colonies after 7 days of incubation on medium containing DCP as the sole P source (Table 4.4, Plate 4.8). The zone of solubilisation showed progressive increase with prolonged incubation. Solubilization index ranged from 1.1 to 6.8. Fast growing isolates N01, B02, L01, I06, Q01, F05, C02, E01, J05, Q03, I01 and B01 recorded the highest SI ranging from 3.8 to 6.8. All of these isolates with the exception of L01 and J05 were identified as rhizobia based on nodulation tests (Table 4.2). Slow growing bacteria isolates J02, N06, J03, K01, H01, I05 and H04 had fairly high SI of 2.4, 2.4, 2.7, 3.0, 3.3, 3.7 and 4.0,

respectively. Four isolates B03, G01, G02, and I02 did not show detectable solubilization of DCP.

Table 4.3: ANOVA of the Phosphorus mobilized from ($CaHPO_4$) and nodulation of groundnut bacteria isolated from P deficient soils of Western Kenya

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Isolate	67	437.8	7.071	45.54	<2e-16***
Residuals	136	21.10	0.155		
Isolate	64	1.22	0.019	3.208	1.18e-08***
Residuals	128	0.776			

***Significant at $P \leq 0.05$

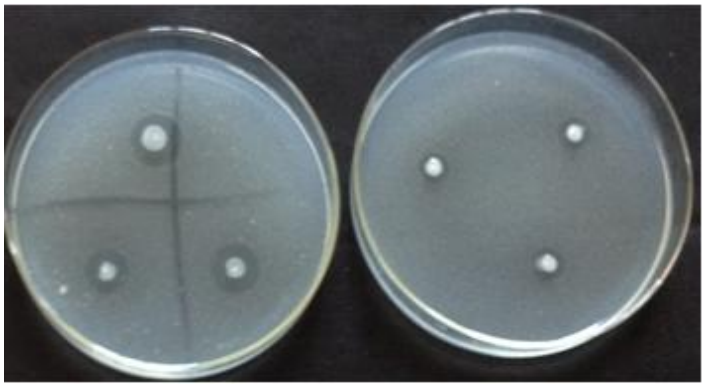

Plate 4.8: Solubilisation halo of the low soluble inorganic phosphate ($CaHPO_4$) for bacteria with high (left) or low (right) efficiency (Source: Author, 2016)

Table 4.4: Phosphate solubilization efficiency of groundnut nodulating bacteria

Isolate	Colony diameter (mm)	Halo zone diameter (mm)	Solubilisation index	Isolate	Colony diameter (mm)	Halo zone diameter (mm)	Solubilisation index
A01	4.7	18.3	4.0 d-i	**H05**	4	5	1.3 stu
A02	5.9	12.1	2.1 l-t	**I01**	2.3	2.3	6.1 ab
A03	3.9	9.0	2.3 k-t	**I02**	2	0	0 u
A04	4	6.0	1.5 p-t	**I03**	4	5	1.3 stu
A05	5	5.7	1.1 tu	**I05**	3	11	3.7 e-k
B01	2.3	15.7	6.8 a	**I06**	3.7	16.3	4.6 c-g
B02	4	18.0	4.5 c-g	**J01**	6.3	16.7	2.7 h-q
B03	4	0.0	0 u	**J02**	4.9	12.1	2.4 j-t
B05	7.9	9.1	1.2 tu	**J03**	4.8	13.1	2.7 h-p
C01	1	3.0	3.0 h-o	**J05**	2	11	5.5 bc
C02	3	15.0	5.0 b-e	**K01**	2	6	3.0 h-o
C03	7.8	14.2	1.8 n-t	**K03**	5.9	11.1	1.9 n-t
C05	4	14.0	3.5 f-k	**K04**	6.7	8.3	1.3 r-u
C06	5	13.0	2.6 i-s	**K05**	7.3	12.7	1.8 n-t
D01	3	11.0	3.7 e-k	**L01**	3.3	14.7	4.5 c-g
D03	5.3	7.7	1.5 p-t	**L03**	4	5	1.3 stu
D05	5.7	9.3	1.7 o-t	**L05**	7.5	9.4	1.3 stu
E01	4	20.9	5.1 bcd	**M01**	4	12	3.0 h-0
E02	3	4.0	1.3 q-u	**M05**	3.2	4.8	1.5 p-t
E03	4	11.0	2.8 h-p	**N01**	3.2	11.8	3.8 e-j
E05	5	13.0	2.6 i-r	**N03**	2	4	2.0 l-t
E06	5	13.0	2.6 i-r	**N06**	5	12	2.4 j-t
F01	7.3	11.7	1.6 o-t	**O01**	4	5	1.3 stu
F03	6.3	11.7	1.9 n-t	**O03**	4.2	13	3.1 h-n
F04	4	6.0	1.5 p-t	**O05**	4	6.4	1.6 p-t
F05	5.3	24.7	4.7 c-f	**P01**	4	14	3.5 f-k
G01	4	0.0	0 u	**P03**	5	18	3.6 f-k
G02	2.3	0.0	0 u	**P05**	5	15	3.0 h-o
G03	3.7	4.3	1.2 tu	**P06**	2	4	2.0 l-t
G05	6.8	11.1	1.6 o-t	**Q01**	5	23	4.6 c-g
G06	4.7	15.3	3.3 f-1	**Q02**	7	10	1.4 p-t
H01	4	13.0	3.3 g-m	**Q03**	3.1	18.9	6.1 ab
H03	4	13.0	3.3 g-m	**Q05**	3	3.7	1.2 tu
H04	3.7	14.3	4.0 d-h	**Q06**	4.8	9.2	1.9 m-t

Means with the same letter in a column are not significantly different at $P < 0.05$

4.10 PCR-RFLP analysis of 16S–23S rDNA IGS regions

PCR amplification of the intergenic spacer (IGS) between the 16S and 23S rDNA genes of the fifty four (54) rhizobia isolates produced a single polymorphic fragment ranging from 750 to1100 bp. Restriction of amplified 16S-23S rDNA IGS region with four endonucleases *Hinf* l, *Msp*1, *Taq*1 and *Csp*61 produced varied number of fragments in all the isolates (Table 4.5). The enzyme *Hinf*1 produced 2 to10 restriction fragments per isolate. These fragments varied in length from 50 and 500 bp (Plate 4.9). Digestion of the IGS PCR products with *Msp*1 gave between 1 and 7 distinct fragments that ranged from 130 and 600 bp (Plate 4.10). The restriction enzyme *Taq*1 yielded 1 to 7 fragments ranging from 130 to 610 bp (Plate 4.11) in all the isolates. *Csp*61 produced 1 to 6 fragments that ranged from 30 to 600 bp in size (Plate 4.12).

Table 4.5: Restriction fragment size range and number determined by PCR-RFLP of the rDNA IGS regions in 54 isolates of groundnut nodulating bacteria

	Restriction Fragment Size Range and Number per isolate								
Isolate	***Hin*f1**	***Msp*1**	***Taq*1**	***Csp*61**	**Isolate**	***Hin*f1**	***Msp*1**	***Taq*1**	***Csp*61**
A01	50-300 (5)	130-600 (6)	250-350 (4)	700 (1)	H04	150-265 (3)	240-600 (3)	130-350 (3)	700 (1)
A02	150 -300 (3)	130-600 (4)	130 - 500 (2)	140 (1)	H05	160-265 (3)	175-300 (3)	400-500 (2)	700 (1)
A03	50-500 (10)	130- 400 (5)	400 (1)	700 (1)	I01	50-250 (3)	240-600 (3)	250-600 (6)	130-700 (3)
A04	50 - 350 (5)	130-400 (5)	400-500 (2)	30-700 (6)	I02	50-350 (5)	130-400 (5)	250-500 (5)	140-450 (2)
A05	160-265 (3)	175-300 (3)	450-500 (3)	130-700 (3)	I03	50-500 (10)	130-600 (6)	500 (1)	130-700 (4)
B02	50 -250 (3)	130-400 (5)	250-400 (4)	700 (1)	I06	50-500 (10)	130-600 (4)	400-500 (2)	30-400 (5)
B03	50-350 (6)	130-450 (6)	130-450 (3)	700 (1)	J01	50-500 (5)	175-300 (3)	250-600 (6)	500 (1)
B05	50 -350 (6)	130-400 (5)	190 -350 (4)	130-450 (2)	J02	50-500 (10)	130-400 (3)	130-610 (7)	700(1)
C01	175 -300 (2)	240-600 (3)	250-600 (7)	130-500 (3)	J03	160-265 (3)	130-450 (6)	190-400 (3)	130-500 (2)
C02	160-265 (3)	130-600 (5)	450-500 (2)	40-500 (2)	K01	150-300 (3)	175-400 (3)	130-610 (5)	140-500 (2)
C03	100-300 (4)	130-600 (4)	250-600 (7)	700 (1)	K03	50-350 (5)	130-400 (4)	130-200 (2)	130-700 (3)
C05	50-350 (6)	240-600 (3)	130-450 (3)	130-700 (3)	K04	160-265 (3)	130-450 (6)	200-350 (3)	700 (1)
D03	160-265 (3)	130-450 (6)	400-500 (2)	700 (1)	L03	50-500 (6)	130-600 (4)	130-350 (3)	140-450 (2)
D05	50-500 (10)	130-600 (4)	250-600 (6)	700 (1)	L05	50-350 (6)	130-450 (6)	130-700 (7)	130-500 (2)
E01	50-350 (6)	130-400 (5)	190-400 (2)	140-500 (2)	M01	50-350 (5)	130-450 (6)	130-350 (3)	30-400 (5)
E02	100-300 (4)	600 (1)	190-350 (4)	700 (1)	M05	50-300 (4)	175-300 (3)	250-600 (6)	130-450 (3)
E03	50-500 (6)	130-400 (5)	130-610 (7)	130-450 (2)	N03	100-300 (4)	175-300 (3)	130-610 (7)	30-400 (5)
F01	160-265 (3)	130-600 (6)	450 (1)	500 (1)	O01	50-500 (6)	130-600 (7)	130-400 (3)	700(1)
F03	160-265 (3)	175-300 (3)	130-350 (3)	30-400 (3)	O03	50-250 (3)	130-400 (5)	190-400 (2)	130-500 (2)
F04	50-350 (5)	130-400 (5)	200-350 (4)	130-450 (2)	O05	50-350 (5)	130-600 (6)	190-375 (2)	130-450 (2)
F05	50-500 (10)	130-400 (5)	500-500 (2)	130 (1)	P01	50-400 (8)	130-600 (4)	400-500 (2)	700 (1)
G01	160-265 (3)	130-450 (6)	130-500 (3)	140-700 (4)	P03	50-350 (5)	130-400 (5)	130-500 (4)	140-500 (2)
G02	160-265 (3)	175-300 (3)	130-610 (7)	30-400 (5)	P05	50-250 (3)	130-450 (6)	130-350 (3)	700 (1)
G03	50-500 (10)	600 (1)	250-600 (6)	500 (1)	Q01	50-350 (5)	130-400 (5)	130-500 (4)	700 (1)
G05	50-350 (6)	130-450 (6)	130-500 (4)	700 (1)	Q02	50-500 (5)	175-300 (3)	250-450 (5)	500 (1)
H01	150-500 (3)	130-500 (6)	200-350 (4)	30-400 (5)	Q05	50-300 (5)	600 (1)	450 (1)	130-450 (2)
H03	100-300 (6)	240-600 (3)	130-500 (3)	130-450 (2)	Q06	50-350 (5)	600 (1)	450-610 (3)	30-400 (5)
Overall	50-500 (10)	130-600 (7)	130-610 (7)	50-700 (6)	Overall	50-500 (10)	130-600 (7)	130-610 (7)	50-700 (6)

Note: Number in bracket is the total number of fragments generated by the respective restriction enzyme.

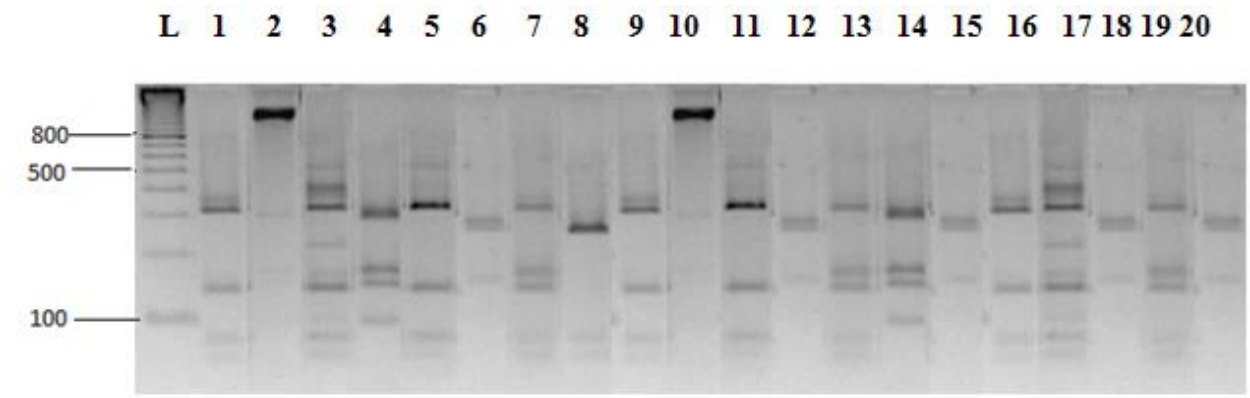

Plate 4.9: Restriction fragment pattern of PCR-amplified 16S-23S rDNA intergenic spacer digested with *Hinf*1 in groundnut nodulating rhizobia

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,

11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

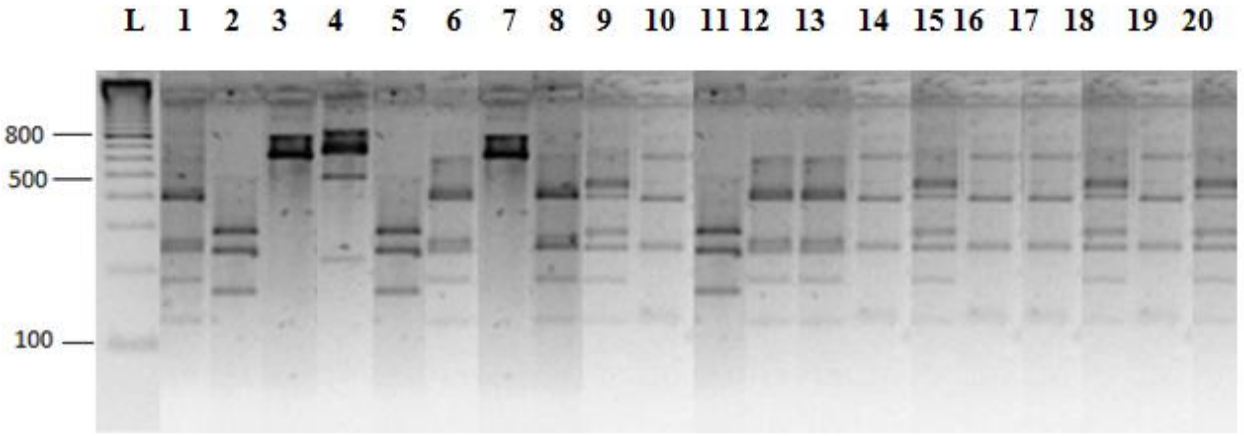

Plate 4.10: Restriction fragment pattern of PCR-amplified 16S-23S rDNA intergenic spacer digested with *Msp*1 in groundnut nodulating rhizobia

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,

11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

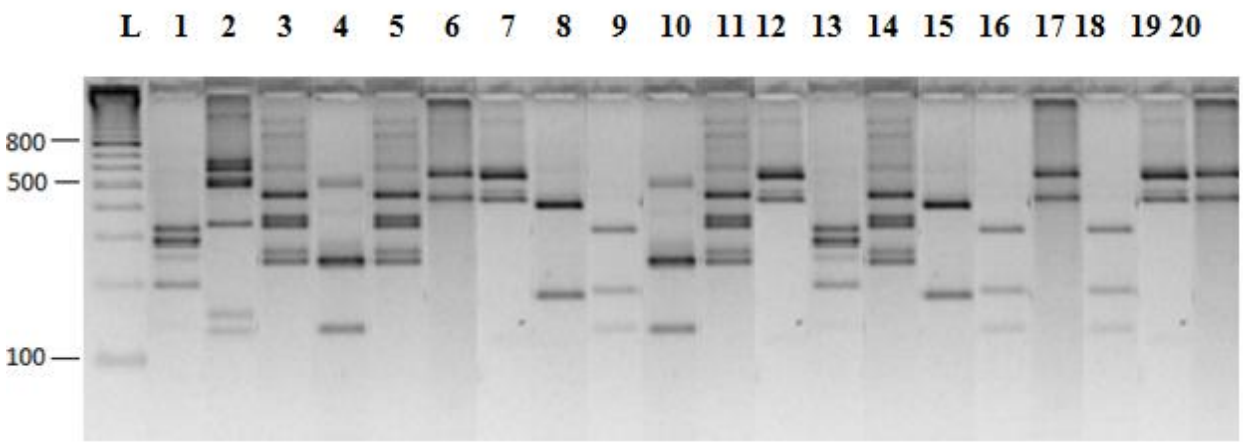

Plate 4.11: Restriction patterns of PCR-amplified 16S-23S rDNA intergenic spacer digested with *Taq* 1 in groundnut nodulating rhizobia

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,

11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

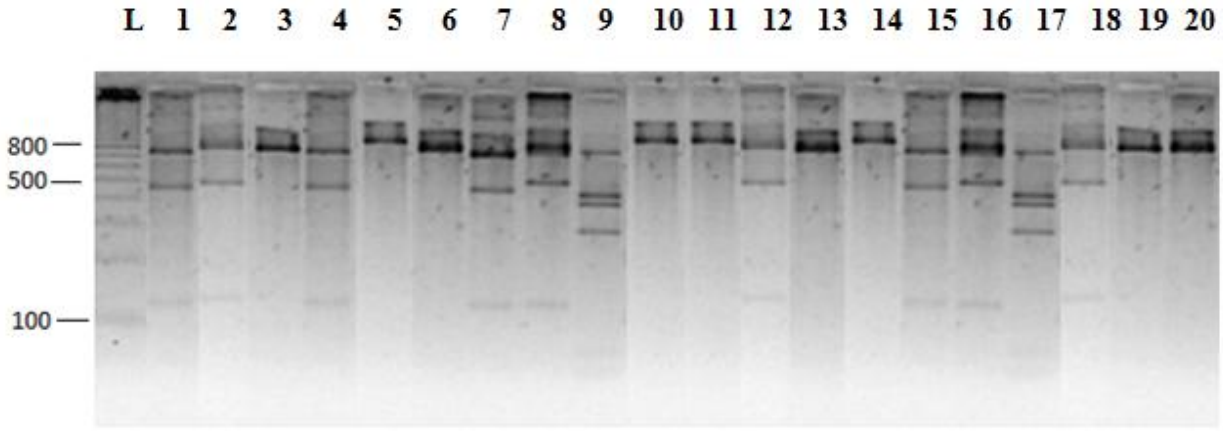

Plate 4.12: Restriction fragments of PCR-amplified 16S-23S rDNA intergenic spacer after digestion with *Csp6*1 in groundnut nodulating rhizobia

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

The various genotypes obtained for each enzyme and each isolate are listed in Table 4.6. Ten different combinations of patterns representing 10 different IGS genotypes (A-J) were detected in the 54 isolates analyzed by PCR-RFLP using four endonucleases (Table 4.6). Among the isolates, none of these genotypes was shared across the four different enzymes, however, enzymes *Taq*1 and *Csp*61 exhibited the same genotypes; H and A, whereas enzymes *Hinf* 1 and *Msp*1 shared genotypes D, C, A and E in some isolates. Digestion with the restriction enzyme *Hinf* 1 produced 10 different RFLP profiles. The five most predominant IGS genotypes were J, D, A, B and G which constituted, 18.5%, 16.6%, 12.9 %, 9.3 % and 7.4 %, respectively of all the analyzed isolates from the studied sites. IGS genotypes C, E, F, H, I each was made up of 5.5% or less. Eight different genotypes (A-H) were produced by digestion of PCR product with *Msp*1 enzyme. IGS genotypes that were most common included B, C, D, E, A and H which had frequencies of 22.2%, 14.8%, 12.9%, 11.1% and 7.4% respectively. Genotypes F and G each had a frequency of 3.7%. Enzyme *Taq*1 also yielded eight different genotypes similar to those of *Msp*1. There was common grouping of the isolates in each of the genotypes obtained using *Taq1*. The top four major IGS genotypes were; A, H, B and G which constituted, 11.1%, 7.4%, and 5.5 %, respectively while C, D, E, F each comprised 3.7% of the isolates. Finally, *Csp6*1 delineated a total of nine different genotypes (A-I). The six main IGS genotypes were I, A, C, H, E and G which recorded, 31.5%, 12.9%, 11.1% , 7.4 % and 5.5 % ,respectively of all the analyzed isolates while IGS genotypes B, D, F each constituted 3.7%. Enzyme *Hinf* I was found to be the most discriminative

with 10 genotypes (A–J) detected among the 54 rhizobia isolates analysed as compared to *Msp*1 (8), *Taq*1 (8) and *Csp*61 (9).

Table 4.6: PCR-RFLP genotypes revealed by 16S-23S intergenic spacer regions

Isolate[a]	Genotypes[b]				Isolate[a]	Genotypes[b]			
	***Hinf*1**	***Msp*1**	***Taq*1**	***Csp*61**		***Hinf*1**	***Msp1***	***Taq*1**	***Csp61***
A01	C	D	-	D	**H04**	-	B	E	G
A02	I	E	B	A	**H05**	J	B	-	I
A03	A	H	I	C	**I01**	G	D	B	F
A04	D	D	H	H	**I02**	D	C	H	H
A05	J	B	-	G	**I03**	A	E	G	I
B02	G	-	-	I	**I06**	A	-	-	G
B03	B	E	-	I	**J01**	F	E	H	I
B05	-	B	E	E	**J02**	A	-	-	C
C01	-	-	F	A	**J03**	J	B	F	C
C02	J	C	C	I	**K01**	I	H	-	A
C03	H	C	B	I	**K03**	D	C	-	-
C05	B	D	-	-	**K04**	J	C	A	E
D03	J	E	A	A	**L03**	E	B	H	-
D05	A	A	I	I	**L05**	B	D	-	H
E01	B	-	-	-	**M01**	D	D	H	A
E02	H	A	-	-	**M05**	-	-	D	I
E03	E	B	A	C	**N03**	H	E	-	-
F01	J	D	G	I	**O01**	E	G	B	I
F03	J	B	C	I	**O03**	G	-	A	I
F04	D	B	-	I	**O05**	D	B	H	-
F05	A	H	G	I	**P01**	-	C	-	A
G01	J	F	-	D	**P03**	D	G	D	B
G02	J	F	-	-	**P05**	G	C	-	I
G03	A	B	-	C	**Q01**	D	H	-	I
G05	B	A	-	H	**Q02**	F	B	-	F
H01	-	E	A	A	**Q05**	C	C	-	E
H03	-	A	-	C	**Q06**	D	E	-	B

a Isolates of groundnut rhizobia from the study sites .
b Different genotypes obtained with each restriction enzyme among the 54 isolates analyzed; isolates with the same letter have the same genotype obtained for one restriction enzyme, vice versa
- Genotype not determined; isolate had unique restriction fragment profile for the given enzyme.

Isolates from different sites of origin were separated into two main groups based on the branch of the dendrogram (Figure 4.3). Enzymes *Hinf* 1, *Msp*1 and *Csp*61 clustered isolates into group I; 44, 45 and 34 and group II composed of 10, 9 and 20 isolates,

respectively. All the isolates from group I and II originated from different sites. *Taq*1 clustered fifty three isolates in group I with an exception of isolate L05. Dendrogram obtained from combined fragments using four enzymes clustered isolates into group I and II with 42 and 12 isolates, respectively.

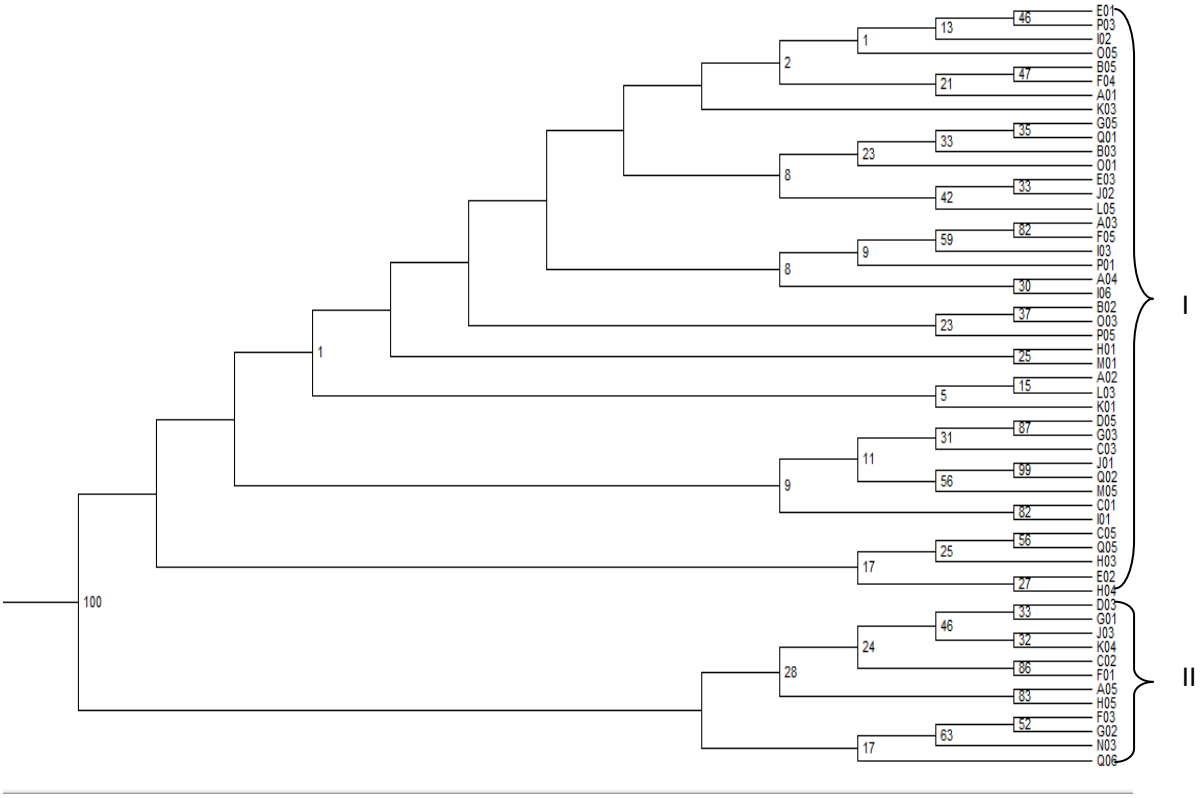

Figure 4.3: UPGMA cluster of groundnut nodulating rhizobia isolates on the basis of 16S-23S IGS fragment patterns generated after digestion with *Hinf* l, *Msp* l, *Taq* 1 and *Csp6* 1.

4.11 RFLP analysis of 16S rRNA genes

PCR amplification of the 16S rRNA locus produced a single band of approximately 1500 bp. Digestion of the 16S rRNA gene amplicons with endonucleases *Hinf* 1, *Msp*1, *Taq*1 and *Csp6*1 each produced multiple fragments. The number of fragments varied with the different restriction enzymes (Table 4.7). The number of fragments generated by each of the four endonucleases ranged between 1 and 8 depending on the isolate. The enzyme *Hinf* 1 produced 1 to 8 restriction fragments per isolate, ranging in size from 50 to 500 bp (Table 4.7, Plate 4.13). Endonucleases *Msp*1, *Taq1, and Csp*61 generated between 2 to 8 restriction fragments ranging from 50 to 700, 150 to 1050 and 150 to 710 base pair in size, respectively (Table 4.7, Plate 4.14 - 4.16). Restriction of 16S rRNA gene with *Hinf* 1, *Msp*1, *Taq*1 and *Csp*61 yielded different banding patterns among the rhizobia isolates.

The number of genotypes per restriction enzyme varied (A-M) with *Hinf* 1, *Msp*1 and *Taq*1 delineating the largest at thirteen (13), and *Csp*61 delineating the least (9) number of genotypes (A-I) (Table 4.8). Genotypes I and J were not shared by any isolate in *Hinf*1 and *Msp*1 respectively. The most frequent IGS genotypes in *Hinf* 1 were C and L including nearly, 12.9 % and 9.3% of the isolates respectively which was followed by A, B, D, E, F, G, H, 1, J, K and M each whose frequency was 7.4% or less. In *Msp*1, genotypes that were most common included E and A which formed 11.1% and 7.4% while genotypes B, C, D, F, G, H, I, J, K, L, M occurred in 5.5% or less of the isolates. Top predominant genotypes in *Taq*1 included, D and C with, 11.1% and 9.3%, respectively whereas IGS genotypes C, D, E and F each represented 5.5 % or less. For *Csp6*1 main genotypes were E, G and C which formed, 11.1%, 9.3% and 7.4 %, respectively whereas A, B, D, F, H, I each constituted 5.5%.

Table 4.7: Restriction fragment size range and number determined by PCR-RFLP of the 16s gene in 54 isolates of groundnut nodule bacteria

	Restriction Fragment Size Range and Number per isolate								
Isolate	***Hin*f1**	***Msp*1**	***Taq*1**	***Csp*61**	**Isolate**	***Hin*f1**	***Msp*1**	***Taq*1**	***Csp*61**
A01	100-450 (4)	130-310 (5)	175-400 (5)	150-400 (6)	**H04**	100 (1)	50-350 (8)	175-400 (2)	200-500 (3)
A02	50-475 (6)	350-700 (2)	150-500 (4)	150-300 (3)	**H05**	100-400 (2)	130-500 (4)	150-1000 (7)	350-710 (2)
A03	50-350 (5)	150-500 (3)	150-1000 (8)	150-350 (7)	**I01**	50-350 (6)	150-500 (3)	175-400 (3)	175-710 (3)
A04	50-475 (6)	50-350 (6)	175-500 (4)	175-710 (4)	**I02**	50-400 (5)	50-350 (8)	150-500 (5)	150-350 (4)
A05	50-400 (4)	50-700 (5)	175-500 (4)	200-450 (5)	**I03**	450 (1)	50-700 (4)	150-1000 (7)	175-710 (5)
B02	50-475 (6)	50-700 (5)	175-1050 (4)	200-450 (5)	**I06**	50-500 (5)	130-500 (4)	150-500 (6)	150-300 (3)
B03	50-475 (7)	130-500 (3)	175-400 (2)	200-450 (5)	**J01**	100-400 (3)	50-700 (4)	175-1000 (2)	150-710 (3)
B05	50-350 (4)	50-450 (6)	175-400 (3)	150-710 (3)	**J02**	100 (1)	130-500 (3)	175-1050 (4)	150-350 (4)
C01	50-475 (5)	150-700 (2)	150-500 (6)	150-710 (5)	**J03**	50-475 (6)	50-700 (4)	150-500 (6)	150-390 (5)
C02	100-400 (3)	130-310 (5)	175-350 (3)	150-500 (3)	**K01**	50-475 (6)	50-450 (6)	175-700 (5)	150-400 (6)
C03	100-450 (4)	130-500 (4)	175-1000 (7)	150-710 (7)	**K03**	50-475 (6)	50-700 (4)	175-500 (4)	150-500 (4)
C05	50-475 (6)	130-500 (2)	175-500 (3)	150-710 (3)	**K04**	50-475 (7)	130-500 (3)	150-1000 (7)	200-450 (5)
D03	50-350 (7)	50-500 (7)	175-250 (3)	150-300 (3)	**L03**	50-475 (7)	50-350 (6)	150-500 (5)	200-450 (5)
D05	50-350 (5)	50-250 (4)	175-400 (3)	175-710 (3)	**L05**	50-400 (4)	150-500 (3)	150-1000 (8)	175-500 (2)
E01	50-475 (5)	50-500 (3)	175-500 (5)	175-450 (6)	**M01**	50-400 (6)	50-700 (4)	150-500 (6)	150-300 (3)
E02	50-500 (7)	50-350 (8)	175-500 (4)	200-500 (3)	**M05**	50-475 (6)	130-250 (3)	150-1000 (7)	175-450 (4)
E03	50-400 (7)	130-310 (5)	150-1000 (7)	230-450 (3)	**N03**	100-400 (3)	50-450 (6)	150-1000 (8)	175-710 (3)
F01	50-400 (6)	130-500 (4)	175-500 (6)	150-390 (6)	**O01**	50-400 (5)	130-290 (4)	150-500 (5)	150-350 (4)
F03	50-350 (4)	50-700 (3)	175-500 (5)	175-450 (7)	**O03**	50-400 (6)	130-500 (4)	175-500 (5)	150-710 (3)
F04	50-500 (6)	150-450 (5)	175-400 (2)	175-310 (2)	**O05**	50-475 (7)	130-700 (4)	150-1000 (7)	350-710 (2)
F05	50-500 (8)	50-350 (8)	175-400 (2)	350-500 (2)	**P01**	50-350 (6)	50-700 (4)	150-500 (6)	150-390 (4)
G01	50-350 (6)	130-150(2)	175-1000 (4)	150-390 (4)	**P03**	50-500 (6)	50-250(4)	150-400 (3)	150-310 (6)
G02	100-400 (3)	150-450 (5)	175-1050 (5)	150-710 (7)	**P05**	50-475 (7)	50-700(4)	175-400 (2)	350-710 (2)
G03	50-475 (6)	50-450 (6)	175-1000 (5)	150-400 (6)	**Q01**	50-475 (5)	150-450(4)	150-500 (6)	175-450 (8)
G05	50-500 (6)	150-450 (5)	175-1050 (5)	50-450(7)	**Q02**	50-475 (6)	130-500(3)	175-400 (4)	150-390 (5)
H01	50-500 (5)	150-290 (5)	150-500 (6)	175-400 (7)	**Q05**	100-450 (4)	50-450(5)	175-1000 (3)	150-710 (3)
H03	100-400 (3)	50-700 (4)	175-500 (3)	150-710 (3)	**Q06**	50-475 (5)	50-700(3)	175-500 (5)	175-710 (4)
Overall	50-500 (8)	50-700 (8)	150-1050 (8)	50-710(7)	**Overall**	50-500 (8)	50-700 (8)	150-1050 (8)	50-710 (7)

Note: Number in bracket is the total number of fragments generated by the respective restriction enzyme

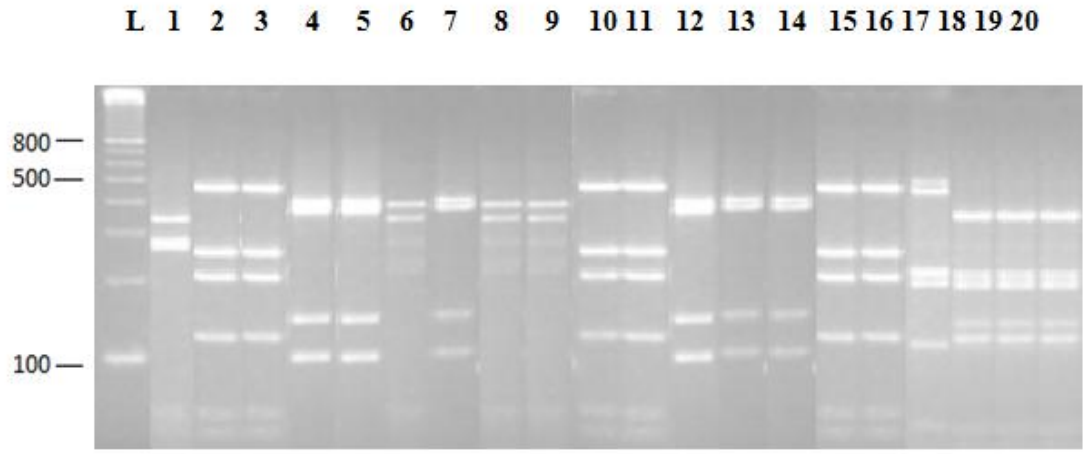

Plate 4.13: Restriction fragments of 16S rRNA gene after digestion with *Hinf*1 in groundnut nodulating rhizobia

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,

11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

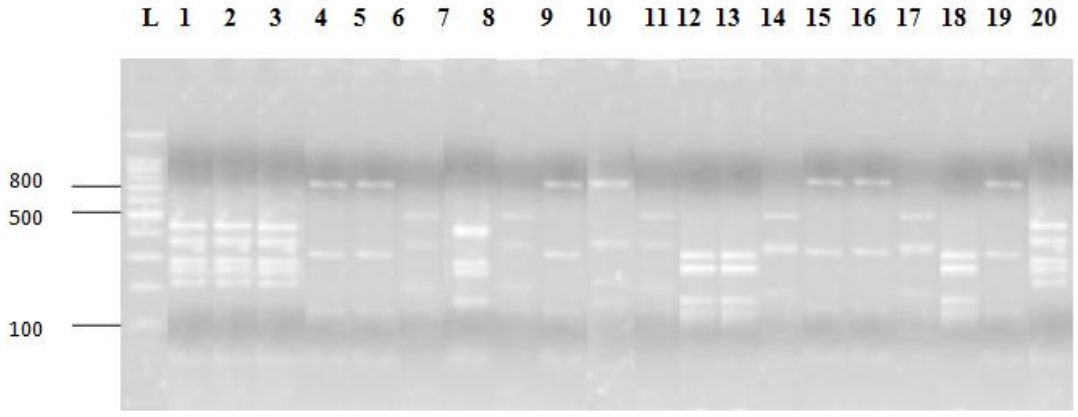

Plate 4.14: Fragments of 16S rRNA gene after digestion with *Msp* 1 in groundnut nodulation rhizobia

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,

11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

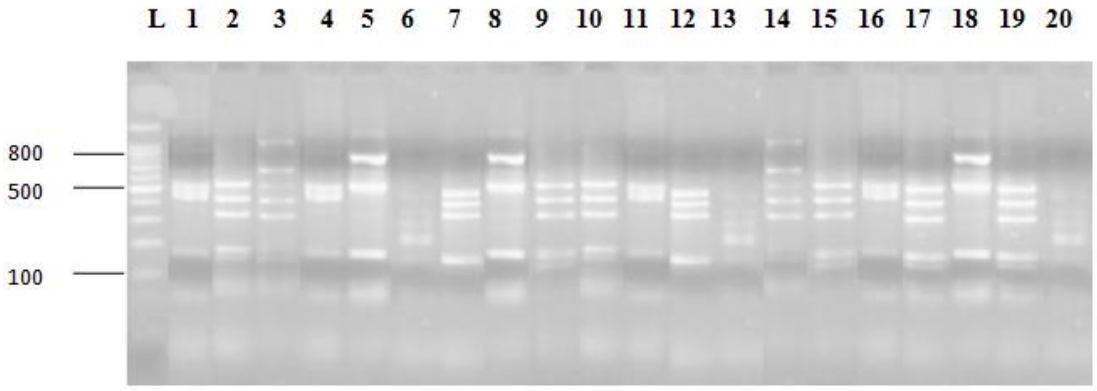

Plate 4.15: Types of restriction patterns of PCR-amplified 16S rRNA gene digested with *Taq* 1

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,

11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

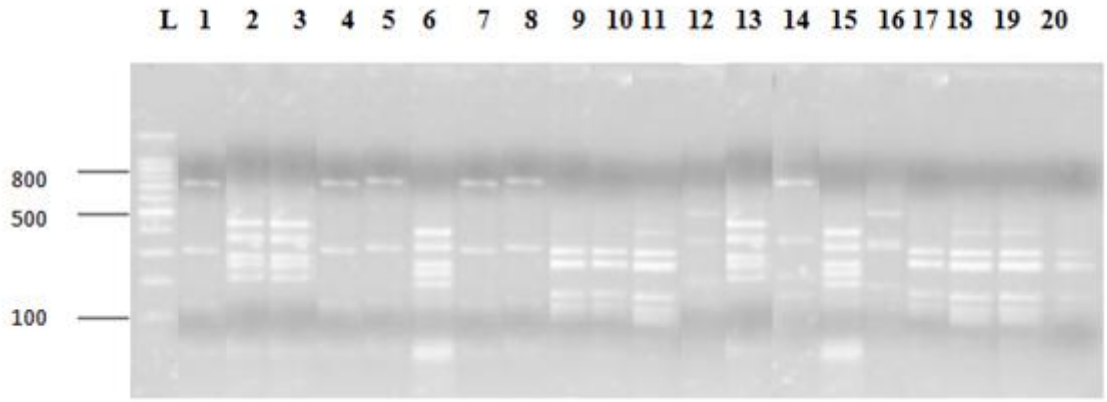

Plate 4.16: Restriction fragments of PCR-amplified 16S rRNA gene digested with *Csp6*1 in groundnut nodulating rhizobia

L-100bpmarker,1-B05,2-K01,3-G03,4-H03,5-J01,6-F01,7-Q05,8-O03,9-M01,10-A02,

11-Q02,12-C02,13-A01,14-C03,15-J03,16-K03,17-I06,18-G01,19-P01,20-D03.

16S rRNA region exhibited high genetic variation with thirteen genotypes found in three enzymes as compared to 16s-23s rDNA which yielded 10 genotypes in only one enzyme (*Hinf*1) (Table 4.8). Cluster analysis indicated isolates from different sites of origin were separated into two main groups based on the main branch of the dendrogram (Figure 4.4). Enzymes *Hinf* 1, *Msp*1, *Taq*1 and *Csp6*1 clustered isolates into group I and II. Group one isolates in all the enzymes originated from different sites. The groupings of isolates in group II differed with enzymes *Msp*1 and *Taq*1 with isolates; A02, C01 and J02 originating from the same locality. Clustering from combined fragments using four enzymes grouped isolates into group I with 42 isolates and 12 isolates in group II. Dendrograms obtained from 16S-23S and 16S rRNA gene showed that isolates C02, HO5, G02, NO3 and O05 were similar in group I while seven isolates were different. Group II depicted 23 isolates as being similar whereas 19 being different from each other. All these isolates originated from different sites.

Table 4.8: PCR-RFLP genotypes revealed by 16s gene

Isolate[a]	Genotypes[b]				Isolate[a]	Genotypes[b]			
	***Hinf*1**	***Msp*1**	***Taq*1**	***Csp*61**		***Hinf*1**	***Msp1***	***Taq*1**	***Csp*61**
A01	K	H	-	D	**H04**	-	A	L	H
A02	C	-	F	C	H05	I	-	D	I
A03	D	M	A	-	I01	-	M	I	F
A04	F	-	H	-	I02	-	A	-	A
A05	-	C	H	G	I03	L	D	D	-
B02	F	C	M	G	I06	M	I	C	C
B03	E	K	L	G	J01	C	E	-	E
B05	J	F	-	E	J02	C	K	M	A
C01	G	-	B	-	J03	C	E	C	-
C02	L	H	K	-	K01	E	F	-	D
C03	K	I	-	-	K03	E	E	-	-
C05	F	-	K	E	K04	-	K	D	G
D03	D	-	-	C	L03	H	-	E	G
D05	G	B	I	F	L05	F	M	A	-
E01	-	-	G	-	M01	-	E	C	C
E02	H	A	-	H	M05	-	-	D	-
E03	J	H	D	-	N03	L	-	A	F
F01	A	J	-	-	O01	H	J	E	E
F03	-	G	G	-	O03	E	-	-	I
F04	B	L	L	-	O05	B	E	D	B
F05	L	A	-	-	P01	A	B	C	-
G01	C	-	-	B	P03	-	D	F	I
G02	A	L	J	-	P05	G	-	L	-
G03	-	F	-	D	Q01	C	-	B	-
G05	L	L	J	-	Q02	C	-	C	-
H01	M	-	B	-	Q05	K	-	-	E
H03	-	E	K	E	Q06	G	G	G	-

a Isolates of groundnut nodulating rhizobia from the study sites .
b Different genotypes obtained with each restriction enzyme among the 54 isolates analyzed; isolates with the same letter have the same genotype obtained for one restriction enzyme, vice versa
- Genotype not determined; isolate had unique restriction fragment profile for the given enzyme.

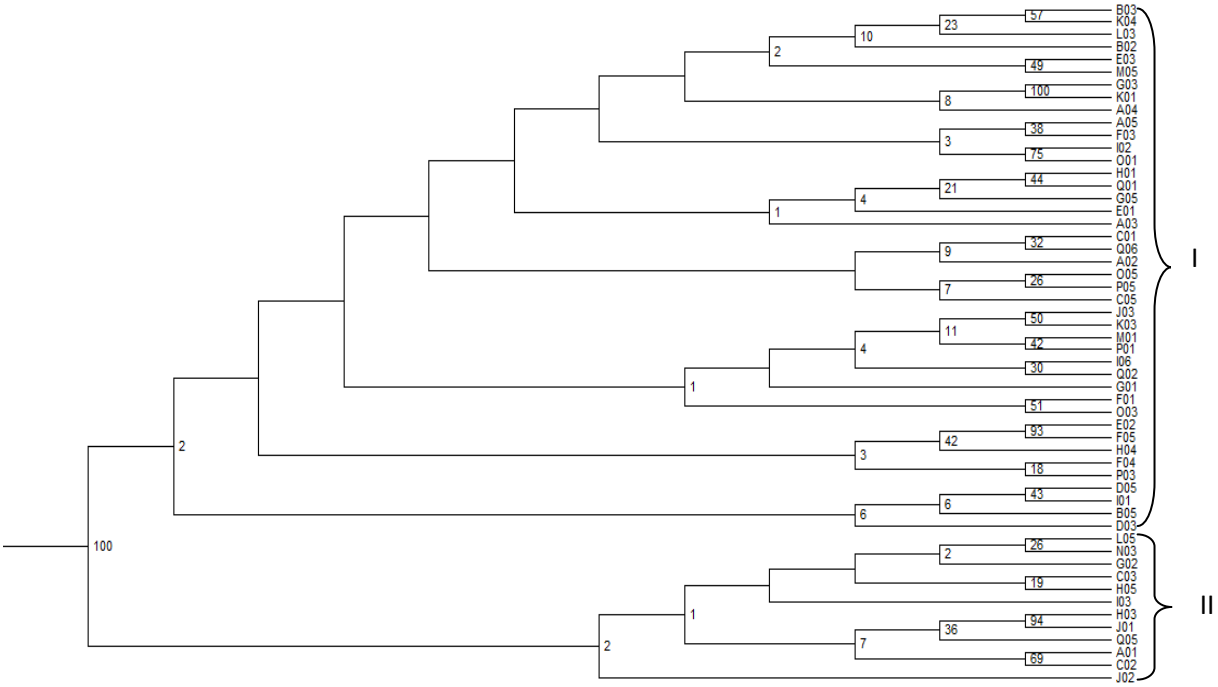

Figure 4.4: Dendrogram showing cluster (UPGMA) of groundnut nodulating rhizobia isolates on the basis 16S rRNA gene with *Hinf* 1, *Msp* l, *Taq*1 and *Csp6*1

CHAPTER FIVE

DISCUSSION

5.1 Phenotypic characterization of bacterial rhizobia isolates

In this study, phenotypic characterization of groundnut nodulating bacteria isolates from P deficient soils in western Kenya was carried out based on the morphological and biochemical attributes. All the isolates produced colonies on YEMA medium with diameter ranging from 2 to 4 mm. The colonies were elevated, had smooth entire margins and a white, creamy, translucent, gummy, glistering appearance. Microscopic observation indicated that 66 of the tested bacteria isolates were gram negative. Differences in colony morphology have been observed indicating that phenotypic diversity exists in bacteria nodulating legume plants similar to what has been observed in other studies (Laurette *et al*., 2015; Kapembwa, Mweetwa, Ngulube & Yengwe, 2016). Variation in colony diameter of the bacteria isolates shows that they belong to different growth categories and may belong to distinct taxonomic groups. Colony size variation has been used as the primary character for differentiating rhizobial isolates of various legumes including common bean and Bambara groundnut (Muthini *et al*., 2014; Benson *et al.*, 2015).

Bacteria isolates nodulating groundnut were grouped into fast and slow types on the basis of their growth on the YEMA medium. This reflects a survival strategy in adverse conditions based on the reduction of the generational interval (Borges, Xavier & Rumjanek, 2010). The presence of both types of rhizobia in different sites in western Kenya is consistent with their appearance in many tropical soils (Sharma *et al.*, 2010).

Some studies by (Sanginga *et al.*, (1989) and Bala *et al.*, (2003) observed that slow-growing rhizobia are predominant in tropical soils. The present study showed the contrary with 81% of the isolates being fast growing. Over 54% of root nodule bacteria isolated from *Cratylia mollis* Mart. ex Benth. *Calliandra depauperata* Benth and *Mimosa tenuiflora* (Willd.) Poir., selected tree legumes and soybean, respectively were fast-growers (Finger-Teixeira, Ferrarese, Soares, da Silva & Ferrarese-Filho, 2010; Boakye, Lawson & Danso, 2016; Kapembwa *et al.*,2016). Several studies (Sanginga *et al.*, 1989; Somasegaran & Hoben, 2012) observed that although *Leucaena leucocephala* was nodulated by both fast and slow-growing rhizobia, effective nitrogen fixing nodules were only formed with the fast-growing rhizobia. These findings indicate that the fast-growing nodule bacteria are predominant and play critical role in N_2 fixation in P deficient soils in western Kenya.

Seventy three percent of the isolates produced mucous while the rest did not. Mucous production is important in maintaining minimum moisture in the immediate environment of the microorganisms. Through its high moisture holding capacity, mucous prevents desiccation and serves as a potential source of energy under conditions of scarcity (Siegel & Weiser, 2015). The ability of the bacterial isolates to produce the mucous *in vitro* is an indication that this process is independent of the plant host and that the kind of polysaccharides making up the mucous are completely dependent on the genotype of the bacteria isolate. Further, the increasing tendency of the bacterial isolates to produce mucous could be a reflection of the adaptation to the acid soils of western Kenya. Seventy percent of bradyrhizobia and indigenous rhizobia isolates from acidic soils of the Brazilian tropical Savannah produced mucous (Finger-Teixeira *et al.*, 2010).

On the basis of rhizobial growth on YEMA medium supplemented with BTB, 51 isolates were classified as acid producers and 17 as alkalizers. These results indicate that groundnut was nodulated by both types of bacteria isolates and acid producers were more prominent than alkalizers in the P-deficient soils in western Kenya. This indicates that acid producers are more important in nodulation and nitrogen fixation in groundnut in western Kenya soils which are acidic. This could be that those acid producers have adapted to acidic soil conditions. Acid producing rhizobia isolates have been observed in tropical soils that tend to be acidic or slightly acidic (Boakye *et al.*, 2016; Kapembwa *et al.*, 2016).

5.2 Biochemical characterization of bacterial isolates

5.2.1 Congo Red Test

Sixty four bacterial isolates exhibited typical characteristics of rhizobia on YEMA medium supplemented with Congo red as white, translucent and elevated colonies. These results were in harmony with those of Singh *et al.*, (2008). However, the isolates were turning yellow when left to grow for five days after the appearance of the colonies indicating acid production. This was also reported in previous studies of Huang *et al.* (2007). In their study, Baoling and others (2007) observed that first growing rhizobia turned yellow after five days of growth as a result of mucoid acid production. Four isolates J05, L01, N06 and P06 absorbed Congo red dye indicating that these isolates may not belong to groundnut nodulating bacteria (Laurette *et al*., 2015). Therefore, molecular characterization and other studies of the non-nodulating bacteria that reside in groundnut

root nodules using DNA sequencing tools is needed to establish their taxonomic status and role in the root nodules.

5.2.2 Hofer's alkaline broth Test

In this study, the isolates B01, D01 and N01 grew in Hofer's alkaline broth at pH 11 suggesting tolerance to alkaline conditions. These results were in contrast with previous studies by Gao, Sun, Li, Wang & Chen, (1994); Küçük, Kivanç & Kinaci, (2006) and Huang *et al.* (2007) which reported failure of bacteria to grow when cultured in Hofer's alkaline solution with a pH of 11. An important attribute for the growth of the organism is pH of the growth media. Slight variations in pH of media may have enormous effect on the growth of the organism. Rhizobia have been reported to show optimal growth at a pH of 6-7 whereas agrobacteria can grow in a pH range of 4-11 (Huang *et al.*, 2007).

5.2.3 Lactose agar test

Sixty eight isolates grew in lactose agar indicating that they were able to use lactose as the sole carbon source. These findings contrast with those of Hunter *et al.*, (2007) who reported fenugreek rhizobia isolates were unable to grow on lactose. The isolates in this study did not produce ketolactase, a compound that gives yellow coloration in Benedict's test. These findings were in agreement with previous studies of Sharma *et al.*, (2010). Failure to form the yellow colour indicates the absence of agrobacteria in the bacteria culture. Agrobacteria utilize lactose to form the reduced product 3-ketolactose, through activity of the enzyme ketolactase that is lacking in rhizobia. Rhizobia do not have this

enzyme and therefore cannot produce 3-ketolactose (Young, Kuykendall, Martinez-Romero, Kerr & Sawada, 2001).

5.3 Confirmation of rhizobia isolates through nodulation test

Ninety five percent of the isolates were confirmed to be rhizobia due to their ability to form effective root nodules. These isolates showed significant variation in nodule number indicating that they were compatible with the test plant. Previous studies (Taktek *et al.*, 2015) also reported that lentil rhizobia nodulated their host very well with different level of infectivity. The cross-section of effective nodules showed red coloration, indicating the presence of leghemoglobin, a trait related to nitrogen efficiency (Abdel Wahab, Zahran & Abd-Alla, 1996). The leaves of the nodulated plants were dark-green, while uninoculated unfertilized control plants showed yellowing typical of nitrogen deficiency. Isolates G05, C06, A05, C02, Q02, F01, I01, B05, L03 and P03 had the highest number of nodules ranging from 40 to 44 indicating that these isolates are symbiotically active.

5.4 Metabolic Characterization

The ability to carry out various enzymatic processes is a vital biochemical characteristic feature of rhizobia. Two enzymatic activities studied were nitrate reductase and urease. Their functions influence nodulation and nitrogen fixation in legumes.

5.4.1 Nitrate reduction

All the sixty eight isolates were able to reduce nitrate to nitrite with fifty two isolates failing to reduce nitrate directly. Two possible explanations could be given. Firstly, it is

possible that there was no reduction of nitrate to nitrite. Secondly, all the nitrite so formed must have been further converted to other products such as nitrous oxide, nitric oxide (Singh *et al.,* 2014). Rapid nitrate utilization was evident in slow growing isolates suggesting quick oxygen utilization. Rapid nitrate utilization by slow growing root nodule bacteria has been reported previously by Gachande & Khansole (2011). Experiments have demonstrated that NO_3^- inhibits nodule formation on legumes primarily as a root-localized effect rather than as a function of whole-plant N nutrition (Eaglesham, 1989; Abdel Wahab *et al.*, 1996). The inhibitory effect of exogenous nitrate on N_2 fixation has variously been attributed to a direct competition between nitrate reductase and nitrogenase for reducing power (Stephens & Neyra, 1983). In addition, it has been found that the decline in total nitrogenase activity upon exposure to nitrate is independent of the N_2-fixing efficiency of the symbiosis (Blumenthal, Russelle & Vance, 1997). Nitrate reductase positive strains of rhizobia can sufficiently infect host legume roots and establish effective symbiosis even in the presence of nitrate levels that would often inhibit these processes (Burns & Hardy, 2012). Therefore, positive nitrate reductase activity is an important characteristic in isolates with potential use as inoculants.

5.4.2 Urea hydrolysis

The ability of some fast and slow growing rhizobia isolates in this study to reduce urea into ammonia shows that they are indeed rhizobia. These findings are similar to a study by Lanier, Jordan, Spears, Wells & Johnson, (2005) who reported a positive urea hydrolysis by some groundnut nodulating rhizobia. The use of urea may allow increased N utilization from fertilizer source without a concomitant decrease in symbiotic N_2

fixation, providing that inter conversion of urea to NO_3- under field conditions can be inhibited. Hence, these urea hydrolyzing isolates when used as inoculants can give promising yield by making the N in applied urea more easily available for seedling growth prior to their initiating symbiotic nitrogen fixation. Excess urea if allowed to accumulate in the rhizosphere could inhibit root hair colonization and establishment of effective symbioses (Pii, Mimmo, Tomasi, Terzano, Cesco & Crecchio, 2015). For instance, it has been found that application of urea at the rate of 90 kg of N ha^{-1} to soybean plants suppressed nodulation by curtailing the enrichment of *Bradyrhizobium* spp. on the host plant (Hirsch & Mauchline, 2015). This situation could be avoided with urea hydrolyzing rhizobia such as those characterized in this study.

5.5 Carbon sources utilization

The bacterial isolates were able to degrade different sources of carbon. The degree of utilization varied from one carbon source to another depending on the isolate. Fast growers showed greater ability to utilize different assortment of carbon sources than slow growers indicating that they possess both uptake systems and catabolic enzymes for most carbohydrates. Slow growing isolates and majority of the fast growing except Q06, F04, C05, C01 and B02 did not utilize citrate as source of carbon suggesting that they may lack enzymes for citrate uptake and breakdown such as permease, citrate lyase and oxaloacetate decarboxylase (Sobczak & Lolkema, 2005). Fast-growing rhizobia isolates utilize a wider range of carbohydrates than slow growers (Kapembwa *et al.,* 2016). Degradation of diverse carbon sources by the bacteria isolates would be useful in preparation of inoculants.

5.6 Temperature assay

The isolates varied in their response to different temperature levels. Sixty seven isolates showed growth at 37°C. All isolates grew at 28°C indicating it was optimal temperature for their growth. This indicates the isolates in this study are able to tolerate a wide temperature regime. Rhizobia are mesophiles that tolerate temperatures ranging between 10°C and 37°C with 28°C as an optimum temperature for growth (Küçük *et al.*, 2006). Increased temperature range of these isolates may be beneficial for their application in temperature stressed conditions since the symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperature (Hungria *et al.*, 2000).

5.7 pH assay

The bacterial isolates differed in their growth response to varying pH levels. Thirty nine isolates representing 57% of the entire collection grew at a pH of 4.0 and majority of them were fast growers. Majority of the isolates that showed tolerance to low pH were sampled from the sites with pH ranging from 4.9 to 6.6. This indicates that these isolates are adapted to acidic soils. The occurrence of acid-tolerant and fast-growing strains nodulating different legumes has been previously reported for tropical soils (Berrada *et al.*, 2012; Boakye *et al.*, 2016). The genetic basis of differences in low pH tolerance could be attributed to expression of acid tolerance genes in these isolates. Transcriptional analysis of ten mesorhizobia isolated from chickpea showed a relationship between higher levels of transcriptional induction of chaperone genes such as *dnaK* and *groESL* upon acidic shock and tolerated acidic pH (Brígido & Oliveira, 2013). All isolates grew

at pH 7.0 and 8.5 indicating that optimal growth is in neutral conditions. The optimum pH for rhizobia ranges between 6.5 and 7.0 (Rodrigues, Laranjo & Oliveira, 2006). The isolates in this study survived on a wide pH range. Therefore, they are potential candidates for further strain improvement targeting use as inoculants in highly acidic or alkaline conditions.

5.8 Phosphate solubilization

Legume nodulating bacteria (LNB) have the ability to solubilize sparingly soluble inorganic phosphates in the soil, increasing the availability of P nutrient to the plants. The isolates in this study demonstrated significant variation in solubilizing insoluble inorganic phosphate. Solubilisation index ranged from 1.1 to 6.8 and fast growing isolates showed greater solubilisation ratio compared to slow growers. This could be attributed to varied ability of the bacteria isolates to produce organic acids. Exudation of gluconic acids has been demonstrated as the main mechanism that bacteria use to adapt to low-P environments (Marra *et al.*, 2011; Kumar & Ram, 2014). Isolates B03, G01, G03 and I02 that originated from sites with relatively high P did not show a clear zone of solubilisation. This shows that genes encoding proteins involved in the biosynthesis of organic acids as well as pyrroloquinoline quinine, cofactors required for phosphate solubilisation are not switched on under sufficient P in the soil (Kumar & Ram, 2014). The solubilization index observed in this study was higher than the previously reported; 0.83-4.68 (Alikhani *et al.*, 2007), 0.33-1.5 (Sridevi & Mallaiah, 2009) and 3.06 (Marra *et al.*, 2011) in rhizobia isolated from different legume hosts, tree legumes and Iranian soils, respectively. This difference in SI values could be ascribed to the varied ability of the

bacteria isolates in acquisition of P under limited sources. Further, the difference observed can be explained by the different calcium phosphate and nitrogen sources used. In the present study, DCP and ammonium sulphate were used as carbon and nitrogen sources while Alikhani *et al.*, (2007) and Sridevi and Mallaiah, (2009) used Sperber and Pikovskaya's media containing yeast at 0.5 g/litre and tricalcium phosphate, respectively. This indicates that yeast may play a role inhibiting the ability of bacteria to solubilize phosphate. In developing efficient growth medium for screening phosphate solubilizing microorganisms, yeast extract was not included as source of nitrogen and vitamin sources because of its inhibitory effect (Taktek *et al*., 2015). It is evident that phosphate solubilisation is a common characteristic among bacteria isolates in P deficient soils in Western Kenya. Indigenous rhizobia isolates; N01, B02, I06, Q01, F05, C02, E01, Q03, I01 and B01 could be key in releasing fixed P in the soils in western Kenya due to their strong solubilising capacity. The isolates could be used to enhance phosphate acquisition in groundnuts grown in P-limiting acid soils of Western Kenya.

5.9 Molecular diversity studies

5.9.1 PCR-RFLP of 16S-23S rDNA IGS

The intergenic spacer between the 16S and 23S rDNA genes may be an effective marker for detecting genetic differences at the intergeneric level and also at the interspecific level. The length of the amplification bands among fifty four groundnut rhizobia isolates ranged from 750 and 1100 bp; which corresponded to the expected size (750-1100) of the 16S–23S rDNA IGS fragment (Normand, Ponsonnet, Nesme, Neyra & Simonet, 1996). Such length variability of the 16S-23S spacer indicates that these rhizobia seem to belong

to several different strains or taxonomic groups. This is consistent with the results of (Kumar *et al*., 2015) between genotypes of distantly or closely related within *R. Leguminosarum*.

One to ten distinct restriction fragments were detected with four different endonucleases and the isolates used. This demonstrates the existence of intragenic diversity among the analyzed isolates. Similar findings were also reported by (Laurette *et al*., 2015). Discrimination of the test isolates into a large number of 16S-23S rDNA genotypes revealed that rhizobia nodulating groundnut are genetically diverse and may belong to different rhizobial groups. The results are consistent with previous findings by (Chen *et al.*, 2004) which also revealed different genotypes of soybean rhizobia despite having been sampled from an unexploited forest region with no history of soybean growing.

The grouping of isolates did not correlate with the geographical origin since isolates from the same origin were included in different groups and isolates from different sites grouped together. This suggests adequate diversity among the analyzed isolates. Similar results have been demonstrated previously (Jebara, Mhamdi, Aouani, Ghrir & Mars, 2001; Laurette et al., 2015).

5.9.2 PCR-RFLP analysis of 16S RNA gene

As a rapid method, PCR-RFLP of the 16S rRNA gene has been used for grouping and identifying rhizobia. In this study PCR amplification of the 16S rRNA locus produced a single fragment of about 1,500 bp in each of the fifty four isolates analysed. This size corresponded to the expected size (1200-1600) of the 16S rRNA genes among bacteria (Terefework *et al.*, 2001). Digestion of the amplified 16S rRNA with four tetrameric

restriction enzymes produced 1 to 8 distinct fragments depending on enzyme-isolate combination. These points to the existence of intragenic diversity within the collection of isolates analyzed. The results are in agreement with those of Porteous, Widmer & Seidler, (2002) that showed combination of a minimum of three restriction endonucleases permitted a good resolution level. Four enzymes were necessary to discriminate isolates used in this study.

In the present study, the relatively large number of 16S RNA gene genotypes observed revealed that rhizobia nodulating groundnut were genetically very diverse and may belong to different rhizobial groups. The 23S rRNA gene, because of its large size and greater phylogenetic information content, gives better resolution than the 16S rRNA gene (Case, Boucher, Dahllöf, Holmström, Doolittle & Kjelleberg, 2007). Discrimination of the test isolates into a larger number of 16S rRNA genotypes in this study disagrees with previous findings probably due to the presence of insertion of tRNA genes. The clustering of isolates did not show any correlation with the geographical origin since isolates from the same origin was included in different group and isolates from diverse origin were found in the same group. This depicts that groundnut nodulating rhizobia population in the studied sites contain a greater diversity. Similar findings have been demonstrated previously (Jebara *et al.*, 2001; Laurette *et al.*, 2015). However, dendrogram from the two regions showed similarity in the groupings of some isolates. This could be explained by the fact that these isolates are diverse.

In Kenya, several studies have investigated the genetic diversity of indigenous rhizobia nodulating cultivated legumes (Wasike *et al*., 2009; Mathu *et al.*, 2012; Herrmann, Chotte, Thuita & Lesueur, 2014). This study revealed that the rhizobia nodulating the groundnut are genetically variable and may belong to different rhizobial groups. The relatively high rhizobia diversity in the sampled sites could be attributed to tolerance to acidic conditions or other environmental reasons not readily explainable. Elsewhere, several authors have reported similar genetic diversity of rhizobia nodulating soybean (Hungria *et al*., 2006; Youseif, El-Megeed, Ageez, Mohamed, Shamseldin & Saleh, 2014; Zhang *et al*., 2014) and common bean (Feng, Poplawsky, Nikolaeva, Myers & Karasev, 2014) using different molecular markers.

Finally, based on the results of the phenotypic and genotypic characterization, ten promising P solubilizing isolates were selected showing ability to nodulate and fix nitrogen in P deficient soils in western Kenya. However, phenotypical characteristics provided little information on the diversity and phylogeny of these bacteria, while molecular analyses revealed large genetic diversity and differences among isolates. The isolates exhibited interesting features such as wide range of carbon-sources utilization, tolerance of acidic pH conditions, high temperature and high effective nitrogen fixation. This study also indicated that most of these isolates possess special characteristics such as phosphate solubilization activities and wide phylogenetic diversity which can make them candidate for multipurpose inoculants production for the legume production system in western Kenya. Isolates N01, B02, I06, Q01, F05, C02, E01, Q03, I01 and B01 could be recommended to farmers for use as groundnut bio-fertilizer.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The study revealed that a high phenotypic diversity of indigenous groundnut rhizobia exists in P deficient soils of Western Kenya

2. There was variation in the nodulation ability of phosphate solubilizing rhizobia (G05, A05, C06, C02, Q02, B05, D01, E02, E05 and F04). These isolates had the best nodulation that produced 40-44 effective nodules per plant.

3. There is a great genetic diversity of the groundnut rhizobia in Phosphorus deficient soils of western Kenya

6.2 Recommendations

1. Ten potential rhizobia isolates (N01, B02, I06, Q01, F05, C02, E01, Q03, I01 and B01) can be recommended for development of inoculants for groundnut in P deficient soils because they demonstrated potential to nodulate fix nitrogen and solubilize P.

2. The IGS and 16S genotypes need to be further characterized by sequencing to ascertain their taxonomic positions

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APPENDICES

Appendix la: Composition of Yeast Mannitol Agar

Ingredients	g/1
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCI	0.1
Yeast extract	0.5
Mannitol	10
Agar	15

Appendix Ib: Dyes incorporated in YEMA

Required concentration in YEMA =25μg^{-1}

Congo Red (CR):

Stock solution: 0.25g of CR was dissolved in 100ml sterile water.

10ml stock solution was per litre of YEMA before autoclaving

Bromothymol Blue (BTB)

Required concentration in YEMA =25μ ml^{-1}

Stock solution 0.5g BTB was dissolved in 100ml ethanol. 5ml stock solution was added per litre of YEM before autoclaving.

Adopted from Somasegaran and Hoben, (1994)

Appendix IIa: Determination of soil pH

The soil pH was determined by adding 25ml of distilled water to 10g of soil (<2mm) in a beaker and the suspension stirred for 10 minutes and then stirred again for 2 minutes. The soil was then measured using a glass electrode on a pH meter (Okalebo *et al.*, 2002).

Appendix IIb: Available phosphorus

Soil extraction for available P was done using the bicarbonate solution (0.5M $NaHCO_3$ at pH 8.5) method (Olsen et al, 1954). The bicarbonate extractant decreases the concentration of Ca as CaCO3 in the calcareous, alkaline and neutral soils containing calcium phosphates. The result is an increase of the P concentration in the solution. In acid soils containing Al and Fe phosphates, P concentration in the solution increases as the pH rises. Precipitation reactions in acid and calcareous soils are reduced to a minimum because the concentration of Al, Fe, and Ca remain at low levels in this extracting solution. P extracted in bicarbonate solution was measured colorimetrically using a spectrophotometer after the development of a blue coloured phosphomolybdate complex.

Appendix III: N- free medium for Grain legumes (Somasegaran & Hoben 1994)

Stock Solution	Element	M	Form	MW	g/1	m	Vol. of stock solution/10L of medium (ml)
1	Ca	1000	$CaCl_2.2H_2O$	147.03	294.1	2.0	5
2	P	500	KH_2PO_4	136.09	136.1	1.0	5
3	Fe	10	Fe-Citrate	355.04	6.7	0.02	5
	Mg	250	$MgSO_4.7H_2O$	246.5	123.3	0.5	
	K	250	$K_2SO_4.H_2O$	174.06	87.0	0.5	
	Mn	1	$MnSO_4.H_2O$	169.02	0.338	0.002	
4	B	2	H_3BO_3	61.84	0247	0.004	
	Zn	0.5	$ZnSO_4.7H_2O$	287.56	0.288	0.001	5
	Cu	0.2	$CuSO_4.5H_4O$	249.69	0.100	0.0004	
	Co	0.1	$CoSO_4.7H_2O$	281.12	0.056	0.0002	
	Mo	0.1	$Na_2MoO_2H_2O$	241.98	0.048	0.0002	

To prepare 10 litres of the nutrient solution, 5.0ml of each stock solution 1 to 4 was added to about 5 litres of distilled water, and then diluted to 10 litres. 1M NaOH was used to adjust pH to 6.8.

Appendix IV: Recommended Protocol for Digestion of PCR Products directly after amplification

1. Add the following reaction components in the order indicated:

PCR reaction mixture 10 µl (~0.1-0.5 µg of DNA)

Water, nuclease-free 16 -17 µl

10X recommended buffer for

restriction enzyme 2 µl*

Restriction enzyme 1-2 µl (10-20 u)

Total volume 30 µl

2. * Only 2 µl of 10X reaction buffer is required for unpurified PCR product in a 30 µl reaction volume.

3. Mix gently and spin down briefly.

4. Incubate at the optimal reaction temperature for 1-16 hours.