ABUNDANCE, GENETIC DIVERSITY AND SYMBIOTIC EFFICIENCY OF COWPEA (*Vigna unguiculata* L.) RHIZOBIA IN SOILS OF SOUTH WESTERN KENYA

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DECLARATION

This thesis is my original work and has not been submitted for an award of a degree in any other University.

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DEDICATION

I would like to dedicate the research findings from this study to anyone who depends on cowpea as a source of livelihood.

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ACRONYMS AND ABBREVIATIONS

AATF – African Agricultural Technology Foundation

ADP – Adenosine diphosphate

AEZ – agro-ecological zone

Al – Aluminium

ATP – Adenosine triphosphate

CPPMU- Central Project Planning and Monitoring Unit

DNA – Deoxyribonucleic acid

Fe- Iron

K – Potassium

LAI- leaf area index

Mg- Magnessium

MLSA – Multilocus sequence analysis

MPN- Most Probable Number

N – Nitrogen

NAAIAP – National Accelerated Agricultural Inputs Access Programme

NCBI – National Center for Biotechnology Information

OC – Organic carbon

P – Phosphorous

PCR – Polymesase Chain Reaction

PGPB – Plant growth promoting bacteria

recA – Recombinase A

RFLP – Restriction fragment length polymorphism

rRNA – Ribosomal ribonucleic acid

SWK – South Western Kenya

WAE- Weeks after crop emergence

GENERAL ABSTRACT

Cowpea is an important food crop in Kenya, but its production is limited by biotic and abiotic constraints. One of the most important abiotic constraints to crop production in South Western Kenya (SWK) is soil acidity, which may limit symbiotic efficiency of indigenous strains of rhizobia and also availability of nutrient elements such as phosphorous. In order to understand the influence of soil ecological conditions on symbiotic performance of cowpea rhizobia, a study was done in soils of SWK and selected reference regions with the following objectives: 1) to characterize the genetic diversity of native cowpea nodulating rhizobia; 2) to determine the abundance and symbiotic efficiency of native cowpea rhizobia; 3) to determine the effects of rhizobia inoculation on nodulation, growth, yield, nitrogen fixation and nodule occupancy of cowpea at two sites in SWK; 4) to determine the effects of phosphatic fertilizer and liming on symbiotic efficiency of native cowpea rhizobia under acid conditions at two sites in SWK. Genetic diversity of cowpea rhizobia was determined through sequence analyses of 16S rRNA and recA genes (objective 1). Abundance of rhizobia was determined through the most probable number (MPN) plant infection technique in germination pouches, symbiotic efficiency of rhizobia was determined through pot experiments in greenhouse using soil samples from seven agro-ecological zones in SWK and two reference regions (Machakos and Kilifi) (objective 2). In objective 3, a field experiment was conducted where four cowpea varieties (KVU 27-1, K80, M66 and Ngor- a landrace) were each inoculated with *Bradyrhizobium* sp. strain USDA 3456 and subjected to three N fertilizer levels (0 kg N ha^{-1}, 20 kg N ha^{-1} and 40 kg N ha^{-1}), using randomized complete block design in a 4x4 factorial arrangement. In objective 4, cowpea varieties (KVU 27-1, M66 and Ngor) were each treated with lime (4 t CaO ha^{-1} or 0 t CaO ha^{-1}) and subjected to three P fertilizer levels (0 kg P ha^{-1},

25 kg P ha^{-1} and 50 kg P ha^{-1}) in a randomized complete block design with a 2 x 3 x 3 factorial arrangement. Data collected included: species diversity of cowpea rhizobia and endophytic bacteria, rhizobial population in soils, nodule numbers and dry weights, leaf area index, shoot dry weight, N-fixed, tissue concentration and uptake of N and P, tissue protein content and grain yield. There is wide genetic diversity of native cowpea rhizobia and endophytic plant growth promoting bacteria in the study area. Cowpea is predominantly nodulated by rhizobial species in the genus *Rhizobium* in soils of the geographic regions covered by this study. Abundance of rhizobia ranged from 0 – 1.0 x 10^5 cells g^{-1} soil, higher rhizobial population was recorded in soils with pH close to 7. Soils that had higher levels of organic carbon, total N and exchangeable aluminium were characterized by lower abundance of rhizobial cells. Rhizobial inoculation had no significant ($P \leq .05$) effects on nodulation, N-fixed, growth and grain yield of cowpea in two acidic soils of SWK. Two nodule endophytes (*Bacillus megaterium* and *Bacillus aryabhattai*) were the main cowpea nodule occupants in the two soils of SWK. Lime application was not beneficial to cowpea plants at moderately acidic soils (pH of 5.6). Phosphorous fertilizer enhanced nodulation, growth and cowpea N uptake in acidic soils of south western Kenya. In conclusion, there is wide genetic diversity of symbiotic and endophytic bacteria in the study regions, which may be utilised in future as bio-inoculants to promote cowpea production. Low pH, high levels of N and Al^{3+} in soils depress abundance of rhizobial cells. Cowpea plants do not respond to rhizobial inoculation in acidic soils of SWK, but phosphorous enhance symbiotic efficiency of cowpea in these soils.

Key words: Cowpea, diversity, nitrogen, phosphorous, rhizobia.

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Background information

Cowpea (*Vigna unguiculata* (L.) Walp) is a food to nearly 200 million people in Africa (AATF, 2012) and provides nutritional balance in African homes, where starchy food made from cassava, yam, banana, millet, sorghum and maize is a staple diet (Bationo *et al*., 2002). The annual world production of dry cowpea grain is over 6.4 million tonnes (Joshi and Rao, 2017), and sub-Saharan Africa contributes about 92% of production area (Nedumaran *et al.*, 2015). In Kenya, cowpea is the second most important legume after common bean, with a market share of 37% of total production volume of pulses and occupying a land area of about 282,000 ha (CPPMU, 2015; Fintac, 2013). Previous studies showed that cowpea production in Kenya was concentrated in Eastern region, which contributed about 85% of total production volumes, while Coast, Western, and Central regions contributed the remaining 15% (Muli and Saha, 2000; Muthamia and Kanampiu, 1996). Owing to the rising interest in African indigenous vegetables in Kenya, cowpea production has spread to most regions. For instance, in a survey conducted at the Northern Rift Valley in Kenya, 40% of households used cowpea as a vegetable (Weller, 2013). South Western Kenya covers among others: South Rift Valley region (Kericho and Bomet Counties), South Nyanza region (Kisii and Homabay Counties) and parts of Kisumu County (Collins *et al.*, 2010; Mahuku and Nzioka, 2011). Although the available legume production statistics show that Nyanza, Western and Rift valley regions of Kenya account for 3.9% of total national cowpea production (Kiambi and Mugo, 2016), there is no published data on cowpea production levels in the individual Counties in South Western Kenya. There is also undocumented inter-county trade in cowpea products (grain and leaf vegetables) within SWK.

Cowpea has high nutritional value; its seed contains 23% protein and 57% carbohydrate, while the leaves contain 27 - 34% protein (Belane and Dakora, 2009). Cowpea is used as fodder, green manure and cover crop for soil conservation. It also has medicinal properties. The leaves and seeds are applied as a poultice to treat swellings and skin infections, leaves are chewed to treat tooth ailments; powdered seeds are applied on insect stings, and roots are used as an antidote for snake bites and for treatment of epilepsy, chest pain and constipation (Brink and Belay, 2006). Cowpea pods have potential export value, due to their recent adoption as vegetable sources in southern Europe (Karapanos *et al.*, 2017).

Despite its increased importance, cowpea production is constrained by biotic and abiotic stresses. Pests are the important biotic stress factors that have been shown to reduce cowpea grain yield by over 70% (AATF, 2012). The most important abiotic stress for cowpea in SWK is soil acidity (NAAIAP), which is associated with deficiency of nitrogen, phosphorous and potassium, reduced abundance of microorganisms such as rhizobia, and decline in plant growth and yield (Miransari, 2016).

1.2 Problem statement and justification

Ecological conditions, particularly moisture and temperature, favor crop production in SWK (Jaetzold *et al*., 2010), but crop yields (CPPMU, 2015) possibly due to soil acidity and deficiencies of N and P (NAAIAP, 2014). The yield gap in cowpea producing areas of Kenya is 2.5 and 5.6 tons ha^{-1} for grain and leaf yield respectively (CPPMU, 2015). The leaf and grain yields of cowpea in SWK can be improved by inoculation with strains of rhizobia which are efficient in nitrogen fixation, but two studies done in central and eastern Kenya showed that the

commercial rhizobial inoculant commonly used by farmers in Kenya had no effects on nodulation, growth and yield of cowpea (Chemining'wa *et al*., 2007; Mathu *et al*., 2012).

There are a number of factors that may have lead to non response of cowpea to rhizobial inoculation. One of the factors may be efficiency of native strains of rhizobia in nitrogen fixation (Ouma *et al*., 2016). The efficient strains of rhizobia can be isolated, their diversity characterized and later used as inoculants to improve cowpea yield. Mathu *et al*. (2012) isolated cowpea rhizobia in parts of Eastern, Coast and Western Kenya, but the study did not characterize the cowpea rhizobia to the species level, which is the research gap partly filled by this study. High population of indigenous rhizobia that are efficient in nodulation may lead to non response of inoculated strains on nitrogen fixation (Kawaka *et al*., 2014). Population and symbiotic efficiency of cowpea rhizobia in Kenya have only been documented in soils of one site in Kilifi (Mathu *et al.,* 2012). The authors reported large populations of rhizobia which fixed more N than commercial *Bradyrhizobium* inoculant strains. There is a wide knowledge gap on abundance of rhizobia in Kenyan soils. Soil acidity which characterizes soils of SWK interferes with signal exchange between nodules and rhizobia, hence reducing ability of rhizobia to nodulate legumes (Ferguson *et al*., 2017). Nitrogen fixing efficiency of rhizobia in acidic soils of SWK may be enhanced by soil amendments such as liming, application of P fertilizer and soil inoculation with efficient strains of rhizobia that are adapted to the acid conditions (Kyei-Boahen *et al*., 2017; Lapinskas, 2007). Although previous researchers have reported non-response of cowpea to rhizobial inoculants, there was need to determine the nodulation and growth response of cowpea under acid under acid conditions and to determine the nodule occupancy which is knowledge gap missing in previous research work on cowpea. Knowledge on nodule occupancy would

contribute to the understanding on whether the inoculated strains of rhizobia have capacity to nodulate cowpea. Despite the role of liming and P in enhancing nitrogen fixation by cowpea, its role in cowpea production in Kenya has been barely documented. Two reports demonstrated that P enhances rhizobial abundance, nodulation, growth and yield of cowpea in soils with pH of 5.5-7.7 in Eastern Kenya (Kimiti and Odee, 2010; Onduru *et al*., 2008). Due to the challenge of soil acidity in SWK, there was need to determine whether the combined effects of lime and P would enhance nodulation, growth and cowpea yield.

Apart from soil fertility improvement, cowpea is a drought tolerant crop whose production is gaining importance in Kenya due to frequent drought conditions induced by climate change. In addition, the crop has export potential due to rising interest in its fresh pods in Eastern Europe (Karapanos *et al*., 2017). Studies that aim to improve the agronomic practices of the crop are therefore justified.

1.3 Study objectives

1.3.1 Overall objective

The overall objective of this study was to determine the abundance, diversity and nitrogen fixing potential of native cowpea rhizobia in South Western Kenya soils.

1.3.2 Specific objectives

The specific objectives of this study were:

i) To characterize the genetic diversity of cowpea rhizobia in soils of South western Kenya based on sequence analyses of 16S rRNA and recA genes.

ii) To determine the abundance and symbiotic efficiency of cowpea rhizobia in soils of South western Kenya

iii) To determine the effects of rhizobia inoculation and nitrogen fertilizer on nodulation, growth, yield, nitrogen fixation and nodule occupancy of cowpea at two sites in South western Kenya

iv) To determine the effects of P fertilizer and liming on symbiotic efficiency of native cowpea rhizobia under acidic soil conditions at two sites in South western Kenya

1.4 Hypotheses

i) Genetic diversity of symbiotic rhizobia of cowpea plant is high owing to the propensity of cowpea to be nodulated by different species of rhizobia

ii) Abundance and symbiotic efficiency of native cowpea rhizobia vary with ecological conditions

iii) Rhizobial inoculation enhances nodulation, nitrogen fixation, growth and nutrient content of cowpea plants

iv) Lime and P fertilizer applications enhance symbiotic efficiency of cowpea rhizobia

CHAPTER TWO: LITERATURE REVIEW

2.1 Botany and ecological requirements of cowpea

Cowpea belongs to the family fabaceae, and its centre of origin is Africa. It is a climbing, trailing or erect annual or perennial herb, cultivated as an annual. It has many lateral and adventitious roots, and stem length may reach 4 m. Flowers are bisexual, and are almost entirely self-pollinated in dry climates, but may exhibit cross pollination (up to 40%) in humid environments. Germination of cowpea takes 3-5 days at a soil temperature of 22^{o}C; optimum growing temperature is 25-35^{o}C, and does well in light textured, free draining soils of pH 5.0 -7.5. It forms N-fixing nodules with *Sinorhizobium fredii* and several *Bradyrhizobium* spp. and its length of growing season varies from less than 60 days for early maturing cultivars to 240 days for late maturing varieties (Brink and Belay, 2006; Jaetzold *et al*., 2010). The altitude for cowpea growth is cultivar dependent, and ranges from 0 – 2000 m above the sea level and the rainfall requirements per growing season is 200 mm, but can be lower for drought tolerant varieties (Infonet-Biovision, 2018).

2.2 Biological nitrogen fixation in legumes

Biological nitrogen fixation is the reduction of atmospheric dinitrogen gas (N_2) into ammonia (NH_3), and it occurs when there are symbiotic relationships between primary producers and microbes; mainly rhizobia, *Frankia* and cyanobacteria (blue-green algae), which posses dinitrogenase enzyme that catalyses the nitrogen fixation process (Groffman and Rosi-Marshall, 2013; Terpolilli *et al*., 2012).

2.2.1 Nodule formation and physiology of biological nitrogen fixation

The first step in legume-rhizobia symbiosis is the signalling between the host and microsymbiont (Bhagwat and Thomas, 1982). During their growth in the rhizosphere of host plants, rhizobia sense compounds such as flavonoids and / or isoflavonoids which are released by the legume roots, and respond by inducing the transcription of nodulation (*nod*) genes (Graham and Vance, 2003). The bacterial genes encoding proteins for nitrogen fixation are called *nif* and *fix* genes, while those that induce nodule formation are called *nod, nol* and *noe* genes (Hans-Walter, 2005). *NifH* is the most studied gene, and is used for phylogenetic analyses (De Meyer *et al*., 2011). The *nod* genes encode about 25 proteins required for bacterial synthesis and export of nod factor (NF), which is a lipochitooligosaccharide molecule. Early in the nodulation process, the NF signals the host plant to initiate root hair deformation (which makes it curl), membrane depolarisation, intracellular calcium oscillations, formation of pre infection threads in the outer cortical cells, and cell division in root cortex, which establishes a meristem and nodule primordium (Gage, 2004). Within the root hair curl, an infection pocket filled with rhizobia is formed, and gives rise to an infection thread, which is a tubular invagination of the cell wall and membrane. The infection thread extends from the infection pocket, through the root hair and into the root cortex, where it passes through the pre infection threads and reaches the growing nodule. As the infection thread grows, it is colonized by rhizobia that are finally released into nodule cells, where they fix nitrogen (Murray, 2011). Nodules are connected to the plant roots via vascular tissues, which supply them with photosynthetic substrates, mainly malate, synthesised from sucrose (Hans-Walter, 2005; Lodwig and Poole, 2003). The bacteria in the plant cells are enclosed by a peribacterial (symbiosome) membrane, the symbiosome separates them from the host plant's cells, and it is here that rhizobia differentiate into bacteroids (Hans-Walter, 2005).

Nodules are of two types namely determinate and indeterminate. Within the two nodule types, there are swollen and non-swollen nodules, but it is yet to be conclusively established whether swelling of nodules enhances N_2 fixation (Oono and Denison, 2010; Terpolilli *et al*., 2012). Indeterminate nodules are elongated and have a persistent meristem that continually give rise to nodule cells which are subsequently infected by rhizobia in the nodule. Examples of plant species with indeterminate nodules are: *Pisum sativum* (pea), *Medicago truncatula*, *Medicago sativa* (alfalfa) and *Trifolium* spp. The determinate nodules are round and do not have a persistent meristem, and are found in legumes which mainly originate from the tropics. The examples are: soybean (*Glycine max*), *Lotus japonicas* and *Vigna unguiculata* (cowpea) (Gage, 2004; Li *et al*., 2012). Determinate nodules also have several bacteroids contained within one peribacterial membrane, while indeterminate nodules have only one bacteroid within the membrane (Lodwig and Poole, 2003).

After nodule development and before nitrogen fixation commences, nodulins, which are nodule-specific proteins are formed. Nitrogenase enzyme is the principal nodulin, which catalyses both the reduction of N_2 into ammonia, and H^+ to molecular hydrogen (Santos *et al*., 2008). Biological nitrogen fixation is summarized in the following equation (Hans-Walter, 2005):

$$N_2 + 4NADH + 4H^+ + 16ATP = 2NH_3 + H_2 + 4\ NAD^+ + 16ADP + 16Pi$$

The reaction is catalysed by two enzymes: dinitrogenase and dinitrogenase reductase. Dinitrogenase contains both Iron and Molybdenum, in a cofactor called FeMoco, and nitrogen fixation takes place while N_2 is bound to dinitrogenase. Dinitrogenase reductase is reduced by electrons donated by a protein that contains ferredoxin, and the reduced enzyme binds ATP and

reduces dinitrogenase, which in turn provides electrons to N_2, reducing it to $2NH_3$ (Zhou *et al*., 2004). The first stable product of biological nitrogen fixation is NH_4^+ and it is transported to the host cell, where it is converted into glutamine and glutamate by the joint action of glutamine synthethase and glutamate synthase (GS-GOGAT) (Hans-Walter, 2005; Lodwig and Poole, 2003; Mokhele *et al*., 2012). It is further converted either to asparagines (amide) or ureides (Allantoin and Allantoic acid) depending on the legume species, and then transported to the other plant parts through the xylem vessel. Legumes with indeterminate nodules such as pea export fixed N in form of amides (asparagines) while legumes with determinate nodules such as soybean and cowpea export fixed N in form of ureides (Hans-Walter, 2005; Lodwig and Poole, 2003).

It was earlier thought that cowpea is nodulated by the slow growing bradyrhizobia (cowpea misclellany group), but recent findings have identified fast growing strains (Chidebe *et al*., 2018; Silva *et al*., 2012). In a related study done in Eastern Kenyan, 97% of isolated strains of cowpea nodulating rhizobia were fast growing (Kimiti and Ondee, 2010).

2.2.2 Symbiotic efficiency of rhizobia

Rhizobia and legume plants are known to interact in a symbiotic way, leading to development of nodules, within which rhizobia convert elemental nitrogen into NH_4^+, which is absorbed by plants for different physiological functions (Berrabah *et al*., 2014; Zhou *et al*., 2004). The formation of nodules with pink/reddish colour is therefore a sign that a particular *Rhizobium* strain has successfully infected the plant cells and is efficiently fixing nitrogen (Hans-Walter, 2005; Sulieman *et al*., 2013). Symbiotic efficiency of rhizobia therefore describes their nitrogen

fixing potential, which can be determined by various methods, one of which is the determination of nodule numbers and weight (Belane *et al*., 2014; Chemining'wa *et al*., 2013). In addition to nodule counts in host plant, molecular analysis of nodule occupancy by rhizobial strains is also useful in determining symbiotic/nodulation efficiency of rhizobia (Atieno *et al*., 2012; Chemining'wa and Vessey, 2006). Due to the role of fixed N in enhancing plant growth (Walker *et al*., 2001), plant growth characteristics such as shoot dry weight have been used to assess the symbiotic efficiency of rhizobia (Argaw, 2012; Belane *et al*., 2014). Tissue N and protein content in legume plants has also been used by different authors in symbiotic efficiency studies (Bradic *et al*., 2003; Mothapo *et al*., 2013). Quantification of the amount of nitrogen fixed (ÖĞÜTÇÜ *et al*., 2008; Pule-Meulenberg *et al*., 2010) in legume plant tissues also gives information on symbiotic efficiency. Leghemoglobin content of nodule has also been used to determine the symbiotic effectiveness of rhizobia (Agrawal and Choure, 2011).

2.2.2.1 Methods of quantifying the amount of nitrogen fixed by rhizobia

Quantification of the amount of nitrogen fixed by rhizobia in legume plants is an important measure of symbiotic efficiency. In summary, the methods used for determining the amount of N- fixed are: $^{15}N_2$ gas enrichment, acetylene reduction activity, N isotope methods (^{15}N isotope dilution and the ^{15}N natural abundance) (Hardason, 2008) the relative abundance of ureides in plant tissue (Herridge and Peoples, 1990) and the N-difference (Gardner *et al*., 2010).

In the N-difference method, total N accumulated by the N_2 fixing plant is compared with that of non N_2 fixing reference plant, with the assumption that both assimilate the same amount of soil mineral N (Herridge *et al*., 2008). The equation for calculating the amount of nitrogen fixed

using this method is (Gardner *et al*., 2010): N fixed = (soil mineral N+ plant N in legume pot/plot) - (soil mineral N + plant N in non-legume pot/plot). The limitation of this method is that there can be differences in root morphologies of N_2 fixing and non N_2 fixing plants, which can affect their relative capacities of exploiting soil N (Peoples *et al*., 2002). It can be used successfully only in soils with limited soil N (Herridge *et al.*, 2008).

The use of $^{15}N_2$ gas entails the enclosure of plants in chambers filled with enriched nitrogen gas, and if nitrogen fixation has occurred, the ^{15}N concentration in a legume plant exposed to $^{15}N_2$ gas is higher than the 0.3663% ^{15}N natural abundance (Hardason, 2008). The practicability of this technique in field conditions has however been put under doubt (Boodley *et al*., 2008).

Nitrogenase enzyme, which reduces N_2 gas into ammonia during the process of biological nitrogen fixation (dos Santos *et al*., 2011) is also known to reduce acetylene (C_2H_2) into ethylene (C_2H_4). The two gases can be detected and quantified using gas chromatography, and can be used for determining the nitrogen fixation capacity of bacterial cultures or plant tissues harbouring N_2 fixing bacteria (Herridge *et al*., 2008; Unkovich *et al*., 2008). Acetylene reduction assay involves uprooting of whole plants to analyse nodulated root systems for ethylene evolution, and this could alter the partial pressure of oxygen around the nodules hence reducing nitrogenase activity (Peoples *et al.*, 2002. The relationship between ethylene production and N_2 fixed is also inconsistent, and therefore this method may not be practical in field experiments (Herridge *et al.,* 2008). The most practical methods in the field are: the ureide-abundance technique and the two forms of ^{15}N isotope techniques (^{15}N-enriched soil and natural- abundance technique) (Boddley *et al.*, 2008).

Legumes export fixed N from root nodules to the sinks either as amides (asparagine and glutamine) or ureides (allantoin and allantoic acid) (Unkovich *et al.,* 2008). In this technique, stem bases of ureide producing legumes such as cowpea and soybean are cut and xylem exudates are collected as bleeding sap; hot water extracts of dried stems or leaves are also collected and analysed for ureides (using calorimetric techniques) and nitrate N (Dakora *et al*., 2008; Herridge, 1982). The relative abundance of ureides in plant tissue is then calculated as follows (Herridge, 1982):

$$\text{Relative abundance of ureides} = \left(\frac{\text{Ureide N}}{\text{Ureide N+Nitrate N}}\right) \text{x } 100$$

The limitation of Ureide assay as a method of determining N_2 fixation is that it needs calibration with another technique (mostly ^{15}N isotope dilution) and also requires many plant samples for analysis (Pauferro *et al*., 2010).

Nitrogen has many isotopes, but the most stable ones are ^{14}N and ^{15}N; and of the N atoms on earth, 99.6337% are ^{14}N, and 0.3663% are ^{15}N (Robinson, 2001; Unkovich *et al*., 2008). The isotopic abundance of the minor isotope (^{15}N) is expressed as a percentage of total N present (atom% ^{15}N) as given in the equation below (Unkovich *et al.,* 2008):

$$\text{Atom\% } ^{15}\text{N} = \left(\frac{^{15}\text{N}}{^{15}\text{N} + ^{14}\text{N}}\right) \text{x } 100$$

^{15}N isotope dilution involves growing both the N_2 fixing and non- N_2 fixing reference plant in soil enriched with equal amount of ^{15}N labelled fertilizer. In the presence of N_2, a fixing plant lowers the ratio of ^{15}N:^{14}N due to incorporation of N from unlabelled air, and this does not occur in the non - N_2 fixing reference plant. The extent to which ^{15}N:^{14}N ratio in the N_2 fixing crop is

decreased relative to the reference plant is used to measure the amount of N_2 fixed (Hardarson, 2008). The percentage of N derived from the atmosphere (%Ndfa) and N_2 fixed by the legume crop in this method is calculated as follows (Hardarson, 2008; Unkovich *et al.*, 2008):

$$\% \text{ Ndfa} = \left(1 - \frac{\text{atom\% } ^{15}\text{N excess } N_2 - \text{fixing plant}}{\text{atom \% } ^{15}\text{N excess reference plant}}\right) \text{x } 100$$

Where atom% ^{15}N excess is the measure of the respective plant sample's ^{15}N content above the atmospheric N_2 (sample atom% ^{15}N-0.3663)

$$\text{Amount of } N_2 \text{ fixed } = \frac{\% \text{ Ndfa x total N} - \text{fixing plant}}{100}$$

The principle behind ^{15}N natural abundance technique is the observation that mostly, the ^{15}N natural abundance of the N in plants derived from the soils is higher than the N derived from the air through BNF, and this difference can be used to quantify the amount of nitrogen fixed (Pauferro *et al.*, 2010; Hardason, 2008). The assumption is that the non-N_2 fixing reference plants used accumulate N only from the soil (Pauferro *et al.*, 2010). The^{15}N natural abundance is expressed in a relative δ (delta) notation, which is the ‰ deviation of the ^{15}N natural abundance of the plant sample from atmospheric N_2, i.e. δ^{15}N (‰) is expressed as: 1000 x (sample atom %^{15}N - 0.3663) / (0.3663) (Naab *et al.*, 2009; Unkovich *et al.*, 2008). The δ^{15}N (‰), % Ndfa (proportion of nitrogen derived from biological nitrogen fixation) and N-fixed (the amount of nitrogen fixed) are determined using the following equations (Belane and Dakora, 2011; Pule-Meulenberg *et al.*, 2010):

$$\delta 15_{\text{N}} \text{ (‰)} = \frac{[15_{\text{N}}/14_{\text{N}}]_{\text{sample}} - [15_{\text{N}}/14_{\text{N}}]_{\text{standard}}}{[15_{\text{N}}/14_{\text{N}}]_{\text{standard}}} \times 1000$$

$$\% \text{ Ndfa} = [(\delta^{15}N_{ref} - \delta^{15}N_{leg}) / (\delta^{15}N_{ref} - \text{B value})] \text{ x } 100,$$

Where: $\delta^{15}N_{ref}$ is the ^{15}N natural abundance of the reference plant; $\delta^{15}N_{leg}$ is the ^{15}N natural abundance of the N_2 fixing legume, and the B value is the ^{15}N natural abundance of the legume being studied depending solely on N_2 fixation for N nutrition.

N- Fixed= % Ndfa x tissue N of the legume

The advantage of ^{15}N natural abundance technique is that no tracer has to be applied. The limitations are that small differences in ^{15}N abundance are measured and there is high variability of ^{15}N in soils (Hardason, 2008).

Studies have shown that cowpea genotypes with high photosynthetic rates and water use efficiency have greater N_2 fixing potential (Belane and Dakora, 2011). Pule-Meulenberg *et al.* (2010) quantified the amount of N_2 fixed in cowpea using ^{15}N natural abundance in Botswana, Ghana and South Africa, and reported variable N-fixed values based on genotype and geographical location. Mathu *et al.* (2012) quantified the amount of nitrogen derived from fixation (% Ndfa) in cowpea plants grown in soils from Kilifi, Bondo, Bungoma, Isiolo and Meru South in Kenya. Crops grown in soils from a site in Kilifi had the highest Ndfa value (98%) and did not respond to commercial rhizobia inoculation.

2.3 Abundance of native rhizobia in soils of various geographical regions

Knowledge of abundance of rhizobia in soils is important as it determines whether or not to inoculate legumes with commercial strains of rhizobia (Chemining'wa *et al*., 2011). Generally, when the population of rhizobia is absent or low in soils, legume crop production can be enhanced by use of commercial rhizobial inoculants (Chemining'wa and Vessey, 2006). There is an inverse correlation between rhizobia inoculation and the increasing numbers of indigenous

rhizobia in soils (Thies *et al*., 1991). High abundance of rhizobia is associated with high symbiotic efficiency of rhizobia, measured based on increased nodule numbers and weight, shoot dry matter, and grain yield (Mathu *et al.,* 2012; Pule-Muelenberg *et al.,* 2010).

Abundance of rhizobia can be determined indirectly by counting the nodule numbers in plants growing in different soils (Chemining'wa *et al*., 2012), and directly by counting number of rhizobia cells. The most commonly used direct method of enumerating rhizobia in soils is the Most Probable Number (MPN) plant-infection technique. The MPN technique involves growing of a legume species inoculated with aliquots of soils from various field sites in growth pouches or leonard jars with sterile media (sand/vermiculite), under nitrogen free solution (Kimiti and Odee, 2010; Maingi *et al*., 2006). Leonard jars/growth pouches that hold the plants are usually arranged in racks and placed in glasshouses or growth chambers at average day and night temperatures of 22^{o}C and 18^{o}C, respectively, and 65%-70% relative humidity (Prevost and Antoun, 2006). Previous studies also show that for MPN experiments, legumes can be grown successfully in greenhouse conditions with ambient light intensity and average daily temperatures ranging from 12^{o}C - 25^{o}C, and plants harvested after 3-5 weeks (Kimiti and Odee, 2010; Thrall *et al*., 2007). The numbers of pouches/leonard jars with nodulated plants are counted at each dilution level of soil inoculum, and then a series of results obtained are checked against those on MPN table to obtain the corresponding number of rhizobia (Prevost and Antoun, 2006). A computer programme called the Most Probable Number Enumeration system (MPNES) is useful for generating MPN tables and computing the individual MPNs of rhizobia (Woomer *et al*., 1990). The number of viable rhizobia/gram of soil or inoculant is determined by multiplying

the MPN estimates by the reciprocal of initial level of 10-fold soil dilution (10^{-1} or 10^{-2}) prepared before the start of serial dilutions (Prevost and Antoun, 2006).

A number of researchers have determined the population of rhizobia and the factors affecting their abundance in soils of various regions and ecological zones. Pigeon pea rhizobia counts in Zimbabwean soils ranged from undetectable to 121 cells/g of soil, while cowpea rhizobia ranged from 16 to 159 cells/g of soil, but the rhizobia strains were not efficient in nodulation (Mapfumo *et al*., 2000). They reported that poor soil organic matter, low soil moisture, low soil pH and low clay content of soil had significant negative effect on rhizobial counts. A study conducted in Embu (Kenya) found that the population of native siratro rhizobia ranged from undetectable to 2.3 $x10^2$ cells g^{-1} of soil, depending on land use; the population of rhizobia was highest in arable land with tea, and the isolated strains had relative symbiotic efficiencies in the range of 27%-112% (Mwenda *et al*., 2011). In another study conducted in Eastern Kenya (Kimiti and Ondee, 2010), the population of native cowpea rhizobia was enhanced by soil amendments with organic manure and P fertilizer, which led to increased shoot biomass of one cowpea genotype. Earlier studies on MPN counts of cowpea nodulating rhizobia in two contrasting agro-ecological regions in Eastern Kenya revealed that in a semi-humid climate, the population counts ranged from $1.04x10^2$ to $7.56x10^3$ cells g^{-1} of soil, while the semi arid to arid conditions of Kiboko had populations of $2.59x10^4$ to $1.89x10^5$ cells/g of soil (Maingi *et al*., 2006). The low population of rhizobia in the semi-arid climate was attributed to soil acidity. Chemining'wa *et al.* (2011) recorded a small cowpea rhizobia population of 78.5 cells/g of soils in Nyeri, Kenya (pH H_2O 4.0), as opposed to $9.0x10^2$ cells/g of soil in Kajiado (pH H_2O 6.4). Alkaline soils were reported to host large populations of native rhizobia in South Eastern Australia (Slattery *et al*., 2004).

However, *Bradyrhizobium* isolates that can survive at soil pH (H_2O) of 3.5 have been isolated (Appunu *et al*., 2009). Mathu *et al.* (2012) reported that the population of native cowpea rhizobia in Chonyi (Coast province), with a pH of 6.06 was 13.5 x 10^3 colony forming units g^{-1} of soil. Inoculation of these soils with three commercial inoculants had no effect on nodulation and biomass yield of cowpea. In contrast, high inoculation response of common bean were reported even when the population of indigenous rhizobia were high (Mnasri *et al*., 2007).

2.4 Characterisation of genetic diversity of rhizobia

Genetic diversity refers to the genetic variation within species. The study of rhizobial diversity is an important step towards identification of new strains and selection of efficient symbiotic associations between legumes and rhizobia, hence maximization of agricultural production (Berrada and Fikri-Benbrahim, 2014). Previous studies on determination of rhizobial diversity involved use of phenotypic, physiological and biochemical characteristics, but current studies engage molecular techniques or a combination of both molecular and non-molecular techniques.

Phenotypic characteristics used for characterising rhizobia are: morphological traits such as mucous production, colony morphology (diameter, form, elevation and optics), growth rate of rhizobia in culture media (fast growers – colonies formed in one or two days, slow growers-colonies formed in 4-10 days (Howieson and Dilworth, 2016). Biochemical and physiological characteristics can also be used for diversity studies of rhizobia. Some of them include: catalase and oxidase enzyme activities, methylene blue and gentian violet treatment, starch hydrolysis, growth on glucose peptone agar, urea hydrolysis, growth on Hofer's alkaline broth, gelatin hydrolysis, citrate utilization, growth in presence of 8% KNO_3, NaCl tolerance, precipitation of calcium glycerophosphate, antibiotic resistance test, utilization of carbon and nitrogen sources,

salt, pH and temperature tolerance (Gauri *et al*., 2011). Although phenotypic, biochemical and physiological methods are useful for characterising and identifying strains of rhizobia; molecular techniques are more reliable and accurate for studying the relationship of closely related bacterial strains and detect higher rhizobial diversity (Aregu, 2013).

Some of the molecular techniques for studying the genetic diversity of cowpea rhizobia include: PCR-RFLP and sequencing of 16S-23S rDNA internal transcribed spacer region (Sarr *et al*., 2011), repetitive sequence based PCR (BOX – PCR) and 16S rRNA gene sequencing (Guimarães *et al*., 2012); PCR- ARDRA (Amplified rDNA Restriction Analysis) and sequence analysis of 16S rRNA (Silva *et al*., 2012); PCR-RFLP of 16S-23S rDNA intergenic (IGS) spacer region (Pule-Meulenberg *et al*., 2010) and multi locus sequence analysis of bacterial house keeping genes such as recA (Glaeser and Kämpfer, 2015). The 16S rRNA gene sequence has been used as a standard genetic marker for identification and taxonomic classification of rhizobia because: it is found in all living organisms and therefore used for comparing phylogenetic relationship between them; the gene sequence is a long stretch (1500 bp) and has both conserved and variable regions that provide enough information for taxonomic purposes (Aregu, 2013). However, the resolution power of 16S rRNA gene sequences is limited in identifying strains or closely related species of recent divergence, hence sequence analyses of other housekeeping and symbiotic genes (multilocus sequence analysis - MLSA) has been recommended (Berrada and Fikri-Benbrahim, 2014). Sequence analysis of 16S-23S rDNA internal transcribed spacer region is also known to give high resolution power in rhizobial taxonomy (Aregu, 2013). Some of the house keeping genes includes recA, gyrB, atpD, and rpoB and encode proteins that serve different functions (BCCM, 2018).

PCR-RFLP analysis of 16S-23S rDNA intergenic spacer (IGS) region applied on crushed nodules of cowpea gave four IGS types in Senegal, and higher strain diversity was observed in water stressed conditions. Sequencing of 16S rRNA gene showed that the IGS types belonged to genus *Bradyrhizobium*. Sequence analysis of 16S-23S rDNA IGS showed that three of the IGS types were close relatives of rhizobial isolates that nodulate *Faidherbia albida* (Krasova-Wade *et al*., 2003). The diversity of cowpea nodulating rhizobia has been shown to decrease as more legumes are introduced in an area (Zilli *et al*., 2004) . In a study conducted to genetically characterise 76 indigenous cowpea rhizobia in five geographic regions of Japan, sequence analysis of the bacterial 16S-23S rDNA internal transcribed spacer (ITS) region clustered all isolates in the genus *Bradyrhizobium,* and were closely related with *Bradyrhizobium japonicum, Bradyrhizobium yuanmingense, Bradyrhizobium elkanii* and *Bradyrhizobium* sp. (Sarr *et al.,* 2011). The species distribution in the five regions varied based on ecological conditions. Silva *et al*. (2012) characterised the diversity of cowpea nodulating rhizobia in Amazon region of Brazil using Amplified rDNA Restriction Analysis (ADRA) and sequencing of 16S rDNA gene. Fast growing isolates in the study had close similarity with *Enterobacter, Rhizobium, Klebsiella* and *Bradyrhizobium*, while slow growing isolates were closely related only to *Bradyrhizobium.* In the same region of Brazil, Guimarães *et al.* (2012) determined the genetic diversity and symbiotic efficiencies of cowpea nodulating rhizobia using repetitive DNA based PCR (BOX-PCR) and 16S rRNA gene sequencing. Most of the strains analyzed belonged to genus *Bradyrhyzobium*, but with high species diversity; other species identified belonged to genera *Rhizobium*, *Burkholderia* and *Achromobacter*, and most nodulating strains showed high symbiotic efficiency. Genetic diversity of native cowpea rhizobia in Senegal were analysed using PCR-RFLP of the 16S – 23S rDNA IGS region and MLSA of six housekeeping genes (Wade *et*

al., 2014). The native strains belonged to genus *Bradyrhyzobium* and closely related with *Bradyrhyzobium yuanmingense* and *Bradyrhyzobium arachidis*. Higher rhizobia diversity was observed in low rainfall areas with alkaline soils. Recent diversity study of cowpea rhizobia in Mozambique (based on MLSA of 16S rRNA, glnII, gyrB, recA, *and* rpoB genes) placed rhizobial isolates into genera *Rhizobium* and *Bradyrhizobium* (Chidebe *et al*., 2018).

Genetic diversity of cowpea rhizobia based on morphological characteristics in Eastern Kenya show that most isolates were fast growing on culture media, meaning that they are likely to be in the genus *Rhizobium* (Kimiti and Odee, 2010; Ondieki *et al*., 2017). PCR-RFLP of the 16S-23S rDNA IGS region analysis conducted on nodules from Chonyi in Kilifi-Kenya grouped indigenous cowpea nodulating rhizobia into six IGS groups, showing a wide diversity of indigenous rhizobial strains which showed high symbiotic efficiency (Mathu *et al.,* 2012). Recent study based on protein profiling and sequence analysis of 16S rRNA gene of cowpea rhizobia in soils of Mbeere and Kilifi in Kenya revealed high species diversity within the genus *Bradyrhizobium*; soil pH and texture were positively correlated with occurrence of rhizobia in these soils (Ndungu et al., 2018). Diversity studies on cowpea rhizobia have focussed on Eastern and Coastal regions of Kenya, but there is no information available for other regions. Multilocus sequence analysis on housekeeping genes other than 16S rRNA has not been utilised on genetic diversity studies in Kenya.

2.5 Nitrogen assimilation and functions in plants

Molecular nitrogen (N_2) is the largest component of the earth's atmosphere, comprising about 78% of the total air volume (Zhou *et al.,* 2004). Despite its abundance, nitrogen is one of the

most deficient nutrient elements in crop production, yet it is required in large amounts by plants and its limitation causes a decline in plant growth and development (Kraiser *et al*., 2011). Plants cannot assimilate N_2 directly, but rather in two ionic forms: nitrate (NO_3^-) and ammonium (NH_4^+) (Havlin *et al.,* 2005). Atmospheric N enters the biological nitrogen cycle in three main ways: biological nitrogen fixation (where N_2 is converted by prokaryotes into NH_3 and then to NH_4^+), atmospheric fixation (lightning and photochemical fixation of N_2 into nitrate), and industrial fixation of N_2 into NH_3 through the Haber-Bosch process (Kraiser *et al.,* 2011). Ammonia obtained through the Haber-Bosch process usually undergoes further chemical reactions to form N-fertilizers. For example, it can be reacted with CO_2 to form urea, or oxidized to nitrate (Zhou *et al.,* 2004). After uptake, NH_4^+ can be converted directly into amino acids, but NO_3^- is first reduced into NH_4^+ before amino acid synthesis (Li *et al.,* 2012). Amino acids are then converted into proteins and nucleic acids. Proteins provide the framework for chloroplasts, mitochondria and other organelles in which biochemical reactions take place, and most enzymes controlling metabolic processes are proteins (Havlin *et al.,* 2005). Nitrogen is a key component of chlorophyll molecule, which is known to convert light energy into chemical energy needed for photosynthesis. This explains why adequate N supply enhances photosynthetic activity, vegetative growth and dark green colour in plants (Havlin *et al*., 2005; Hudson *et al*., 2011).

2.6 Effects of starter nitrogen on nodulation, growth and yield parameters in legume crops

Although rhizobia fix atmospheric nitrogen for use by host legumes, plants would require nitrogen for initial growth before nodules are formed. Therefore, nitrogen fertilizer is sometimes applied as a starter dose, especially when soil N is low (about 20 kg N ha^{-1} within 0-15 cm layer) (Hasan *et al*., 2010). Application of 60 kg N ha^{-1} of N fertilizer gave the highest nodule numbers

in cowpea (18.7 nodules plant^{-1}) in non-inoculated plots of N deficient regions of Sierra Leone (Haque *et al*., 1980). Starter-N enhanced biomass production and amount of N-fixed in soybean grown in South Dakota U.S.A, where cold and wet climate delays onset of crop emergence and N fixation; nonetheless, increased N rates decreased ureide levels which is an indicator of a decrease in N-fixation (Osborne and Riedell, 2011). Research findings show that the symbiotic association between common bean and its rhizobia may require 40 kg N ha^{-1} starter dose of nitrogen fertilizer for yield enhancement (Brito et al., 2011b). Similarly, rhizobia inoculation and starter nitrogen fertilizer (23 kg N ha^{-1}) enhanced nodule numbers, dry matter and grain yield in common bean (Daba and Haile, 2000). The balance in N demand is likely to be met from external sources like nitrogen fertilizer. Application of starter nitrogen (50-75 kg ha^{-1} of urea) enhanced nodule numbers, growth and grain yield of chickpea (*Cicer arietinum* L.); however, 100 kg ha^{-1} of urea depressed nodulation (Namvar *et al*., 2011). Low rates of N (20 kg N ha^{-1}) enhanced chlorophyll content, activities of nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, protein content and seed yield of lentil (Suryapani *et al*., 2013). Hasan *et al*. (2010) reported an increase in green and dry matter yield and crude protein in cowpea forage due to application of N fertilizer. On the other hand, application of starter N (26 kg N ha^{-1}) had no effect on growth and yield parameters in cowpea, common bean (*Phaseolus vulgaris* L.), lima bean (*Phaseolus lunatus* L.), green gram (*Vigna radiata* L.), pigeon pea (*Cajanus cajan* L.) and lablab (*Lablab purpureus* L.) at the University of Nairobi's field station in Kenya (Chemining`wa *et al*., 2007). Delay in nodulation under high rates of starter N was also reported in red clover in Canadian soils (Thilakarathna *et al*., 2012).

2.7 Effects of rhizobia inoculation on growth, symbiotic efficiency and yield parameters in legume crops

Leguminous plants form symbiotic associations with nitrogen fixing bacteria, which are collectively called rhizobia (Trabelsi *et al*., 2011). Legume crops are inoculated with commercial strains of rhizobia if the compatible strains of rhizobia are absent in soil, or if the population of indigenous rhizobia in soils are low or symbiotically inefficient (Mnasri *et al.,* 2007; Chemining'wa and Vessey, 2006; Thies *et al.,* 1991). Responses of legumes to rhizobia inoculation have been investigated by various authors. *Rhizobium* inoculation enhanced N content, N harvest index, shoot dry matter and seed yield of soybean in Turkey, with late maturing cultivars being more responsive to inoculation (Sogut, 2006). Genotypic differences in biological nitrogen fixation (measured as %Ndfa), grain yield and tissue N in cowpea has been reported (de Freitas *et al*., 2011). Although rhizobia inoculation enhanced tissue N in cowpea, non-inoculated plants gave similar grain yield as inoculated plants in a study conducted in Brazil (de Freitas *et al*., 2011). In Eastern Kenya, rhizobia inoculation enhanced seed yield of cowpea (Onduru *et al.,* 2008). However, inoculation of legume crops with commercial strains of rhizobia does not always give positive results, more so if the indigenous rhizobia are abundant and efficient in nitrogen fixation (Chemining'wa *et al.,* 2007). In a study conducted in Ghana, native strains of cowpea rhizobia gave higher symbiotic effectiveness than inoculated strains (Fening and Danso, 2002). Chemining'wa *et al*. (2007) reported that rhizobia inoculated cowpea plants had similar nodule numbers, shoot biomass and seed yield compared to un-inoculated plants in Kenya. Similar findings on cowpea were reported in soils of Chonyi in Kilifi, Kenya (Mathu *et al*., 2012). For inoculants to be effective, strains of rhizobia contained in them must be highly competitive in nodulation and efficient in nitrogen fixation compared to the native strains

(Trabelsi *et al.,* 2011). Research findings however show that co-inoculation of *Bradyrhizobium* sp. with plant growth promoting bacteria (for example *Paenibacillus graminis* and *Paenibacillus durus*) can increase nodulation, plant tissue N content and shoot dry weight, and delay nodule senescence in cowpea compared to inoculation of *Bradyrhizobium* species alone (Rodrigues *et al*., 2013).

2.8 Effects of phosphorous fertilizer and liming on growth, symbiotic efficiency and yield of legume crops

Plants absorb P either in form of $H_2PO_4^-$ or HPO_4^{2-}, the former is predominant at pH below 7.2 while the latter is predominant at pH above 7.2 (Havlin *et al.,* 2005). Phosphorous plays a role in energy storage and transfer as adenosine di-phosphate (ADP) and adenosine tri-phosphate (ATP) (Nyoki and Ndakidemi, 2014). The process of energy transfer is called phosphorylation, and it involves the transfer of energy rich $H_2PO_4^-$ molecules from ATP to energy requiring substances in plants, adenosine tri-phosphate is converted into ADP in the process. When the terminal $H_2PO_4^-$ molecule from either ADP or ATP is split off, large amount of chemical energy (12000 cal mol^{-1}) is liberated (Havlin *et al.,* 2005). Orthophosphate (pi), together with CO_2 and H_2O, is a primary substrate of photosynthesis (Rychter and Rao, 2005). Phosphorous also plays a central role in partitioning triose phosphates (end products of photosynthesis) between starch and sucrose biosynthetic pathways (Versaw and Harrison, 2002). It is an essential element in DNA and RNA, and it is estimated that in photosynthetic organisms, DNA and RNA constitute 0.095g and 0.091g of P per gram of dry matter, respectively (Raven, 2013). DNA and RNA contain the genetic code of the plant to produce proteins and other compounds essential for plant structure, seed yield, and genetic transfer. Phosphorous being a structural component of these nucleic acids

is therefore essential for vigorous growth and development of reproductive parts like fruits and seeds (Havlin *et al.,* 2005). Phosphorous is known to enhance root biomass (Wissuwa *et al*., 2005), crop maturity in grain crops, straw strength in cereals and N_2 fixing capacity in legumes (Havlin *et al.,* 2005). The role of P in symbiotic process is in energy generation required for reduction of N_2 into ammonia (Dashora, 2011; Sulieman *et al*., 2013). Application of P fertilizer has been found to increase the amount of N_2 fixed in cowpea by 30 - 40% (Vesterager *et al*., 2008). In white clover, P deficiency occurred after plants had formed nodules, nodule growth stopped and the proportion of plant N derived from symbiotic N_2 fixation declined at low P rates (Almeida *et al*., 2000).

Previous studies showed that application of 17 kg P ha^{-1} increased nodulation, shoot dry weight and tissue N content of cowpea in Mozambique (Kyei-Boahen *et al*., 2017). Earlier findings linked P deficiency to decline in nodule and shoot biomass (Alkama *et al*., 2009). Phosphorous enhanced the leaf area index, dry matter accumulation and grain yield of cowpea at a rate of 30 kg ha^{-1} in Nigeria (Ahamefule and Peter, 2014). Similarly, P fertilizer enhanced cowpea nodulation by native rhizobia, leaf area, total biomass and decreased incidence and severity of brown blotch disease (Owolade *et al*., 2006). In addition, P fertilizer also increased the number of pods per plant, grain and stover yield and 100 seed weight of two cowpea genotypes in savannah region of Nigeria, and the highest response was observed when plants were treated with 60 kg P ha^{-1} (Singh *et al.,* 2011). Phosphorous fertilizer has been reported to enhance water stress tolerance in cowpea (Uarrota, 2010). However, the response of cowpea to P fertilizer is genotype dependent, probably because of genotypic differences in root uptake efficiency of P (Gitte *et al*., 2003). Similar genotypic differences in P uptake, P use efficiency in P deficient

soils and N_2 fixation have been reported in cowpea and common bean (Jemo *et al*., 2006; Tajini and Drevon, 2014). Application of 40 kg P ha^{-1} and cowpea inoculation with *Bradyrhizobium japonicum* were reported to improve uptake of mineral nutrients (N, P, K, Mg, Ca and Na) in Tanzania (Nyoki and Ndakidemi, 2014). In Eastern Kenya, combined application of 45 kg P ha^{-1} and *Bradyrhizobium* inoculation increased cowpea grain yield by 54% (Onduru *et al.*, 2008). There is a strong positive correlation between N_2 fixing efficiency and uptake of mineral nutrients (P, K, Mg, S, Na, Fe, Cu, Zn, Mn and Bo) in cowpea genotypes (Belane and Dakora, 2014).

In acid soils, inorganic P can either precipitate as Fe/Al-P secondary minerals or is adsorbed to surfaces of Fe/Al oxide and clay minerals thereby making Al^{3+}, Mn^{2+} and Fe^{3+} ions soluble which causes plant toxicity (Havlin *et al.*, 2005). Soil acidity is also known to reduce the survival and persistence of rhizobia, hence curtail their symbiotic efficiency (Appunu *et al*., 2009). Lime contains Ca^{2+} and or Mg^{2+} ions, which displace Al^{3+} and Fe^{3+} in the negatively charged soil colloids, thus making P available for plant use (Kisinyo *et al*., 2012). Liming also enhances the solubility of molybdenum, which is a component of the nitrogenase enzyme that catalyses N_2 fixation reactions (Havlin *et al.*, 2005). Liming reduced the concentration of exchangeable, extractable and monometric aluminium in soils (Slattery *et al*., 1995). Liming and goat manure application reduced exchangeable acidity and increased available P, exchangeable Mg^{2+}, K^{+} and Ca^{2+} in soils and increased soybean yield in Embu, Kenya (Sefarim *et al*., 2013). Lime application (4t ha^{-1}) raised soil pH and also increased available P when it was combined with P fertilizer in soils where *Sesbania sesban* was grown in Western Kenya (Kisinyo *et al*., 2012). Lime application at a rate of 1t ha^{-1} enhanced cowpea yield, and increased soil calcium and

magnesium in Northern Brazilian Amazon (Costa, 2012). Kernel yield and the amount of N-fixed in groundnut increased in response to lime application in Zambia (Reddy *et al*., 1998). Liming increased the population of native rhizobia in soils from 4 rhizobia cells g^{-1} of soil to 7250 rhizobia cells g^{-1} of soil and consequently enhanced nodulation and seed N in legume crops in Australia (Fettel *et al*., 2007). Similar research work in Australia showed that liming increased the numbers of *Bradyrhizobium* spp, and enhanced nodule and shoot dry matter of *Ornithopus* spp by 57 and 28%, respectively (Hartley *et al*., 2004)

CHAPTER THREE: GENETIC DIVERSITY OF COWPEA (*Vigna unguiculata* L.) NODULATING RHIZOBIA IN SEVEN GEOGRAPHIC REGIONS OF KENYA

Abstract

Leaf and grain yield of cowpea in Kenya is low and can be improved by crop inoculation with efficient strains of rhizobia. Biofix is the only available commercial rhizobial inoculant for cowpea in Kenya, but previous studies show that it is inefficient in nitrogen fixation. Efficient rhizobial strains can be identified after genetic diversity studies of native rhizobia have been done, and their symbiotic efficiency verified. However, information on the genetic diversity and symbiotic efficiency of native rhizobia that nodulate cowpea in most regions of Kenya is limited. The objective of this study was to determine the genetic diversity of cowpea nodulating rhizobia in soils from 21 sites distributed in five geographic regions of south western Kenya and two other reference regions with long history of cowpea cultivation. The method used in the study was sequencing and phylogenetic analyses of 16s rRNA and rec A genes. Cultural and biochemical methods were also used in the initial characterisation of cowpea rhizobia. Based on 16s RNA sequence analysis, 25 isolates in this study were closely related to known nitrogen fixing bacteria. Twenty one of them belonged to the genus *Rhizobium*, two were placed in the genus *Bosea* and two others belonged to genera *Bradyrhizobium* and *Mesorhizobium*. All rhizobial isolates were gram negative. All cowpea nodule isolates, except two, were fast growing (acid producing). Generally, there was congruence in phylogenetic grouping of rhizobial isolates in both 16s RNA and recA trees. However, incongruence in species identification of three isolates of rhizobia was observed in sequence analyses of 16s RNA and recA genes, but 16s RNA gene gave ≥ 99% sequence homology to known species in GenBank, and may have given better species identification. One isolate may represent a novel species in the genus *Rhizobium,*

because sequences of both genes did not have close similarity to any known species in NCBI database. Forty three nodule isolates had high similarity to plant growth promoting bacteria, 84% of them were identified as *Bacillus megaterium* and *Bacillus aryabhattai* which also had wide geographical distribution. Their role in legume-rhizobium symbiosis needs to be investigated further. Among the seven geographic regions, Nyakach Central was species rich and had the highest species diversity of 2.15 on Shannon's index. This site was characterized by soil pH close to neutral and relatively high phosphorous level. It was concluded that the genetic diversity of cowpea nodulating rhizobia and plant growth promoting bacteria in the seven geographic regions of Kenya is high, and *Rhizobium* sp. is more competitive in nodulating cowpea. There is a need to establish the symbiotic efficiency of the cowpea nodule isolates through field and greenhouse experiments.

Keywords: Sequencing, recA, 16S rRNA, *Rhizobium*, plant growth promoting bacteria

3.1 Introduction

Nitrogen (N) is one of the most deficient nutrients in Kenyan soils (NAAIAP, 2014), yet it plays significant roles in most physiological processes in plants including photosynthesis (Havlin *et al*., 2005). Nitrogen deficiency can be corrected by application of inorganic fertilizers, but farming in Kenya is practiced by small-scale farmers who account for 75% of total agricultural production (Salami *et al.*, 2010), and have limited financial resources for purchasing farm inputs. Integrated nutrient management approaches that focus on optimization of biological N fixation through *Rhizobium*-legume symbioses, could be a better way of N replenishment in soils (IAEA, 2008). Cowpea-rhizobia symbioses can produce surplus N amounting to 60-70kg ha^{-1} after a cropping season (Sigh *et al*., 2009), and may contribute 11-20% of N requirement to companion

crops (Senaratne *et al*., 1995). Efficiency of rhizobia-legume symbioses can be achieved by isolating and characterizing the diversity native strains of rhizobia in diverse soil conditions and reintroduction of superior strains in form of commercial inocula. Conditions such as pH and levels of available N, P and K influence the survival of rhizobial species in soil. For example, *Sinorhizobium* sp. may be predominant in alkaline soil while *Bradyrhizobium* sp. is abundant at pH close to 7 (Zhang *et al*., 2011). Therefore in order to maintain survival of rhizobial strains introduced in form of inoculants in soil, diversity studies in various ecological conditions would be useful in understanding the soil ecological conditions favouring particular species. Characterisation of cowpea rhizobia was done in soils of Eastern Kenya using cultural and biochemical methods, where 97% of isolates were speculated to belong to genus *Rhizobium* due to production of acidic reactions in culture media (Kimiti and Ondee, 2010). However, cultural and biochemical techniques cannot give accurate species identification. Recent studies at two agro-ecological zones of Kenya (Coastal lowland 4- Kilifi and upper midland-Mbeere) showed wide diversity of cowpea rhizobia within genus *Bradyrhizobium* (Ndungu *et al*., 2018). The study was however based on protein profiling and sequencing of 16S rRNA gene of rhizobial isolates, but the current trend in rhizobial diversity studies involves the use of multilocus sequence analyses of various housekeeping and symbiotic genes which include recA, NifH, nodC and gyrB (Berrada and Fikri-Benbrahim, 2014; Laguerre *et al*., 2001). 16S rRNA gene sequencing is reported to give more precision in identification of rhizobial species, and recA is useful in discriminating the identity of two closely related bacterial species (Guimarães et al., 2012; Zbinden et al., 2011). The objective of this study was to characterise the genetic diversity of cowpea nodulating rhizobia in seven geographical regions of Kenya by sequence analyses of two house- keeping genes (16S rRNA and recA).

3.2 Materials and methods

3.2.1 Soil sampling, soil analyses and nodule harvesting

Soil samples were collected from three farmer's fields located in five regions in south western Kenya (Kericho East, Kericho West, Bomet Central, Nyamira north, and Pap-Onditi in Nyando Sub-county) and two other regions where cowpea is commonly grown (Mwala in Machakos county and Fumbini in Kilifi county). In each of the farms, soil samples were collected from two sites; with and without a history of cowpea cultivation. Soil samples were also collected from experimental plots previously inoculated with a commercial strain of rhizobia and also non inoculated plots in two sites located at Bomet Central and Kericho East. Soil sampling was done randomly within a radius of 6 m at a depth of 15-20 cm using a soil auger, where a total of 24 soil cores were collected and mixed to obtain 2 kg of a composite sample (Maingi *et al*., 2006; Mwenda *et al*., 2011). The total number of soil samples was 42. The samples were analyzed for pH (H_2O), percent (%) organic carbon (OC), total N (%) , P (ppm) and Al (cmol kg^{-1}) (Okalebo *et al*., 2002) before the onset of the experiment. The pH, organic carbon, total nitrogen, phosphorous and aluminium in the study area ranged from 4.11 to 7.1, 0.33% to 4.19%, 0.05% to 0.97%, 1.50 mg kg^{-1} to 101.36 mg kg^{-1} and 0.06 cmol kg^{-1} to 0.84 cmol kg^{-1}, respectively.

Cowpea variety (K80) was used as a "trap" host plant for cowpea rhizobia from each soil sample. Its seed was obtained from the Kenya Agricultural and Livestock Research Organization (KALRO) in Katumani. Trapping of rhizobia in soils, nodule harvesting and storage was done as described (Howieson and Dilworth, 2016; Sarr *et al*., 2011; Vessey and Chemining'wa, 2006), with minor modifications. Cowpea seeds were surface sterilized by immersion in 3% sodium hypochlorite for 1 minute followed by 70% ethanol for 30s and then rinsed five times in distilled

water. Four cowpea seeds were then sown in 1 litre pots filled with sterile vermiculite in greenhouse, and then thinned to two upon emergence. Ten-fold dilution of each of the 42 soil samples were prepared under aseptic conditions in the laboratory, by diluting 100 g of a soil sample in 900ml of sterile water, then 2 ml of a soil diluent was inoculated onto cowpea seedlings immediately after emergence. Nutrient solution used was prepared as described (Broughton and Dilworth, 1970). Day temperatures in greenhouse ranged from 28^{o}C to 30^{o}C. Nodule harvesting was done 8 weeks after inoculation, where plants were carefully uprooted, roots separated from shoots and then washed before harvesting the nodules. Ten nodules were harvested at random from pots containing each soil sample, put in a cool box and immediately transferred to the laboratory. Nodules were then surface sterilized by immersion in 70% ethanol for 1 minute, immersed in 3% sodium hypochlorite for 3 minutes and then rinsed 6 times in sterile distilled water. Nodules were then stored under 40% glycerol at temperatures below -20^{o}C until DNA extraction.

3.2.2 Isolation of rhizobia and cultural characterization of rhizobia isolates

Five nodules were randomly selected from the nodules previously harvested from each soil sample, sterilized in 70% ethanol for 2 minutes and rinsed in 3 changes of nanopure water. Each nodule was crushed using a plastic pestle in an Eppendorf tube containing 100 µl of 40% glycerol and 20 µl of the resulting cell suspension was streaked onto yeast extract mannitol agar (YEMA) containing 0.1% congo red (Mothapo et al., 2013; Somasegaram and Hoben, 1994) and incubated at 28^{o}C for10 days. A single colony from a group of similar colonies which did not absorb congo red dye was re-isolated on tryptone yeast (TY) agar (Beringer, 1974) and incubated at 28^{o}C for 2-4 days. Pure overnight cultures were made by aseptically transferring single

bacterial colonies with a loop from TY agar plates into 10 ml TY yeast broth and incubating them at 27^{o}C on a rotary shaker for 200 rpm until they turned turbid (about 24-48 hours). For long term storage, 700 µl of overnight cultures were combined with 300 µl 40% glycerol in a cryovial and stored at -80^{o}C. These frozen stocks were used for both DNA extraction and further cultural tests. A Gram staining reaction was carried out on a loopful of pure rhizobia culture grown on TY agar using gram staining kit (TCS biosciences, UK). Isolates of rhizobia were cultured on YEMA with bromothymol blue pH indicator for 5-7 days at temperature of 28^{o}C; fast and slow growing isolates turned media yellow and blue respectively (Somasegaram and Hoben, 1994). Further authentication of rhizobial isolates was done through Ketolactose test (Bhatt *et al*., 2013; Sharma *et al*., 2010). Lactose was replaced with mannitol in YEMA to make ketolactose media, and then a loopful of bacterial cultures from frozen stocks was streaked on plates containing the media. Bacterial colonies that turned yellow when Benedict's reagent was added to plates with ketolactose agar media were confirmed to be *Agrobacterium*, while rhizobia did not change colour of the media.

3.2.3 DNA extraction and polymerase chain reaction

An overnight culture of rhizobia was grown in TY broth, and used for DNA extraction using Gene Elute bacterial genomic DNA kit for gram positive bacteria (Sigma Aldrich ltd). Quality of DNA was measured using nanodrop 1000 spectrophotometer (labtech ltd, UK) and was found to be within the required 260 nm/280 nm absorbance ratios of 1.7-2.0. Primers that target 16S rRNA and recA genes in rhizobial DNA, were subjected to 50 µl polymerase chain reactions (PCRs) that consisted of 25µl of 2x PCRBIO Taq Mix (PCR biosystems ltd), 2 µl of each of the 10 µM forward and reverse primer, 5 µl DNA and 16 µl of nano pure water. Primer sequences

and PCR conditions are shown (Table 3.1). The amplified PCR products (5 µl) were separated on 1% agarose gel stained with GelRed dye (Biotium, USA), run at 90 V for 40 minutes in TE buffer, and then finally visualized on Syngene G: BoxChemi XL Gel documentation system to confirm the success of PCR amplification. A 1 kb hyperladder was used as a molecular weight marker (Bioline, UK). The PCR products were purified using QIAquick kit (Qiagen ltd) before sequencing.

Table 3.1: Primers used in this study

Primer	Target gene	Sequence (5´- 3´)	PCR conditions	Reference
recA 41F	recA	TTCGGCAAGGGMTCGRTSATG	95°C 2 min 35 cycles (95°C 15s, 60°C 15s, 72°C 15s) and final extension of 72°C for 5 min	(Pablo *et al*., 2005)
recA 640R	recA	ACATSACRCCGATCTTCATGC		
27F	16s rRNA	AGA GTTTGATCCTGGCTCAG	94°C 5mins; 35 cycles (94°C 40s, 65°C 40s, 72°C 1.5mins) and Final extension of 72°C for 7 minutes	(Guimarães *et al*., 2012; Lane, 1991)
1492R	16s rRNA	GGTTA CCTTGTTACGACTT		

3.2.4 DNA sequencing and phylogenetic analyses

Samples for sequencing were prepared as follows: 15µl of each pure DNA sample obtained after PCR product purification was pipetted in duplicate into eppendorf tubes; then DNA was mixed with 2µl of forward and reverse primers in separate tubes. Samples were then sent for sequencing in both forward and reverse directions at Eurofins genomics (Germany). Both recA and 16s rRNA genes were sequenced with the same primers used for PCR. Forward and reverse nucleotide sequences were aligned, similarities verified, and then edited to enhance quality using Bioedit software, version 7.2.5 (Hall, 1999). Sequences were then submitted for comparison with National Center for Biotechnology Information (NCBI) GenBank sequences using nucleotide Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi? PAGE

_TYPE=BlastSearch). All evolutionary analyses were done in MEGA 6 software (Tamura *et al*., 2013). Alignment of sequences was done using Clustal W (Thompson *et al*., 1994). Phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) and Kimura 2 parameter model (Kimura, 1980) was used to compute evolutionary distances with default parameters. A bootstrap confidence analysis (Felsenstein, 1985) was conducted with 1000 replicates.

3.2.5: Statistical analyses

Species richness and diversity indices (Shannon's and Simpson's) of bacterial isolates were determined in each geographic region, based on sequence analyses of 16s rRNA gene. Species richness (S) was determined by counting the total number of different species in each geographic region. Shannon's diversity index (H') was determined using the formulae: $H' = -\sum Pi \ln(Pi)$), where *Pi* is the proportion of individuals belonging to species *i* and ln is the natural logarithm. Simpson's index of diversity (D_1) was calculated using the formulae:$1 - \sum Pi^2$ (Morris *et al*., 2014), where Pi is the proportion of individuals belonging to species *i.*

3.3 Results

3.3.1 Cultural characteristics and phylogenetic analysis of rhizobia and selected plant growth promoting bacteria

The total number of bacterial isolates from cowpea nodules in the six geographic regions of Kenya was 157. Based on 16s rRNA sequence analyses and BLAST results from NCBI GenBank, 25 of these isolates were closely related to known symbiotic bacteria distributed in genera *Rhizobium* (21 isolates), *Bosea* (2 isolates), *Bradyrhizobium* (1 isolate) and

Mesorhizobium (1 isolate) (Table 3.2). Forty three isolates were plant growth promoting bacteria, while the rest (89 isolates) were *Agrobacterium tumefasciens* (Syn. *Rhizobium radiobacter*) and other pathogenic bacteria.

All rhizobial isolates, *Rhizobium pusense* and four selected plant growth promoting bacteria (PGPB) were gram positive and had colony sizes of 1-7 mm on YEMA. Isolates that tested positive to ketolactose were strains of *Rhizobium pusense;* and all isolates except *Bosea* sp. produced acids on culture media (bromothymol pH indicator turned yellow) (Table 3.2).

Phylogenetic analysis of 16s rRNAgene sequences clustered the isolates into three main groups (Fig. 3a). The first group of the 16S rRNA tree had four clusters; clade 1a had nine isolates that clustered with reference species *Rhizobium tropici* CIAT 899 at 78% bootstrap support. Five of the nine isolates had 99-100% sequence similarity to *Rhizobium tropici* strains LNP6, 233, B28 and ALSG5A1 (Table 3.2 and Fig. 3a). Two isolates had 99 and 100% similarity to *Rhizobium miluonense* strain LJ8 while isolates 16c and 30b had 100% sequence similarity to *Rhizobium* spp. Clade 1b had five isolates that clustered at 98% bootstrap support; three of the isolates were 99-100% similar to *Rhizobium miluonense* strains NS-35 and CC-B-L1, and the remaining isolates were 99% similar to *Rhizobium tropici* strain B28 and *Rhizobium* sp. (Table 3.2 and Fig. 3a). Clade 1c consisted of isolates that had 99% sequence similarity to *Rhizobium alamii* and *Rhizobium sullae* which appear to be recent descendants of a common ancestor (99% bootstrap value). The last cluster of group I (1d) consisted of four isolates with 98-100% similarity to *Rhizobium phaseoli* strain GYS7 (2 isolates), *Rhizobium grahamii* and *Rhizobium tibeticum* (Table 3.2 and Fig. 3a). In general, this group consisted purely of isolates in the family Rhizobiaceae (Weir, 2016), and had 8 species within the genus *Rhizobium*.

All isolates except 31b in Group II had 100% sequence similarity to *Rhizobium pusense* (Table 3.2, Fig. 3a). High bootstrap value of 99% supports clustering of *Agrobacterium* sp. with *Rhizobium pusense,* and their relatedness is further confirmed by positive test to ketolactose in culture media (Table 3.2). Group 3 consisted of diverse isolates that had sequence similarities of 97-100% to species distributed in 8 genera (*Bosea, Labrys, Mitsuaria, Pseudacidovorax, Mesorhizobium, Brevibacillus, Paenibacillus* and *Bradyrhizobium*). The group had two subgroups; in subgroup 3a, *Bosea* sp. and *Labrys neptuniae* shared common ancestry with *Bradyrhizobium japonicum,* a reference isolate obtained from Rothamsted research centre (U.K) (Fig. 3a).

Polymerase chain reaction amplification of recA gene in all the isolates was successful, but 16 gene sequences were omitted in phylogenetic analysis due to sequencing failure or low quality as revealed by bioedit software. Rec A tree was split into four main groups (Fig. 3b). Subgroup 1d of group 1 in 16s tree (Fig. 3a) formed its own group – G 3 with 97% bootstrap support in rec A tree (Fig. 3b). It was, however, noted that isolates 9c and 91b in group 3 of reA tree had closest sequence similarity of 98% to *Rhizobium* sp. (Table 3.3), as opposed to 98% and 100% sequence similarity to *Rhizobium phaseoli* in the 16s rRNA sequence analysis (Table 3.2). Group 1 of rec A tree (Fig. 3b) split into three sub-groups: Isolates 21c, 23c and 42a clustered together with 80% bootstrap support, this clustering is in agreement with 16s rRNA tree; isolates 37a, 30b and 98b formed subgroup 1b with 80% bootstrap support, but isolates 30b and 98b grouped with isolates in cluster 1a of 16s rRNA tree; 1c is a clade with isolates 33b and 43b grouped at 100% bootstrap support, which is in agreement with 16s rRNA tree. In group 2, isolates 11b, 99b, 16c

and 17c grouped with reference species *Bradyrhizobium japonicum* just like in 16s rRNA tree. Similarly, all the strains that had close sequence similarity to *Rhizobium pusense* grouped together in both recA and 16s rRNA trees (Fig. 3a and 3b).

Although there were some subgroup similarities between recA and 16s rRNA phylogenetic trees, there was incongruence in taxonomic positions of some isolates. Within the genus *Rhizobium*, isolate 23c had close similarity of 99% to *Rhizobium miluonense* strain LJ8 in 16s rRNA gene, but 97% similarity to *Rhizobium tropici* strain NCSU 2459 in recA gene (Fig. 3a and 3b, Table 3.2 and 3.3). Similarly, isolates 42a and 43b had closest similarities to *Rhizobium miluonense* strain LJ8 and *Rhizobium sullae* strain SCAU26 respectively in 16s rRNA gene, but the respective classification based on rec A gene placed them as *Rhizobium multihospitium* strain CCBAU 31043 and *Rhizobium mesosinicum* strain CCBAU. Finally, isolates 11b and 99b were identified as *Labrys neptuniae* strain Liujia-146 (100% similarity) and *Pseudacidovorax* sp. (99% similarity), respectively, in the 16s rRNA gene, but had 88% and 92% similarity to *Bradyrhizobium* sp. and *Variovorax paradoxus* respectively in the rec A gene.

Despite the existence of incongruence, rec A gene refined the taxonomy of three isolates (30b, 16c and 17c). While 16s rRNA gene would only classify them to the genus level, rec A gene sequences of the isolates were closely related to *Rhizobium tropici* and *Bosea thiooxidants*, respectively (Table 3.3, Fig. 3b). Isolate 37a may represent a novel species in the genus *Rhizobium*, because sequences of both genes did not have close similarity to any known species in NCBI database

Table 3.2: Cultural characteristics and species identification of nitrogen fixing and selected endophytic bacteria in seven geographic regions of Kenya based on 16S rRNA gene sequences

Isolate code	Gram test	Colony size on YEMA[†]	Ketolactose test[‡]	Colony colour on YEMA and Bromothymol Blue (5-7 days)	Closest species on GenBank[§]	GenBank Accession number	Similarity
42a	Negative	7 mm	-	Yellow	*Rhizobium miluonense* LJ8	KF515658.1	100%
77a	Negative	3 mm	+	Yellow	*Rhizobium pusense* YIC4260	KU685529.1	100%
50a	Negative	3 mm	-	Yellow	*Rhizobium tropici* 233	EU488749.1	99%
47a	Negative	4 mm	-	Yellow	*Rhizobium miluonense* NS-35	KU305717.1	99%
37a	Negative	3 mm	-	Yellow	*Rhizobium* sp.	KF836032.1	99%
38a	Negative	6 mm	-	Yellow	*Rhizobium miluonense* NS-35	KU305717.1	100%
87a	Negative	5 mm	-	Yellow	*Rhizobium miluonense* CC-B-L1	JN896360.1	99%
61a	Negative	1 mm	+	Yellow	*Rhizobium pusense* ZJY-286	KP282790.1	99%
1b	Negative	5 mm	-	Yellow	*Rhizobium multihospitium* NS-28	KU305703.1	99%
96a	Negative	1 mm	-	Yellow	*Rhizobium pusense* SM-T3	KF876889.1	100%
11b	Negative	2 mm	-	Yellow	*Labrys neptuniae* Liujia-146	NR 043801	100%
100a	Negative	5 mm	+	Yellow	*Rhizobium pusense M-T3*	KF876889.1	100%
24b	Negative	3 mm	+	Yellow	*Rhizobium pusense SM-T3*	KF876889.1	100%
26b	Negative	2 mm	-	Yellow	*Rhizobium grahamii* CFN 234	JF424610.1	99%
21c	Negative	4mm	-	Yellow	*Rhizobium tropici* B28	JX010975.1	99%
20b	Negative	2 mm	-	Yellow	*Rhizobium tropici* B28	JX010975.1	99%
31b	Negative	4 mm	-	Yellow	*Rhizobium* sp.	KM891589.1	99%
30b	Negative	1 mm	-	Yellow	*Rhizobium* sp.	KJ185035.1	100%
76b	Negative	1 mm	-	Yellow	*Brevibacillus brevis* N-421	KJ735916.1	99%
72b	Negative	3 mm	-	Yellow	*Rhizobium tropici* LNP6	GQ181036.1	99%
79b	Negative	3 mm	+	Yellow	*Rhizobium pusense* SM-T3	KF876889.1	100%
33b	Negative	1.5mm	-	Yellow	*Rhizobium alamii* CCBAU 15292	GU552885.1	99%

Table 3.2 *cont.*

43b	Negative	3 mm	-	Yellow	*Rhizobium sullae* SCAU26	FJ785219.1	99%
55b	Negative	4 mm	+	Yellow	*Rhizobium pusense* SM-T3	KF876889.1	100%
56b	Negative	3mm	-	Yellow	*Mitsuaria chitosanitabida* R8-376	JQ659937.1	99%
91b	Negative	5 mm	-	Yellow	*Rhizobium phaseoli* GYS7	JQ342895.1	100%
98b	Negative	2-4mm	-	Yellow	*Rhizobium tropici* LNP6	GQ181036.1	100%
99b	Negative	1 mm	-	Yellow	*Pseudacidovorax* sp.	HQ834240.1	99%
17c	Negative	2 mm	-	Blue	*Bosea* sp.	KP125320.1	100%
9c	Negative	4-6 mm	-	Yellow	*Rhizobium tibeticum* CC-RB301	JN896365.1	99%
10c	Negative	1-2mm	-	Yellow	*Rhizobium phaseoli GYS7*	JQ342895.1	98%
1c	Negative	4 mm	+	Yellow	*Rhizobium pusense* SM-T3	KF876889.1	100%
13c	Negative	2 mm	-	Yellow	*Paenibacillus typhae* P30E	KF010804.1	99%
16c	Negative	4mm	-	Blue	*Bosea* sp.	KM025198.1	98%
25c	Negative	1mm	-	Yellow	*Bradyrhizobium* sp.	KF114656.1	97%
23c	Negative	2 mm	-	Yellow	*Rhizobium miluonense* LJ8	KF515658.1	99%
47c	Negative	2 mm	-	Yellow	*Mesorhizobium* sp.	EU874894.1	99%
2b	Negative	4mm	-	Yellow	*Rhizobium tropici* ALSG5A1	KU598665.1	99%
16b	Negative	3 mm	-	Yellow	*Rhizobium* sp.	DQ507206.1	100%

[†]Yeast mannitol extract agar; [‡] - absence of *Agrobacterium*, + presence of *Agrobacterium*; [§] National Centre for Biotechnology Information (NCBI) GenBank (USA).

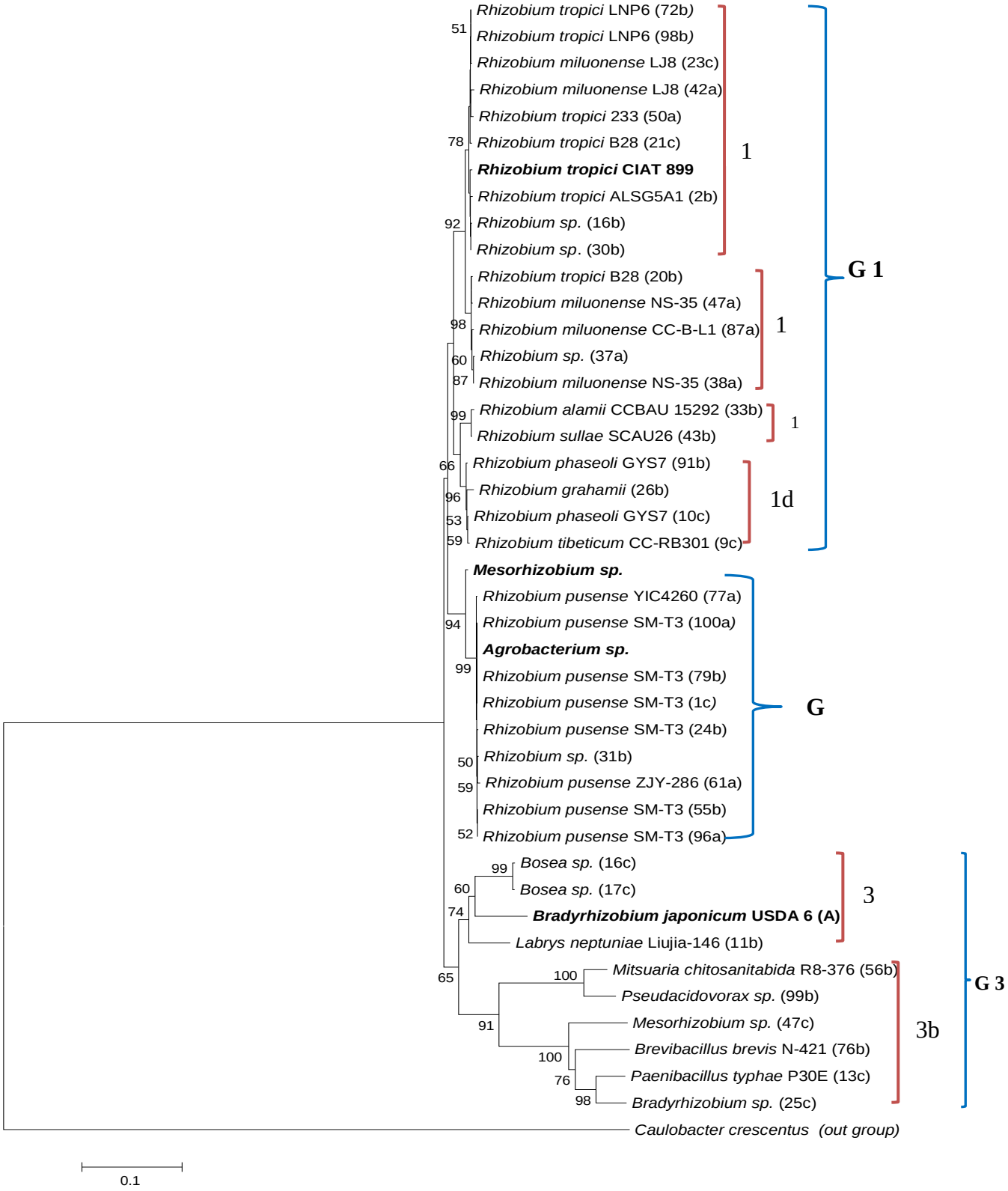

Fig. 3a: Neighbour joining tree of 16S rRNA sequences showing phylogenetic relationships of isolates of bacteria found in cowpea nodules in soils from seven geographic regions in Kenya and reference strains in bold letters. Bootstrap values greater than 50% are shown on the corresponding nodes. The values shown in brackets represent sampling points, also used for isolate identification. The scale represents nucleotide substitutions per site.

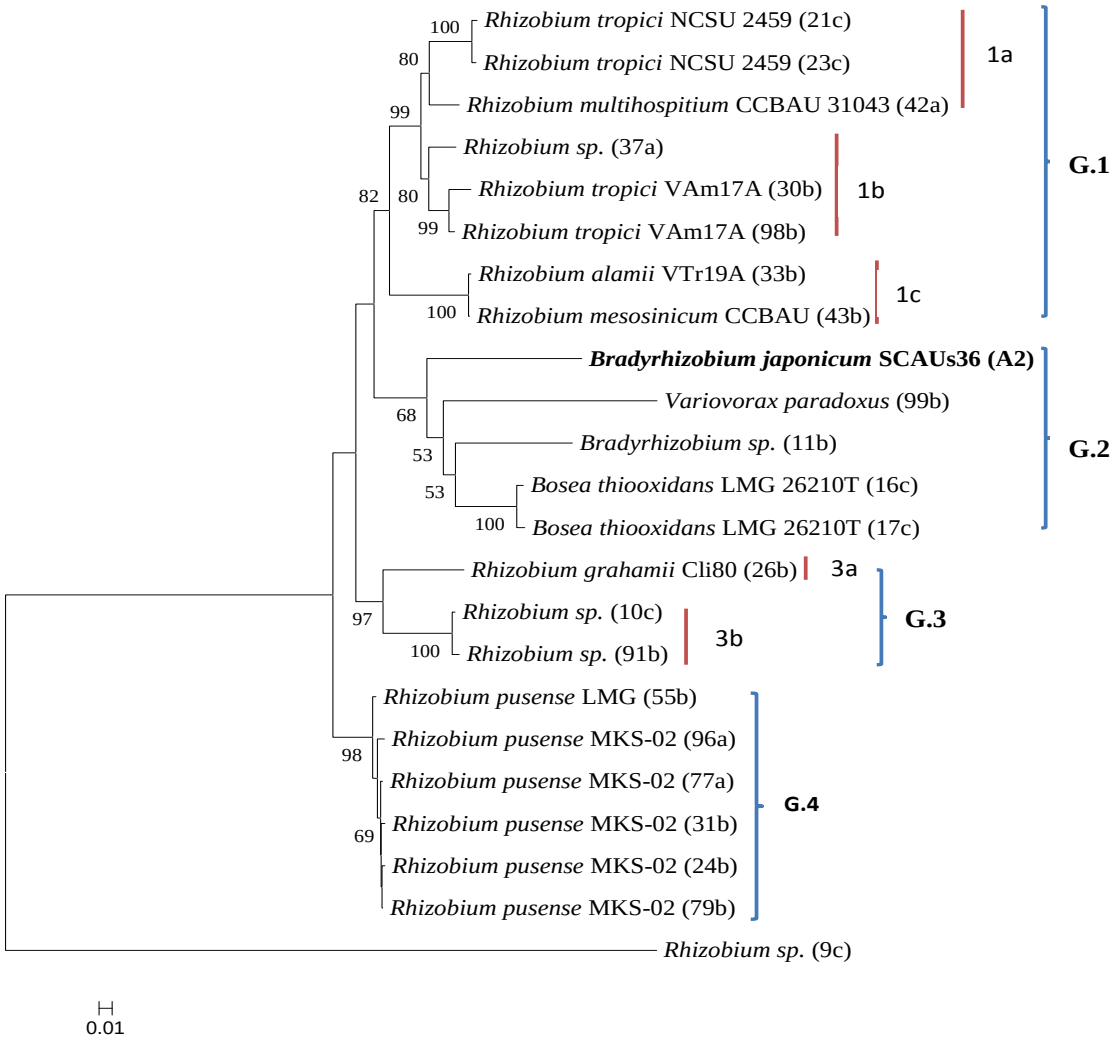

Fig. 3b: Neighbour joining tree of recA sequences showing phylogenetic relationships of bacterial isolates found in cowpea nodules in soils from seven geographic regions of Kenya and a reference isolate (in bold letters). Bootstrap values greater than 50% are shown on the corresponding nodes. The values shown in brackets represents sampling sites. The scale represents nucleotide substitutions per site.

Table 3.3: Species of symbiotic and endophytic bacteria isolated from cowpea nodules in seven geographic regions of Kenya based on NCBI† BLAST results of recA gene sequences

Strain code	Closest Species on NCBI GenBank	Similarity	Accession Number
9c	*Rhizobium* sp.	98%	KR400574.1
10c	*Rhizobium* sp.	98%	KR400574.1
11b	*Bradyrhizobium* sp.	88%	EU288698.1
16c	*Bosea thiooxidans* LMG 26210T	95%	FR871216.1
17c	*Bosea thiooxidans* LMG 26210T	95%	FR871216.1
21c	*Rhizobium tropici* NCSU 2459	97%	KJ535983.1
23c	*Rhizobium tropici* NCSU 2459	97%	KJ535983.1
24b	Rhizobium pusense MKS-02	99%	HF563598.1
26b	*Rhizobium grahamii* Cli80	95%	JF424623.1
30b	*Rhizobium tropici* VAm17A	98%	LC107304.1
31b	*Rhizobium pusense* MKS-02	99%	HF563598.1
33b	*Rhizobium alamii* VTr19A	96%	LC107315.1
37a	*Rhizobium* sp.	98%	GU433533.1
42a	*Rhizobium multihospitium* CCBAU 31043	96%	GU433534.1
43b	*Rhizobium mesosinicum* CCBAU	96%	EU120732.1
77a	*Rhizobium pusense* MKS-02	99%	HF563598.1
79b	*Rhizobium pusense* MKS-02	99%	HF563598.1
91b	*Rhizobium* sp.	98%	KR400574.1
96a	*Rhizobium pusense* MKS-02	99%	HF563598.1
98b	*Rhizobium tropici* VAm17A	99%	LC107304.1
99b	*Variovorax paradoxus*	92%	CP003911.1
A2	*Bradyrhizobium japonicum* SCAUs36	100%	KP219178.1

† National Centre for Biotechnology Information (USA)

3.3.2 16s rRNA phylogenetic analysis of plant growth promoting bacteria

A high number of isolates that had close similarity to plant growth promoting bacteria (PGPB) were also isolated from cowpea nodules. The neighbour joining 16s rRNA phylogenetic tree (Fig. 3c) clustered the PGPB into three groups. The first group (G1) makes up 84% of all the isolates of PGPB. These isolates clustered at bootstrap support of 98%, and had 99-100% identity to three species within the genus *Bacillus* namely: *Bacillus megaterium, Bacillus aryabhattai and Bacillus flexus* (Fig. 3c and Table 3.4). Only one strain of *Bacillus flexus* was isolated. Group 2 (G2) consisted of one isolate that had 99% identity to *Paenibacillus*

illinoisensis strain IHB. Five isolates clustered together to form group three and they had 99 – 100% similarity to diverse species distributed in five genera, namely: *Chryseobacterium taiwanense, Methylobacterium radiotolerans, Stenotrophomonas rhizophila, Burkholderia cepacia* and *Mitsuaria chitosanitabida* (Fig. 3c).

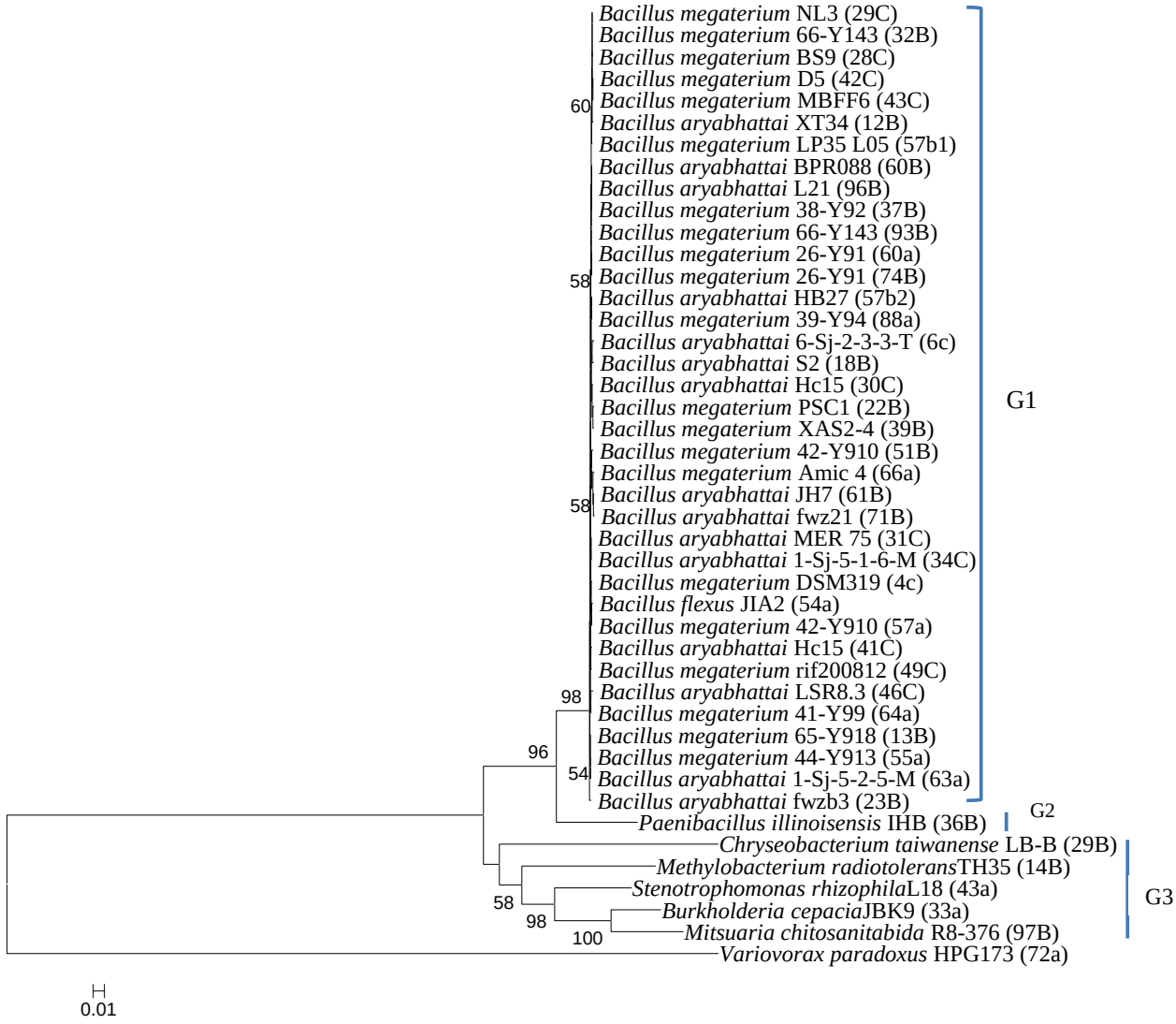

Fig. 3c: Neighbour joining tree of 16S rRNA sequences showing phylogenetic relationships of isolates of plant growth promoting bacteria found in cowpea nodules in soils from seven geographic regions of Kenya. Bootstrap values greater than 50% are shown on the corresponding nodes. The values shown in brackets are strain laboratory codes which represent farms where samples were collected. The scale represents nucleotide substitutions per site.

Table 3.4: Identification of plant growth promoting bacteria in six geographic regions of Kenya based on 16s rRNA sequences found in National Centre for Biotechnology Information (NCBI) GenBank

Strain code	Closest species and strain name in NCBI	Accession number	% Similarity
4C	*Bacillus megaterium* DSM319	CP001982.1	100
6C	*Bacillus aryabhattai* 6-Sj-2-3-3-T	KJ009550.1	100
12B	*Bacillus aryabhattai* XT34	KP797990.1	99
13B	*Bacillus megaterium* 65-Y918	KU647258.1	99
14B	*Methylobacterium radiotolerans* TH35	LC026010.1	99
18B	*Bacillus aryabhattai* S2	KX158860.1	99
22B	*Bacillus megaterium* PSC1	KU196781.1	100
23B	*Bacillus aryabhattai* fwzb3	KF208486.1	99
28C	*Bacillus megaterium* BS9	KR063189.1	99
29B	*Chryseobacterium taiwanense* LB-B	AB495176.1	99
29C	*Bacillus megaterium* NL3	KU862862.1	100
30C	*Bacillus aryabhattai* Hc15	JF899293.1	100
31C	*Bacillus aryabhattai* MER_75	KT719649.1	99
32B	*Bacillus megaterium* 66-Y143	KU647259.1	99
33a	*Burkholderia cepacia* JBK9	CP013732.1	99
34C	*Bacillus aryabhattai* 1-Sj-5-1-6-M	KJ009458.1	100
37B	*Bacillus megaterium* 38-Y92	KU647231.1	100
39B	*Bacillus megaterium* XAS2-4	JF496306.1	99
41C	*Bacillus aryabhattai* Hc15	JF899293.1	100
42C	*Bacillus megaterium* D5	KC441754.1	100
43a	*Stenotrophomonas rhizophila* L18	JN700131.1	100
43C	*Bacillus megaterium* MBFF6	HQ840732.1	99
46C	*Bacillus aryabhattai* LSR8.3	KT718049.1	99
49C	Bacillus megaterium rif200812	FJ527647.1	99
51B	*Bacillus megaterium* 42-Y910	KU647235.1	100
54a	*Bacillus flexus*JIA2	KX607116.1	100
55a	*Bacillus megaterium* 44-Y913	KU647237.1	99
57a	*Bacillus megaterium* 42-Y910	KU647235.1	100
57b1	*Bacillus megaterium* LP35_L05	KM350269.1	100
57b2	*Bacillus aryabhattai* HB27	KM659228.1	99
60a	*Bacillus megaterium* 26-Y91	KU647219.1	99
60b	*Bacillus aryabhattai* BPR088	KU161294.1	99
61b	*Bacillus aryabhattai* JH7	KX230137.1	100
63a	*Bacillus aryabhattai* 1-Sj-5-2-5-M	KJ009467.1	100
64a	*Bacillus megaterium* 41-Y99	KU647234.1	99
66a	*Bacillus megaterium* Amic_4	KX228234.1	100
71b	*Bacillus aryabhattai* fwz21	KF208483.1	99
72a	*Variovorax paradoxus* HPG173	JQ291591.1	99
74b	*Bacillus megaterium*26-Y91	KU647219.1	100

Table 3.4 *cont*.

88a	*Bacillus megaterium*39-Y94	KU647232.1	100
93b	*Bacillus megaterium* 66-Y143	KU647259.1	100
96b	*Bacillus aryabhattai* L21	KU179335.1	99
97b	*Mitsuaria chitosanitabida* R8-376	JQ659937.1	99

3.3.3 Species distribution, richness and diversity indices of rhizobia and plant growth promoting bacteria

Species distribution, richness and diversity indices of isolates of rhizobia and plant growth promoting bacteria was assessed in the seven geographic regions of Kenya, based on 16s rRNA sequence analyses and BLAST results from NCBI GenBank (Table 3.5). Results of 16s rRNA BLAST were used due to better sequencing success compared to recA gene.

Generally, the isolates had close similarity to 26 species of rhizobia and PGPB, but 69% of them were isolated only in specific regions. *Brevibacillus brevis* was isolated only in Ainamoi sub-county of Kericho; *Rhizobium tibeticum, Paenibacillus typhae and Bradyrhizobium* sp. were isolated only at Kipsitet, Kericho; *Stenotrophomonas rhizophila and Mesorhizobium* sp. were found only in Bomet Central; *Rhizobium grahamii* was isolated in Nyamira North; *Rhizobium alamii, R. sullae, Mitsuaria chitosanitabida* and *Pseudacidovorax* sp. were isolated in Nyakach Central; *Rhizobium multihospitium, Labrys neptuniae, Burkholderia cepacia, Bacillus flexus* and *Variovorax paradoxus* were isolated only in Machakos, and finally *Methylobacterium radiotolerans* and *Chryseobacterium taiwanense* were isolated only in Kilifi, at the Kenyan coastal lowlands (Table 3.5). Nyakach Central and Machakos had higher species richness and diversity compared to any other region on Shannon's index, and also higher species diversity on Simpson's index over all regions except Kipsitet in Kericho County. The two regions also had

the highest number of localised species. Although Kipsitet had species richness of 8, it had higher species diversity, based on both indices, than Kilifi, which had species richness of 14. Bomet Central had the least species number and diversity in both Shannon's and Simpson's indices.

Table 3.5 Species distribution, species richness and diversity indices of bacterial isolates from cowpea plants in soils sampled from seven geographic regions of Kenya

	Geographic region						
Species†	**Kericho (Ainamoi)**	**Kericho (Kipsitet)**	**Bomet Central**	**Nyamira North (Ekerenyo)**	**Nyakach Central (Pap-Onditi)**	**Machakos (Mwala)**	**Kilifi (Fumbini)**
Rhizobium miluonense	1	0	1	0	0	0	3
Rhizobium pusense	1	1	1	1	1	3	0
Rhizobium tropici	2	0	0	0	1	1	2
Rhizobium sp.	0	0	0	0	1	1	3
Rhizobium multihospitium	0	0	0	0	0	1	0
Labrys neptuniae	0	0	0	0	0	1	0
Rhizobium grahamii	0	0	0	1	0	0	0
Rhizobium alamii	0	0	0	0	1	0	0
Rhizobium sullae	0	0	0	0	1	0	0
Brevibacillus brevis	1	0	0	0	0	0	0
Mitsuaria chitosanitabida	0	0	0	0	2	0	0
Rhizobium phaseoli	0	1	0	0	1	0	0
Pseudacidovorax sp.	0	0	0	0	1	0	0
Bosea sp.	0	1	0	0	1	0	0
Rhizobium tibeticum	0	1	0	0	0	0	0
Paenibacillus typhae	0	1	0	0	0	0	0
Bradyrhizobium sp.	0	1	0	0	0	0	0
Mesorhizobium sp.	0	0	1	0	0	0	0
Bacillus megaterium	1	1	0	1	5	4	4
Bacillus aryabhattai	1	1	0	2	4	2	0
Methylobacterium radiotolerans	0	0	0	0	0	0	1
Chryseobacterium taiwanense	0	0	0	0	0	0	1
Burkholderia cepacia	0	0	0	0	0	1	0
Stenotrophomonas rhizophila	0	0	1	0	0	0	0
Bacillus flexus	0	0	0	0	0	1	0
Variovorax paradoxus	0	0	0	0	0	1	0
Species richness	7	8	4	5	19	16	14
Shannon diversity index	1.75	2.08	1.09	1.33	2.15	2.13	1.67
Simpson's index of diversity	0.82	0.88	0.56	0.72	0.85	0.86	0.8

† Species names based on 16s rRNA National Centre for Biotechnology information nucleotide BLAST results.

3.4 Discussion

The use of cultural and biochemical methods may complement molecular approaches of identifying rhizobia and other bacteria. Species from genus *Agrobacterium* and *Rhizobium* exhibited similar cultural characteristics in this study, and are known to be phylogenetically related (Deng *et al*., 1995). Ketolactose test was useful in distinguishing eight *Agrobacterium tumefasciens* (Syn. *Rhizobium radiobacter*) isolates from rhizobia, as these isolates had 99-100% sequence similarity to *Rhizobium* sp. in the NCBI Genbank. *Rhizobium pusense* also tested positive to ketolactose, which confirms its sequence similarity to *Rhizobium radiobacter* (Panday *et al*., 2011) or *Agrobacterium pusense* (Aguilar *et al*., 2016); it also grouped with *Agrobacterium* sp. in a 16s RNA phylogenetic tree.

Previous studies have shown that cowpea is commonly nodulated by slow growing alkaline producing species and strains within the genus *Bradyrhizobium (*Appunu *et al.,* 2009*;* Bejarano *et al.,* 2014*;* Sarr *et al.,* 2011*).* However, most of the isolates from cowpea nodules across all the geographic regions in the current study were predominantly in the genus *Rhizobium* and were fast growing and acid producing (bromothymol blue indicator turned yellow in culture media). Earlier research findings showed that 97% of cowpea nodulating rhizobia in parts of eastern Kenya were fast growing (Kimiti and Ondee, 2010). Isolation of fast growing strains of cowpea nodulating rhizobia has also been reported in Brazil (Silva *et al*., 2012) and China (Zhang *et al*., 2007). Cowpea was nodulated by nitrogen fixing bacteria distributed in genera *Rhizobium*, *Bradyrhizobium*, *Bosea*, and *Mesorhizobium*, which confirms reports of its symbiotic promiscuity (Guimarães *et al*., 2012; Jaramillo *et al*., 2013). The only commercial inoculant available for use in cowpea production in Kenya is *Bradyrhizobium* sp. USDA 3456, which is often inefficient in nitrogen fixation (Chemining'wa *et al*., 2007; Mathu *et al*., 2012). Given the symbiotic

promiscuity of cowpea, there may be need to test and select efficient strains of native cowpea rhizobia in Kenya across genera other than *Bradyrhizobium*.

Sequence analyses of other house - keeping genes in addition to 16s rRNA have been proposed for refining the phylogenetic analysis and taxonomy of rhizobia (Martens *et al*., 2008). The reason is that 16s rRNA gene may not give wide genetic diversity of rhizobia compared to genes such as recA, gyrB and atpD (Delamuta *et al*., 2012). In addition, it is highly conserved in some genera of rhizobia and has low rate of evolution (Azevedo *et al*., 2015). However, recA sequence failure was recorded in 16 isolates of rhizobia and selected PGPB in this study. Nonetheless, recA gene defined the species of isolate 30b as *Rhizobium tropici* and isolates 16c and 17c as *Bosea thiooxidants,* which had been classified only to the genus level in the 16s gene. This observation is in agreement with the view that 16s rRNA gene could be a limited phylogenetic marker due to its low resolution at the species level (Glaeser and Kampfer, 2015). Five isolates (23c, 42a, 43c, 11b and 99b) that had congruence in phylogenetic grouping in 16s rRNA and recA trees had incongruent taxonomic positions. Isolates 23c, 42a and 43c were all placed in the genus *Rhizobium* in the analysis of both genes, but species differences were distinct. Rec A gene has better species discrimination of closely related bacteria than 16s rRNA gene, as long as sequence homology to known species is over 94% (Zbinden *et al*., 2011). Although recA gene met this criterion, 16s rRNA gene had ≥ 99% sequence similarity for the three species, hence more house - keeping genes need to be analysed. The 16s rRNA gene is still useful in species identification of bacteria (Das *et al*., 2014); this may be true for isolates 11b and 99b which had 100% and 99% identity to *Labrys neptuniae* Liujia-146 and *Pseudacidovorax* sp. respectively. In recA gene, both isolates had 88% and 92% similarity to *Bradyrhizobium* sp. and *Vaviovorax paradoxus* respectively. Generally, phylogenetic grouping of most isolates was consistent in both 16s rRNA and recA trees, except 26b, 10c

and 91b. This observation indicates that genetic rearrangements could have occurred in the course of evolution (Laguerre *et al*., 2001).

The number of isolates that had close identity to plant growth promoting bacteria (PGPB) was unexpectedly high. Based on 16s rRNA gene, sequence similarity of all isolates to known species of PGPB in NCBI database was 99-100%, which is above the 98.65% threshold for species identification (Kim *et al*., 2014). Although PGPB were isolated from cowpea nodules, they are endophytic bacteria that are not known to fix nitrogen (Palaniappan *et al.*, 2010). However, the presence of nitrogen fixation –*nifH* gene in some strains of *Bacillus megaterium* and *Paenibacillus massiliensis* has been reported (Ding *et al*., 2005). Their entry into nodules is possibly via infection threads alongside rhizobia (Leite *et al*., 2017). Strains of *Bacillus megaterium* and *Bacillus aryabhattai* constituted about 84% of all isolates of PGPB, and grouped together in the 16s rRNA tree irrespective of geographic origin. Plant growth promoting bacteria are known to enhance plant growth through solubilisation of fixed phosphorous in soils, production of plant growth hormones such as indole -3- acetic acid (IAA), tolerance to abiotic stresses through the action of 1-aminocyclopropane - 1 - carboxylate (ACC) deaminase, production of siderophones associated with enhanced iron availability and enhancement of resistance to pathogens (Da Costa *et al*., 2016; De Souza *et al*., 2015; Vejan *et al*., 2016). *Bacillus megaterium* is known to produce IAA (Stajković *et al*., 2011) and, like *B. aryabhattai,* increases root elongation and shoot growth, which is associated with production of cytokinins (Ortíz-Castro *et al.*, 2008; Siddikii *et al*., 2010). Earlier research work on PGPB in Kenya showed that co-inoculation of *Bradyrhizobium japonicum* and *Bacillus subtilis* enhanced shoot dry matter of soybean (Atieno et al., 2012). Similar studies have shown that co-inoculation of *Bradyrhizobium* sp., *Paenibacillus graminis* and *Paenibacillus durus* increases symbiotic efficiency in cowpea (Rodrigues *et al*., 2013). A recent study (Korir *et al.*, 2017), confirmed

that co-inoculation of *B. megaterium, Paenibacillus polymyxa* and rhizobia enhances nodulation and shoot dry weight of common bean in Kenya. There is therefore need to conduct further studies on plant growth promoting potential of the isolates obtained from cowpea nodules, and select efficient strains for use in the manufacture of commercial inoculants.

Most of the species of rhizobia and PGPB were limited in their geographic distribution, except *Rhizobium miluonense, R. tropici, R. phaseoli, Bosea sp., Bacillus megaterium* and *B. aryabhattai*. These bacterial isolates need screening for potential use as commercial inoculants, since one of the considerations when screening strains for such use is wider ecological adaptation (Slattery and Pearce, 2002). Available phosphorous and pH were the two soil properties that had positive correlation with most species of rhizobia and PGPB in this study. Nyakach Central, a region which had the highest species numbers and diversity on Shannon's index had pH of 5.87 – 7.1 in five of six sampling sites. Furthermore, 56% of isolates of rhizobia and PGPB in this region were obtained in soils with the highest soil phosphorous levels of 72.97 – 126 ppm. Higher bacterial diversity is associated with pH closer to neutral (Xia *et al*., 2015), and the optimum growth of rhizobia has been recorded at pH of 6-8 (Bhargava *et al*., 2016). Adequate phosphorous nutrition is known to increase population of rhizobia and PGPB in soils (Fatima *et al.*, 2006). Some strains of rhizobia and PGPB are known to solubilise fixed P in soils (Qin *et al*., 2011) and this could also explain the abundance of P in Nyakach Central.

3.5 Conclusions

It was concluded that there is wide genetic diversity of rhizobia and plant growth promoting bacteria in the seven geographic regions of Kenya. Among all the bacterial isolates, *Rhizobium tropici, Bacillus megaterium and Bacillus aryabhattai* had wider geographic distribution. Soils that had the highest

species diversity of rhizobia and plant growth promoting bacteria were characterised by pH between 5.8 and 7.1, and high level of available phosphorous. Symbiotic efficiency of bacterial isolates needs to be determined and other housekeeping genes should be used to refine their phylogeny.

CHAPTER FOUR: ABUNDANCE AND SYMBIOTIC EFFICIENCY OF COWPEA (*Vigna unguiculata* L.) RHIZOBIA IN SEVEN AGRO-ECOLOGICAL ZONES OF KENYA

ABSTRACT

A study was conducted to establish the abundance and symbiotic efficiency of native rhizobial species in seven agro-ecological zones of Kenya. Using soil samples from 44 sites, abundance of rhizobia was determined by the plant infection technique with cowpea as a trap plant. A greenhouse experiment was conducted to evaluate the symbiotic efficiency of indigenous rhizobia (nodule number, nodule and shoot dry weight). The relationships between rhizobial abundance, symbiotic efficiency and soil chemical conditions were done using simple linear regression. Spearman's rank correlation coefficient was used to determine the relationship between rhizobial species and soil chemical conditions. Rhizobial species identified in chapter three were used for the correlation analyses. Results showed that 23% of the sampled sites had high abundance of indigenous cowpea rhizobia (> 1 x 10^3 cells g^{-1} soil), and two of the sites recorded the highest nodule numbers and dry weights. Abundance of rhizobia was positively correlated with soil pH, but had significant negative correlation with exchangeable aluminium (Al^{3+}) ($P<.0001$), total nitrogen (N) and organic carbon. Generally, 70% of soils with high abundance of rhizobia had moderate to neutral pH of 5.47 - 6.75, and Al levels below 0.21 cmol kg^{-1}. *Rhizobium miluonese* had a significant ($P\leq.05$) positive correlation with Al, suggesting that it may be tolerant to the element. There was a strong positive correlation between abundance of rhizobia and cowpea nodule numbers and dry weight. In general, sites that had less than 58 cells of rhizobia g^{-1} of soil exhibited low symbiotic efficiency. It was concluded that soils with low concentration of Al^{3+} and soil pH between 5.4 and 6.8 favoured the proliferation of rhizobia. The negative correlation between organic carbon and rhizobial population need to be investigated.

Keywords: Abundance, aluminium, cowpea, rhizobia, soil pH, symbiotic efficiency.

4.1 Introduction

Cowpea is an important crop in Kenya as a source of food and feed, and for its positive role in soil fertility improvement. However, its leaf and grain yields are low. The average grain yield per annum is about 0.5 t ha^{-1} (CPPMU, 2015) against a potential of 3 t ha^{-1} (Brink and Belay, 2006). Fresh Leaf yield of the crop is 2.6 t ha^{-1}, but the potential is 8.4 t ha^{-1} (AFA, 2015; Kabululu, 2008). Most farmers rarely apply inorganic fertilizers to increase cowpea yield, probably because it is known to be adapted to low input environments (Pule-Meulenberg *et al.,* 2010). However, soil fertility has been on the decline in Kenyan soils (Odendo *et al*., 2011), and this could be one of the reasons for the huge yield gap in cowpea production. One way of restoring soil fertility in cropping systems that incorporate cowpea plants is by focussing on enhancing the efficiency of legume-rhizobia symbioses, which are expected to yield surplus N for use by the host legume plant and other crops in rotation (Nebiyu *et al*., 2014). When native soil rhizobia are inefficient in nitrogen fixation, efficient strains may be introduced in form of commercial inocula. Nonetheless, previous studies show that inoculation of cowpea plants with commercial strains of rhizobia in most Kenyan soils does not increase cowpea yield (Chemining`wa *et al.*, 2007; Mathu *et al.*, 2012). Although there is an indication that abundance and nitrogen fixing potential of cowpea rhizobia in most of the crop's production areas of Kenya is high (Maingi et al., 2006; Mathu *et al.*, 2012), there is need to explore whether there are other soil factors that limit symbiotic efficiency of native cowpea rhizobia. Research work done in Eastern Kenya revealed that soil amendments with phosphorous and organic manure enhanced abundance and symbiotic efficiency of cowpea rhizobia (Kimiti and Ondee, 2010; Onduru *et al*., 2008). Furthermore, most of the soils in the study area are acidic (NAAIAP., 2014), a condition known to limit symbiotic efficiency of rhizobia (Ferguson *et al*., 2013). This study aims at filling the knowledge gap on the abiotic factors limiting

abundance, species distribution and nitrogen fixing potential of native cowpea rhizobia in Kenya. The study was conducted to determine the abundance and symbiotic efficiency of native rhizobia, and to determine the relationship between abundance, symbiotic traits and chemical properties in seven agro-ecological zones of Kenya.

4.2 Experimental site, soil sampling and analyses

Greenhouse experiments were conducted in 2014-2015 period at the University of Nairobi's Kabete Field Station to determine the population size and symbiotic efficiency of indigenous strains of cowpea nodulating rhizobia in soils sampled from seven agro-ecological zones (AEZs). The AEZs are distributed in six Counties of Kenya where cowpea is grown. A total of 44 soil samples were taken from 22 small holder farms. In each farm, soil samples were collected from two sites (with and without a history of cowpea cultivation). Soil sampling at each site was done randomly within a radius of 6 m at a depth of 20 cm using a soil auger, whereby a total of 24 soil cores were collected and mixed to obtain 2 kg of a composite sample (Maingi *et al*., 2006; Mwenda *et al*., 2011). The composite samples were immediately transported to the laboratory and stored at a temperature of 5^{o}C, and then chemical analyses were done before the onset of greenhouse experiments. Soil samples were analyzed for macronutrients (total N, available P, K, Ca and Mg), exchangeable Al, pH (H_2O), and organic carbon (%) using the procedures described previously (Okalebo *et al*., 2002).

Soil pH in the study area ranged from 4.11 at site N12 in Kilifi located in agro-ecological zone (AEZ) CL4 to 7.1 at site P1 in zone LM4. Mean total N ranged from 0.04% at site 5c in LH2 (Bomet County) to 0.97% in zone LH1 in Kericho County. Phosphorous (P) content ranged from 1.50 mg kg^{-1} at site N26 in Kericho County (AEZ LH1/UM2) to 101.36 mg kg^{-1}) at zone LM4. The Organic carbon levels ranged

from 0.33% in CL4 (Kilifi) to 4.19% in LH1 (Kericho). The sites varied in exchangeable ions; exchange Al^{3+} (0.06-0.84 cmol kg^{-1}), K^{+} (0.30-2.80 cmol kg^{-1}), Ca^{2+} (1.00-10.00 cmol kg^{-1}) and Mg^{2+} (0.40-3.05 cmol kg^{-1}) (Table 4.1).

4.3 Determination of population size and nodulation efficiency of rhizobia

Population size (abundance) of cowpea nodulating rhizobia in sampled soils was determined using the plant infection technique in 16 x 20 cm germination pouches obtained from Mega international, USA. The experiment was done under a glasshouse with an average day temperature of 28-30^{o}C. Seeds of cowpea variety K80 were surface sterilised and pre-germinated as per the procedures previously described (Kimiti and Ondee, 2010; Maingi *et al*., 2006). Cowpea seeds of uniform colour and size were selected, and then surface sterilised by immersion in 3% solution of sodium hypochlorite for five minutes and finally rinsed in eight changes of sterile distilled water. They were then sown lightly in wet sterile vermiculite contained in plastic tray and incubated at 28^{o}C for 48 hours. Seedlings with 1-1.5 cm long radicles were selected and transferred aseptically using a pair of forceps into germination pouches with nitrogen free nutrient solutions. The contents of nitrogen free solution were: $CoCl_2.6H_2O$ – 0.004 mg, H_3BO_3 – 2.86 mg, $MnCl_2.4H_2O$- 1.81mg, ZnSO4.7H_2O – 0.22 mg, $CuSO_4.5\ H_2O$- 0.08 mg, H_2MoO_4. H_2O - 0.09 mg, $MgSO_4.7\ H_2O$ - 492.96 mg, K_2HPO_4 -174.18 mg, KH_2PO_4 -136.09 mg, $CaCl_2$ -110.99 mg, $FeC_6H_5O_7$. H_2O – 5 mg and distilled water – 1 litre (Prevost and Antoun, 2006). Prior to the onset of the experiment, the glasshouse floor was disinfected using 0.5% sodium hypochlorite and 70% ethanol for working benches (https://ag.umass.edu/greenhouse-floriculture/fact-sheets/cleaning-disinfecting-greenhouse). One week after seed establishment in germination pouches, seedlings were inoculated using 1 ml of soil inoculum prepared from each soil sample following the protocols described earlier (Prevost and Antoun, 2006; Somasegaram and Hoben, 1994). A 10-fold dilution of each soil

sample was prepared by placing 10 g of soil into 90 ml of distilled water in a 500 ml conical flask, and then corked and dispersed for 10 minutes using a wrist motion shaker at 400 oscillations minute^{-1}. Dilution series from 10^{-1} – 10^{-6} were then made from each soil solution and 1 ml of soil solution from each dilution level was inoculated in quadruplicate into the root zone of cowpea seedlings grown in germination pouches. A positive control (inoculation with *Bradyrhizobium* sp. USDA 3456) and a negative control (without inoculation and with N fertiliser - 0.75g L^{-1} of KNO_3) was also included. The *Bradyrhizobium* inoculant was obtained from Mea Ltd, Kenya.

4.3.1 Crop management and data collection

The levels of nutrient solutions in growth pouches were monitored daily and maintained at optimum concentrations at the plant's root zone. Data collected were: population of rhizobia in cells gram^{-1} of soil, nodule number and nodule dry weight. Population of rhizobia in all soils sampled was determined as described by Somasegaran and Hoben (1994). Four weeks after inoculation, plant roots were scored for presence or absence of nodules. The scores obtained were checked against those on the most probable number (MPN) table for 10-fold dilution to obtain the most likely number (m). The population of rhizobia or the most probable number (MPN) of rhizobial cells g^{-1} of soil in a particular soil was finally calculated using the formulae: $\frac{mxd}{v}$, where: m is the most likely number obtained from MPN table, d is the reciprocal of the lowest dilution used, and v is volume of soil diluent inoculated in each pouch. Nodule numbers and weights were determined in plants that received the lowest dilution (10^{-1}). Nodules were separated from roots and counted, put in khaki papers and oven-dried at a temperature of 60^{o}C for 72 hours before dry weight determination

4.4 Species of rhizobia used for correlation analyses

Bacterial isolates that had sequence identity of 98% -100% to known nitrogen fixing species of rhizobia from previous diversity study (Chapter three if this thesis) were selected for correlation analysis. The species selected were: *Rhizobium miluonense, Rhizobium tropici, Rhizobium multihospitium, Rhizobium grahamii, Rhizobium alamii, Rhizobium phaseoli, Rhizobium tibeticum, Rhizobium* sp. and *Bosea* sp. Isolates identified as *Mesorhizobium* sp. and *Bradyrhizobium* sp were excluded because they grouped with plant growth promoting bacteria in the 16S rRNA phylogenetic tree.

4.5 Statistical analyses

Data collected (nodule numbers, nodule dry weight and shoot dry weight) were subjected to analysis of variance (ANOVA) using Genstat statistical software (16^{th} edition, VSN International, U.K). Means were compared using Tukey's test at $P \leq .05$. Relationship between abundance of rhizobia and nodule numbers, nodule dry weights, shoot dry weights and soil chemical characteristics were determined using simple linear regression in Sigma plot version 10.0.0 (Systat software Inc., USA). Correlation between rhizobial species and soil chemical conditions was determined using Spearman's rank correlation coefficient (r_s) in Genstat software (16^{th} edition, VSN International, UK). Two tailed t- test was run to evaluate significance of relationship at 5% level of significance.

4.6 Results

4.6.1 Abundance and symbiotic efficiency of cowpea nodulating rhizobia

Ten sites had high population of rhizobia (over 1.0×10^3 cells g^{-1} of soil), half of which had no known history of legume cultivation (Table 4.1 and 4.2). The highest population of cowpea rhizobia (1.0×10^5 cells g^{-1} soil) was recorded at AEZ UM4 in Machakos County. Cowpea plants grown in this soil had

high nodule numbers but low nodule and shoot dry weights (Table 4.2). Site N18 in AEZ LM4 (Kisumu County) was one of the ten sites with high population of rhizobia. Cowpea plants grown in its soil had the highest nodule numbers and nodule dry weights (48 nodules $plant^{-1}$ and 71.75 mg $plant^{-1}$ respectively), and relatively high shoot dry matter (Table 4.2). Although site N15 in zone LM4 had the highest cowpea shoot dry weight, it had moderate abundance of rhizobia (580 cells $gram^{-1}$ of soil) in spite of its cowpea cultivation history (Table 4.2). Sixty three percent of sites with high abundance of rhizobial cells, above 1.0×10^3, had moderately acidic to neutral soils pH of 5.47- 6.75 and Al levels below 0.20 cmol kg^{-1} of soil; 50% of the same sites had no history of legume cultivation (Table 4.1 and 4.2). Cowpea nodulating rhizobia were not detected in four sites, two of which were located in AEZ LH1 in Kericho County, and had strongly acidic soils (pH 4.8 and 4.9) and also high Al^{3+} content (0.70 and 0.75 cmol kg^{-1} of soil) (Table 4.1 and 4.2). In general, sites that had undetectable levels to 58 cells of rhizobia g^{-1} of soil exhibited low symbiotic efficiencies (low nodule numbers, nodule and shoot dry weights) (Table 4.2).

Table 4.1: Geographical description, legume cultivation history and soil chemical characteristics of the study area

Site code	County	Agro-ecological zone[†]	Legume cultivation History	pH	%O.C	%N	P (mg kg^{-1})	K (cmol kg^{-1})	Ca (cmol kg^{-1})	Mg (cmol kg^{-1})	Al (cmol kg^{-1})
1c	Bomet	LH2	None	5.26	2.57	0.31	8.85	0.80	2.80	0.93	0.30
2c	Bomet	LH2	Beans, Lucerne	5.58	3.23	0.90	77.48	2.30	7.40	2.15	0.32
3c	Bomet	LH3	Beans	5.26	2.70	0.31	14.36	1.75	5.60	1.87	0.60
4c	Bomet	LH3	None	6.37	2.65	0.16	11.6	2.80	8.00	3.05	0.10
5c	Bomet	LH2	Beans	5.49	2.82	0.04	13.53	2.05	6.00	1.60	0.20
6c	Bomet	LH2	None	5.91	3.43	0.50	8.05	2.30	3.20	0.65	0.18
7c	Nyamira	UM2	Cowpea	5.20	2.82	0.25	9.51	0.75	2.50	0.83	0.50
8c	Nyamira	UM2	None	5.72	3.25	0.20	16.02	2.80	8.00	2.67	0.30
9c	Nyamira	UM2	Cowpea	4.97	2.90	0.28	6.51	1.05	3.80	1.30	0.84
10c	Nyamira	UM2	None	5.60	3.82	0.39	11.69	1.70	4.50	1.67	0.21
11c	Nyamira	UM2	Cowpea	6.32	3.53	0.24	19.50	0.40	4.30	1.60	0.22
12c	Nyamira	UM2	None	5.10	2.60	0.17	6.84	0.60	1.80	0.47	0.70
13c	Kericho	LH1	None	4.85	4.19	0.42	8.18	0.60	2.10	0.50	0.75
14c	Kericho	LH1	Cowpea, beans	4.89	2.80	0.77	9.96	1.05	4.40	1.54	0.70
15c	Kericho	LH1	None	4.80	4.04	0.97	8.68	1.70	5.40	2.00	0.75
N19	Kericho	UM2	None	6.19	3.20	0.12	17.2	0.50	4.21	1.21	0.30
N20	Kericho	UM2	Beans, cowpea	5.47	3.53	0.28	8.51	1.60	5.00	1.40	0.10
N21	Kericho	UM2	None	5.74	2.50	0.27	31.44	0.80	3.40	1.93	0.20
N22	Kericho	UM2	Cowpea, beans	6.68	2.70	0.16	20.87	2.70	7.40	3.10	0.42
N23	Kericho	UM2	Cowpea, beans	6.47	2.67	0.27	30.25	2.00	6.80	2.26	0.15
N24	Kericho	UM2	None	5.77	1.90	0.17	15.20	1.05	3.30	0.95	0.36
N25	Kericho	LH1/UM2[‡]	Cowpea, beans	5.30	3.08	0.42	10.35	2.55	7.60	2.10	0.19
N26	Kericho	LH1/UM2	None	5.30	3.45	0.11	1.50	1.85	5.40	1.80	0.44
N27	Kericho	UM2	None	5.72	3.54	0.29	9.15	2.70	7.00	2.33	0.30
P1	Kisumu	LM4	None	7.10	1.89	0.24	126.00	2.80	10.00	3.89	0.45
P2	Kisumu	LM4	Cowpea, beans, green gram, groundnut	5.87	1.14	0.14	72.97	2.05	6.20	2.15	0.15
N15	Kisumu	LM4	Cowpea, green grams, beans	6.00	1.52	0.15	101.36	1.70	5.50	1.83	0.15
N16	Kisumu	LM4	None	6.75	1.77	0.18	2.68	2.40	8.40	3.00	0.15

Table 4.1 cont'd

N17	Kisumu	LM4	Cowpea, ground nuts, crotalaria	5.02	0.97	0.15	35.4	0.60	2.00	0.76	0.65
N18	Kisumu	LM4	None	6.12	0.60	0.11	8.10	0.40	1.50	0.50	0.20
S1	Machakos	UM4	None	5.47	2.60	0.13	5.01	1.05	3.00	0.86	0.21
S2	Machakos	UM4	Cowpea, pigeon pea	6.50	2.00	0.13	5.01	1.30	3.80	1.27	0.21
S3	Machakos	UM4	None	6.20	1.80	0.13	10.10	1.20	2.90	1.04	0.07
S4	Machakos	UM4	Cowpea	5.21	2.56	0.08	22.38	0.95	2.70	0.90	0.21
S5	Machakos	UM4	Cowpea, pigeon pea	6.62	2.60	0.15	89.68	1.80	5.00	1.67	0.11
S6	Machakos	UM4	None	5.86	2.40	0.15	42.75	0.90	4.70	2.00	0.06
N7	Kilifi	CL4	Cowpea	6.87	1.20	0.11	26.20	0.50	1.40	0.50	0.15
N8	Kilifi	CL4	None	6.28	0.91	0.11	12.25	1.00	3.20	1.30	0.44
N9	Kilifi	CL4	None	6.61	0.75	0.10	2.20	0.60	1.90	0.51	0.15
N10	Kilifi	CL4	Cowpea	5.75	0.50	0.07	11.69	0.50	1.90	0.63	0.10
N11	Kilifi	CL4	Cowpea	4.19	0.43	0.07	5.01	0.50	1.40	0.55	1.20
N12	Kilifi	CL4	None	4.11	0.33	0.10	10.85	1.40	4.00	1.33	1.20
N13	Kilifi	CL4	Cowpea	4.94	0.47	0.07	4.32	0.40	1.20	0.40	0.88
N14	Kilifi	CL4	Cowpea	5.45	0.61	0.05	41.75	0.30	1.00	0.44	0.26

† LH- lower highland, UM- upper midland, LM- lower midland, CL- coastal lowland, source: (Jaetzold *et al*., 2006; Jaetzold *et al*., 2009; Jaetzold *et al*., 2010; Jaetzold *et al*., 2012); ‡ Transitional zone between LH1 and UM2; OC – organic carbon

Table 4.2: Site differences in nodule numbers, nodule dry weight, and shoot dry weights of cowpea and abundance of native soil rhizobia in a greenhouse experiment conducted at the University of Nairobi's Kabete Field Station in 2015, using soils sampled from 44 sites distributed in seven agro-ecological zones of Kenya.

Site code	AEZ[†]/County	Nodule number plant^{-1}	Nodule dry matter (mg plant^{-1})	Shoot dry matter (g plant^{-1})	Abundance (cells g^{-1} soil[‡])
1C	LH2- Bomet	1.50_{f}	3.75_{efg}	1.13_{g}	6
2C	LH2- Bomet	3.00_{ef}	5.75_{efg}	1.40_{fg}	31
3C	LH3- Bomet	3.00_{ef}	5.75_{efg}	1.43_{efg}	17
4C	LH3- Bomet	32.38_{ab}	58.75_{ab}	3.40_{abcdef}	1.0×10^4
5C	LH2- Bomet	1.00_{f}	0.75_{g}	2.00_{cdefg}	Undetected
6C	LH2- Bomet	27.75_{abcd}	17.25_{cdefg}	1.60_{defg}	1.7×10^4
14C	LH1- Kericho	1.75_{f}	1.00_{g}	$2.70_{abcdefg}$	Undetected
15C	LH1- Kericho	1.25_{f}	1.25_{g}	2.37_{bcdefg}	Undetected
N19	UM2- Kericho	16.62_{bcdef}	41.50_{abcde}	3.33_{abcdef}	100
N20	UM2- Kericho	28.25_{abc}	23.25_{bcdefg}	2.33_{bcdefg}	1.7×10^3
N21	UM2- Kericho	22.75_{bcdef}	10.75_{defg}	3.90_{abc}	58
N22	UM2- Kericho	14.25_{bcdef}	20.25_{cdefg}	$3.10_{abcdefg}$	58
N23	UM2- Kericho	4.88_{cdef}	41.00_{abcde}	$3.17_{abcdefg}$	3.1×10^2
N24	UM2- Kericho	6.12_{cdef}	40.00_{abcde}	4.30_{ab}	58
N25	LH1/UM2- Kericho	19.62_{bcdef}	15.50_{defg}	3.83_{abc}	1.0×10^3
N26	LH1/UM2- Kericho	10.62_{bcdef}	44.00_{abcd}	3.33_{abcdef}	5.8×10^2
N27	UM2- Kericho	11.00_{bcdef}	10.25_{defg}	$2.60_{abcdefg}$	1.0×10^3
N10	CL4- Kilifi	16.00_{bcdef}	39.50_{abcdef}	2.43_{bcdefg}	310
N11	CL4- Kilifi	25.38_{abcde}	$36.25_{abcdefg}$	$2.50_{abcdefg}$	1.7×10^3
N12	CL4- Kilifi	14.12_{bcdef}	14.50_{defg}	$2.90_{abcdefg}$	58
N13	CL4- Kilifi	4.38_{def}	29.50_{bcdefg}	$3.10_{abcdefg}$	58
N14	CL4- Kilifi	19.50_{bcdef}	$37.00_{abcdefg}$	$3.23_{abcdefg}$	100
N7	CL4- Kilifi	13.88_{bcdef}	53.75_{abc}	3.60_{abcde}	3.1×10^2
N8	CL4- Kilifi	25.50_{abcde}	46.25_{abcd}	3.93_{abc}	3.1×10^4
N9	CL4- Kilifi	13.12_{bcdef}	39.50_{abcdef}	2.33_{bcdefg}	1.7×10^2
N15	LM4- Kisumu	7.38_{cdef}	44.75_{abcd}	4.63_{a}	5.8×10^2
N16	LM4- Kisumu	48.75_{a}	12.00_{defg}	3.63_{abcd}	5.8×10^3
N17	LM4- Kisumu	21.25_{bcdef}	39.50_{abcdef}	$3.03_{abcdefg}$	5.8×10^3
N18	LM4- Kisumu	48.38_{a}	71.75_{a}	$3.13_{abcdefg}$	3.1×10^4
P1	LM4- Kisumu	10.62_{bcdef}	21.75_{bcdefg}	1.50_{defg}	5.8×10^2
P2	LM4- Kisumu	19.62_{bcdef}	21.75_{bcdefg}	$2.53_{abcdefg}$	1.0×10^3

Table 4.2 cont'd

S1	UM4- Machakos	16.75_{bcdef}	22.50_{bcdefg}	$2.63_{abcdefg}$	1.0×10^3
S2	UM4- Machakos	24.50_{bcdef}	47.00_{abcd}	$3.20_{abcdefg}$	5.8×10^2
S3	UM4- Machakos	17.75_{bcdef}	33.25_{bcdefg}	$2.60_{abcdefg}$	5.8×10^2
S4	UM4- Machakos	27.12_{abcd}	29.75_{bcdefg}	$2.50_{abcdefg}$	1.7×10^3
S5	UM4- Machakos	22.00_{bcdef}	29.25_{bcdefg}	$3.17_{abcdefg}$	1.0×10^3
S6	UM4- Machakos	32.75_{ab}	28.75_{bcdefg}	1.77_{cdefg}	1.0×10^5
10C	UM2- Nyamira	4.75_{cdef}	10.50_{defg}	$3.03_{abcdefg}$	6
11C	UM2- Nyamira	6.00_{cdef}	14.00_{defg}	3.57_{abcdef}	6
12C	UM2- Nyamira	4.38_{def}	20.25_{cdefg}	3.50_{abcdef}	58
7C	UM2- Nyamira	5.75_{cdef}	9.75_{defg}	2.00_{cdefg}	6
8C	UM2- Nyamira	1.50_{f}	1.75_{fg}	$2.70_{abcdefg}$	Undetected
9C	UM2- Nyamira	5.75_{cdef}	9.75_{defg}	$2.70_{abcdefg}$	17

Means followed by the same subscript letter in a column are not significantly ($P \leq .05$) different according to Tukey's test, † Agro-Ecological Zone; ‡ low – less than 10^2 cells g^{-1} soil, moderate – 10^2 to 10^3 cells g^{-1} soil, high- over 10^3 cells g^{-1} soil (Drew *et al*., 2012).

4.6.2 Simple linear regression between abundance of rhizobia, nodulation traits and soil chemical conditions

The results of simple linear regression show that there were significant positive relationships between abundance of rhizobia and: nodule number (R^2= 0.67, P<.0001), nodule dry weight (R^2 = 0.41, P<.0001) and soil pH (R^2 = 0.10, P=.03) (Figure 4.1). There were significant negative relationships between abundance of rhizobia and exchangeable Al (R^2 = 0.10, P<.03), total N (R^2 = 0.11, P<.02) and organic carbon (R^2 = 0.12, P<.01) (Figure 4.1). A strong negative relationship between soil pH and exchangeable Al (R^2 = 0.51, P<.0001) and a weak negative relationship between pH and available P in soils (R^2 = 0.12, P<.018) were observed in the study (Figure 4.2).

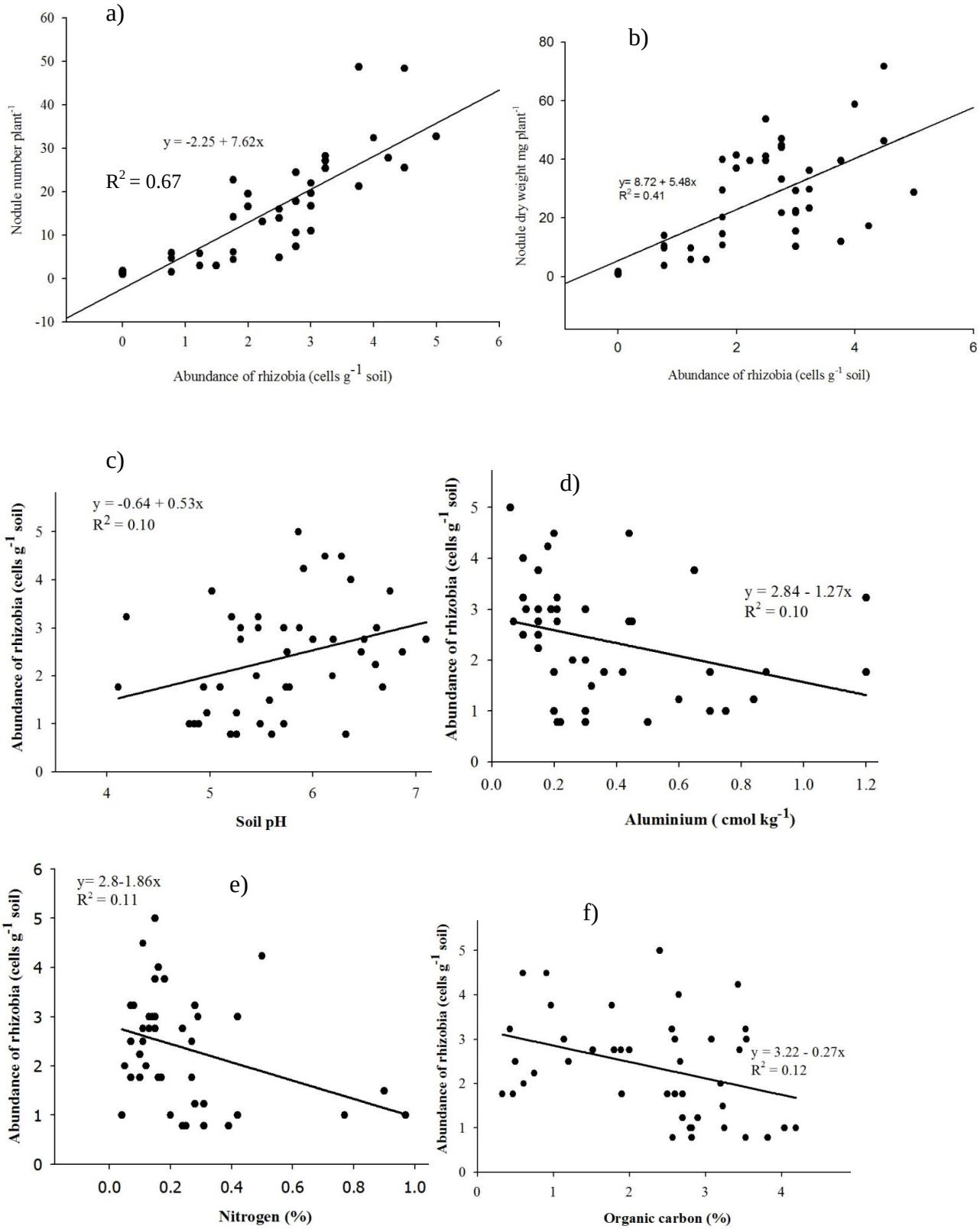

Figure 4.1: Simple linear regressions between abundance of rhizobia versus symbiotic traits and soil chemical conditions. The values on the X-Axis on Figure 4.1a and b, Y- axis on Figure 4.1c-f represent powers of ten of rhizobial cells ($10^0 – 10^5$).

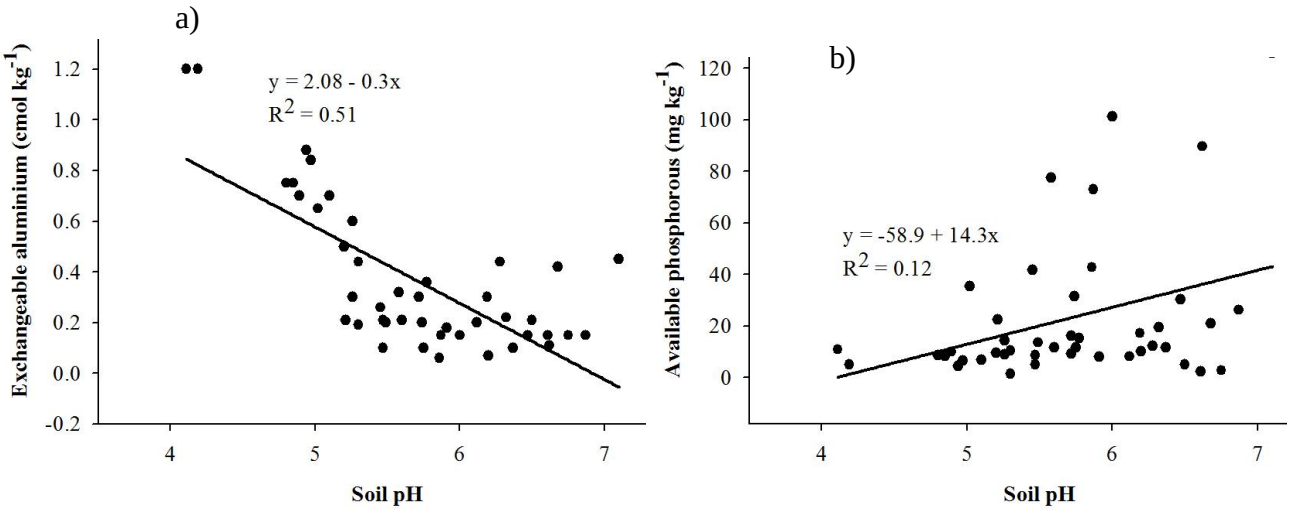

Figure 4.2: Simple linear regression between soil pH and exchangeable Al and available P

4.6.3 Correlation between soil chemical conditions and rhizobial species

There were significant positive correlations between exchangeable Al, pH, N, OC and occurrence of *Rhizobium miluonense*, *Bosea* sp. and *Rhizobium grahamii* respectively (Table 4.3). Available phosphorous (P) in soils also had significant positive correlations with *R. alamii* and *R. phaseoli*. There were significant negative correlations between exchangeable Al, N, available P and occurrence of *R. tibeticum*, *R. multihospitium*, *Rhizobium* sp. and *R. tropici* respectively (Table 4.3).

Table 4.3: Spearman's rank correlation coefficient between soil chemical characteristics and occurrence of native species of rhizobia in soils collected form 44 sites distributed in seven agro-ecological zones of Kenya

	Al	*B. sp*	N	OC	P	*R.ala*	*R. gr*	*R. ma*	*R.mil*	*R. mu*	*R. ph*	*R. sp*	*R. tro*
Al	1												
B. sp	0.02	1.00											
N	-0.12	-0.19	1.00										
O C	-0.10	-0.04	0.74	1.00									
P	0.00	-0.21	0.02	0.09	1.00								
R. ala	-0.31	-0.10	0.06	-0.08	0.42*	1.00							
R. gr	-0.08	-0.14	0.33*	0.33*	0.12	-0.10	1.00						
R. tib	-0.42*	-0.10	-0.03	0.03	-0.20	-0.07	-0.10	1.00					
R. mil	0.34*	-0.26	-0.09	-0.02	0.10	-0.17	-0.26	-0.17	1.00				
R. mu	0.31	-0.14	-0.41*	-0.21	0.00	-0.10	-0.14	-0.10	-0.26	1.00			
R. ph	0.10	-0.14	0.19	-0.08	0.33*	-0.10	0.43*	-0.10	-0.26	-0.14	1.00		
R. sp	0.08	-0.14	-0.08	-0.33	-0.37*	-0.10	-0.14	-0.10	-0.26	0.43*	-0.14	1.00	
R. tro	0.24	0.15	-0.12	-0.16	-0.40*	-0.17	-0.26	-0.17	0.42*	-0.26	-0.26	-0.26	1.00
pH	-0.81**	0.33*	0.04	0.09	0.01	0.14	0.08	0.31	-0.19	-0.45*	-0.12	-0.04	-0.37*

B. sp – Bosea sp, OC- organic carbon, *R. ala - Rhizobium alamii, R.gr- Rhizobium grahamii, R.tib – Rhizobium tibeticum, R. mil- Rhizobium miluonense, R. mu- Rhizobium multihospitium, R. ph- Rhizobium phaseoli, R.sp – Rhizobium sp, R.tro – Rhizobium tropici;* ** correlation significant at $p<0.001$, * correlation significant at $p\leq0.05$.

4.7 Discussions

Site N18 which is located in AEZ LM4 in Kisumu County had high abundance of rhizobia (3.1×10^4) in its soil, which possibly led to the observed high nodule numbers and nodule dry matter. High abundance of rhizobia in soils is associated with enhanced symbiotic efficiency (Mathu et al., 2012), as demonstrated by the significant positive correlation between rhizobial abundance and nodule number and dry weight, respectively. Similar observations were reported in previous research work (Thrall *et al.*, 2007; Wongphatcharachai *et al.,* 2015). Although sites with lower abundance of rhizobia are expected to show low symbiotic efficiency (Argaw and Tsigie, 2015), site N15 that had 580 cells g^{-1} of soil recorded the highest shoot dry matter. The population of rhizobial strains in this site may possess high nodulation competitiveness and also high nitrogen fixing potential (Mapfumo *et al.,* 2000). In contrast, site S6 at zone UM4 appeared to possess symbiotically inefficient strains of rhizobia since it had a high population of rhizobia but registered low nodule and shoot dry weight. Since soil physiochemical conditions in this

site (pH of 5.86 and low levels of Al^{3+}) were favourable for proliferation of rhizobia, absence of cowpea cultivation history may explain the low nodule and shoot weights in cowpea (Mothapo et al., 2013). In soils that had high rhizobial population of over 1000 cells g^{-1} of soil, high nodule occupancy with commercial inoculant application was reported (Hungria *et al*., 2003). Soils with similar rhizobial population recorded high grain yield with application of 40 kg N ha^{-1} (Argaw and Muleta, 2017), which further confirms that high abundance of rhizobia may not always enhance biological nitrogen fixation.

There were significant negative correlations between abundance of rhizobia and concentration of Al^{3+} in soils. Soil pH and abundance of rhizobia had significant positive correlation; therefore high levels of Al^{3+} and low soil pH were associated with depressed abundance of rhizobia in soils. The end result is low symbiotic efficiency of rhizobia because abundance of rhizobia was positively correlated with nodulation. Low pH is known to reduce the growth rate of rhizobia in soils which may lead to delayed nodulation and depressed nodule numbers (Ferreira *et al.,* 2016; Segundo *et al.,* 1999). The most probable cause of delayed nodulation in acidic soils is disruption of signal exchange between legumes and rhizobia (Ferguson *et al.,* 2013), more so in the initial step where the host legume and rhizobial strains must recognise each other so that nodulation process can begin (Nelson and Sadowsky, 2015). Acidic soils are associated with low nodule numbers, low population of rhizobia, poor plant growth and reduced activity of nitrogenase enzyme (Ferguson *et al.,* 2013; Lombardi *et al*., 2009; Rice *et al*., 2000). It was however observed that *Rhizobium tropici* had a negative correlation with soil pH, and may suggest that it is tolerant to the acidic conditions in some of the study sites. *Rhizobium tropici* is known to thrive well under both acidic and other environmental stresses (Ribeiro *et al.,* 2012; Riccillo *et al*., 2000; Santasup *et al*., 2001). On the other hand, very high pH may also be characterised by salinity and sodicity in soils, which can curtail nodulation and nitrogen fixation (L'taief *et al*., 2007; Rao *et al*.,

2002). Earlier authors reported optimum nodulation and cowpea growth at pH of 6.6-7.6 (Joe and Allen, 1980). However, *Sinorhizobium* sp. can tolerate alkaline soils (Zhang et al., 2011), while strains of *Bradyrhizobium japonicum* and *Rhizobium tropici* tolerant to low pH conditions have been identified (Indrasumunar *et al*., 2011; Morón *et al*., 2005).

Low soil pH is known to enhance solubility of Al^{3+} (Havlin *et al*., 2005). Aluminium ions inhibits plant root growth (Panda and Matsumoto, 2007) by binding to cell wall of plant root cells, causing rigidity and rupturing of the cells (Kopittke *et al*., 2008). In addition, it causes thickening of rhizobial infection threads, hence interfering with bacterial release from the threads (Sujkowska-Rybkowska *et al*., 2012). Aluminium ions inhibit growth of rhizobial cells (Paudyal *et al*., 2010) and consequently the population of rhizobia in soils decline (Andrade *et al*., 2002). The overall effect of high concentration of Al^{3+} is reduction in nodule numbers, nitrogen fixation and plant growth (Mendoza-Soto *et al*., 2015; Shamsuddin *et al*., 1992). Some authors have reported strains of common bean rhizobia tolerant to high concentrations of Al^{3+} (Avelar Ferreira *et al*., 2012), which is a potential research area. Correlation analysis in this study showed that *Rhizobium miluonense* can tolerate soils with high levels of Al. Isolation of Al tolerant rhizobia is of great significance in Kenya and tropical regions, since it's a major abiotic stress factor in their soils (Brunner and Sperisen, 2013). The use of Al tolerant rhizobial species in strongly acid soils has been suggested as one way of mitigating adverse effects of Al^{3+} on N_2 fixation (Jaiswal *et al.*, 2018). Liming and increasing the organic carbon content are other suggested management options in soils with high Al content (Andrade *et al*., 2002; Jaiswal *et al*., 2018).

Abundance of rhizobia in soils was also reduced in soils with high nitrogen content due to the negative correlation between soil N and rhizobial abundance. High soil nitrogen has been reported to decrease legume nodulation by rhizobia (Argaw and Tsigie, 2015; Vargas *et al*., 2000). Reduced nodule numbers are associated with low abundance of rhizobia in soils because rhizobial cells multiply in nodules and are then released into the soil upon nodule senescence (Denison and Kiers, 2011). Nitrate N is known to reduce nodule formation to a larger extend (Saito *et al*., 2014). The mechanisms behind reduction of nodulation by nitrate is possibly inhibition of synthesis of Nod gene - inducing flavonoids and reactive oxygen species (Van Noorden *et al.*, 2016). Reactive oxygen species is also thought to play a role in nodule development (Pauly *et al.*, 2006). Organic carbon had an inverse correlation with abundance of rhizobia in this study, which contradicts previous findings (Thrall *et al*., 2007). However, similar findings have been reported in a population of *Bradyrhizobium* sp. (Yan *et al*., 2014). Previous research work also showed that the population and symbiotic efficiency of rhizobia was high at soil organic carbon between 2-3%, but declined as organic carbon increased (Swanepoel *et al*., 2011). In the current study, there were sites with organic carbon content greater than 3%. Phosphorous content was not significantly correlated with abundance of rhizobia, which contradicts previous findings (Yan *et al*., 2014). This could suggest that P may only play a role in enhancing the physiological process of nitrogen fixation (Tang *et al*., 2001), but play a minimal role in enhancing proliferation of rhizobial communities in some soils. However, P content was positively correlated with the occurrence of *Rhizobium alamii* and *Rhizobium phaseoli*. This suggests that P effects are dependent on the species of rhizobia.

4.8 Conclusions

Abundance and symbiotic efficiency of rhizobia were high in moderate to slightly acidic soils with low concentration of Al^{3+}. *Rizobia miluonense* and *Rizobia tropici* may possess some level of tolerance to

Al^{3+} and soil acidity, respectively, which are the key abiotic stresses in tropical soils. Strains of both species should be screened for tolerance to Al and soil acidity. Contrary to previous findings, OC had inverse correlation with abundance and distribution of most rhizobial species.

CHAPTER FIVE: EFFECTS OF RHIZOBIA INOCULATION ON SYMBIOTIC TRAITS, GROWTH AND YIELD OF COWPEA (*Vigna unguiculata* L.) IN SOILS OF SOUTH WESTERN KENYA

Abstract

A field experiment was conducted to determine the effects of rhizobia inoculation and nitrogen (N) fertilizer on nodulation, growth, yield, N fixation and nodule occupancy of cowpea at moderate and strongly acidic soils of South Western Kenya. The experimental sites were located at Bomet central and Kericho East. Four cowpea varieties (K80, KVU 27-1, M66 and Ngor) received each of the following treatments: inoculation with *Bradyrhizobium* sp. USDA 3456 and N fertilizer (0 kg N ha^{-1}, 20 kg N ha^{-1}, 40 kg N ha^{-1}). N fertilizer served as experimental control. The experimental design used was randomized complete block design in a 4 x 4 factorial arrangement. N-fixed by cowpea plants in inoculated and untreated plots was determined using the ^{15}N natural abundance technique. Nodule occupancy was done by sequence analyses of 16S rRNA gene in bacterial isolates from cowpea nodules. Rhizobial inoculation significantly ($P \leq .05$) increased cowpea nodulation twice out of five sampling times in moderately acidic soils (Bomet central), but increased cowpea nodules only once in strongly acidic soils (Kericho East). *Bradyrhizobium* inoculation had no significant ($P \leq .05$) effects on growth, tissue N or on amount of N fixed at the experimental sites. The quantity N-fixed by the four cowpea varieties in the acid soils was between 9.8 - 19.8 mg N $plant^{-1}$, which was less than 2 kg N ha^{-1}. None of the bacterial isolates from cowpea nodules had similarity to inoculated *Bradyrhizobium* sp. or any species in *Rhizobiaceae* family, possibly due to antagonistic effects of nodule endophytes on rhizobia. Nodules were dominated by two species of endophytic plant growth promoting bacteria (PGPB): *Bacillus megaterium* and *Bacillus aryabhattai.* It was concluded that under the prevailing soil conditions in South

Western Kenya, cowpea plants do not respond to *Bradyrhizobium* inoculation, and amount of N-fixed by rhizobia is low. Two species of endophytic PGPB are predominant in the acid soils and their role in cowpea production need be determined.

Keywords: *Bradyrhizobium* inoculant, N-fixed, plant growth promoting bacteria, ^{15}N natural abundance

5.1 Introduction

Cowpea (*Vigna unguiculata* L.) is the second most important legume crop after common bean in Kenya (Fintrac, 2013). It is predominantly grown as an indigenous vegetable in regions located west of the Rift valley (CPPMU, 2015). Drought conditions and unpredictable rainfall patterns caused by climate change are associated with yield loss and crop failure in most areas of South western Kenya, which include Kericho county (Takeshi *et al*., 2017; Thornton, 2010). One of the mitigation strategies against climate change induced drought is growing of tolerant crops such as cowpea (Shiferaw *et al*., 2014). However, crop production in Western Kenya is limited by decline in soil fertility probably due to continuous cropping without replenishment of soil nutrient elements (Kimetu *et al.*, 2008; Odendo *et al*., 2011) Consequently, there has been deficiency of nitrogen (N) and phosphorous in these soils (NAAIAP, 2014). Crop production in Western Kenya could also be constrained by soil acidity, which is associated with aluminium toxicity and phosphorous deficiency (NAAIAP, 2014; Zheng, 2010). Soil acidity and phosphorous deficiency are known to reduce population size and symbiotic efficiency of rhizobia (Fatima et al., 2006; Ferguson et al., 2013). This may lead to decline in crop yields due to the role of rhizobia in N fixation.

Cowpea is a leguminous crop which form symbiotic associations with nitrogen fixing bacteria, which can fix about 150 kg N ha^{-1} in one cropping season (Pule-Meulenberg et al., 2010). Cowpea nodules also

host non rhizobial endophytes which may have plant growth promoting activities such as phosphate solubilisation, which can also boost crop yield (Leite et al., 2017). Nitrogen fixation by cowpea rhizobia can be optimized by crop inoculation with efficient strains of rhizobia (Kyei-Boahen *et al*., 2017). Currently, the only available commercial rhizobial inoculum of cowpea in Kenya is *Bradyrhizobium* sp. USDA 3456, marketed by MEA limited as Biofix. However, its efficacy in N_2 fixation in SW Kenya has not been well established. However, previous research findings in acidic soils of Central Kenya show that this strain may not be efficient in N-fixation (Chemining'wa *et al*., 2007), though the observations were only based on nodulation and growth data. Nodule occupancy tests based on restriction fragment length polymorphism (RFLP) of the 16S-23S rDNA region of rhizobial DNA, confirmed that *Bradyrhizobium* sp. strain USDA 3456 was inefficient in nodulation of cowpea in five geographic regions of Kenya (Mathu et al., 2012). However, the study did not report the rhizobial species dominant in cowpea nodules. The study objectives were to determine the effects of *Bradyrhizobium* inoculation on nodulation, growth, yield and N-fixation in cowpea, and to characterise the species of cowpea nodulating rhizobia in inoculated and un-inoculated plots at two sites in S.W Kenya.

5.2 Materials and methods

5.2.1 Experimental sites and soil analyses

A field experiment was conducted in two farms located at two sites in S.W Kenya (Agricultural Training Centre-ATC farm in Bomet Central and Nile heritage farm in Kericho East). The ATC farm is located at an altitude of 1920 m above the sea level. It receives an average annual rainfall of 1302 mm and its agro-ecological zone is LH2 (Jaetzold *et al*., 2010). Nile heritage farm is located at an altitude of 2182 m above the sea level with an average annual rainfall of 2090 mm and mean annual temperature of 17.2^{o}C. It is found in agro-ecologial zone LH1 (Jaetzold *et al*., 2010). Prior to field experiments, soil samples

were collected from field experimental sites at a depth of 20 cm following procedures described by Havlin et al. (2005). The samples were analyzed for pH (H_2O), organic carbon (%), total N (%) , available P (mg kg^{-1}), available K (cmol kg^{-1}) and also exchangeable Al (cmol kg^{-1}) using the protocols described by previous authors (Okalebo *et al.*, 2002). Population of cowpea rhizobia was also determined in the soil samples using the most probable number (MPN) plant infection technique in germination pouches (Somasegaram and Hoben, 1994), under glasshouse conditions at the University of Nairobi. Cowpea variety K80 was used as a trap plant for rhizobia in soils. Isolation of bacteria in cowpea nodules and molecular studies were done at the School of Biological Sciences, University of Reading (U.K).

Bomet Central (agricultural training centre farm) had the following soil chemical properties: pH 5.58 (moderately acidic), organic carbon 2.57%, total N 0.21%, available P 8.85 mg kg^{-1}, available K 0.80 cmol kg^{-1} and exchangeable Al 0.30 cmol kg^{-1} of soil. Rhizobial population was 6 cells g^{-1} of soil. In Kericho East, pH was 4.85 (strongly acidic), organic carbon 4.19%, total N 0.42%, available P 8.19 mg kg^{-1}, available K 0.60 cmol kg^{-1} and exchangeable Al was 0.75 cmol kg^{-1} of soil. Rhizobial cells were not detected by MPN plant infection method in the experimental site at Kericho East.

5.2.2 Treatments and field experimental design

Cowpea varieties M66, K80, KVU 27-1 and Ngor used in the study were chosen based on their ecological requirements: Variety M66 is grown in medium to higher altitudes, KVU 27-1 is grown in medium altitudes, K80 is a dry land variety (http://www.infonet-biovision.org/default /ct/120/crops), and Ngor is a local variety commonly grown by farmers in S.W Kenya. The four varieties are dual purpose (grown for grain and leaves). Each of the four cowpea genotypes received the following treatments:

rhizobia inoculation, 20 kg N ha^{-1}, 40 kg N ha^{-1} or untreated (neither rhizobia inoculation nor nitrogen fertilizer was supplied). The experimental design used was randomized complete block design in a factorial arrangement, and treatments were replicated three times. Nitrogen fertilizer was supplied in form of calcium ammonium nitrate, and peat based cowpea rhizobial inoculant (*Bradyrhizobium* sp. USDA 3456, Mea Ltd Kenya) was applied as a seed dress, at the rate of 0.2 kg ha^{-1}. The size of each experimental plot was 2.5 m x 2.5 m. Prior to planting, all plots received 25 kg ha^{-1} of P fertilizer (in form of triple superphosphate). Seed rate was 25 kg ha^{-1}, and plant spacing was 50 cm x 20 cm. Two seeds were placed per hill, but one plant was retained per hill after emergence. As a precaution to avoid cross contamination, plots that were inoculated with rhizobia received the treatment last. Crops were weeded using a hand hoe as from the 4^{th} week after emergence until its canopy could smother weeds. Agrochemicals that included Tata alpha® (lambda-cyhalothrin) and Oshothane® (mancozeb) were sprayed following manufacturer's instructions for crop protection against pests and diseases, respectively.

5.2.3 Field data collection and analysis of biological nitrogen fixation

The data collected were: number of active nodules, nodule dry weight (mg $plant^{-1}$), leaf area index (LAI), shoot dry weight (g $plant^{-1}$), shoot and grain nitrogen content (%), proportion of nitrogen derived from atmosphere (Ndfa), nitrogen fixed (mg $plant^{-1}$) and grain yield (tons ha^{-1}). Data collection was done at 50% flowering stage (10 weeks after emergence) and pod filling stage (16 weeks after crop emergence). In Kericho East, data was collected beginning from the 6^{th} week after emergence in short rains season of 2012, due to predicted crop loss as a result of supernormal rains. During the data collection date, six plants were selected at random from the inner rows of each plot, shoots were separated from roots at crown level and oven dried at 60^{o}C for 72 hours, and then shoot dry matter was

determined. Immediately after harvesting the shoots, cowpea root cores (6.5 cm in diameter and 15 cm deep) (Chemining'wa and Vessey, 2006) were taken using a soil corer. A total of six cores were taken per plot and transported to the laboratory, where the soil was carefully removed with flowing water, and then nodules were removed. Active nodules with pink colour were counted, and then oven dried at 60^{o}C to constant weight and dry weight determined. Leaf area index (LAI) was determined at 50% flowering stage of plants, using the cork borer method (Law-Ogbomo and Remison, 2008), where leaf discs were punched using a cork borer, and the relationship between area and dry weight of the disc was used to determine the leaf area. The leaf area was divided by the ground area occupied by a plant in the field to determine LAI. The oven dried plant shoots harvested at the flowering stage (Hue *et al*., 2000) were analysed for tissue N using Kjeldahl method (Muñoz-Huerta *et al*., 2013). Grain samples were analysed for total N using the same procedure. All the pods from the three inner rows were harvested at the end of the season, shelled and oven dried at 60^{o}C for 72 hours before weighing to obtain grain yield.

During the early pod filling stage in the short rains season of 2013, three plant shoots were sampled at random from three inner rows in plots that were inoculated with *Bradyrhizobium* sp. and control plots that did not receive any treatment, at both sites. *Zea mays* grown at adjacent plots were also sampled for use as reference plants for ^{15}N isotope analysis. Shoot samples were oven dried at a temperature of 60^{o}C until constant in weight, weighed and then finely ground. Half a gram of ground shoots of each sample was weighed into 5 ml Eppendorf tube and sent to the University of California Davis for determination of isotope ratios of $^{15}N/^{14}N$ and nitrogen content, using PDZ Europa ANCA-GSL elemental analyzer connected to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK). ^{15}N abundance ($\delta^{15}N$) was expressed in per mil (‰) relative to international standard (atmospheric nitrogen) as shown (Perkins *et al*., 2014): $\delta^{15}N$ (‰) = [($R_{sample}/R_{standard}$) -1]*1000, where R is $^{15}N/\ ^{14}N$. The

proportion of nitrogen derived from atmospheric nitrogen fixation in cowpea shoots was calculated using the equation: Ndfa (%) = 100 ($\delta^{15}N_{ref}$ - $\delta^{15}N_{fixing\ plant}$)/ ($\delta^{15}N_{ref}$ -*B)* (Boddey *et al*., 2001). $\delta^{15}N_{ref}$ is the ^{15}N natural abundance of the reference plant, $\delta^{15}N_{fixing\ plant}$ is the ^{15}N natural abundance of the N_2 fixing legume and *B* value is ^{15}N natural abundance of cowpea plant depending solely on N_2 fixation for nitrogen nutrition. The B value used in this study was -1.759 (Naab *et al*., 2009). The amount of nitrogen fixed in cowpea shoots was calculated as (Peoples *et al*., 2002): N-fixed = % Ndfa x shoot N content.

5.2.4 Nodule harvesting and isolation of symbiotic and endophytic bacteria from cowpea nodules

In the short rains season of 2013, nodules were harvested from the four cowpea varieties (K80, M66, KVU27-1 and Ngor) in field experiment plots at Kericho East and Bomet Central, when plants had attained 50% flowering stage. Nodule harvesting, sterilization and storage were done as described in previous studies (Mathu et al., 2012; Sarr et al., 2011; Vessey and Chemining'wa, 2006). Nodule samples were collected randomly from three plants within inner rows of plots that were inoculated with *Bradyrhizobium* sp. and also control plots with no treatment applied. Harvested nodules were washed, put in a cool box and immediately transferred to the Tea Research Institute of Kenya laboratory, where they were surface sterilized by immersion in 70% ethanol for 1 minute, then immersed in 3% sodium hypochlorite for 3 minutes and rinsed 6 times in sterile distilled water. Nodules from each of the four varieties in inoculated and untreated plots that had been replicated thrice at experimental sites were pooled and five representative samples were selected at random and stored in universal bottles with 40% glycerol at a temperature of -20^{o}C. A total of 40 nodules from each of the two experimental sites were stored. Nodule samples were then taken for isolation and DNA extraction of symbiotic and endophytic bacteria at the University of Reading (UK). During isolation, nodules were sterilized in 70% ethanol for

2 minutes and finally rinsed in 3 changes of nanopure water. Each nodule was crushed using a plastic pestle in an Eppendorf tube containing 100 µl of 40% glycerol and 20 µl of the resulting cell suspension was streaked onto yeast extract mannitol agar (YEMA) containing 0.025% Congo red dye (Mothapo et al., 2013; Somasegaram and Hoben, 1994), and incubated at 28^{o}C for 10 days. Bacterial colonies were not obtained from nodule samples from Kericho East. Twelve distinct colonies (based on colour, size and shape) isolated from Bomet Central were re-isolated on tryptone yeast (TY) agar (Beringer, 1974) and incubated at 28^{o}C for 2-4 days. Pure overnight cultures were then made by aseptically transferring single bacterial colonies with a loop from TY agar plates into 10 ml TY broth and incubating them at 27^{o}C on a rotary shaker at 200 rpm until they turned turbid (about 24-48 hours). The overnight cultures were used for DNA extraction.

5.2.5 DNA extraction and polymerase chain reaction

An overnight culture of bacteria was grown in TY broth, and used for DNA extraction using Gene Elute bacterial genomic DNA kit for gram positive bacteria (Sigma Aldrich Ltd). Quality of DNA was measured using nanodrop 1000 spectrophotometer (labtech Ltd, UK) and was within the required 260 nm/280 nm absorbance ratios of 1.7-2.0. Universal primers that target 16S rRNA gene of bacteria were subjected to 50 µl polymerase chain reaction (PCR) reactions that consisted of: 25 µl of 2x PCRBIO Taq Mix (PCR biosystems Ltd); 2 µl of each of the 10 µM forward and reverse primer; 5 µl DNA and 16 µl of nanopure water. Primer sequences and PCR conditions are shown (Table 5.1). The amplified PCR products (5 µl) were separated on 1% agarose gel stained with GelRed dye (Biotium, USA), run at 90 V for 40 minutes in TE buffer, and then finally visualized on Syngene G: BoxChemi XL Gel documentation system to confirm the success of PCR amplification. A 1 kb hyperladder was used as a

molecular weight marker (Bioline, UK). The PCR products were purified using QIAquick kit (Qiagen Ltd) before sequencing.

Table 5.1: Primers used in this study

Primer	Target gene	Sequence (5´- 3´)	PCR conditions	Reference
27F	16s rRNA	AGA GTTTGATCCTGGCTCAG	94^{o}C 5mins; 35 cycles (94^{o}C 40s, 65^{o}C 40s, 72^{o}C 1.5mins) and Final extension of 72^{o}C for 7 minutes	(Guimarães *et al*., 2012; Lane, 1991)
1492R	16s rRNA	GGTTA CCTTGTTACGACTT		

5.2.6 DNA sequencing, identification of isolates and phylogenetic analyses

Samples for sequencing were prepared as follows: 15 µl of each pure DNA sample obtained after PCR product purification was pipetted in duplicate into Eppendorf tubes; then DNA was mixed with 2 µl of a forward and reverse primer (Table 4.1) in the separate tubes. Samples were then sent for sequencing in both forward and reverse directions at Eurofins genomics (Germany). Forward and reverse nucleotide sequences of each DNA sample were aligned and edited for similarities using Bioedit software, version 7.2.5 (Hall, 1999). Identification of bacterial isolates was then done by submitting their edited sequences for comparison with National Centre for Biotechnology Information (NCBI) GenBank sequences, using nucleotide Basic Local Alignment Search Tool (BLASTN). Phylogenetic analyses were then done in MEGA 6 software (Tamura *et al*., 2013), where DNA sequences of bacterial isolates and reference strains were first aligned using MUSCLE (Edgar, 2004). Evolutionary history of the isolates were then inferred using maximum likelihood tree based on Kimura 2- parameter model (Kimura, 1980). A bootstrap confidence analysis (Felsenstein, 1985) was conducted with 1000 replicates.

5.2.7 Statistical analyses

Data collected from field were subjected to analysis of variance using GenStat statistical software, 16th edition (VSN International Ltd). Means were compared using Fischer's Protected LSD at 5% level of

significance.

5.3 Results

5.3.1 Effects of rhizobia inoculation and N fertilizer on active nodule numbers and dry weight of four cowpea varieties

Interactions between cowpea varieties and nitrogen sources were significant ($P \leq .05$) for nodulation parameters only at Bomet Central (Table 5.2). Application of 20 kg N ha^{-1} increased number of active nodules in cowpea variety K80 during the short rains of 2013 Rhizobia inoculation increased nodule dry weights in varieties K80 and K66 during the 2012 long and short rain seasons, respectively However, in the 2013 short rains season, untreated plants of the local variety Ngor and K80 had higher nodule dry weights than other treatments Application of 40 kg N ha^{-1} depressed nodule numbers and dry weights in variety Ngor and K66 in all the seasons.

Table 5.2: Influence of variety and soil amendments on nodule numbers and dry weight in a field experiment conducted in Bomet Central over three seasons between 2012 and 2013.

	Parameter, season, year and sampling time			
	active nodules plant^{-1}	Nodule dry weight in mg plant^{-1}		
Treatment	Short rains† - 2013 (10 WAE‡)	Long rains - 2012 (10WAE)	Short rains - 2012 (16WAE)	Short rains-2013 (10WAE)
K80 §	2.11_{bc}	19.0_{bc}	0.00_{d}	24.56_{a}
KVU 27-1§	0.39_{d}	17.22_{cd}	0.00_{d}	0.44_{c}
Ngor §	2.22_{bc}	15.67_{cde}	1.00_{d}	27.89_{a}
M66 §	0.39_{d}	7.78_{fgh}	2.56_{cd}	4.89_{c}
K80 + 20 kg N ha^{-1}	6.56_{a}	10.56_{efg}	1.78_{cd}	5.44_{c}
KVU 27-1 + 20 kg Nha^{-1}	0.11_{d}	7.67_{fgh}	1.22_{cd}	0.78_{c}
Ngor + 20 kg N ha^{-1}	2.56_{b}	3.11_{hi}	0.00_{d}	4.44_{c}
M66 + 20 kg N ha^{-1}	1.09_{bcd}	8.33_{fgh}	0.89_{d}	3.72_{c}
K80 + 40 kg N ha^{-1}	1.56_{bcd}	10.44_{efg}	1.89_{cd}	1.44_{c}
KVU 27-1 + 40 kg N ha^{-1}	0.89_{cd}	7.00_{fghi}	1.11_{cd}	0.78_{c}
Ngor + 40 kg N ha^{-1}	0.11_{d}	1.22_{i}	0.00_{d}	0.67_{c}
M66 + 40 kg N ha^{-1}	0.33_{d}	1.78_{i}	1.22_{cd}	0.67_{c}
K80 + Inoculation§§	2.39_{bc}	35.00_{a}	4.11_{bc}	7.44_{bc}
KVU 27-1 + Inoculation	1.44_{bcd}	23.89_{b}	7.11_{b}	1.56_{c}
Ngor + Inoculation	1.61_{bcd}	12.78def	1.44cd	1.89_{c}
M66 + Inoculation	2.28_{bc}	6.11ghi	10.22a	15.11_{b}
Mean	1.63	11.72	2.16	6.36
P value	*P*<.001	*P*<.001	*P*=.004	*P*<.001
$LSD_{0.05}$	1.52	5.80	3.08	8.86
CV (%)	12.20	29.70	27.50	25.60

† Short rains season extended into dry season of the following year; ‡ Weeks after crop emergence; § neither nitrogen fertilizer nor rhizobia inoculation was supplied; §§ inoculation with *Bradyrhizobium* sp. USDA 3456. Means followed by same letter within a column are not significantly different at $P \leq .05$ (Fischer's protected LSD test).

At Bomet Central, it was generally observed that rhizobia inoculation enhanced cowpea nodule numbers compared to the control in only two sampling times, both of which were in the short rains season. In four out of five sampling times, control and inoculated plots had plants with similar nodule numbers (Table 5.3). Rhizobia inoculation also enhanced nodule dry weights only during two sampling times at

the same site. At Kericho East, rhizobia inoculation enhanced cowpea nodule numbers compared to the control only during the short rains season of 2012, but had no significant effect on nodule dry weight (Table 5.4). Generally, application of 40 kg N ha^{-1} reduced nodule numbers and dry weight in cowpea plants in both sites during most of the sampling times (Table 5.3 and 5.4).

Table 5.3: Effects of rhizobia inoculation and nitrogen fertilizer on cowpea nodule numbers and dry weight in a field experiment conducted at Bomet Central during the 10th and 16th week after emergence (WAE) in three rain seasons between 2012 and 2013

	Active nodules $plant^{-1}$ (10WAE)			Active noduless $plant^{-1}$ (16WAE)		Nodule dry matter $plant^{-1}$ (10WAE)			Nodule dry matter $plant^{-1}$ (16WAE)	
Treatment	Long rains (2012)	Short rains (2012)	Short rains (2013)	Long rains (2012)	Short rains (2012)	Long rains (2012)	Short rains (2012)	Short rains (2013)	Long rains (2012)	Short rains (2012)
Control†	11.33_{ab}	2.69_{ab}	1.28_{bc}	8.86_{a}	0.61_{b}	14.92_{b}	4.62	14.11_{a}	67.58_{a}	0.89_{b}
20 kg N ha^{-1}	8.83_{b}	2.47_{b}	2.58_{a}	5.33_{ab}	0.89_{b}	7.42_{c}	4.85	3.60_{bc}	25.11_{b}	0.97_{b}
40 kg N ha^{-1}	7.81_{b}	1.81_{b}	0.72_{c}	3.25_{b}	0.75_{b}	5.11_{c}	1.39	0.89_{c}	16.08_{b}	1.06_{b}
Inoculation‡	14.33_{a}	4.14_{a}	1.93_{ab}	8.69_{a}	2.92_{a}	19.44_{a}	4.99	6.5_{b}	40.17_{b}	5.72_{a}
Mean	10.56	2.78	1.63	6.53	1.29	11.72	3.96	6.36	37.24	2.16
P value	*P*=.007	*P*=.02	*P*<.001	*P*=.006	*P*=.003	*P*<.001		*P*<.001	*P*=.002	*P*<.001
$LSD_{0.05}$	3.83	1.54	0.76	3.58	1.34	2.9	Ns	4.43	26.41	1.54
CV (%)	23.10	19.70	12.2	27.40	21.10	29.7	29.2	25.6	27.4	27.5

† Neither nitrogen fertilizer nor rhizobia inoculation was supplied; ‡ inoculation with *Bradyrhizobium* sp. USDA 3456. Means followed by same letter within a column are not significantly different at *P*<.05; Ns – treatment effects not significant at *P*≤.05.

Table 5.4: Effects of Rhizobia inoculation and nitrogen fertilizer on nodule numbers and dry weight of four cowpea varieties in a field experiment conducted in Kericho East over three seasons between 2012 and 2013

	Parameter, season, year and sampling time					
	Active nodules plant^{-1}			Nodule dry weight plant^{-1}		
Treatment	Long rains 2012 (10WAE)	Short[1] rains 2012 (6WAE)	Short rains 2013 (10WAE)	Long rains 2012 (10WAE)	Short rains 2012 (6WAE)	Short rains 2013 (10WAE)
Control	4.19	3.08_{b}[5]	2.08_{ab}	15.17_{a}	6.78_{ab}	3.72_{ab}
20 kg N ha^{-1}	2.14	1.36_{c}	1.56_{ab}	3.56_{b}	2.03_{b}	2.53_{bc}
40 kg N ha^{-1}	3.28	0.53_{c}	0.75_{b}	4.42_{b}	3.90_{ab}	1.06_{c}
Inoculation	5.31	4.69_{a}	2.81_{a}	18.11_{a}	8.69_{a}	5.31_{a}
Mean	3.73	2.42	1.80	10.32	5.35	3.15
P value		*P*<.001	*P*<.01	*P*<.001	*P*<.001	*P*<.01
LSD	NS	1.35	1.34	7.1	5.43	2.49
CV (%)	26.7	17.60	19.80	28.9	33.3	25.80

WAE – weeks after crop emergence, means followed by same letter within a column are not significantly different at *P*<.05 (Fischer's protected LSD test).

5.3.2 Nodulation characteristics of four cowpea varieties in a field experiment conducted in Bomet Central and Kericho East over three seasons between 2012 and 2013

In Bomet Central, cowpea variety K80 had significantly ($P \leq .05$) higher nodule numbers than other varieties during the long and short rains season of 2012 and 2013 respectively (Fig. 5.1). During the late pod filling stage (16 WAE) of the 2012 long rains season, all the varieties except M66 had similar number of active nodules. Cowpea variety M80 had higher nodule dry weight than the other three varieties during the 10th WAE in the 2012 long rains season (Fig. 5.2). However, the local variety (Ngor) had higher nodule dry weight than the other varieties during the 16th WAE of the same season (Fig. 5.2).

In Kericho East, there were no significant varietal differences in the number of active nodules over all the seasons (Fig. 5.3). The nodule dry weight of variety K80 was significantly lower than for other

varieties during the long rains season of 2012 (Fig. 5.4). In the 2012 short rains season, cowpea variety KVU 27-1 had significantly higher nodule dry weight than K80; variety Ngor had the least nodule weights in the 2013 short rains season (Fig. 5.4). In general, cowpea varieties K80 and KVU 27-1 had better nodulation in Bomet Central and Kericho East respectively. Nodulation was generally depressed during the short rains compared to the long rains season.

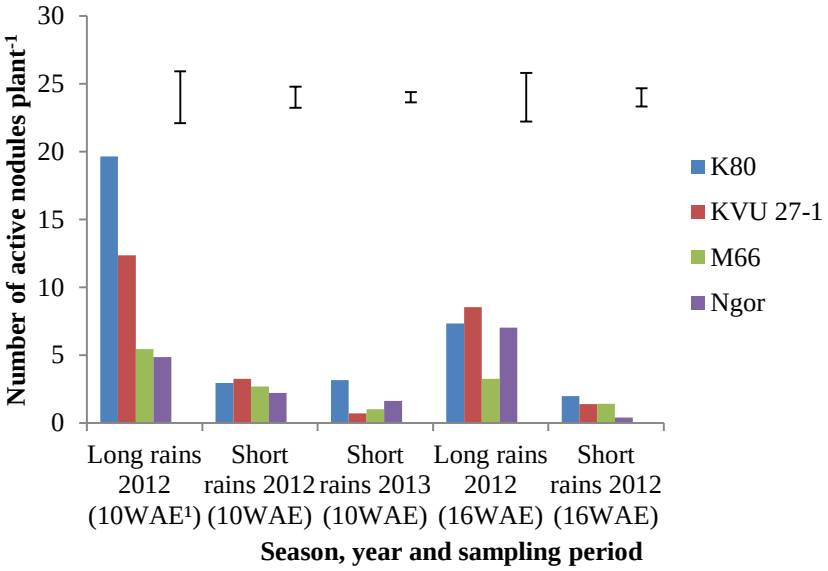

Fig. 5.1: Number of active nodules of four cowpea varieties in a field experiment conducted in Bomet Central over three seasons between 2012 and 2013. Error bars shows differences within varietal means at $P \leq .05$, according to Fischer's protected least significance difference (LSD) test. [1]Weeks after crop emergence

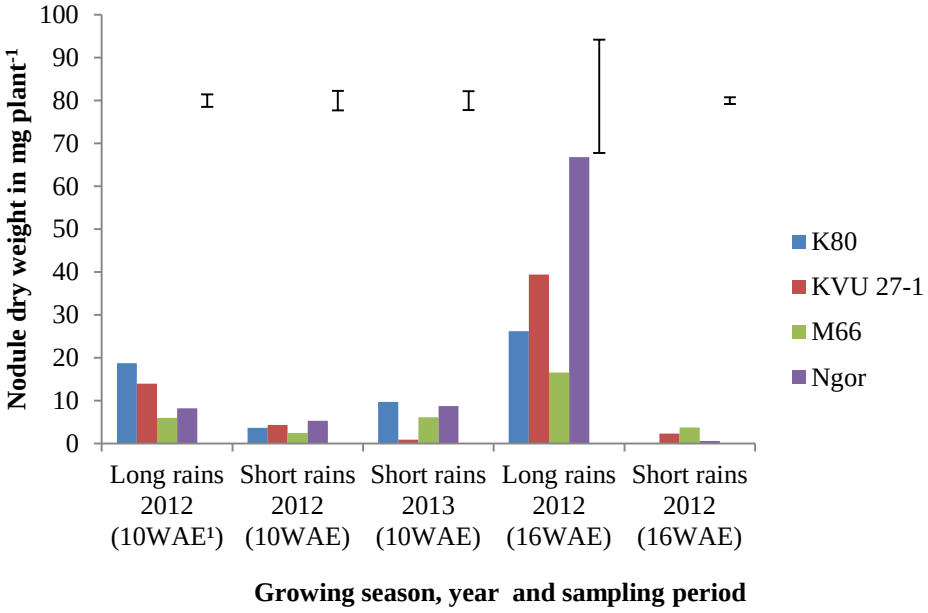

Fig 5.2: Nodule dry weights of four cowpea varieties in a field experiment conducted in Bomet Central over three seasons between 2012 and 2013. Error bars shows differences within varietal means at $P \leq .05$, according to Fischer's protected LSD test. [1]Weeks after crop emergence

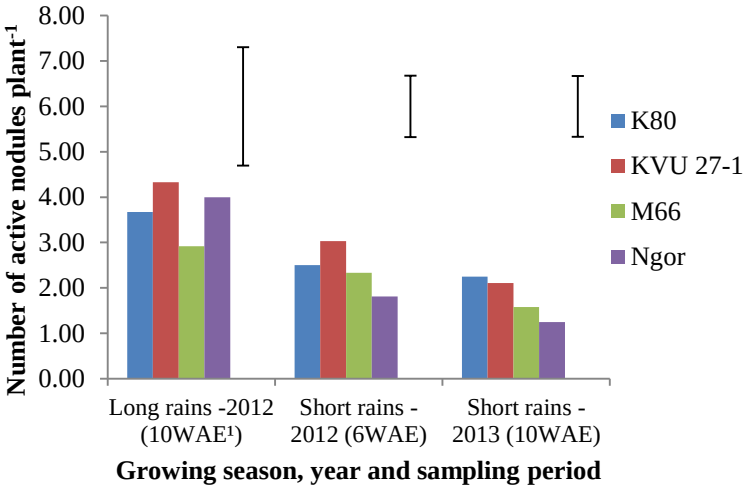

Fig 5.3: Number of active nodules of four cowpea varieties in a field experiment conducted in Kericho East over three seasons between 2012 and 2014. Error bars show differences within varietal means at $P \leq .05$, according to Fischer's protected LSD test. [1]Weeks after crop emergence

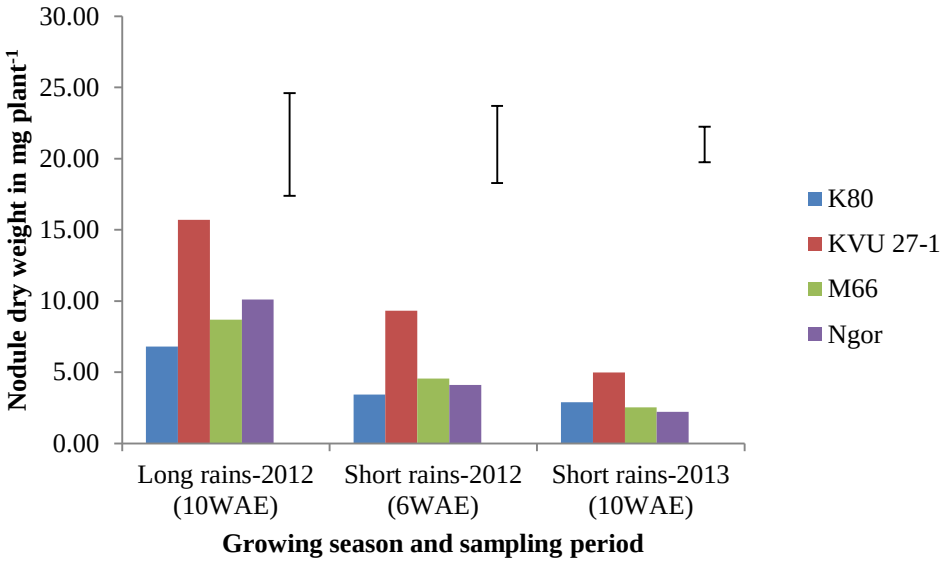

Fig 5.4: Nodule dry weights of four cowpea varieties in a field experiment conducted in Kericho East over three seasons between 2012 and 2013. Error bars shows differences within varietal means at $P \leq .05$, according to Fischer's protected least significance difference (LSD) test. [1]Weeks after crop emergence check labelling of the graphs

5.3.3 Effects of rhizobia inoculation and nitrogen fertilizer on growth, grain yield and tissue nitrogen of four cowpea varieties in Kericho East and Bomet Central

Rhizobia inoculation and nitrogen fertilizer had no significant ($P \leq .05$) effects on shoot dry matter of cowpea in both sites (Table 5.5). Most of the data on cowpea were not obtained in Kericho East, due to crop damage by hailstorms during the long rains season.

Table 5.5: Effects of rhizobia inoculation and nitrogen fertilizer on shoot dry weight of four cowpea varieties in a field experiment conducted in Bomet Central and Kericho East over three seasons between 2012 and 2013

	Site, sampling period and growing season						
	Bomet Central					Kericho East	
	10 WAE			16 WAE		10 WAE	
Treatment	Long rains (2012)	Short rains (2012)	Short rains (2013)	Long rains (2012)	Short rains (2012)	Long rains (2012)	Short rains (2013)
Control	15.69	10.57	5.13	47.50	13.48	0.92	2.14
20 kg N ha^{-1}	16.79	10.51	7.92	48.00	13.87	0.91	2.87
40 kg N ha^{-1}	15.00	13.46	5.28	39.10	17.42	0.91	2.18
Inoculation	17.07	8.93	5.96	36.10	14.15	1.11	2.70
Mean	16.14	10.87	6.07	42.68	14.73	0.96	2.47
$LSD_{0.05}$	Ns	Ns	Ns	Ns	Ns	Ns	Ns
CV (%)	13.5	13.6	16.3	9.6	13.6	14.2	16.3

Ns: Treatment effects were not significant

Rhizobia inoculation and nitrogen fertilizer had no significant effects on leaf area indices (LAI) and grain yield of cowpea in all seasons and in both sites (Table 5.6). There were no significant ($P \leq .05$) treatment effects on shoot and grain nitrogen content of cowpea plants in both sites (Table 5.7 and 5.8).

Table 5.6: Effects of rhizobia inoculation and nitrogen fertilizer on leaf area index and grain yield of four cowpea varieties in a field experiment conducted in Bomet Central and Kericho East over three seasons between 2012 and 2013

	Site, parameter, season and year						
	Bomet Central					Kericho East	
	LAI†			Grain yield (tons ha^{-1})		LAI	Grain yield (tons ha^{-1})
Treatment	Long rains (2012)	Short rains (2012)	Short rains (2013)	Long rains (2012)	Short rains (2013)	Short rains (2013)	Short rains (2013)
Control	0.72	0.14	0.53	0.85	1.17	0.21	0.27
20 kg N ha^{-1}	0.69	0.23	0.7	0.99	1.37	0.25	0.31
40 kg N ha^{-1}	0.57	0.16	0.46	1.02	1.48	0.18	0.23
Inoculation	0.60	0.15	0.68	1.16	1.47	0.32	0.28
Mean	0.65	0.17	0.59	1.01	1.37	0.24	0.27
LSD	Ns[5]	Ns	Ns	Ns	Ns	Ns	Ns
CV (%)	9.1	6.8	10.9	14.3	9.9	7.4	11

† Leaf area index

Table 5.7: Effects of rhizobia inoculation and nitrogen fertilizer on shoot and grain nitrogen content of four cowpea varieties in a field experiment conducted at Bomet Central during the short rains season of 2013

	Shoot nitrogen content					Grain nitrogen content				
	Variety (V)					Variety (V)				
N treatment	K80	KVU 27-1	Ngor	M66	Mean (N)	K80	KVU 27-1	Ngor	M66	Mean (N)
Control	0.80	0.77	0.82	0.82	0.80	1.87	1.69	2.08	1.59	1.81
20 kg N ha^{-1}	0.79	0.81	0.77	0.84	0.80	1.89	1.79	1.59	1.66	1.73
40 kg N ha^{-1}	0.86	0.76	0.79	0.79	0.80	1.96	1.79	2.00	1.86	1.90
Inoculation	0.79	0.84	0.78	0.86	0.82	1.96	1.81	1.62	2.11	1.87
Mean (V)	0.81	0.79	0.79	0.83		1.92	1.77	1.82	1.81	
$LSD_{0.05}$ (N)		Ns				Ns				
$LSD_{0.05}$ (V)		Ns				Ns				
$LSD_{0.05}$ (NxV)		Ns				Ns				
CV (%)		5.80				13.00				

Ns: Treatment effects not significant

Table 5.8: Effects of rhizobia inoculation and nitrogen fertilizer on shoot and grain nitrogen content of four cowpea varieties in a field experiment conducted in Kericho East during the short rains season of 2013

	Shoot nitrogen content (%)					Grain nitrogen content (%)				
	Variety (V)					Variety (V)				
N source	K80	KVU 27-1	Ngor	M66	Mean (N)	K80	KVU 27-1	Ngor	M66	Mean (N)
Control	0.85	0.80	0.86	0.85	0.84	1.72	1.88	1.86	1.46	1.73
20 kg N ha^{-1}	0.79	0.87	0.86	0.85	0.85	1.94	1.93	1.79	1.58	1.81
40 kg N ha^{-1}	0.81	0.77	0.86	0.85	0.82	1.65	1.85	1.93	1.83	1.81
Inoculation	0.88	0.87	0.84	0.86	0.86	2.01	1.74	1.73	1.71	1.80
Mean (V)	0.83	0.83	0.86	0.85		1.83	1.85	1.83	1.64	
$LSD_{0.05}$ (N)		Ns				Ns				
$LSD_{0.05}$ (V)		Ns				Ns				
$LSD_{0.05}$ (NxV)		Ns				Ns				
CV (%)		5.2				11.6				

5.3.4 Effects of rhizobia inoculation on biological nitrogen fixation in four cowpea varieties at Bomet Central and Kericho East

Rhizobia inoculation did not significantly ($P \leq .05$) increase the proportion of nitrogen derived from the atmosphere (Ndfa) or the amount of nitrogen fixed (N-fixed) in four cowpea varieties at both experimental sites (Table 5.9). The Ndfa ranged from 2.78 to 13.12% and 0 to 8.23% in Bomet central and Kericho East, respectively. The amount of N-fixed ranged from 6.84 to 29.92 mg plant^{-1} in Bomet and 0 to 22.93 mg plant^{-1} in Kericho East. It was, however, observed that there was no nitrogen fixation by rhizobia in inoculated plots with local variety of cowpea (Ngor) at Kericho East. It was further observed that the mean N-fixed by a cowpea plant was higher in Bomet Central than in Kericho East by 10.06 mg N plant^{-1} (Table 5.9).

Table 5.9: Influence of rhizobia inoculation on nitrogen derived from atmosphere (Ndfa) and nitrogen (N) fixed by four cowpea varieties in a field experiment conducted in Bomet Central and Kericho East during the short rains season of 2013

	Bomet Central		Kericho East	
Treatment	Ndfa (%)	N-fixed (mg plant^{-1})	Ndfa (%)	N-fixed (mg plant^{-1})
K80 - inoculation†	4.99	19.89	3.67	9.06
KVU 27-1- inoculation	2.84	6.84	2.05	3.84
M66 – inoculation	8.36	23.70	4.15	11.86
Ngor – inoculation	2.79	21.55	2.04	5.61
K80 + inoculation‡	5.93	29.92	4.18	16.32
KVU 27-1 + inoculation	2.78	14.23	8.23	22.93
M66 + inoculation	6.35	19.66	6.48	8.36
Ngor + inoculation	13.12	22.71	0.00	0.00
Mean	5.90	19.81	3.85	9.75
$LSD_{0.05}$ (variety x inoculation)	Ns	Ns	Ns	Ns

†- inoculation: plants were not inoculated with a commercial strain of rhizobia,
‡+ inoculation: plants inoculated with *Bradyrhizobium* sp. USDA 3456, Ns: treatment effects not significant at $P \leq .05$.

5.3.5 Phylogenetic affiliation of isolates of bacteria from cowpea nodules in field experimental sites

Twelve isolates that had close sequence similarity to 16 S rRNA sequences of plant growth promoting bacteria were obtained from nodules of three cowpea varieties, which were either inoculated or un-inoculated in Bomet Central. None of the isolates was obtained from nodules of cowpea variety K80 (Table 5.10, Fig. 5.5). There were no successful cultures of bacterial isolates from nodules harvested in Kericho East. Isolates obtained from nodules in Bomet Central had 99-100% sequence similarity to strains of *Bacillus megaterium, Bacillus aryabhattai or Bacillus altitudinis* (Table 5.10). None of the isolates had similarity to the *Bradyrhizobium* sp. inoculated to plants in the field experiment (Table 5.10). In a phylogenetic tree, six isolates grouped at 69% bootstrap support with four type strains of *B. megaterium* and *B. aryabhattai*; four isolates grouped at 76% bootstrap value to one type strain of *Bacillus altitudinis* and other reference strains of *B. megaterium and B. aryabhattai* obtained from NCBI GenBank; two isolates grouped with *Bacillus altitudinis* strain H82 at 88% bootstrap value (Fig. 5.5).

Table 5.10: Phylogenetic affiliation of bacterial isolates from nodules of three cowpea cultivars that received two treatments in a field experiment conducted at Bomet Central in Kenya during short rains season of 2013

Isolate name	Host cowpea variety	Treatment	Closest species and strain on NCBI nucleotide BLAST[1]	GenBank accession number	Similarity (%)
28c	M66	Inoculated†	*Bacillus megaterium* strain 66-Y143	KU647259.1	99
29c	M66	Inoculated	*Bacillus aryabhattai* strain JN5	KX399857.1	100
30c	M66	Inoculated	*Bacillus aryabhattai* strain Hc15	JF899293.1	100
31c	Ngor	Inoculated	*Bacillus megaterium* strain C414	KY515438.1	100
34c	Ngor	Inoculated	*Bacillus aryabhattai* strain 1-Sj-5-1-6-M	KJ009458.1	100
41c	KVU 27-1	Inoculated	*Bacillus aryabhattai* strain Hc15	JF899293.1	100
42c	M66	Control‡	*Bacillus megaterium* strain LNL6	GQ181059.1	100
43c	M66	Control	*Bacillus megaterium* strain MBFF6	HQ840732.1	99
44c	M66	Control	*Bacillus altitudinis* strain H82	KC934848.1	100
46c	Ngor	Control	*Bacillus aryabhattai* strain LSR8.3	KT718049.1	99
47c	Ngor	Control	*Bacillus altitudinis* strain CORSS02	MF425586.1	100
49c	KVU 27-1	Control	*Bacillus megaterium* strain rif200812	FJ527647.1	99

[1]Nucleotide blast (BLAST N) was done using 16s RNA gene sequences of bacterial isolates; † plants inoculated with *Bradyrhizobium* sp. USDA 3456; ‡ plants were not inoculated with commercial rhizobia.

5.4 Discussion

Inoculation of cowpea plants with commercial *Bradyrhizobium* enhanced nodule numbers and dry weights in cowpea plants, though not consistently in all sampling times during the research period. Increase in nodule numbers and dry weights in cowpea are in agreement with the work of other researchers (Farias *et al*., 2016; Kyei-Boahen *et al*., 2017). In Bomet Central, rhizobia inoculation enhanced nodule dry weights in two genotypes, K80 and M66. Varietal difference in legume nodulation has been reported previously (Pule-Meulenberg *et al*., 2010), and it is a trait that can be exploited in breeding programs for improving N_2 fixation. It was further observed that untreated cowpea local variety Ngor and improved variety K80 had high nodule dry weights during periods of low soil moisture (short rains season) in Bomet Central. This suggests that native rhizobia may have nodulation preference for the two varieties under moisture stress conditions. Although moisture stress is known to limit biological nitrogen fixation (Hossain *et al*., 2016), there are strains of cowpea rhizobia that have high nodulation efficiency under water limited conditions (Krasova-Wade *et al*., 2006). Nodulation efficiency in drought conditions could be attributed to high antioxidant and acid phosphatase activities in nodules (Mouradi *et al*., 2017). Varietal differences in cowpea nodulation were observed in the two experimental sites. Varieties K80 and KVU 27-1 appeared to nodulate better in Bomet Central and Kericho East, respectively. Variation in nodulation of cowpea under different ecological conditions was also reported in a study involving nine cowpea genotypes in diverse regions of Ghana and South Africa (Pule-Meulenberg *et al*., 2010). Bomet Central receives less rainfall and has higher mean annual temperature than Kericho East (Jaetzold *et al*., 2010), and that could explain why K80, which is a dry land variety nodulated better in this site. Application of 40 kg N ha^{-1} reduced nodule numbers and weight. High soil N is known to reduce nodulation and nitrogen fixation in cowpea and other legumes (Ayisi *et al*., 2000; Namvar *et al*., 2011; Sarr *et al*., 2015). Some of the possible reasons for reduced nodulation due to high N concentration could be inhibition of cell division in root cortex during initial stages of nodule

development (Gentili *et al*., 2006) and reduction in activity of nitrogenase enzyme in root nodules (Xia *et al*., 2017). It was however observed that 20 kg N ha^{-1} enhanced the number of active nodules during the short rains of 2013 in variety K80 at Bomet Central. Most researchers have reported that a small concentration of starter nitrogen fertilizer may enhance nodulation and legume plant growth (Argaw and Muleta, 2017; Brito et al., 2011a; Namvar et al., 2011). This is because legume plants may not meet their N requirements from biological nitrogen fixation alone (Salvagiotti et al., 2008). However, response of legume plants to starter nitrogen may be attained in soils with NO_3^-N levels below 20 kg N ha^{-1} (McKenzie *et al*., 2001).

Rhizobia inoculation and application of nitrogen fertilizer had no significant effects on shoot dry matter, leaf area index, grain yield and tissue N of cowpea plants in this study. Similar findings were reported in earlier studies done in seven geographic regions of Kenya (Chemining'wa et al., 2007; Mathu et al., 2012). It was further observed that rhizobia inoculation had no significant effects on Ndfa and N-fixed in this study, which may erroneously suggest that indigenous rhizobia could have been efficient in nitrogen fixation. However, the mean Ndfa value attained in the experimental sites was below 6%, which is very low compared to 83-98% reported in other agro-ecological zones of Kenya (Mathu et al., 2012). The amount of nitrogen fixed was on average less than 20 mg $plant^{-1}$, which was also low compared to 401-934 mg $plant^{-1}$ obtained in other regions of Africa (Pule-Meulenberg *et al*., 2010). These findings confirm that neither indigenous nor commercial cowpea rhizobia in the study area are efficient in nitrogen fixation. Although nitrogen content may have been sufficient for cowpea production in the experimental sites, the site with higher total N (0.42%) had plants with very low shoot dry matter and marginal grain yield. It is hypothesised therefore that low available P in both sites, and low pH coupled with higher levels of exchangeable Al^{3+} in Kericho East, could be responsible for low symbiotic

efficiency as it is in agreement with findings from a similar study (Ferguson *et al*., 2013). In previous research work where cowpea showed significant increase in nodulation, growth and grain yield in Kenya, soil pH was higher than 5.5 and soil was amended with P fertilizer (Kimiti and Odee, 2010; Onduru *et al*., 2008). Phosphorous is fixed by Al^{3+} in acid soils hence is unavailable for plant use (Havlin *et al*., 2005), yet its deficiency is known to reduce nodule numbers, nodule weight and nitrogen fixation (Jakobsen, 1985; Schulze and Drevon, 2005). Phosphorous has been reported to enhance cell division in early stages of nodule development (Gentili *et al*., 2006), which may explain why nodulation declines with its deficiency. Phosphorous deficiency may also be associated with decline in activity of nitrogenase enzyme (HØGh-Jensen *et al*., 2002). The most important role of P in N_2 fixation may be ATP synthesis, which generates chemical energy required for the physiological process (Havlin *et al*., 2005).

None of the cowpea nodules harvested from the two experimental sites was occupied by any known species of rhizobia. Since isotopic analysis showed evidence of nitrogen fixation in the two sites, there is likelihood that N_2 fixing rhizobia from the study sites did not grow on culture media. In nodules occupied by both rhizobia and endophytic bacteria, the latter has been reported to produce antagonistic compounds, or trigger the production of inhibitory compounds by the host legume that will negatively affect the growth of rhizobia (Muresu et al., 2008). The remedy would be direct sequencing of rhizobial DNA without first isolation in culture media, but the method would not capture bacterial diversity in nodules (Muresu et al., 2008). Alternatively, the nodule occupancy is very low for rhizobial strains in the study sites. Three species of plant growth promoting bacteria (PGPB), namely: *Bacillus aryabhattai, Bacillus altitudinis and B. megaterium* were isolated from cowpea nodules, which is consistent with findings from a similar study (Leite *et al*., 2017). Nitrogen fixing genes (NifH) have been isolated from

PGPB that include *Bacillus marisflavi, Paenibacillus massiliensis and Bacillus megaterium* in China (Ding *et al*., 2005). It is however unclear whether the isolates of PGPB in this study were involved in N_2 fixation. The PGPB isolated in this study serve several functions in crop production. *Bacillus megaterium* is known to solubilise phosphorous in soil (Elkoca *et al*., 2007). Apart from P solubilisation, *Bacillus altitudinis* produces indole acetic acid (IAA) which is a growth hormone, and siderophore (Sunar *et al*., 2015). The latter chelates iron and makes it available for microbial and plant use (Ahmed and Holmström, 2014). *Bacillus aryabhattai* promotes plant growth through production of growth hormones (IAA, gibberellic acid and cytokinin) and also enhances heat stress tolerance in plants (Park *et al*., 2017). In general, rhizobia and PGPB play synergistic roles in growth and development of legumes (Mishra *et al*., 2014).

5.5 Conclusions

Application of *Bradyrhizobium* inoculant and nitrogen fertilizer has no significant effects on growth, grain yield, tissue N or nitrogen fixation of cowpea in soils with similar physiochemical conditions with the two sites in South Western Kenya. Two bacterial species of cowpea nodule endophytes (*Bacillus megaterium* and *Bacillus aryabhattai*) are dominant in acid soils of South Western Kenya. There is need to establish whether PGPB plays a role in plant growth and nitrogen fixation of cowpea.

CHAPTER SIX: INFLUENCE OF P FERTILIZER AND LIMING ON NODULATION, GROWTH AND NUTRIENT CONTENT OF COWPEA (*Vigna unguiculata* L.) IN ACIDIC SOILS OF SOUTH WESTERN KENYA

Abstract

Cowpea production in South Western Kenya (SWK) is constrained by soil acidity which is associated with deficiency of phosphorous (P). Phosphorous deficiency limits nitrogen (N) fixing efficiency of rhizobia, crop growth and yield. A field experiment was conducted at Kericho East and Bomet Central in SWK, to determine the effects of liming and P fertilizer on nodulation, growth, yield and nutrient content of cowpea. Three cowpea varieties (KVU 27-1, M66 and Ngor) were each treated with: lime (0 t CaO ha^{-1} and 4 t CaO ha^{-1}) and P fertilizer (0 kg P ha^{-1}, 25 kg P ha^{-1} and 50 kg P ha^{-1}). A randomized complete block design in a 2 x 3 x 3 factorial arrangement was used for treatment layout. Data collected were: nodule number and weight, leaf area index, shoot dry weight, tissue N and protein content, shoot and grain N and P uptake and grain yield. Liming had no effects on cowpea nodulation, but enhanced grain N and P uptake and grain yield of variety KVU 27-1 at Kericho East. Application of 50 kg P ha^{-1} enhanced nodulation at both sites, growth of cowpea at Kericho East in all seasons and shoot protein content of variety KVU 27-1 at Bomet Central. However, KVU 27-1 had the highest grain protein content at Kericho East without P fertilizer. Application of 25 and 50 kg N ha^{-1} enhanced N and P uptake in cowpea. Grain yield of variety M66 was higher in plots without lime or P fertilizer at Bomet Central. Magnesium and K concentration in cowpea shoots were positively correlated with cowpea growth, protein content, N and P uptake. It was concluded that liming was not beneficial to nodulation at the study sites and may not be a requirement for cowpea production at Bomet Central. Phosphorous fertilizer enhanced most agronomic traits of cowpea in the study area.

Key words: cowpea varieties, lime, nutrient uptake, phosphorous nutrition, soil pH

6.1 Introduction

Cowpea is one of the most important legume crops in Kenya, and it is grown as a food crop whereas its leaves are used mainly as a vegetable (Saidi *et al*., 2010). It has a high nutritional value; its seed contains 23% protein and 57% carbohydrate, while the leaves contain 27 - 34% protein (Belane and Dakora, 2009). It is a potential export crop as its pods are currently being promoted for use as a vegetable in Eastern Europe (Karapanos *et al*., 2017). Integration of cowpea into existing cropping systems can enhance soil fertility as its rhizobia can fix up to 201 kg N ha^{-1} in a cropping season (IAEA, 2008), and the crop can leave a net fixed N deposit of 60-70 kg ha^{-1} in soils (Sigh *et al*., 2011). The annual production volume of cowpea leaves and grain in Kenya fluctuates depending on environmental conditions (CPPMU, 2011), but the production area is about 281,000 hectares (CPPMU, 2015). Although there is no published data on cowpea production level in individual counties of South Western Kenya (SWK) (Kericho, Bomet, Kisii, Nyamira and Homabay) and parts of Kisumu county, farmers produce cowpea as a vegetable for use especially during the dry season. Production of food crops that include cowpea in SWK is however constrained by low soil pH and phosphorous (P) deficiency (NAAIAP, 2014). One of the causes of P and other nutrient deficiencies in the region could be continuous cropping without replenishment of soil with external sources of fertilizer (Jaetzold *et al*., 2010). Soil acidity in SWK could be another cause of P deficiency, due to a possibility of this element being fixed by aluminium (Al^{3+}) or Iron (Fe^{3+}) in such soils (Havlin *et al*., 2005). Soil acidity is also known to adversely affect the survival and persistence of rhizobia, hence curtail their symbiotic efficiency (Appunu *et al*., 2009). Phosphorous deficiency can be corrected by application of inorganic P fertilizer or organic P resources such as rock phosphate, and soil liming. Lime contains Ca^{2+} and/or Mg^{2+} which displace Al^{3+} and Fe^{3+} hence P becomes available for plant use (Kisinyo *et al*., 2012). Similarly, molybdenum which enhances the activity of the nitrogenase enzyme in nodules becomes

available when acid soils are limed (Havlin *et al.*, 2005). Previous research work showed that combined application of 45 kg P ha^{-1} and *Bradyrhizobium* inoculant increased cowpea grain yield by 54% in mildly acidic soils of Eastern Kenya (Onduru *et al*., 2008). Maize plants grown at soil pH of 5.3 and supplied with 2 t ha^{-1} of lime and low rates of P fertilizer (30 kg P ha^{-1}) had the highest dry matter yield compared to those supplied with 100 kg P ha^{-1} at a similar lime level in Western Kenya (Opala., 2011). However, the optimum lime and P level for production of various cowpea genotypes under acidic soils of South Western Kenya has not been documented. The objective of this study was to determine the effects of liming and three levels of P fertilizer on nodulation, growth, grain yield and nutrient content of three cowpea varieties in acid soils of SWK.

6.2 Materials and methods

6.2.1 Experimental sites and soil analyses

A field experiment was conducted at two sites (Farmers Training Centre at Bomet Central and Kerego-Kericho East) located in SWK. Bomet Farmers Training Centre is located at an altitude of 1920 m above the sea level, receives an average annual rainfall of 1302 mm and its agro-ecological zone is LH2; Kerego is located at an altitude of 2182 m above the sea level, with an average annual rainfall of 2090 mm and mean annual temperature of 17.2^{o}C and lies in agro-ecological zone LH1 (Jaetzhold *et al.*, 2010). Before planting, soils were sampled at a depth of 20 cm from each site, and analysed for organic carbon, soil pH, total N, available P and exchangeable cations (K^{+}, Ca^{2+}, Mn^{2+}, Mg^{2+} and Al^{3+}) at the Soil Science Laboratory of the Faculty of Agriculture, University of Nairobi. Available P was analysed using Mehlich-1 method (Mehlich, 1953). Organic carbon was analysed using Walkley-Black method, total N was analysed using Kjeldahl method, and finally exchangeable cations were extracted using ammonium acetate as documented by Okalebo *et al*. (2002). Population of rhizobia was determined at

the experimental sites using the most probable number (MPN) plant infection technique in germination pouches under glasshouse conditions, at the Department of Plant Science and Crop protection (University of Nairobi), following protocols described previously (Somasegaram and Hoben, 1994).

Soil pH in the study sites was strongly and moderately acidic (Horneck *et al*., 2011) at Kericho East and Bomet Central, respectively (Table 6.1). Available P in the two sites was below the 20 mg kg^{-1} required for optimum crop production (Pierzynski, 2000); while mg levels of 0.47 cmol kg^{-1} in Kericho East, was considered low for crop production (Horneck *et al*., 2011). Rhizobial cells in soils of Kericho East were not detected by the MPN plant infection technique.

Table 6.1: Soil chemical characteristics and rhizobial population in the experimental sites

	Bomet Central (FTC†)	Kericho East (Nile heritage farm)	Critical level
pH (H_2O)	5.58	4.85	5.5-6.5 (Davis et al., 1991)
Organic carbon (%)	2.57	3.98	1.5 % (Joe and Allen, 1980)
Total N (%)	0.31	0.42	0.2 – 0.3% (Sichone and Mweetwa, 2018)
Available P (mg kg^{-1})	8.85	8.18	10-12 mg kg^{-1} (Sichone and Mweetwa, 2018)
Exchangeable K (cmol kg^{-1})	0.8	0.6	0.15 cmol kg^{-1} (NicodemusEzeh et al., 2007)
Exchangeable Ca (cmol kg^{-1})	2.8	2.1	2.6 cmol kg^{-1} (Adeleke and Akinrinde, 2011)
Exchangeable Mg (cmol kg^{-1})	0.93	0.47	0.26 cmol kg^{-1} (Adeleke and Akinrinde, 2011)
Exchangeable Na (cmol kg^{-1})	0.35	0.35	Less than 15% -ESP‡ (McIntyre, 1979)
Exchangeable Al (cmol kg^{-1})	0.3	0.75	1.0 cmol kg^{-1} (Simon et al., 2014)
Population of rhizobia	6 cells g^{-1} of soil	Undetected	100 cells g^{-1} of soil (Drew et al., 2012)

† Farmers Training Centre, ‡ Exchangeable sodium percent

6.2.2 Treatments and experimental design

The cowpea genotypes used in this study were M66, KVU 27-1 and Ngor. Variety M66 is a variety adapted to medium to high altitudes, KVU 27-1 is adapted to medium altitudes (http://www.infonet-biovision.org/PlantHealth/Crops/Cowpea). Ngor is a landrace commonly grown by farmers in the study

sites, and distributed through the local seed supply system. Each of the three cowpea genotypes was subjected to the following treatments: P fertilizer in form of triple superphosphate (TSP) at rates of 0 kg P ha^{-1}, 25 kg P ha^{-1} and 50 kg P ha^{-1}; liming with calcium oxide (CaO), at rates of 0 t ha^{-1} and 4 t ha^{-1}. Lime was applied two weeks before planting. The experimental design used was randomized complete block design in 2 x 3 x 3 factorial arrangement, and treatments were replicated three times. The size of each experimental plot was 2.5 m x 2.5 m. Prior to planting, all the plots received starter N fertilizer (in form of calcium ammonium nitrate) at the rate of 20 kg N ha^{-1}. Seed rate was 25 kg ha^{-1}, and seeds were sown at spacing of 50 cm x 20 cm.

6.2.3 Crop husbandry and data collection

Crops were weeded using a hand hoe as from the 4^{th} week after emergence until their canopies could smother weeds. Agrochemicals that included Tata alpha® (lambda-cyhalothrin) and Oshothane® (mancozeb) were sprayed following manufacturer's instructions for crop protection against pests and diseases which included aphids, and leaf spots. The data collected included: nodule numbers, nodule and shoot dry weight, leaf area index (LAI), shoot and grain N and P concentration, shoot and grain N and P uptake, shoot and grain protein content, and grain yield (kg ha^{-1}). Additional nutrient elements (K, Ca, Mg, Zn and Mn) on cowpea shoots were also analysed. Data collection was done at 50% flowering stage of the crop, except grain yield, N, P and protein content done after grain harvesting. Data on nodulation and growth parameters at Kericho East were collected at early vegetative stage (6^{th} week after emergence) in the long rains season of 2012 due to supernormal rains which were damaging to the crop.

Six plants were harvested above ground at random from the inner rows of each plot at each sampling period and put in paper bags. Immediately after harvesting above ground biomass, cowpea root cores

(6.5 cm in diameter and 15 cm deep) (Chemining'wa and Vessey, 2006) were taken using a hand hoe. A total of six root cores were taken per plot and transported to the laboratory alongside above ground biomass. Soil was carefully removed with flowing water, and roots were separated from the nodules. Active nodules with white-pink colour were counted, put in paper envelopes and oven dried alongside above ground biomass at a temperature of 60^{o}C to constant weight. Nodule and shoot dry weights were determined afterwards. Six cowpea shoots were sampled and their leaf area determined using the cork borer method (Law-Ogbomo and Remison, 2008), in which leaf discs were punched using a cork borer, and the relationship between area and dry weight of the discs used to estimate the leaf area. The leaf area was divided by the ground area occupied by the six plants in the field, to obtain the LAI. The oven dried plant shoots harvested at the 50% flowering stage (Hue *et al*., 2000) were used for plant tissue analyses. Shoot and grain analyses for N, P, K, Ca, Mg, Zn and Mn were done following procedures described previously (Okalebo *et al*., 2002). Cowpea N and P uptake was calculated by multiplying the total N and P concentration by the shoot dry weight (Opala., 2011). During crop harvest, 12 plants were selected at random from the three inner rows of each plot, their pods harvested, shelled and grains oven dried at 60^{o}C to 13 % moisture content. Grain weights were taken using an electronic balance.

6.2.4 Statistical analyses

Data collected were subjected to analysis of variance (ANOVA) using Genstat software 16th Edition (VSN International, U.K). Whenever treatment effects were significant, means were compared using Fischer's protected least significance difference test at $P \leq 0.05$. Correlation analyses between shoot nutrient content and agronomic parameters were done using Pearson's correlation coefficient (*r*).

6.3 Results

6.3.1 Effects of phosphorous (P) fertilizer and liming on nodulation and growth of three cowpea (*Vigna unguiculata* L.) varieties at Bomet Central and Kericho East

Agricultural lime, P fertilizer and cowpea variety interactions for nodule numbers, nodule and shoot dry weights were significant ($P \leq .05$) only during the long rains season of 2012 in Bomet Central (Table 6.2). Improved cowpea varieties (KVU 27-1 and M66) had the highest numbers of active nodules in unlimed plots supplied with 50 kg P ha^{-1}. However, application of 50 kg P ha^{-1} depressed nodule numbers in unlimed plots with variety Ngor (Table 5.2). The highest nodule dry weight was recorded on variety Ngor, in unlimed plots supplied with 25 kg P ha^{-1}. Compared to the control plots without P fertilizer, application of 50 kg P ha^{-1} consistently enhanced nodule numbers and dry weight in cowpea during all sampling periods at both Kericho East and Bomet Central (Tables 6.3 and 6.4). Lime application did not affect cowpea nodulation at both sites. In general, nodule numbers and dry weights were very low in Kericho East compared to Bomet Central (Tables 6.3 and 6.4). Application of 25 kg P ha^{-1} without liming enhanced shoot dry weight in cowpea variety M66 at Bomet Central during the long rains season of 2012 (Table 6.2). In general, application of 25 kg P ha^{-1} increased shoot dry weight in cowpea plants compared to other P rates at Bomet Central (Table 6.5). Phosphorous fertilizer had no significant effects on shoot dry matter of cowpea at Bomet Central during the rest of the growing seasons. Similarly, leaf area index (LAI) of cowpea plants was not enhanced by any treatment at the same site in all growing seasons. In Kericho East, plants supplied with 50 kg P ha^{-1} had consistently higher shoot dry matter and LAI than those not supplied with P fertilizer in all the seasons (Table 6.5). In addition, lime application increased shoot dry matter of cowpea plants at Kericho East during the 2012 short rains season (Table 6.6). However, lime, P fertilizer and variety interactions were not significant for shoot dry matter and LAI of cowpea at Kericho East.

Table 6.2: Influence of lime, phosphorous fertilizer and variety interactions on nodulation and shoot dry weight of cowpea in a field experiment conducted at Bomet Central during the long rains season of 2012

	Parameter		
Treatment	Nodule number plant^{-1}	Nodule dry weight mg plant^{-1}	Shoot dry weight (g plant^{-1})
0t ha^{-1} lime + 0 kg P ha^{-1} + KVU 27-1	11.56bc	31.67d	15.09cd
0t ha^{-1} lime + 0 kg P ha^{-1}+ M66	5.00c	24.00d	14.82cd
0t ha^{-1} lime + 0 kg P ha^{-1}+ Ngor	4.22c	10.56d	7.14g
0t ha^{-1} lime + 25 kg P ha^{-1}+ KVU 27-1	14.44abc	56.11bcd	17.53bc
0t ha^{-1} lime + 25 kg P ha^{-1} + M66	14.44abc	41.33cd	20.87a
0t ha^{-1} lime + 25 kg P ha^{-1} + Ngor	21.78ab	133.78a	20.38ab
0t ha^{-1} lime + 50 kg P ha^{-1} + KVU 27-1	27.44a	108.22abc	18.81ab
0t ha^{-1} lime + 50 kg P ha^{-1} + M66	27.78a	80.00abcd	13.21def
0t ha^{-1} lime + 50 kg P ha^{-1} + Ngor	5.78c	37.1 d	14.03de
4t ha^{-1} lime + 0 kg P ha^{-1} + KVU 27-1	7.11bc	11.89d	10.03fg
4t ha^{-1} lime + 0 kg P ha^{-1} + M66	11.33bc	22.57d	15.16cd
4t ha^{-1} lime + 0 kg P ha^{-1} + Ngor	7.67bc	33.56d	13.08def
4t ha^{-1} lime + 25 kg P ha^{-1} + KVU 27-1	18.00abc	67.44abcd	13.11def
4t ha^{-1} lime + 25 kg P ha^{-1} + M66	12.11bc	33.89d	20.37ab
4t ha^{-1} lime + 25 kg P ha^{-1} + Ngor	8.44bc	36.67d	17.83abc
4t ha^{-1} lime + 50 kg P ha^{-1} + KVU 27-1	9.67bc	24.56d	18.96ab
4t ha^{-1} lime + 50 kg P ha^{-1} + M66	16.22abc	38.67cd	11.36ef
4t ha^{-1} lime + 50 kg P ha^{-1} + Ngor	19.00abc	119.00ab	12.07def
Mean	13.40	50.60	15.21
P value	*P*=.02	*P*=.03	*P*=.003
$LSD_{0.05}$	14.89	70.14	3.27
CV (%)	20.70	21.80	13.00

Means followed by same letter in a column are not significantly different at $P \leq .05$ (Fischer's protected LSD test).

Table 6.3: Effects of phosphorous fertilizer on nodule numbers and weight during the 50% flowering stage of cowpea plants in a field experiment conducted at Bomet Central between 2012-13

	Active nodules plant^{-1}			Nodule dry weight (mg plant^{-1})		
Treatment	Long rains (2012)	Short rains (2012)	Short rains (2013)	Long rains (2012)	Short rains (2012)	Short rains (2013)
0 kg P ha^{-1}	7.81b	2.96b	3.22b	22.37b	5.37b	4.71b
25 kg P ha^{-1}	14.87a	4.24b	7.32a	61.54a	20.91ab	21.59a
50 kg P ha^{-1}	17.65a	6.97a	7.82a	67.93a	33.69a	23.00a
Mean	13.40	4.73	6.12	50.61	20.00	16.40
P value	*P*=.007	*P*=.01	*P*=.04	*P*=.01	*P*=.006	*P*=.004
$LSD_{0.05}$	6.08	2.65	3.89	28.63	16.83	16.69
CV (%)	20.70	26.10	30.00	21.80	30.00	30.00

Means followed by same letter in a column are not significantly different at *P*≤.05 (Fischer's protected LSD test).

Table 6.4: Effects of phosphorous fertilizer on nodule numbers and weight in a field experiment conducted at Kericho East at active vegetative and 50% flowering stages of cowpea plants in 2012 and 2013

	Active nodules plant^{-1}			Nod dry weight mg plant^{-1}		
Treatment	Long rains (2012-vegetative stage)	Short rains (2012-flowering stage)	Short rains (2013-flowering stage)	Long rains (2012-flowering stage)	Short rains (2012-flowering stage)	Short rains (2013-flowering stage)
0 kg P ha^{-1}	0.192b	2.76b	0.73b	0.15b	3.00b	1.92b
25 kg P ha^{-1}	1.041b	5.93a	1.45b	1.20b	11.69a	3.07b
50 kg P ha^{-1}	2.483a	8.26a	2.96a	4.80a	15.30a	6.61a
Mean	1.24	5.65	1.71	2.05	10.00	3.87
P value	*P*<.001	*P*<.001	*P*=.007	*P*<.001	*P*<.001	*P*=.01
$LSD_{0.05}$	0.99	2.53	1.36	1.75	5.12	3.28
CV (%)	20.50	23.40	19.70	25.30	28.50	30.00

Means followed by same letter in a column are not significantly different at *P*≤.05 (Fischer's protected LSD test).

Table 6.5: Effects of P fertilizer on shoot dry weight and leaf area index of cowpea plants during the active vegetative and 50% flowering stages in a field experiment conducted at Bomet Central and Kericho East between 2012-13

	Bomet Central	Kericho East				
	Shoot dry weight (g plant^{-1})				Leaf area index	
Treatment	Long rains (2012-flowering stage)	Long rains (2012-vegetative stage)	Short rains (2012-flowering stage)	Short rains (2013-flowering stage	Short rains (2012-flowering stage)	Short rains (2013-flowering stage)
0 kg P ha^{-1}	12.55c	0.20c	1.20c	0.76b	0.03c	1.52b
25 kg P ha^{-1}	18.35a	0.30b	2.91b	1.34a	0.07b	2.37ab
50 kg P ha^{-1}	14.74b	0.52a	4.12a	1.54a	0.13a	3.34a
Mean	15.21	0.34	2.74	1.21	0.08	2.41
P value	*P*<.001	*P*<.001	*P*<.001	*P*=.002	*P*<.001	*P*=.005
$LSD_{0.05}$	1.34	0.09	0.73	0.42	0.04	1.06
CV (%)	13.00	30.00	13.60	15.70	1.70	23.2

Means followed by similar letters in a column are not significantly different at *P*≤.05 (Fischer's protected LSD test).

Table 6.6: Influence of lime rate on shoot dry matter of cowpea plants in a field experiment conducted in Kericho East in short rains season of 2012

Lime rate	Shoot dry matter
0 t ha^{-1}	2.33b
4 t ha^{-1}	3.15a
Mean	2.74
P value	*P*=.009
$LSD_{0.05}$	0.6
CV (%)	13.6

Means followed by different letters in a column are significantly different at *P*≤.05 (Fischer's protected LSD test)

6.3.2 Effects of liming and P fertilizer application on nutrient content and grain yield of three cowpea varieties at Bomet Central and Kericho East

Phosphorous fertilizer rate and variety interactions were significant for shoot and grain nitrogen and protein content (Table 6.7). Application of 50 kg P ha^{-1} enhanced shoot N and crude protein of cowpea

variety KVU 27-1 at Bomet Central . At Kericho East, local variety Ngor had higher grain N and crude protein content with supply of 25 kg P ha^{-1}. It was further observed that cowpea variety KVU 27-1 had higher N and crude protein content in plots wihout P fertilizer at the same site (Table 6.7). Liming and application of P fertilizer enhanced grain N and P uptake of cowpea plants at Kericho East (Table 6.8 and Fig 6.1). Cowpea variety M66 had highest grain yield at Bomet Central without any treatment applied, but no grain was harvested from the same variety at Kericho East without the soil amendments (Table 6.9). Cowpea variety KVU 27-1 had the highest grain yield at Kericho East when soil was limed and supplied with the highest P rate. However, grain yield was not recorded in the same variety without lime application or P fertilizer application at the same site .

Table 6.7: Influence of P fertilizer and variety interactions on shoot and grain nutrient content of cowpea plants in a field experiment conducted at Bomet Central and Kericho East during the short rains season of 2013

	Bomet Central		Kericho East	
Treatment	Shoot N (%)	Shoot CP† (%)	Grain N (%)	Grain CP (%)
0 kg P ha^{-1} + KVU 27-1	1.75_{abcd}	9.54_{abcd}	1.90_{a}	10.37_{a}
0 kg P ha^{-1} + M66	1.52_{cd}	8.27_{cd}	1.39_{d}	7.59_{d}
0 kg P ha^{-1} + Ngor	1.75_{abcd}	9.54_{abcd}	1.77_{ab}	9.65_{ab}
25 kg P ha^{-1} + KVU 27-1	1.58_{bcd}	8.58_{bcd}	1.72_{abc}	9.37_{abc}
25 kg P ha^{-1} + M66	1.87_{abc}	10.17_{abc}	1.67_{bc}	9.08_{bc}
25 kg P ha^{-1} + Ngor	1.63_{bcd}	$8.9b_{cd}$	1.90_{a}	10.34_{a}
50 kg P ha^{-1} + KVU 27-1	2.04_{a}	11.13_{a}	1.71_{abc}	9.37_{abc}
50 kg P ha^{-1} + M66	1.48_{d}	8.04_{d}	1.56_{cd}	8.48_{cd}
50 kg P ha^{-1} + Ngor	1.93_{ab}	10.49_{ab}	1.68_{bc}	9.15_{bc}
Mean	1.73	9.41	1.7	9.27
P value	*P*=.05	*P*=.05	*P*=.01	*P*=.01
$LSD_{0.05}$	0.39	2.12	0.2	1.067
CV (%)	19.2	19.2	9.8	9.8

† Crude protein. Means followed by a similar letter in a column are not significantly different at $P \leq .05$ (Fischer's protected LSD test).

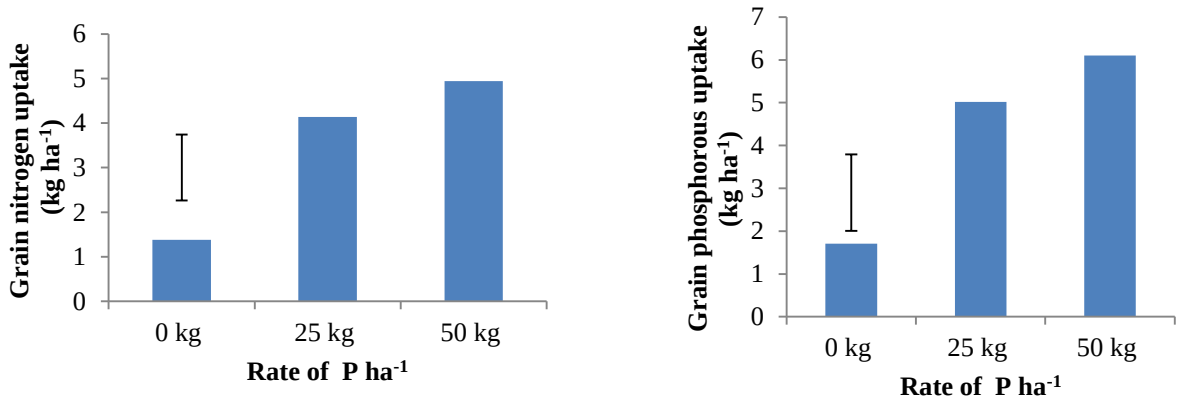

Fig 6.1: Effects of phosphorous fertilizer on grain nitrogen and phosphorous uptake of cowpea plants at harvest in a field experiment conducted in Kericho East during the short rains season of 2013. LSD bars show differences in means of P fertilizer rates at P≤.05 (Fischer's protected LSD test).

Table 6.8: Influence of lime rate on nutrient uptake of cowpea grain in a field experiment conducted at Kericho East during the short rains season of 2013

Lime rate	Grain N uptake	Grain P uptake
0 t ha^{-1}	2.60b	3.25b
4 t ha^{-1}	4.38a	5.30a
Mean	3.49	4.27
P value	*P*=.005	*P*=.007
$LSD_{0.05}$	1.26	1.45
CV	26.4	27.5

Means followed by different letters in a column are significantly different at *P*≤.05 (Fischer's protected LSD test)

Table 6.9: Influence of P fertilizer and variety interactions on grain yield of cowpea plants in a field experiment conducted at Bomet Central and Kericho East during two rain seasons between 2012-13

	Grain yield (tons ha^{-1})		
	Bomet Central		Kericho East
Treatment	Long rains (2012)	Short rains (2013)	Short rains (2013)
0t ha^{-1} lime + 0 kg P ha^{-1} + KVU 27-1	1.48 bc	0.85	0.00 f
0t ha^{-1} lime + 0 kg P ha^{-1}+ M66	2.49 a	1.66	0.00 f
0t ha^{-1} lime + 0 kg P ha^{-1}+ Ngor	0.43 e	0.91	0.14 cdef
0t ha^{-1} lime + 25 kg P ha^{-1}+ KVU 27-1	0.91 cde	0.94	0.29 abc
0t ha^{-1} lime + 25 kg P ha^{-1} + M66	1.75 abc	1.60	0.06 ef
0t ha^{-1} lime + 25 kg P ha^{-1} + Ngor	1.79 ab	1.54	0.13 cdef
0t ha^{-1} lime + 50 kg P ha^{-1} + KVU 27-1	1.68 abc	1.03	0.18 cdef
0t ha^{-1} lime + 50 kg P ha^{-1} + M66	0.99 bcde	2.81	0.23 bcde
0t ha^{-1} lime + 50 kg P ha^{-1} + Ngor	1.06 bcde	1.32	0.40 ab
4t ha^{-1} lime + 0 kg P ha^{-1} + KVU 27-1	1.33 bcd	1.10	0.19 cdef
4t ha^{-1} lime + 0 kg P ha^{-1} + M66	1.77 abc	1.31	0.07 def
4t ha^{-1} lime + 0 kg P ha^{-1} + Ngor	0.51 de	0.99	0.07 def
4t ha^{-1} lime + 25 kg P ha^{-1} + KVU 27-1	1.52 bc	0.95	0.28 abc
4t ha^{-1} lime + 25 kg P ha^{-1} + M66	1.65 abc	1.46	0.27 abcd
4t ha^{-1} lime + 25 kg P ha^{-1} + Ngor	1.05 bcde	1.35	0.33 abc
4t ha^{-1} lime + 50 kg P ha^{-1} + KVU 27-1	1.03 bcde	2.16	0.47 a
4t ha^{-1} lime + 50 kg P ha^{-1} + M66	1.81 ab	2.17	0.32 abc
4t ha^{-1} lime + 50 kg P ha^{-1} + Ngor	1.04 bcde	0.88	0.21 bcde
Mean	1.35	1.39	0.20
P value	P=.02		P=.03
$LSD_{0.05}$	0.87	NS	0.21
CV (%)	30.00	20.40	7.00

Means followed by same letters in a column are not significantly different at $P \leq .05$ (Fischer's protected LSD test).

6.3.3 Correlation analyses

There were significant positive correlations between protein content and N uptake, as well as shoot K, N, Ca, Mg and Mn content (Table 6.10). Cowpea LAI was positely correlated with N and P uptake, shoot dry weight and content of K and Mg. There were significant positive correlations between N uptake and P uptake, shoot dry weight and K, N and Mg content . Nodule dry weight of cowpea was significantly positively correlated with shoot concentration of Mn. Phosphorous uptake of cowpea also had a significant positive correlation with shoot content of K, P and Mg. Shoot dry weight of cowpea had significant positive correlation with shoot Mg content .

Table 6.10: Pearson correlation coefficient for shoot nutrient content and agronomic parameters of three cowpea varieties in a field experiment conducted at Bomet Central in the short rains season of 2013

	K (%)	P (%)	N (%)	Ca (%)	Mg (%)	Zn (mg kg^{-1})	Mn (mg kg^{-1})
Protein (%)	0.27*	0.19	1.00***	0.41***	0.33*	0.23	0.30*
LAI†	0.25*	0.10	0.15	-0.05	0.27*	0.02	-0.17
N uptake (g $plant^{-1}$)	0.29*	0.14	0.35**	0.01	0.43***	0.19	-0.09
Nodule dry weight $plant^{-1}$	-0.17	-0.12	0.04	0.01	-0.15	-0.11	0.35**
P uptake (g $plant^{-1}$)	0.30*	0.47***	0.07	0.05	0.35**	0.16	-0.25
Shoot dry matter $plant^{-1}$	0.15	0.04	-0.06	-0.19	0.29*	0.11	-0.21

† Leaf area index; * correlation significant at $P \leq .05$, ** correlation significant at $P \leq .01$, *** correlation significant at $P < .001$

6.3.4 Varietal differences in nodulation, growth, yield and nutrient content of cowpea in field experiment conducted at Kericho East and Bomet Central in rain seasons between 2012-13.

Cowpea variety KVU 27-1 had significantly ($P \leq .05$) higher nodule number, nodule and shoot dry weight during two rain seasons at Kericho East (Fig. 6.2). The grain protein levels of cowpea variety KVU 27-1 and local variety Ngor were significantly ($P \leq .05$) higher than M66 at Kericho East during the short rains season of 2013 (Fig. 6.2). At Bomet Central, varietal differences for three parameters were

significant only during the long rains season of 2012 (Fig. 6.3). The improved cowpea varieties (KVU 27-1 and M66) had significantly higher shoot dry matter than Ngor, but the latter had higher leaf area index than the improved varieties (Fig. 6.3). Grain yield was higher in variety M66 than in varieties KVU 27-1 and Ngor (Fig. 6.3).

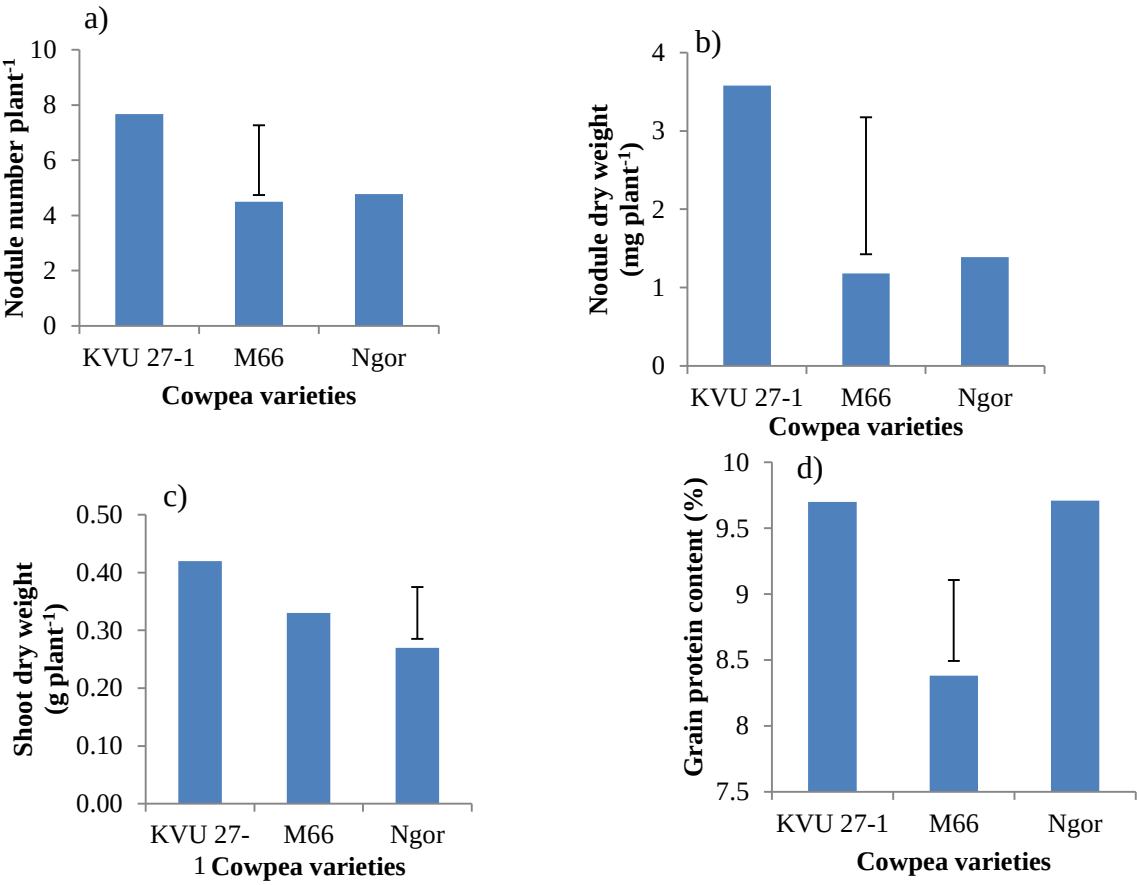

Fig. 6.2: Varietal differences in nodulation, growth and grain nutrient content of cowpea plants in a field experiment conducted at Kericho East between 2012-14; a - sampling done during the 10th week after crop emergence in the short rains season of 2012, b & c - sampling done during the 6th week after crop emergence in long rains season of 2012, d - sampling done at harvest in the short rains season of 2013. LSD bars show differences in varietal means at ($P \leq .05$) (Fischer's protected LSD test).

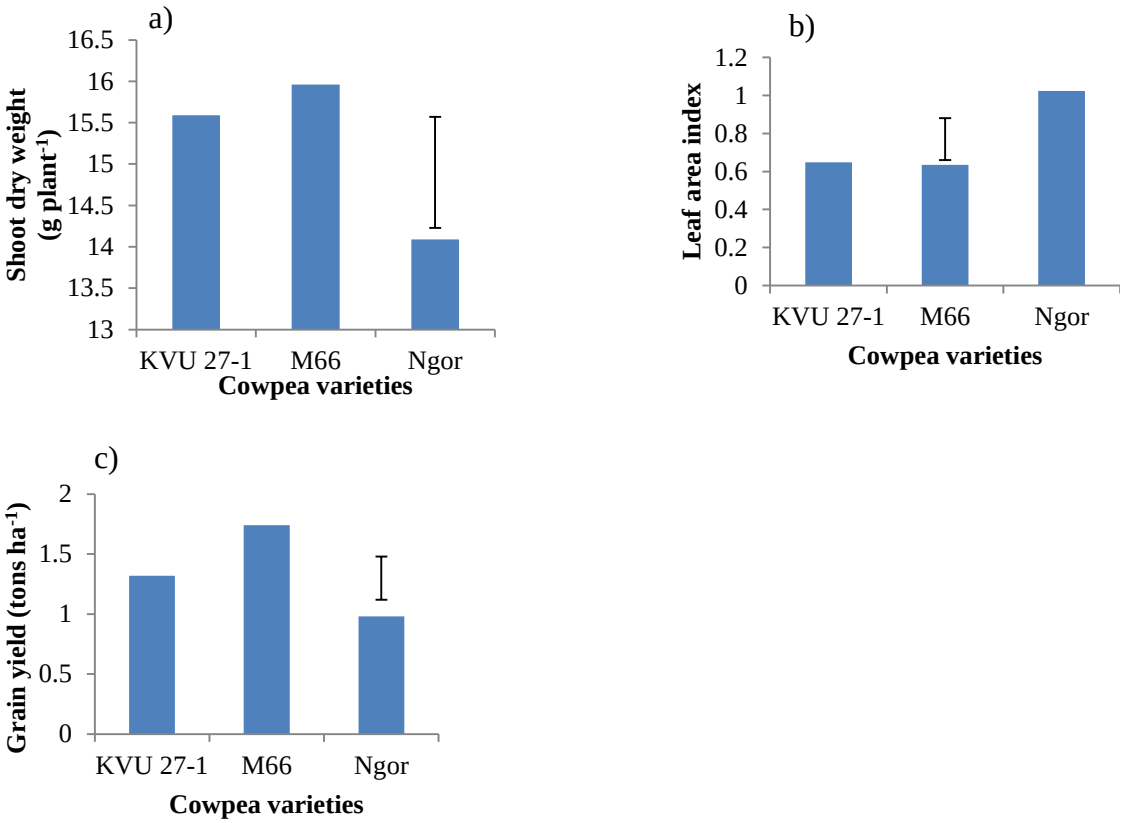

Fig 6.3: Varietal differences in growth and grain yield of cowpea plants in a field experiment conducted in Bomet Central during the 10th week after crop emergence in the long rains season of 2012. LSD bars show differences in varietal means at ($P \leq .05$) (Fischer's protected LSD test)

6.4 Discussion

Liming had no effects on nodulation in the study sites, even in the strogly acid soils (pH 4.85) of Kericho East, but it enhanced shoot dry matter in one site only once over the three rain seasons. In contrast, lime application enhanced nodulation and growth of cowpea in moderately acidic soils with pH of 5.4 in Nigeria (Bello *et al*., 2018). Similarly, lime application enhanced growth of common bean (*Phaseolus vulgaris*) and also growth and nutrient content of *Sesbania sesban* under similar conditions of soil acidity (Kassa *et al*., 2014; Kisinyo *et al*., 2012). At pH of 4.85 recorded at Kericho East, it would be expected that Al^{3+} would become soluble and cause plant toxicity which is characterised by inhibition of uptake, translocation and utilisation of P by plants (Havlin *et al*., 2005; Haynes, 1982) ,

hence decline in physiological processes such as N_2 fixation. Liming would often reverse these conditions in soils (Kisinyo *et al*., 2012) and consequently plant growth and nodule formation would increase. The non significant effects of lime on nodulation and minimal effects on growth of cowpea in the site with strogly acid soils may suggest that Al concentration of 0.75 cmol kg^{-1} may not cause toxicity in cowpea. Nodulation was responsive to application of 50 kg P ha^{-1} in general at both sites. Enhanced nodulation in cowpea in response to high rate of P fertilizer is in agreement with findings reported in Ghana (Karikari *et al*., 2015). However, nodulation response to P fertilizer rate was genotype dependent, as also reported in Nigeria (Nkaa *et al*., 2014). Lower P rates (25 kg ha^{-1}) enhanced nodule weights in the local variety Ngor, but 50 kg P ha^{-1} enhanced nodule numbers in improved varieties (M66 and KVU 27-1) at Bomet Central. This suggests that the cowpea landrace requires low P input for effective nodulation at this site, and confirms previous findings that some cowpea genotypes require low nutrient input for growth (Pule-Meulenberg et al., 2010).

Cowpea plant growth responded positively to 50 kg P ha^{-1} at Kericho East in all the seasons, but lower P rate (25 kg P ha^{-1}) increased shoot dry matter at Bomet Central. Although both sites had slight variation in available Mehlich 1- P (8.19 mg kg^{-1} and 8.85 mg kg^{-1} respectively), the possible explanation for cowpea response to low P rate at Bomet Central could be lower solubility of Al at its pH of 5.58, hence minimal P fixation (Hargreaves, 2015; Havlin *et al*., 2005). Consequently, shoot P uptake was not enhanced by liming or P fertilizer at Bomet Central. However, lime application and P fertilizer ehanced grain N and P uptake of cowpea in the strogly acidic soils of Kericho East. Liming may have raised the soil pH thus increasing the available P (Kisinyo *et al*., 2012), hence increase in its uptake. The possible role of P in N uptake is increased root growth which would facilitate N absorption (Wen *et al*., 2016). Nonetheless, the increased grain N uptake due to liming in Kericho East did not translate into

increased grain N and protein content. Application of 25 kg P ha^{-1} significantly increased grain protein content of local cowpea variety Ngor at Kericho East. Phosphorous is an important component of ATP and nucleic acids, which are essential for protein synthesis (Nyoki and Ndakidemi, 2014; Raven, 2013). In contrast, cowpea variety KVU 27-1 had higher grain N and protein content in plots without P fertilizer at Kericho East. Previous research work in South Africa also reported varietal differences in protein content of cowpea in the absence of fertilizer application (Adeyemi *et al*., 2012). Nonetheless, KVU 27-1 responded to the highest P rate and liming for grain yield at Kericho East, but there is an inverse correlation between grain yield and protein content (Kyei-Boahen *et al*., 2017; Martos-Fuentes *et al*., 2017). Therefore on smallholder farms with mimimal application of P fertizer, families can still obtain sufficient protein from cowpea variety KVU 27-1 under similar ecological conditions as Kericho East. At Bomet Central, M66 gave the highest grain yield in the absence of P fertilizer or liming. Available P in this site was low (8.85 mg kg^{-1}), thus M66 may have high phosphatase ativity, which is associated with high P acquisition in soils low in P (Makoi *et al*., 2010). Generally, cowpea variety KVU 27-1 performed better than other varieties in terms of nodulation and growth at Kericho East, while M66 had higher growth and grain yield at Bomet Central. This confirms that agronomic traits in cowpea are controlled by genotype and environment interactions (Horn *et al*., 2018; Martos-Fuentes *et al*., 2017).

In general, cowpea plants at Bomet Central had higher nodulation, shoot dry weight and 1.19 more tons of grain ha^{-1} than Kericho East. Kericho East had very low population of rhizobia undetected by MPN technique. However, cowpea plants were able to nodulate, but rhizobial cells in this site may posess low symbiotic efficiency, which is characterised by low nodule and shoot dry weight (Ohlson *et al*., 2018). Correlation analyses showed significant positive correlation between Mg^{2+} and N and P uptake, growth

parameters and protein content of cowpea. Soils of Kericho East were deficient in Mg^{2+}, and this may partly explain the poor growth and yield of cowpea in this site. Magnesium is important for chlorophyll and nucleic acid synthesis, a co-factor in many enzymes controlling phyiological processes in plants and enhances crop tolerance to abiotic stresses (Senbayram *et al*., 2016; Tanoi and Kobayashi, 2015). Potassium had positive correlation with N and P uptake, LAI and protein content of cowpea. This may be due to the fact that it is involved in many plant physiological processes such as photosynthesis, regulation of enzymes synthesis, cell signalling and tolerance to biotic and abiotic stresses (Oosterhuis *et al*., 2014). Manganese also had significant positive correlation with nodule dry weight, which is consistent with findings of previous authors (Vadez *et al*., 2000). Fertilizers in Kenya contains mainly N and P, but there is need to include other nutrient elements such as K, Mg and Mn for optimum production of leguminous crops.

6.5 Conclusions

Lime application had no influence on nodulation and protein content of cowpea in the study sites, but enhanced grain N and P uptake at Kericho East. However, Liming was not necessary at Bomet Central. Generally, cowpea required 50 kg P ha^{-1} and 25 kg P ha^{-1} for nodulation and shoot growth of cowpea at Kericho East and Bomet Central respectively. Effects of P fertilizer on protein content and yield of cowpea depended on genotype and site. Variety KVU 27-1 required high rate of P fertilizer to enhance its protein content at Bomet Central, but its protein content was higher in the absence of the fertilizer at Kericho East. Grain yield of M66 was also higher at Bomet Central without any input of P. Nodulation, growth and grain yield of cowpea was generally low in Kericho East even with the soil amendments. In order to optimize cowpea production in Kericho East, a similar study should incorporate efficient rhizobial inoculants strains and Mg fertilizer which was deficient in the site.

CHAPTER SEVEN: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 Discussion

Abundance, symbiotic efficiency and genetic diversity of cowpea rhizobia in south western Kenya and the reference regions in this study depends on soil chemical conditions. Soil pH had positive correlation with abundance of rhizobia in soils, which suggests that soil acidity reduces rhizobial population. The most probable explanation for reduced rhizobial population in acidic soils could be due to solubility of Al^{3+} (Havlin *et al*, 2005), which had significant negative correlation with abundance of rhizobia. Aluminium ions also had significant negative correlation with most species of rhizobia and plant growth promoting bacteria. Once abundance of rhizobia is reduced, nodulation is also reduced because there were significant positive correlations between rhizobial abundance and nodule numbers and dry weight. The most commonly used remedy for reducing soil acidity and hence reducing solubility of Al^{3+} is liming of soils. However, the use of aluminium tolerant species of rhizobia as inoculants needs further research. *Rhizobium miluonense* had positive correlation with Al^{3+}, and may be considered for screening for aluminium tolerance in future studies. *Rhizobium tropici* was also abundant in acidic soils. Sequence analysis of recA gene in addition to 16S rRNA proved useful in refining species identification of rhizobia and plant growth promoting bacteria. Sequence analysis of 16S rRNA gene identified two isolates as *Bosea* sp, but recA refined their species identification as *Bosea thioxidants,* which confirmed that multilocus sequence analysis is useful for phylogenetic studies of bacteria. Cowpea is predominantly nodulated by *Rhizobium* sp. in the seven agro-ecological zones covered by this study as opposed to *Bradyrhizobium* sp. reported in a related study (Ndungu *et al*., 2018). However, earlier research work by Kimiti and Odee (2010) revealed that 97% of cowpea nodulating rhizobia isolated from one farm in Eastern Kenya produced acidic reactions in culture media, which is a characteristic of *Rhizobium* sp.

The only commercial cowpea inoculant in Kenya marketed as Biofix, which contains *Bradyrhizobium* sp. USDA 3456 was inefficient in nodulation and nitrogen fixation in two acidic soils of SWK. This suggests that the ecological conditions in SW Kenya may not favour its infectiveness. Previous work also confirmed nodulation inefficiency when Biofix inculant was applied to cowpea (Mathu et al., 2012). Nodulation tests for efficient strains of rhizobia isolated in this study and other previous studies may serve useful as alternatives to *Bradyrhizobium* sp. USDA 3456. Lime application had no significant effects on nodulation of cowpea in two acidic soils with pH of 4.85 and 5.68, and had no effect on cowpea growth and yield at pH of 5.68. Phosphorous fertilizer also had no significant effect on cowpea yield at the same pH of 5.68. In addition, high P rate (50 kg P ha^{-1}) enhanced growth and yield of cowpea in strongly acidic soils of SWK (pH 4.85). These observations may suggest that at pH of 5.68, solubility of Al^{3+} may be minimal hence minimal P fixation, hence the available P is efficiently utilised by the plants.

7.2 Conclusions

It was concluded that low soil pH and high concentration of Al^{3+} are the major abiotic factors limiting abundance and symbiotic efficiency of rhizobia, but *Rhizobium miluonense* and *Rhizobium tropici* are likely to be tolerant to these abiotic stress factors. Over 90% of cowpea nodulating rhizobia in the seven agro-ecological zones covered by this study belong to the genus *Rhizobium.* Two species of plant growth promoting bacteria (*Bacillus aryabhattai* and *Bacillus megaterium*) dominate cowpea nodules across the seven agro-ecological zones. It was also concluded that *Bradyrhizobium* sp. USDA 3456 has low symbiotic efficiency in acidic soils of south western Kenya. Lime application is not necessary for cowpea production at pH of 5.68 in lower highland 2 of south western Kenya. Application of 50 kg P ha^{-}

[1] increases nodulation under acidic soils of SWK, and also increases growth and yield of cowpea in soils with pH of 4.85 at lower highland 1 in south western Kenya.

7.3 Recommendations

It is recommended that:

1) *Rhizobium tropici* and *Rhizobium miluonense* should be screened for their tolerance to low pH and Al^{3+}
2) Symbiotic and other housekeeping genes need to be used to refine the taxonomy of rhizobial isolates in this study
3) Symbiotic efficiency of rhizobial isolates in this study need to be determined under field conditions against commercial rhizobial strains, and efficient strains should be tested for potential use as bio-inoculants
4) Plant growth promoting activities and role of *Bacillus megaterium* and *Bacillus aryabhattai* in cowpea growth needs to be determined
5) The response of cowpea to rhizobia inoculation in soils amended with agricultural lime and P fertilizer need to be determined in the acidic soils of SWK
6) Farmers producing cowpea in strongly acidic soils should apply P fertilizer to enhance yield of cowpea, but more P rates should be incorporated in future experiments to determine the optimum P rate for cowpea production in the acidic soils.

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