

**INFLUENCE OF BIOCHAR AMENDMENT ON THE EFFECTIVENESS OF ELITE  
KENYAN RHIZOBIA NODULATING COMMON BEAN (*Phaseolus vulgaris L.*)**

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## DECLARATION

This thesis is my original work and has not been presented for award of a degree/Research in any other academic institution.

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## **DEDICATION**

I dedicate this work to my late mother, Rosemary Wanjiku Koinange for her love, care and happiness which she selflessly offered to our family in her short-lived life and to my family for their understanding and support during this study period. Above all I extend my sincere gratitude to the Almighty God for making this journey possible.

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## GENERAL ABSTRACT

Symbiotic nitrogen fixation (SNF) can provide an agronomic and economically sustainable alternative to declining soil fertility and high cost of chemical fertilizers faced by smallholder farmers in Kenya. The aim of the study was to identify highly effective indigenous rhizobia for production of commercial bean inoculants and to investigate the influence of bio-char as a soil amendment on the effectiveness of both indigenous Kenyan rhizobia and commercial inoculant in symbiosis with common bean in low fertile soils of Western Kenya. Bio-prospecting was conducted in Kenya to collect rhizobia isolates capable of nodulating and fixing N in symbiotic association with common bean. Three hundred and eighty rhizobia isolates were recovered from nodules of wild and cultivated legume hosts growing along a transect of different agro-ecological zones covering about 1045 km transect. These isolates were authenticated and tested for effectiveness on climbing bean (*Phaseolus vulgaris L.*) var. Kenya Tamu in sterile vermiculite, scaling down the number of test isolates to sixteen for further study. In the greenhouse experiment conducted at the Kabete campus, University of Nairobi, using soil from Butula in West Kenya two bean cultivars, climbing bean var. Kenya Tamu and bush bean var. New Rosecoco were inoculated with the 16 promising isolates which were compared to two widely used commercial inoculant strains, USDA 2667 and CIAT 899. Out of this experiment, eight most promising isolates including, NAK 5, 45, 67, 92, 104, 157, 186 and 191 were identified to be tested in field trials. Results show that the isolates did not yield significant differences under field conditions and warrants further comparison under different field conditions. To investigate the influence of bio-char on the effectiveness of indigenous rhizobia and commercial strain nodulating common bean, a two season field experiment was conducted in Butere and Nyabeda, Western Kenya. There were no significant differences and interactions observed in biomass production and grain yield from common beans inoculated with indigenous rhizobia isolates and CIAT 899 with or

without biochar addition in both seasons in both Butere and Nyabeda. Most significant differences were between managements receiving mineral N and those receiving inoculant suggesting effective management of N fertilizer and little difference between the candidate elite strains. While the effect of biochar addition was not significant in relation to inoculation, there are very strong trends suggesting benefits from adding 2 t ha<sup>-1</sup> biochar. Genetic diversity and phylogeny of the elite Kenyan rhizobia isolates nodulating common bean and soybean (Waswa *et al.*, 2014) was assayed using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the 16S-23S rDNA intergenic spacer region and 16S rRNA gene sequencing. A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis confirmed the greater relationship of indigenous strains of *Bradyrhizobium* to reference strains of *B. elkanii*, *B. japonicum* and *Bradyrhizobium spp.* and a greater relationship of indigenous strains of *Rhizobium* to reference strains of *Rhizobium sp.*, *Rhizobium tropici* and *Rhizobium phaseoli*. The study results suggest that continual isolation and characterization to identify elite isolates and use of biochar, as a soil amendment, offers an opportunity for improvement of SNF with lesser limitations geographically to the areas of use.

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## **ABBREVIATIONS AND ACRONYMS**

BNF	Biological Nitrogen Fixation
CAN	Calcium Ammonium Nitrate
CFU	Colony Forming Unit(s)
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxy Ribonucleic Acid
EDTA	Ethyl Diamine Tetracetic Acid
IGS	Intergenic Spacer
LR	Long rains
M	Molar
MIRCEN	Microbiology Resource Centre
MPN	Most Probable Number
N2Africa	Putting nitrogen fixation to work for smallerholder farmers in Africa
N	Nitrogen
NF	Nitrogen Fixation
PCR	Polymerase Chain Reaction
P	Phosphorous
pH	Hydrogen potential
RFLP	Restriction Fragment Length Polymorphism
rpm	rotation(s) per minute
SR	Short rains
SSA	Sub-Saharan Africa
TBE	Tris Borate EthylDiamineTetracetic Acid
USDA	United States Department of Agriculture.
V	volts

## CHAPTER 1

### GENERAL INTRODUCTION

#### 1.1 Significance of Biological Nitrogen Fixation (BNF).

Nitrogen (N) is essential for the normal plant growth. N is a basic constituent of proteins and nucleic acids and is also a basic constituent of several other compounds of primary importance to plant metabolism such as chlorophyll, nucleotides, proteins, alkaloids, enzymes, hormones and vitamins (Defoer and Bundelman, 2000). Deficiency in mineral nitrogen often limits plant growth and symbiotic relationships have evolved between plants and a variety of nitrogen-fixing organisms (Freiberg, 1997).

Biological nitrogen fixation involves conversion of atmospheric nitrogen (N) to organic ammonia molecules, a form of N that can be utilized by plants (Vessy *et al.*, 2005). It is the incorporation of atmospheric Nitrogen in the tissue of plants mainly belonging to the family Fabaceae (legumes). BNF is beneficial to the plants, humans and animals in that it avails nitrogen readily to the plants that in turn uses it to synthesize proteins that are transferred along the food chain. It enhances the ability of plants' nitrogen assimilation in the form of ammonium (NH<sub>4</sub>). The assimilated form of N is in turn used to synthesis building blocks for intermediate metabolism (alkaloid, pyrimidine, purine, and amino acid biosynthesis). BNF influences photosynthesis because nitrogen is one of the components used in synthesizing chlorophyll. Increased plant protein levels and reduced depletion of soil N reserves are obvious consequences of legume N fixation.

It has been estimated that the 80-90% of the N available to plants in the natural ecosystem originates from biological nitrogen fixation (Rascio and Rocca 2008). According to Tate,

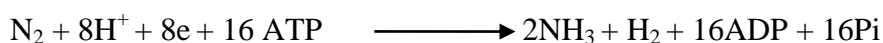
(1995) rhizobial symbiotic association with the more than 100 cultivated important legumes contribute nearly half the annual quantity of BNF entering soil ecosystem.

BNF reduces the use of inorganic fertilizers in order to nourish the plants with nitrogen and farmers benefit through reduced operating costs (Werner & Newton, 2006, Peoples *et al.*, 1995; Walley *et al.*, 2007; Gehring, 2003). Yield increases of rotation crops planted after harvesting of legumes are often equivalent to those expected from application of 30 to 80 kg of fertilizer-N/ ha (Zahran, 1999). Besides, inorganic fertilizers may have a detrimental effects on the environment such as leaching into ground water or soil acidification.

## **1.2 Mechanisms of Biological Nitrogen Fixation**

Biological nitrogen fixation, a process utilized only by certain prokaryotes, is catalyzed by a two-component nitrogenase complex (Yan *et al.*, 2010). Nitrogenase, an oxygen labile enzyme highly conserved in symbiotic diazotrophs, catalyzes the simultaneous reduction of one N<sub>2</sub> and 2 H<sup>+</sup> to ammonia and a molecule of hydrogen gas (Stacey, 1992). Nitrogen fixation may be performed by the free-living or symbiotic bacteria (Wang, 2006). Ammonium (NH<sub>4</sub>) is believed to be an intermediate in assimilation of combined oxygen as well as biological nitrogen fixation and there is a great mass of evidence in support to this. For instance amides, the conjugate base of ammonia, account for a large percentage of the nitrogen present in nodules (Wilson & Burris, 2000).

The BNF mechanism can be represented as:



Adenosine triphosphate (ATP) is required for cleavage and reduction of nitrogen to ammonia. Oxidative degradation of sugars and other related molecules generates the ATP (Rees *et al.*,



2005). The host plant manufactures the required sugars after which they are transferred to the nodules. Mo-Fe protein (Molybdoferredoxin) and Fe protein (Azoferreredoxin) are the two metalloproteins that are contained by nitrogenase enzyme. Substrate reduction takes place in Mo-Fe protein that has an active site. Electron transfer is coupled to ATP hydrolysis by Fe protein that acts as an electron donor. Nitrogenase has four basic mechanisms at the protein level. First, a complex reaction between the reduced Fe protein with the Mo-Fe protein and two bound ATP is formed. The second mechanism involves transfer of electrons between the two proteins merged to the hydrolysis of ATP. The next step involves Fe-protein dissociation accompanied by re-reduction, via flavodoxins or ferredoxins and exchange of ATP for ADP. Lastly, the cycle is repeated until sufficient numbers of protons and electrons are accumulated in order for available substrates to be reduced (Mulongoy, 2008).

### **1.3 Factors affecting BNF**

The major determinants of nitrogen fixation include edaphic, climatic and biotic factors. Edaphic factors include drought, soil acidity, P deficiency, excess mineral N, excessive soil moisture and deficiency of molybdenum, calcium, cobalt and boron. Availability of light and extreme temperatures are the two climatic determinants of BNF while excessive defoliation of host plant, pests and diseases and crop competition are the major biotic influencers (Mulongoy, 2008). As emphasized by Howieson and Ballard (2004), the manifestation of stresses are more often dependent upon the host-rhizobia symbiont. For example, symbioses with medics are particularly sensitive to soil pH below 6 (Munns, 1970), whereas the lupin symbiosis with *Bradyrhizobium sp.* will function optimally between pH 4 and 6, but is sensitive to pH above 6 (Tang and Robson, 1993).

Generally, the optimal soil pH for BNF is usually in the range of 6 to 7. A soil pH significantly outside this range (<5 or >8) is detrimental because it disrupts infection,

thereby limiting nodule development (Werner and Newton, 2006). Soil pH also affects the amount of nitrogen fixed. For example, in a very acidic soil (pH 4.4), BNF can be reduced up to 30% (Abendroth *et al.*, 2005; Dakora, 2008). Soil acidity also causes Ca deficiency and manganese and aluminum toxicity hence negatively affecting nodulation. In contrast, soil acidity affects rhizobial survival while alkalinity affects competition for nodulation. However, this is primarily a host effect as proliferation of the micro-symbionts for clover and medic under alkaline conditions has been reported (Ballard and Charman, 2000; Brockwell, 2001; Denton *et al.*, 2000).

Clay content in soils also affects the survival of rhizobia. Clay exhibits a protective effect on rhizobia and this has been well documented (Lahav, 1962; Marshall, 1975; Fuhrmann *et al.*, 1986).

The concentration of minerals in the soil also influences N fixation. As such, low concentration of Ni, As, Pd, Pb, and Zn stimulate N-fixation in soil Nostoc. Nevertheless, long exposure to Pb, Zn, and Cd inhibit fixation (Belnap, 2001). Various microelements such as Co, B, Mo, and Cu are also essential for N-fixation. Phosphorus deficiency is a major constraint of effective nitrogen fixation because phosphorus is an important nutrient in the process of nodulation and nitrogen fixation (World Bank, 2006). Under P shortage conditions, legumes may lose the distinct advantage of unlimited source of symbiotic N (Suliman *et al.*, 2008). The more the supply of phosphorus to its optimal, the more abundant are the nodules (Gowariker *et al.*, 2009).

Increasing salt concentrations may have a detrimental effect on soil microbial populations as well, either due to direct toxicity or through osmotic stress (Yadav *et al.*, 2010). Salinity causes various forms of stress, such as an osmotic effect and ionic toxicity, and affects the overall metabolic activities. One of the first results of this metabolic disturbance is the over-

production of reactive oxygen species, inducing the oxidative integrity and nodule functioning. Symbiosis response to this constraint includes morphological modification, such as the change of nodule cortex structure and biochemical adaptations, such as the modulation of antioxidant enzyme expression in nodules (Abdelly *et al.*, 2008).

Temperature influences BNF since symbiotic systems have different ability to tolerate low (<25° C) and high (>35° C) temperatures (Belnap, 2001). There is evidence that persistence and survival of rhizobial strains in soils is influenced by soil temperatures. For instance, in the hot, dry Sahel-savannah of West Africa cowpea rhizobia grow at 37° C subsequently, 90% of strains isolated from the region grow well at 40° C (Mohammadi *et al.*, 2012). A continually cool root zone temperature can significantly delay the onset of nitrogen fixation compared to an optimum soil temperature (Abendroth *et al.*, 2006). Nitrogen fixation is dependent on photosynthesis, and this makes light an essential part in influencing fixation. A drop in light levels causes a decline in BNF (Rao, 1988). It is important to take into account that these stresses are synergistic and it is important to recognize those situations acting together rather than in isolation (Howieson and Ballard, 2004).

#### **1.4 Rhizobial Ecology and Genetic Diversity**

Rhizobia exhibit a wide range of geographical locations as well as specificity to the hosts. Different strains of rhizobia differ in their symbiotic capabilities, for instance while some strains can be used as commercial inoculants most cannot. Some of the rhizobia strains have a detrimental effect in the soil while some do not (Granada *et al.*, 2014). For instance, *Sinorhizobium sp.* 9702-M4 with 16s rRNA gene has the ability to synthesis an extracellular polymer that enhances the transportation of toxic metals, cadmium and lead as well as hydrophobic pollutants in soil (Wolde-meskel, 2005).

Studies have shown that tropical rhizobia are diverse with sub-groups of varied symbiotic specificity and effectiveness (Thies *et al.*, 1991; Mpeperekwi *et al.*, 1996). Studies by Bala and Giller (2007) showed rhizobia of the same phylogenetic grouping nodulating *Caliandra calothyrsus*, *Glycidia sepium* and *Leucaena leucocephala* in some soils, but failing to nodulate at least one of the hosts in others, suggesting that rhizobial phylogeny and host range (infectiveness) are only weakly linked.

Symbiotic nitrogen fixation (SNF) require that host and microorganism are compatible and also that the soil environment be appropriate for the exchange of signals that precede infection (Hirsch *et al.*, 2003; Zhang *et al.*, 2002). Earlier reviews have chronicled the influence of biotic and abiotic soil factors on rhizobia ecology (Amarger, 2001; Sessitsch *et al.*, 2002). Tools, such as intrinsic antibiotic resistance (Beynon and Josey, 1980), serology (Bohlool and Schmidt, 1973; Purchase and Vincent, 1977; Purchase *et al.*, 1951), and multi-locus enzyme electrophoresis (Pinero *et al.*, 1988; Eardly *et al.*, 1990) have all helped toward a more detailed examination of rhizobial population structure in soil, and how this is influenced by host and environment. However, only with the development of molecular (Hirsch *et al.*, 2003; Thies *et al.*, 2001) and computational tools have considered large populations of rhizobia on a routine basis.

Originally, rhizobia diversity have been determined using characteristics such as growth rate, colony morphology (size, shape, color, texture and general appearance) and antibiotic resistance (Graham *et al.*, 1991). However, these methods are not sufficiently discriminative to account for all the variation exhibited in the target species. They cannot delineate sources of observed phenotypic variation into its components that may be due to environmental factors or underlying genetic factors.

Present studies and research conducted on the genetic diversity of rhizobia yielded efficacy of molecular tools in determining the genetic diversity of rhizobia. Among the molecular tools used in determining the genetic diversity include extraction of nucleic acids (DNA/RNA), polymerase chain reaction (PCR), DNA sequencing, electrophoresis of nucleic acids, nucleic acid hybridization, restriction fragment length polymorphism (RFLP), ribotyping, amplified fragment length polymorphism (AFLP), subtractive hybridization, GUS marker gene technology, and terminal-restriction fragment length polymorphism analysis (T-RFLP). Among these, the most commonly used tools include extraction of nucleic acids, PCR and RFLP (Nour *et al.*, 1994; Sessitsch *et al.*, 1997; Thies *et al.*, 2001). The intergenic spacer between 16S and 23S rRNA genes was described to be more variable (Massol-Deya *et al.*, 1995). PCR-RFLP analysis of the 16S-23 rRNA intergenic region and sequence analysis of the 16S rRNA gene are essential tools in clustering genetically related rhizobia. These techniques have been frequently used in microbial taxonomy to determine inter and intra-specific relationships (Abaidoo *et al.*, 2000; Doignon-Bourcier *et al.*, 2000; Sarr *et al.*, 2005). The 16S rRNA gene sequences are an indispensable parameter in rhizobial taxonomy and methods based on differences in ribosomal RNA genes have been frequently applied to species identification (Laguerre *et al.*, 1994). Nevertheless, the conservative nature of 16S rRNA genes limits its use for discrimination at the strain level.

### **1.5 Production of common bean and their response to rhizobial inoculation.**

Common beans (*Phaseolus vulgaris L.*) are important grain legumes in human diets. The crop is mainly cultivated by women, and it is the primary staple food for over 200 million people in sub-Saharan Africa (Orlando, 2012). Cultivation of common bean in Africa is widespread, but production (approximately 80 percent of African bean production) is concentrated in 10 countries. In terms of area, Kenya is the leading producer of common bean

in Africa followed by Uganda and then Tanzania (FAO, 2008). However, in terms of production, Kenya comes second after Uganda. Common bean is considered as a short-season crop with most bush varieties maturing in 65 to 110 days from emergence to physiological maturing (Buruchara, 2007). Maturity may extend to 200 days among climbing varieties (Gomez, 2004).

Common bean fixes about 10 kg shoot N per ton dry matter. Since belowground N represent 25-50% of total plant N, 15 kg total N is fixed per ton of shoot dry matter for common bean (Murray *et al.*, 2008). Ideally, inoculating common bean variety GLP-2 with commercial inoculants increases shoot dry matter per plant (Chemining'wa *et al.*, 2011). However, reports have shown that common bean (*Phaseolus vulgaris L.*) often shows poor nodulation and reduced BNF (Graham *et al.*, 1982; Serraj and Sinclair, 1998 and Giller, 2001). Failure of the beans to respond to inoculants can be attributed to the failure to select inoculant rhizobia from indigenous strains well adapted to a range of environmental conditions (Maingi *et al.*, 2000). Deficient levels of P and low pH interfere with nodulation and rhizobia population sizes. As reported by Chemining'wa *et al.*, (2011) in a study conducted in central Kenya, soils that have low pH and P have reduced nodulation as compared to sites whose soil has a high pH and P. The competition between indigenous rhizobia and introduced strains for nodulation is a key determinant in successful inoculation. Considering variables such as seed and pod numbers per plant, *Rhizobium* inoculation has failed to yield any significant influence on the said parameters. This can be attributed to high populations of ineffective and more competitive indigenous rhizobia (Masandu and Ogendo, 2001).

Nitrogen fixation with common bean can be improved through selection of both effectively nodulating and competitive rhizobia strains (Shamseldin and Werner, 2004), selection of the strains more adapted to environmental stresses such as salinity (Shamseldin and Werner,

2005), improve dual-strains inoculum of *Rhizobium* with some of diazotrophs that increase the common bean nodulation (Straub *et al.*, 1987), understanding genetic diversity of rhizobial strains (Moawad *et al.*, 2004) and knowledge on soil fertility status.

## **1.6 Biochar amendments.**

According to Lehmann and Joseph, ( 2009) biochar is described as biomass that has been pyrolysed in a zero or low oxygen environment, produced with the intent to deliberately apply to soils. Owing to the inherent properties of biochar, application to soil at a specific site is expected to sustainably sequester carbon and improve soil functions while avoiding short- and long-term detrimental effects to the wider environment as well as human and animal health (Verheijen *et al.*, 2009).

As is evident in the *terra preta* (soils particular to the Amazonian Basin) soils, the addition of biochar darkens the colour of soils (Sohi *et al.*, 2009). Expectation of increased soil fertility benefits arise from studies of the *terra preta* soils that contains high proportions of black carbon (Haumaier *et al.*, 1995; Glaser *et al.*, 2002; Lehmann *et al.*, 2003; Lehmann and Rondon, 2006). The evident fertility of the *terra preta* soils is generally attributed to increased soil organic matter content as compared to adjacent soils and are now used to produce fruit crops (Krull, 2009).

### **1.6.1 Properties of Biochar**

The relative stable nature of biochar facilitates the ability to sequester carbon in the soil and as such, biochar is very relevant in solving climate change issue due to its long turnover time in soil. Despite the difficulties experienced in estimating the period in which newly created biochar can remain viable in the soil, most of its proponents estimate the time to be up to five thousand years.

The pyrolysis process greatly affects the qualities of biochar and its potential value to agriculture in terms of agronomic performance or in carbon sequestration. The process and process parameters, principally temperature and furnace residence time, are particularly important; however, the process and process conditions also interact with feedstock type in determining the nature of the product. These variables together are key influences on the chemical, biological and physical properties, which limit the potential use for biochar products (Sohi *et. al.*, 2009). Analysis conducted by Wang *et al.*, (2013) on crop residues and wood material based biochar, demonstrated a significant level of variance in the chemical and physical properties of the resulting biochar. The biochars from five crop-residues (rice straw, maize straw, wheat straw, coconut shell, and rice husk) and two wood-based biochars (elm and bamboo) were pyrolyzed at 500 °C to 700 °C for 4, 8 and 16 hours. An increase in pyrolysing temperature to 700 °C from 500 °C causes the respective biochars to increase their pH, surface area, ash content, and total Ca and P contents and a decrease in cation exchange capacity, yield, total N and total acid (Wang *et. al.*, 2013). Subsequently, prolonging residence time from 4 to 8 or 16 hours increases the ash content and surface area of the biochars and a decrease in yield. An increase in temperature results to the formation of more aromatic and recalcitrant structures in the biochars (Sohi *et. al.*, 2009). Crop residue biochars have a higher ash percentage, CEC, and total contents of P, N, Mg, and Ca besides having higher pH, yield, and contents of total acid, C, Na, and K as compared to wood-based biochars. Increasing pyrolysis temperature from 300 to 800°C decreased the yield of biochar from 67 to 26% and increased the carbon content from 56 to 93% (Tanaka, 1963).

Kuwagaki (1990) proposed that seven properties should be measured for a quality assessment for agronomically-used biochar: pH, volatile compound content, ash content, water holding capacity, bulk density, pore volume, and specific surface area. In general, the carbon content of biochar is inversely related to biochar yield.



### 1.6.2 Biochar feedstocks

Current results suggest that the type of feedstock used for pyrolysis is more important where biochar is to be applied as a soil conditioner (Sohi *et al.*, 2009). Indeed, feedstock is a primary factor governing the chemical and physical properties of biochar (Sohi *et al.*, 2009).

An array of feedstocks include but not limited to waste-streams such as wood chips, sawmill waste, hurricane debris, poultry litter (Das *et al.*, 2008), used cardboard products, sewage sludge (Shinogi *et al.*, 2002) and urban lawn debris (such as, grass clippings, tree branches and leaves). Feedstocks can also come from crop residues such as nut shells and rice husks (Demirbas, 2006; Demirbas *et al.*, 2006), corn stover, bagasse from the sugarcane and olive waste (Yaman, 2004), wheat straw and other agricultural by-products. Analyzing feedstocks in terms their lifecycle and overall sustainability is very critical in selecting the most appropriate types (Lal, 2005).

It is essential to consider the amount of toxins in feedstocks before using them because some feedstocks such as sewage sludge and landfill and industrial waste may contain heavy metals. Before setting up a biochar operation it is essential to consider all their aspects on a business/local community level (Lehmann and Joseph, 2012).

The elemental ratios of carbon, oxygen and hydrogen are key feedstock parameters in commercial use and the quality of fuel products (Friedl *et al.*, 2005). The proportions of hemi-cellulose, cellulose and lignin content determine the ratios of volatile carbon (in bio-oil and gas) and stabilised carbon (biochar) in pyrolysis products. Feedstocks with high lignin content produce the highest biochar yields when pyrolysed at moderate temperatures; approximately 500 °C (Fushimi *et al.*, 2003; Demirbas, 2006).

For a particular feedstock, the elemental composition of the pyrolysis products can still be greatly affected by the processing temperature and pyrolysis residence time (Sohi *et al.*, 2009). For instance, there was a corresponding impact on the pH of the biochar from 7.6 at 310°C to 9.7 at 850°C (Kuwagaki and Tamura, 1990).

### **1.6.3 Production of biochar**

There are four major techniques of producing biochar which are but not limited to slow pyrolysis, flash pyrolysis, fast pyrolysis, and hydrothermal carbonization. The slow pyrolysis differs from fast pyrolysis in that the latter involves heating biomass at a rate of 500 °C to 700 °C while the former takes place at a rate of 300 °C (Ronsse *et al.*, 2013). The feedstock undergoes a series of pretreatment stages in order to increase yield. The steps include drying, reducing the feedstock into appropriate size and freeing its alkaline compounds (Yronwode, 2011). Drying feedstock may produce volatile organic compounds, as such, all the exhaust fumes ought to be monitored to avoid poisoning and hazardous environment impact (Cummer and Brown, 2002).

### **1.6.4 Biochar as a soil amendment**

Soil amendment is the addition of any material to the soil with the aim of improving its physical properties such as permeability, water retention, water infiltration, structure, aeration and drainage. Soil amendments can be classified into two broad categories which include, inorganic and organic amendments (Sohi *et al.*, 2009). The central quality of biochar and char that makes it attractive as a soil amendment is its highly porous structure, potentially responsible for improved water retention and increased soil surface area (Sohi *et al.*, 2009). Addition of biochar to soil has also been associated with increased nutrient use efficiency, either through nutrients contained in biochar or through physico-chemical processes that

allow better utilisation of soil-inherent or fertilizer-derived nutrients (Sohi *et al.*, 2009). Besides, biochar's enhanced nutrient retention capacity reduces the total fertilizer requirement (Chan *et al.*, 2007).

As is evident in the *terra preta*, the addition of biochar darkens the colour of soil, especially in soils that are low in organic matter (Sohi *et al.*, 2009). Since dark soils absorb more solar energy they may, depending on water content and plant cover, display higher soil temperatures (Krull *et al.*, 2004). This will affect rate processes, enhancing the cycling of nutrients and potentially extending the growing season in seasonal climates.

The soil habitat is greatly modified by biochar since its material properties are not similar to the uncharred organic matter in the soil (Schmidt and Noack, 2000). The chemical stability of the biochar makes it difficult for microorganisms to utilize the N or C as a source of energy and probably nutrients contained in the C structures. As a result, biochar can be used to control emission of CH<sub>4</sub> and N<sub>2</sub>O by the soil (Sohi *et al.*, 2009). Research conducted by Rondon *et al.* (2006) in infertile tropical soils showed reduced N<sub>2</sub>O and increased water retention, cation exchange capacity, soil pH, and potassium availability with addition of biochar in the soil. Evidence from *terra preta*'s visual observation showed that biochar could stabilize other organic matter (Lehmann and Sohi, 2008; Sohi *et al.*, 2006). As such, biochar-based soil management practices could increase the net carbon gain.

Base cations (primarily Ca, Mg, and K) in biomass are transformed during pyrolysis into oxides, hydroxides, and carbonates (ash) that are mixed with the biochar. Due to the presence of these bases most biochars function as a liming agent when applied to soil. Biochar is a low density material that reduces soil bulk density (Laird *et al.*, 2010; Rogovska *et al.*, 2010) and thereby increases water infiltration, root penetration, and soil aeration. Furthermore, biochar has been shown to increase soil aggregate stability (Glaser *et al.*, 2002), although the

mechanism for this effect is not yet clear (Brodowski *et al.*, 2006). In an experiment conducted by Lehmann and Joseph, (2009) using high and low temperature biochars made from poultry litter and green waste and without applying any fertilizer also showed a 70% soil moisture increase in the soil.

The surface of biochar has carboxylic groups, which contribute to the negative charge and this attributes to the increase in CEC (Liang *et al.*, 2006). Application of biochar in soil may be done in conjunction with fertilizer management due to its low mineral nutrients available in its ash content, as such; biochar reduces the loss of  $\text{NH}_3$  through volatilization by effectively absorbing it (Lee *et al.*, 2010). Moreover, it decreases nutrient leaching while increasing fertilizer efficiency and reducing the contamination of underground water sources (Laird *et al.*, 2010).

A number of studies have illustrated that biochar can increase the cation exchange capacity of the soil. Once fresh biochar is exposed to oxygen and water in the soil environment, spontaneous oxidation reactions occur, resulting in an increase in the net negative charge and hence an increase in cation exchange capacity (Joseph *et al.*, 2009). As such, aged biochar particles are associated with high concentrations of negative charge, potentially promoting soil aggregation and increasing nutrient availability to plants (Liang *et al.*, 2006; Major *et al.*, 2010). However, Granatstein *et al.*, (2009) found that cation exchange capacity did not change significantly as a result of biochar application, although there was a trend of increasing cation exchange capacity when added to soils with a low initial cation exchange capacity. Inyang *et al.*, (2010) also measured the anion exchange capacity in bagasse biochars and suggest that the addition of biochar would significantly enhance the exchange capacities (cation and anion) of soils and improve their nutrient holding capacities. The physical quality of soil is also augmented by the biochar due to its high porosity, hence increasing the

hydraulic activity and the water-holding capacity of the soil (Laird *et al.*, 2010). The improved stability and structure formation of the soil decrease magnitude of soil loss due to erosional processes. However, scanty information and evidence exist, at present, on the impact of biochar on soil erosion (Stavi and Lal, 2012).

Biochar addition may affect the soil biological community composition as demonstrated for the biochar rich *terra preta* soils in the Amazon (Yin *et al.*, 2000; Kim *et al.*, 2007; O'Neill *et al.*, 2009; Grossman *et al.*, 2010) and has been shown to increase soil microbial biomass (Liang *et al.*, 2010; O'Neill *et al.*, 2009; Jin, 2010). Brussaard *et al.*, (2007) suggest that organic amendments are perhaps the most important means of managing biodiversity in soils. The microbial reproduction rate has also been shown to increase in some biochar-amended soils (Pietikäinen *et al.*, 2000; Steiner *et al.*, 2004)

However, biochar has also been shown to change soil biological community composition and abundance (Pietikäinen *et al.*, 2000; Yin *et al.*, 2000; Kim *et al.*, 2007; O'Neill *et al.*, 2009; Liang *et al.*, 2010; Grossman *et al.*, 2010; Jin, 2010). Such changes may well have effects on nutrient cycles (Steiner *et al.*, 2008) or soil structure (Rillig and Mummey, 2006) and, thereby, indirectly affect plant growth (Warnock *et al.*, 2007).

## **1.7 Problem Statement and Justification**

Smallerholder farming systems in sub-saharan Africa (SSA) are characterized by use of suboptimal inorganic levels due to their high prices and reduced economic return (Henao and Baanante, 2001). Declining soil fertility, high fertilizer costs and intensification of agriculture coupled with the reduction in farm sizes are major limitations to crop production in smallholder farms in Kenya (Maobe *et al.*, 2000; Cheruiyot *et al.*, 2001; Chemining'wa *et al.*, 2004). Chemical fertilizers do not improve soil physical structure or enhance soil biological

activity and by themselves are often insufficient to maintain soil fertility (Wallace and Knausenberger, 1997). According to Chemining'wa et al., (2007) reduced legume production is as a result of declining soil fertility and poor N availability for plants growth. Bationo *et al.*, (2011) also reported that low soil N is a major constraint to food production in Eastern and Central Africa. As a result, cheaper sources of nitrogen (Otieno *et. al.*, 2009) and alternative soil amendments strategies need to be sought if yields are to be sustained and food security attained.

Successful nitrogen fixation depends on the interaction between legume genotype, *Rhizobium* strain and environment (Giller, 2001). Reports have shown that common bean (*Phaseolus vulgaris* L.) show poor nodulation and limited response to rhizobia inoculation in terms of fixation rates (Giller, 2001, Graham *et al.*, 1982 and Serraj and Sinclair, 1998). The failure of common bean to respond to inoculants has been attributed to the failure to select the *Rhizobium* strains from indigenous populations which are well adapted to the actual environmental conditions (Maingi *et al.*, 2000), unsatisfactory host-microsymbiont interactions (Graham 1981) and environmental constrains such as acidity. Low fertile and highly acidic soils in Western Kenya often hamper BNF as reported by Chemining'wa *et al.*, (2011). The lack of effective rhizobial inoculants for leguminous crops adapted to African soils is also a major constraint to food production (Burton, 1981). Little is also known about the genetic diversity of indigenous rhizobia in Kenyan soils which can be efficiently exploited for improved agricultural productivity.

The legume-rhizobia symbiosis represents the most important route for sustainable nitrogen input into agroecosystems (Lindstorm *et al.*, 2010) compared to the application of N fertilizer (Hamdi, 1999; Bationo *et al.*, 2007). Inoculation with efficient strains of rhizobia may increase yields in areas where the bacteria are not already present (Giller, 2001). Full nitrogen

benefits can only be achieved in the presence of efficient rhizobia strains, which can be native to the soil or introduced in the form of commercial inoculants. The occurrence of a wide diversity of strains increases the opportunity for a legume host to find a compatible rhizobium in any particular soil and as such, continual isolation and characterization to identify new isolates offers the opportunity of improving BNF. A wide diversity of isolates ensures a sustainable source of replacement strains and may be developed into strains for commercial use (Musiyiwa *et al.*, 2005).

There is therefore a need to identify and assess the genetic diversity of indigenous rhizobia in Kenyan soils which are well adapted and can be efficiently exploited in their ability to biologically fix nitrogen for improved agricultural productivity.

There is a growing interest in using biochar as a soil amendment for improving soil quality. It has been previously shown that biochar addition to soil increases N fixation by both free-living and symbiotic diazotrophs (Ogawa, 1994; Rondon *et al.*, 2007). Therefore, the use of biochar as a soil amendment may improve survival of the rhizobia in the low fertile and acidic soils of West Kenya.

## **1.8 Objectives**

### **1.8.1 Broad objective**

Identify elite indigenous Kenyan *Rhizobium* for production of commercial bean inoculants with sustainable soil management practises.

### **1.8.2 Specific objectives**

1. To assess the effectiveness of elite indigenous Kenyan rhizobia isolates nodulating common bean (*Phaseolus vulgaris* L.) in the greenhouse.

2. To investigate the influence of biochar on the effectiveness of indigenous rhizobia nodulating common bean in low fertility soils.
3. To assess the genetic diversity of elite indigenous rhizobial isolates from diverse agro-ecosystems in Kenya.

### **1.9 Hypotheses.**

1. Elite indigenous rhizobia recovered from different Kenyan soils and ecological zones do not significantly differ in their effectiveness.
2. Biochar soil amendment has a significant effect on the nodulation and nitrogen fixation by indigenous rhizobia in common beans.
3. There is substantial genetic diversity among the elite indigenous strains from Kenyan soils and ecological zones.

### **1.10 Presentation of the Thesis**

The thesis is divided into six chapters addressing the evaluation of indigenous rhizobia on the performance of common bean (*Phaseolus vulgaris* L.) and the effect of biochar amendment in low fertility soils. Chapter 1 presents the Introduction and Literature Review. Chapter 2 describes the research approaches applied. Chapter 3 elaborates findings on the effectiveness of elite indigenous rhizobia isolates nodulating common bean. The observations of the effect of biochar as a soil amendment on performance of inoculated beans grown in farmers' field in Western Kenya are discussed in Chapter 4. The genetic diversity of the best performing rhizobia isolates with *Phaseolus vulgaris* L. and *Glycine max* is discussed in Chapter 5 while Chapter 6 presents a synopsis of the research findings, conclusions and recommendations.



## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Rhizobial isolates, origin and maintenance of cultures

The origin of the indigenous rhizobia isolates used in this study are presented in Table 2.1. These were selected from the original 380 rhizobia isolates which were collected from different regions of Kenya and screened in the greenhouse using sterile horticultural vermiculite held in pots (Waswa *et al.*, 2014) and N-free nutrient solution (Broughton and Dillworth, 1970). The isolates were maintained under refrigeration in Yeast Extract Mannitol Agar slants (Bohloul and Schmidt, 1970), which had the following composition: (Mannitol 10.0g/l, K<sub>2</sub>HPO<sub>4</sub> 0.5g/l, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.2g/l, NaCL 0.1g/l, Yeast Extract 1.0g/l and Agar 15g/l).

Table 2. 1: Indigenous rhizobia isolates screened in the greenhouse where non-sterile soil was used as media, showing the host the isolates were isolated and geographical area they were collected.

CODE	Geographical area (County)	Ecological zone	Host	Growth characteristic
NAK 5	Embu	montaine	<i>Macropitilium atropurpureum</i> *	Fast
NAK 8	Embu	montaine	<i>Macropitilium atropurpureum</i> *	Fast
NAK 22	Embu	montaine	<i>Macropitilium atropurpureum</i> *	Fast
NAK 45	Taita	montaine	<i>Macropitilium atropurpureum</i> *	Fast
NAK 67	Mt. Elgon (Busia)	montaine	<i>Phaseolus vulgaris</i> *	Fast
NAK 74	Nairobi	Wetland margin	<i>Phaseolus vulgaris</i> *	Fast
NAK 83	Bungoma	Midlands	<i>Glycine max</i>	Slow
NAK 92	Mumias (Kakamega)	Midlands	<i>Phaseolus vulgaris</i> *	Fast
NAK 104	Butula	Midlands	<i>Phaseolus vulgaris</i> *	Fast
NAK 157	Kwale	Coastal plain	<i>Phaseolus vulgaris</i> *	Fast
NAK 185	Kikuyu (Kiambu)	Escarpment forest	<i>Sesbania sesban</i>	Fast
NAK 186	Kikuyu (Kiambu)	Escarpment forest	<i>Sesbania sesban</i>	Fast
NAK 188	Kikuyu (Kiambu)	Escarpment forest	<i>Crotalaria sp.</i>	
NAK 189	Kikuyu (Kiambu)	Escarpment forest	<i>Clover sp.</i>	Fast
NAK 191	Naivasha	Salt lake margin	<i>Crotalaria pseudospartium</i>	Fast
NAK 208	Nairobi	Wetland margin	<i>Glycine max</i>	Fast

Source: N2Africa Rhizobium Database(Woomer *et al.*, 2013)

## **2.2 Site description and soil analysis**

Experiments were performed to identify best performing indigenous rhizobia associated with common bean from MIRCEN-N2Africa culture collection, University of Nairobi (Woomer *et al.*, 2014) and to investigate the influence of biochar on the effectiveness of indigenous rhizobia nodulating common beans in farmer's field in West Kenya. Greenhouse studies were conducted at University of Nairobi field station, Kabete Campus, using potted soil from Butula, West Kenya. The field experiments were conducted at two sites in West Kenya, Butere and Nyabeda, both situated within smallholder farming communities. Nyabeda is located at N 00° 08' 21.2" and E 034° 24' 32.3", 1331 m above sea level and Butula is located at N 00° 11' 47.8" and E 034° 30' 17.5" at the same elevation. The top soils (0–15 cm) from each field site (Butula, Butere and Nyabeda) were sampled before planting of by sampling randomly eight cores per replicate using a 3.5 cm soil auger, they were bulked and sub-sampled. Chemical characterization of soils was done for nutrient composition as described by Okalebo *et al.* (2002). The sites had no known recorded history of inoculation with *Rhizobium* strains.

## **2.3 Greenhouse experiments**

### **2.3.1 Assessment of indigenous rhizobial populations.**

The most-probable-number (MPN) method described by Woomer *et al.* (1990) was used to determine the number of viable and infective indigenous rhizobia populations in soil used for the greenhouse as well as the field experiments (Butere and Nyabeda). Seeds were surface sterilized and pre-germinated in sterile vermiculite. Two seeds were planted into the growth porches containing sterile N-free nutrient solution (Broughton and Dillworth, 1970; Somasegaran and Hoben, 1985) with four replicates per dilution. A 10-fold serial dilution was done for each soil by adding 1 g of soil into 9 ml of sterile water. This was mixed

thoroughly on a shaker for 20 minutes to disperse the soils. Dilutions were continued up to  $10^{-6}$  for each of the soils. Each growth porch was inoculated with 1 ml of the appropriate soil dilution. Non-inoculated control was included. The plants were then grown in the greenhouse for 28 days, regularly adding N-free nutrient solution (Broughton and Dillworth, 1970; Somasegaran and Hoben, 1985) as required, after which they were observed for nodulation. The presence and absence of nodules after 28 days were recorded according to Woomer *et al.*, 1990 and Olsen and Rice, 1996. Based on the formula by Somasegaran and Hoben (1985), the number of rhizobia per gram of soil was estimated by:



Figure 2.1: Picture of growth porches with 2 plants in each porch containing sterile N free nutrient solution with 4 replications per dilution

$m \times d \div v$  Where;

m = likely number from the MPN table for the lowest dilution series

d = lowest dilution (first unit)

v= volume of aliquot

Populations estimates were then assigned using the MPNES computer program (Woomer *et al.*, 1990)

## **2.3.2 Performance of indigenous isolates in potted soil from Butula.**

### **2.3.2.1 Experimental design and treatments**

The experiment was arranged as a split-plot in a Randomized Complete Block Design (RCBD) with four replicates. Bean cultivars, climbing and bush beans, were the main plots and the inoculation treatments and the controls formed the sub-plots. The inoculation treatments included the 16 rhizobial isolates, a plus-nitrogen control with no inoculation, a non-inoculated control with no nitrogen and two reference strain controls (CIAT 899 and USDA 2667). The plus-nitrogen control contained N applied as a 0.77g CAN per pot. Sympal, a basal dressing fertilizer, was also added to all pots at the rate of 500 kg ha<sup>-1</sup>. The treatments were randomized in each block.

### **2.3.2.2 Preparing Greenhouse pots**

A total of 160 plastic, three-liter pots were cleaned and decontaminated using commercial bleach. Pots contained sterile gravel at the bottom layer and 2 kgs of field soil from Butula. The soil was covered with a plate, to minimize airborne contamination, that had a hole for the plants and another where a pipe was inserted for watering the plants. Sterilization of both the pots and gravel was done by thoroughly washing with clean water and later soaking them in 3.85% sodium hypochlorite overnight and allowing the reagent to volitalize.

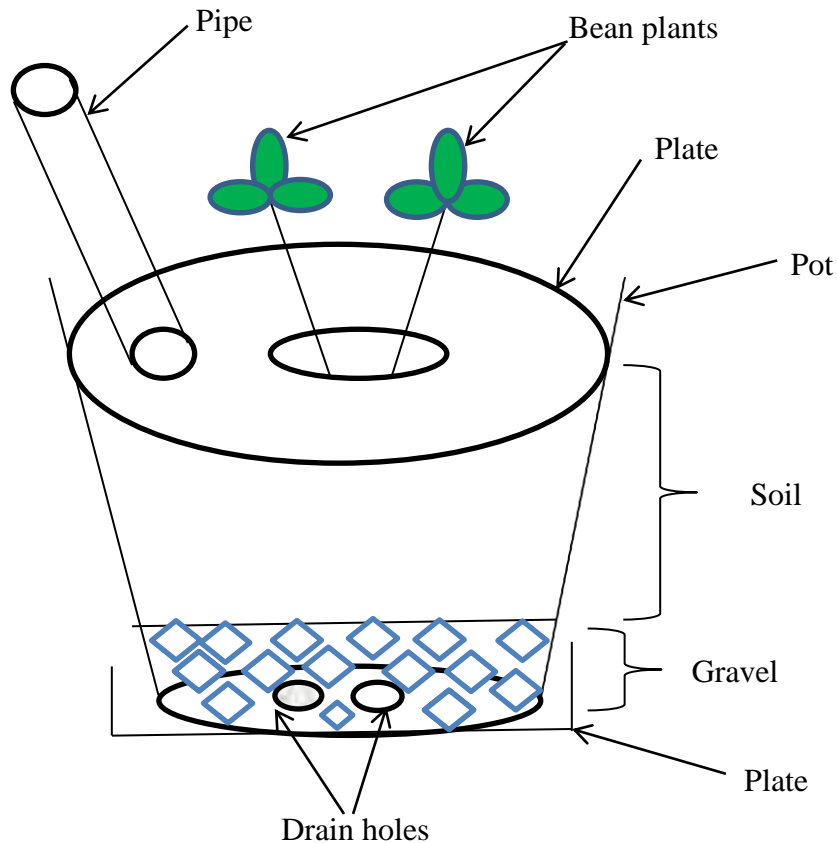


Figure 2.2: Arrangement of gravel and soil in pots used for effectiveness testing of indigenous rhizobia isolates in the greenhouse.

### 2.3.2.3 Culturing the rhizobia.

The rhizobia isolates and the two reference strains were cultured seven days prior to planting by growing the rhizobia in Yeast Extract Mannitol broth (Mannitol 10.0g/l,  $K_2HPO_4$  0.5g/l,  $MgSO_4 \cdot 7H_2O$  0.2g/l, NaCL 0.1g/l, Yeast Extract 1.0g/l). They were incubated at 28°C on a rotary shaker for 5 days to reach early turbidity (approximately  $1 \times 10^9$  cells  $ml^{-1}$ ).

### 2.3.2.4 Surface-sterilizing and pre-germination of seeds

Surface sterilization of the common bean seeds was performed by rinsing the seeds in 95% ethanol for 10 seconds and then soaking in 3.85% sodium hypochlorite solution for 5 minutes. The seeds were then rinsed with six changes of sterile distilled water. Aseptic procedures were observed throughout the rinsing. The seeds were sown in sterile horticultural

grade vermiculite held in shallow autoclavable polypropylene tray and then incubated at 28 °C for 2 days until the radicles were 0.5 - 1.0 cm long and ready for planting.

#### **2.3.2.5 Inoculating and planting of seeds.**

Three well-spaced holes were made in the potted soil to a depth that could accommodate the pre-germinated seeds below the surface (about 3 cm). Three well germinated seeds were placed in each hole with the radicle entering first using a sterile forcep. After placement of the seed, the holes were covered with the potted soil. Emerging seedlings were thinned to two uniform plants per pot after 10 days. The shoot of the unwanted plant was excised aseptically using sterile scissors to avoid contamination and disturbing the rooting medium during thinning. Inoculation with rhizobial culture was done after 7 days by dispensing 1 ml of rhizobial broth around the root of each plant in each pot. A sterile pipette for each rhizobial culture was used.

#### **2.3.2.6 Destructive Sampling**

Sampling was done at 40 days after planting by harvesting the two plants in each pot. Shoots of each treatment were oven dried at 70°C for 48 hours. The number of nodules per plant and shoot dry weight were determined and compared with those in the control treatments. The best performing rhizobia isolates, those that out-performed the commercial strains, were selected to be tested on-farm in Western Kenya.

### **2.4 On-farm Field trials**

#### **2.4.1 Experimental design and treatments.**

The experiment was set up as a Split-Plot in a Randomized Complete Block Design (RCBD) with four replicates. Each plot (15 m<sup>2</sup>) consisted of five rows, spaced 0.6 m apart. Plots,

within each block, were 0.5 m apart and the distance between blocks was 1 m. The bean cultivars were the main plots and the inoculation treatments were the sub-plots. Treatments included bean rhizobia isolates (8 isolates selected from the greenhouse pot experiment), two non-inoculated controls with and without N and two reference strains (USDA 2667 and CIAT 899). Treatments with biochar included most promising indigenous bean rhizobia isolates, CIAT 899 (*R. tropici*), two non-inoculated controls, with nitrogen and with no nitrogen. The position of these treatments were randomized within each block.

## **2.4.2 Culturing of rhizobia, carrier preparation and inoculant preparation.**

### **2.4.2.1 Culturing of rhizobia isolates**

Rhizobia isolates were cultured 21 days prior to planting by growing the rhizobia in 100 ml Erlenmeyer flasks containing 70 ml of yeast-mannitol broth as described in Section 2.3.2.3.

### **2.4.2.1 Preparation of Carriers**

The filter mud was ground using a hammer mill then sieved to pass through a 2.12  $\mu\text{m}$  mesh. The filter mud was wetted to about 45% moisture holding capacity and later packed into 50 g autoclavable polyethylene bags as described by Woomer *et al.* (2011). The bags were immediately sealed to maintain the moisture content and left to stay for 2 days allowing spores to grow. Packaged carrier materials used in this experiment were subjected to triple sterilization under conditions of 121  $^{\circ}\text{C}$  for 3 hours, to ensure proper sterility of the carrier.

### **2.4.2.2 Preparation of rhizobial inoculants.**

Each package of sterilized carrier material was then injected with the appropriate rhizobial broth (approximately  $1 \times 10^9$  cells  $\text{ml}^{-1}$ ). The volume of bacterial broth added was 50% of the water holding capacity of the filter mud. Rhizobial broths were injected aseptically using



sterile syringes into the bags that contained carrier materials then mixed thoroughly. The punctured area was wiped with 70% alcohol and sealed. The packets were stored at room temperatures (23°C) for 14 days before they were used to allow hardening of the inoculant rhizobia. This handling resembles the curing interval of commercial inoculants.

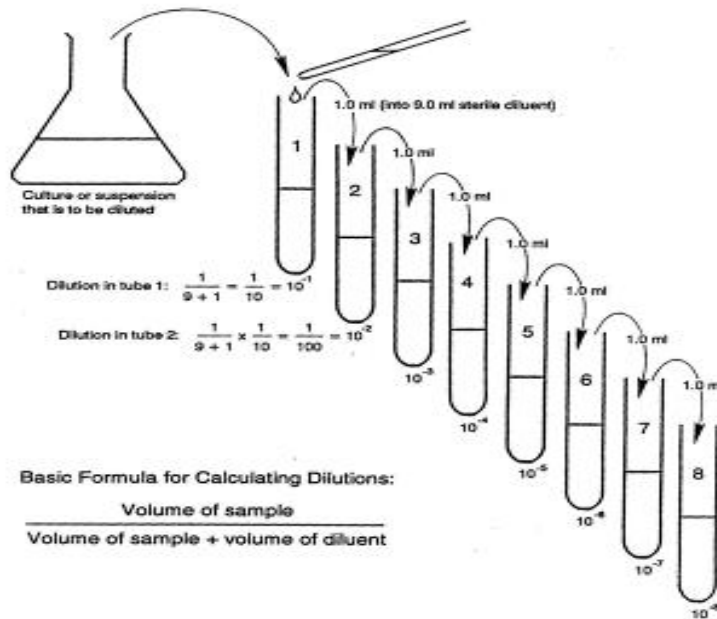


Figure 2.3: Procedure for 10-fold serial dilution

### 2.4.3 Testing the quality of the inoculants

After 14 days from inoculation of the filter mud carrier as described in Section 2.4.2, ten samples, representing each rhizobial isolate and reference strain, were picked randomly to determine the number of viable rhizobia and contaminants. These were determined through the drop-plate method described by Miles and Misra, (1938). A 10-fold serial dilution was done for each packet as shown in Figure 2.3 by adding an initial 1 g of inoculant into 9 ml of sterile water. This was mixed thoroughly on a shaker for 20 minutes to disperse the inoculant. Dilutions were continued up to  $10^{-7}$  for each sample ensuring each dilution was thoroughly mixed. A sterile pipette tip was used for each dilution level. Drops of 20  $\mu\text{l}$  for dilutions of  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  replicated three times were plated on YEM agar containing congo red for

all the samples. All the plates were incubated at 28°C for at least 24 hours before colonies were counted and the CFU g<sup>-1</sup> determined. From this value, the rhizobia population was calculated. Only the number of colonies that grew in the range of 5 to 55 colonies were counted.

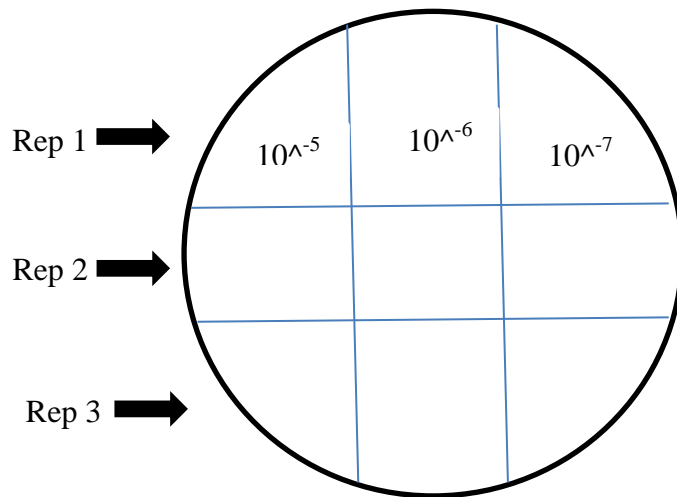


Figure 2.4: A drop plate showing three dilutions each with three replicates.

Viable population of rhizobia per gram of inoculants was calculated using the following formula:

$$\text{No. of cells per g} = 1/\text{drop volume} \times \text{number of colonies} \times \text{dilution factor}$$

#### 2.4.4 Inoculating and planting of seeds

The seeds were inoculated with filter mud-based inoculants using a solution of gum arabic (40%, w/v) as a sticker. Based on the viable counts of inoculants (approx.  $1.0 \times 10^9$  rhizobia g<sup>-1</sup>) and on the average weight of the individual seed, seed lots were inoculated to give a population of  $10^6$  rhizobia cells/seed. The seeds were planted immediately after inoculation at spacing of 10 cm by 60 cm and 15 cm by 60 cm for bush beans (New Rosecoco) and climbing bean (Kenya Tamu) respectively.

#### **2.4.5 Application of inputs.**

Biochar was added at the rate of 2 t ha<sup>-1</sup> to treatments receiving biochar. CAN was applied to the N fertilized treatments at the rate 0.375 kg per plot (78 kg N ha<sup>-1</sup>) split in three doses. Sympal, a commercially-available fertilizer blend for legumes (0-23-15 + Ca, Mg and S), was also added to all plots at the rate of 0.375 per plot (200 kg ha<sup>-1</sup>) as basal dressing. All the inputs were applied in the furrow and incorporated to a depth of 3 cm.

#### **2.4.6 Sampling and harvesting**

The first sampling was recovered at 48 days from planting, coinciding with 50% flowering of the plants. The second sampling was done at crop maturity, 90 and 120 days from sowing, for New Rosecoco and Kenya Tamu respectively. The first rows on either side of the plots were omitted to eliminate contamination. During the first sampling, plants were carefully uprooted, samples were taken from an area of 0.3m<sup>2</sup>. Roots were separated from the shoots and then soil was washed off in gently running water to ensure that roots and nodules remained intact and nodules were recovered and counted. Shoots and nodules were oven dried for 48 hours at 70 °C and dry weight recorded. At harvest, beans were harvested from an area of 5.4 m<sup>2</sup> and the dry weight of plant biomass and grain yield recorded.

### **2.5 Genetic diversity of elite indigenous rhizobial isolates**

#### **2.5.1 Culturing of the rhizobia isolates**

The rhizobia isolates and the two reference strains (USDA 110 and CIAT 899) were cultured seven days prior to DNA extraction by growing the rhizobia in Yeast Extract Mannitol broth (Mannitol 10.0g/l, K<sub>2</sub>HPO<sub>4</sub> 0.5g/l, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.2g/l, NaCL 0.1g/l, Yeast Extract 1.0g/l). They were incubated at 28°C on a rotary shaker for 5 and 7 days to reach early turbidity for fast and slow growers respectively (approximately 1 x 10<sup>9</sup> cells ml<sup>-1</sup>).

## **2.5.2 Total genomic DNA extraction from liquid bacterial cultures**

Liquid culture (1.2 ml), from each of the pure rhizobial cultures, was centrifuged for 5 minutes at 3000 rpm at room temperature and the supernatant poured out. The pellet was then cleaned with 500 µl of TE 1X, centrifuged for 5 minutes at 13000 rpm and the supernatant poured out. The pellet was resuspended in 540 µl of TE 5 X and incubated for 5 minutes at 70 °C. 2 µl of Proteinase K and 30 µl of 10% SDS (w/v) were added and incubated for 15 minutes at 70 °C. 600 µl of phenol:chloroform:isoamylalcohol 25:24:1 (v/v/v) was added and centrifuged at 13000 rpm for 5 minutes at room temperature. The supernatant was transferred to a clean tube and extracted with an equal volume of chloroform isoamylalcohol 24:1 (v/v) to wash off residual phenol. The supernatant was transferred into another clean 1.5 ml tube and the volume noted after centrifuging for 5 minutes at 13000 rpm at 20 °C. Ice-cold isopropanol equaling 100 µl was added, mixed gently and incubated overnight at -20 °C for DNA precipitation. The samples were then centrifuged for 15 minutes at 13000 rpm at 4 °C. The supernatant was poured out and the pellet washed by adding 100 µl of 70% ethanol and centrifuged for 15 minutes at 13000 rpm at 4 °C. The supernatant was poured off and the pellet air-dried. The pellet was re-suspended with 50 µl of sterile distilled water and stored at -20 °C.

## **2.5.3 PCR-RFLP analysis of the 16S-23S rDNA intergenic spacer region**

### **2.5.3.1 PCR amplification**

A fragment of the intergenic region between the 16S and 23S rDNA (930-1050 bp) was amplified by PCR using two primers: FGPS 1490-72; 5'-TGCGGCTGGATCCCCTCCTT-3' (Normand *et al.*, 1996) derived from the 3' end of the 16S rDNA corresponding to positions 1,521-1,541 of *E. coli* and from the 5' end of the 23S rDNA (FGPL 132-38; 5' CCGGGTTTCCCCATTCGG-3') corresponding to positions 114-132 of *E. coli* (Ponsonnet

and Nesme, 1994). PCR amplification was carried out in a 25  $\mu$ l reaction volume containing 2  $\mu$ l of total DNA extract, 1.0  $\mu$ M of each primer and one freeze dried bead (Ready-to-Go PCR beads, Pharmacia Biotech) containing 2.5 U of *Taq* DNA polymerase, 10 mM Tris- HCL, (pH 9 at RT), 50 mM KCL, 1.5 mM MgCl<sub>2</sub> and 200  $\mu$ M of each dNTP. PCR amplification was performed in a Bio-Rad PCR system thermal cycler adjusted to the following program: initial denaturation for 5 minutes at 94 °C, 35 cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 58 °C, extension for 30 seconds at 72 °C and final extension for 7 minutes at 72 °C. PCR amplified DNA was visualized by electrophoresis of 3  $\mu$ l of the amplified DNA on 2% (w/v) horizontal agarose gel (SIGMA®) in TBE buffer (1.1 w/v Tris-HCL; 0.1% w/v Na<sub>2</sub>EDTA 2H<sub>2</sub>O; 0.55% w/v Boric acid), prestained with 3.5  $\mu$ l of ethidium bromide. The gel was photographed under UV illumination with Gel Doc (BIO-RAD) Software (USA).

### **2.5.3.2 Restriction fragment analysis**

Aliquots (10  $\mu$ l) of PCR products were digested with 5 U (1  $\mu$ l of a 5 U/ $\mu$ l solution) of *Msp* I restriction endonuclease, restriction buffer (2 $\mu$ l) and sterile micropure water (2  $\mu$ l) in a total volume of 15  $\mu$ l for 2 hours at 37°C. The RFLP profiles were then visualised by gel electrophoresis on a 3% agarose gel (w/v) pre-stained with 0.117  $\mu$ g ml<sup>-1</sup> ethidium bromide. 7  $\mu$ l of the restricted product with 7  $\mu$ l of loading dye were loaded in the agarose gel and run at 100 V for 3 hours. The gel was then visualized under UV trans-illumination and photographed using gel documentation system. Strains that had identical restriction fragment profiles were classified into the same genotypic/intergenic spacer (IGS) group.

## **2.5.4 Sequence analysis of the 16S rRNA gene**

### **2.5.4.1 PCR of the 16S rRNA gene**

PCR was carried out on the 13 DNA samples. Primers used for sequencing were the following: the forward primer 27F (5'AGAGTTTGATCCTGGCTCAG3') corresponding to positions 27–46 of the *E.coli* of the 16S rRNA gene sequence and the reverse primer 1492R (5'TACGGCTACCTTGTTACGACTT 3') corresponding to positions 1,525–1,506 of *E.coli* (Lane 1991). PCR mix for one sample included: PCR master 12.5 µl, Forward primer 27F 1 µl, Reverse primer 1 µl, sterile distilled water 7.5 µl and 3 µl DNA template. PCR amplification was performed in a Bio-Rad PCR system thermal cycler adjusted to the following program: initial denaturation for 5 minutes at 94 °C, 35 cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 58 °C, extension for 30 seconds at 72 °C and final extension for 7 minutes at 72 °C.

### **2.5.4.2 Purification of PCR product**

High pure PCR product kit version 15, (Roche 2010) was used for purification of PCR product. This kit includes binding buffer, washing buffer and elution buffer. PCR product (25 µl) was mixed well with 125 µl binding buffer, centrifuged for 1 minute at 13000 rpm and flow through was discarded. Then, 125 µl of washing buffer were added each to the mixture and centrifuged for 1 minute at 13000 rpm and flow-through was discarded respectively. Finally, 25 µl of elution buffer was added, centrifuged for 1 minute at 13000 rpm and purified PCR products were collected.

After purification, the PCR products were again subjected to electrophoresis to check for purity and molecular size.

#### **2.5.4.3 Partial 16S rRNA gene sequencing**

Purified PCR products were sequenced for the DNA region coding for the 16S rRNA gene in an ABI 377 (PE-Applied Biosystems sequence analyzer) at the Segolip unit of the BeCA hub, ILRI.

#### **2.5.5 Data Analysis**

The generated sequences were submitted to the GenBank database through BLAST to search for significant 16S rRNA alignments. A phylogenetic tree was constructed based on the partial 16S rRNA gene sequences of the indigenous rhizobial isolates and rhizobial reference strains from the GenBank. The sequences of the rhizobial strains were aligned pairwise and compared to type strains in the GenBank database. A dendrogram was inferred with Neighbour-Joining Algorithm (Saitou and Nei 1987) using ClustalX software (Thompson *et al.*, 1997) and the phylogenetic tree reconstructed with PHYLIP (Felsenstein, 1993), package and a bootstrap analysis using 100 replications.

## CHAPTER 3

### EFFECTIVENESS OF ELITE KENYAN RHIZOBIA NODULATING COMMON BEAN (*Phaseolus vulgaris* L.).

#### Abstract

Identification of elite strains of rhizobia in Kenya that effectively nodulates common beans is intended to provide superior rhizobia for use as commercial legume inoculants. Greenhouse and field experiments were conducted to evaluate the effectiveness of Kenyan native rhizobia in symbiotic association with two common bean cultivars, Kenya Tamu (climbing bean) and New Rosecoco (bush bean). In the greenhouse experiment conducted at University of Nairobi, Kabete using representative soil from Butula in Busia County, the two bean cultivars were inoculated with 16 elite indigenous isolates which were compared to two strains widely used commercial inoculants namely USDA 2667 (*R. leguminosarum* bv. *phaseoli*) and CIAT 899 (*R. tropici*). In the field experiments, bean cultivars were inoculated with eight most promising indigenous isolates (NAK 5, NAK 45, NAK 67, NAK 92, NAK 104, NAK 157, NAK 186 and NAK 191) identified in the greenhouse experiment. The experiments were designed as split-plot in a Randomized Complete Block Design. In the greenhouse, the interaction between common bean and rhizobia isolates was significantly different in terms of plant biomass at  $p=0.02$  and  $p=0.03$  in the first and second experiment respectively. Three of the isolate (NAK 5, NAK 186 and NAK 191) outperformed commercial strain (CIAT 899) and eight (NAK 104, NAK 92, NAK 5, NAK 208, NAK 189, NAK 188, NAK 186 and NAK 191) outperformed USDA 2667 with Kenya Tamu variety in the two greenhouse experiments. Six isolates (NAK 22, NAK 83, NAK 104, NAK 45, NAK 5 and NAK 92) outperformed CIAT 899 and two isolates (NAK 5 and NAK 92) outperformed USDA 2667 New Rosecoco variety. The rhizobia isolate NAK 5 outperformed CIAT 899 and USDA 2667



for both Kenya Tamu and New Rosecoco varieties in terms of plant biomass. There were no differences observed among the eight Kenyan isolates and the standard strains in terms of shoot dry weight, nodule dry weight and grain yield over two seasons conducted at Butere and Nyabeda, west Kenya. There were also no interactions observed between rhizobia isolates and bean varieties in both seasons at both Nyabeda and Butere. Indications are that the isolates have not yielded significant differences under field conditions and warrants further comparison under different field conditions. There were also indications of native rhizobia obscuring response to inoculation.

### **3.1 Introduction**

Common bean (*Phaseolus vulgaris L.*) is an important grain legume in human diets and a major food crop in SSA (Katungi, 2009; Chemining'wa *et al.*, 2011 and Kaizzi *et al.*, 2012). Beans production has continuously exhibited a downward trend due to several biotic, abiotic and socio-economic constraints (Kambewa, 1997; Xavery *et al.*, 2006). According to Chemining'wa *et al.*, (2007) the reduced legume production is as a result of declining soil fertility and poor N availability for plants growth. Substantial N inputs are required for optimum plant growth and adequate food, feed and fibre production. Nitrogen in agriculture is obtained from soil through mineralization of soil organic matter and from external sources, both organic and inorganic and its application to crops often results in yield improvement (Giller, 2001). Nitrogen deficiency may exert pronounced adverse effects on crop development and yield.

Legume-rhizobia symbiosis represents the most important route for sustainable nitrogen input into agroecosystems (Lindstorm *et al.*, 2010) than the application of inorganic N fertilizers (Hamdi, 1999; Bationo *et al.*, 2007). Grain legumes can yield up to about 300 kg N ha<sup>-1</sup> year<sup>-1</sup>, while tree legumes can fix as much as 600 kg N ha<sup>-1</sup> year<sup>-1</sup> with well matched symbionts

(Giller, 2001). However, common bean show poor nodulation and reduced BNF (Graham *et al.*, 1982; Serraj and Sinclair, 1998 and Giller, 2001) fixing 15 kg total N per ton of shoot dry matter (Murray *et al.*, 2008). This has been ascribed to unsatisfactory host-microsymbiont interactions (Graham, 1981; Moawad *et al.*, 2004; Shamseldin and Werner, 2005) and environmental agronomic factors such as salinity of soils (Graham, 1992) and soil acidity (Arons and Graham, 1991), high temperature in the rhizosphere (Karanja and Wood, 1988), susceptibility to bacteriophage (Mendum *et al.*, 2001), losing of symbiotic plasmid (Noel *et al.*, 1986), defect in *nod* genes based on genetics rearrangement between plasmid and genomic DNA (Girard *et al.*, 1991), low phosphorus (Powell, 1980), competition with native rhizobial strains (Anyango *et al.*, 1998) and high available nitrogen (Crespo *et al.*, 1987). There have also been reports of inoculant strains losing their symbiotic properties (Weaver and Wright, 1987).

The host-rhizobia symbiosis requires a precise matching to enable maximization of symbiotic nitrogen fixation. The greatest challenge therefore, is to match legume crops and rhizobia for optimal effectiveness either by having plant genotypes adapted to local rhizobial populations or by inoculation with effective rhizobia strains which are highly competitive and adapted to prevailing environmental conditions (Lindstorm *et al.*, 2010). For example, a wide diversity of soybean-nodulating rhizobia has been found in Kenyan soils (Waswa *et al.*, 2014). However, only six out of the 100 isolates obtained from the study were symbiotically more effective than the standard commercial strain USDA 110. This emphasizes the possibility of closer matching of strain and crop for improved symbiotic efficiency by careful strain selection. It is therefore important to continually isolate and identify higher nitrogen-fixing isolates to be used as inoculant strains from the wide diversity of indigenous rhizobia (Lindstorm *et al.*, 2010). A wide diversity of isolates ensures a sustainable source of

replacement strains and which may be developed into strains for commercial use (Musiyiwa *et al.*, 2005).

One empirical approach to identification of elite rhizobia strain involves the stepwise collection, isolation and authentication of native rhizobia, the screening of the isolates against reference strains for symbiotic effectiveness, the assessment of their competitive abilities and the evaluation of their performance under a range of field conditions (Howieson *et al.*, 2000). This stepwise approach assists in eliminating the worst performing isolates from further consideration. This approach was used in this study to evaluate the effectiveness of Kenya's native rhizobia on farmer accepted varieties of common beans and is intended to result in improved legume inoculants by identifying elite indigenous rhizobia.

## **3.2 Material and methods**

### **3.2.1 Greenhouse experiments**

#### **3.2.1.1 Assessment of indigenous rhizobial populations.**

The experiment was conducted at University of Nairobi field station, Kabete Campus. The most-probable-number (MPN) method described by Woome *et al.* (1990) was used to determine the number of viable and infective indigenous rhizobia populations in the red clayey loam soils from Butula, Nyabeda and Butere as described in Section 2.3.1.

#### **3.2.1.2 Performance of indigenous isolates in potted soil from Butula.**

The experiment was conducted in the greenhouse at University of Nairobi field station, Kabete Campus. The effectiveness of 16 rhizobial isolates in symbiotic association with two common bean varieties, Kenya Tamu (climbing bean) and New Rosecoco (bush bean), were evaluated in greenhouse potted soil. The experiment was arranged as a Split-Plot in a Randomized Complete Block Design (RCBD) with four replicates as described in Section

2.3.2.1. The isolates including NAK 5, NAK 8, NAK 22, NAK 45, NAK 67, NAK 74, NAK 83, NAK 92, NAK 104, NAK 157, NAK 185, NAK 186, NAK 188, NAK 189, NAK 191 and NAK 208 were isolated from a range of agroecological zones in Kenya including the coastal plain and its mangrove swamps; the uplands and Rift Valley highlands; the Afro-montane zone of Mount Elgon and the Lake Victoria Basin, covering about 1045 km as shown in Table 2.1. The isolates were maintained and grown in Yeast Extract Mannitol.

Red clayey loam collected from a farm in Butula, west Kenya was used in this experiment and the chemical analysis done for its nutrient composition as described by Okalebo *et al.* (2002).

Greenhouse pots were sterilized and filled with sterile gravel at the bottom layer and 2 kgs of soil as shown in Figure 2.3. The rhizobia isolates and the two reference strains were cultured seven days prior to planting to reach early turbidity (approximately  $1 \times 10^9$  cells  $\text{ml}^{-1}$ ). Surface sterilization of the bean seeds was performed as described in Section 2.3.2.4 and were sown in sterile horticultural grade vermiculite held in shallow autoclavable polypropylene tray and then incubated at  $28^\circ\text{C}$  for 2 days until the radicles were 0.5 - 1.0 cm long and ready for planting. The soil was fertilized with Sympal, a commercially-available fertilizer blend for legumes (0-23-15 + Ca, Mg and S) at a rate of  $500 \text{ kg ha}^{-1}$  mixed with two kg of soil  $\text{pot}^{-1}$ . Planting was done by placing three well germinated seeds in each of the well-spaced holes (about 3 cm) in each pot and covering them with the potted soil as described in Section 2.3.2.5. Pots were regularly irrigated with rhizobia-free water.

Emerging seedlings were thinned to two uniform plants per pot after 10 days. Inoculation was done after 7 days by dispensing 1 ml of rhizobial broth around the root of each plant in each pot.

Sampling was done at 40 days after planting by harvesting the two plants in each pot. The number of nodules per plant and shoot dry weight were determined and compared with those in the control treatments as described in Section 2.3.2.6. The best performing isolates were selected for field testing.

### **3.2.2 On-farm Field trials**

Eight best performing isolates rhizobia were selected from the potted soil experiment for assessment under field under farmer conditions. A two-season field experiment was established in west Kenya at Nyabeda and Butere farms during 2013 long rains (March to August) and 2013-2014 short rains (September to January). Chemical analysis of the red clayey loam soils from these farms was done for nutrient composition as described by Okalebo *et al.* (2002). The eight indigenous isolates and two reference strains (USDA 2667 and CIAT 899) were compared on climbing (Kenya Tamu) and bush (New Rosecoco) beans.

The experiment was arranged as a Split-Plot in a Randomized Complete Block Design (RCBD) with four replicates. The bean cultivars, Kenya Tamu and New Rosecoco, were the main plots and the inoculation treatments were the sub-plots. Inoculation treatments included indigenous isolates, two non-inoculated controls with and without N and two standard reference strains (USDA 2667 and CIAT 899). CAN was applied to the N fertilized treatments (+N) at the rate 0.375 kg per plot (78 kg N ha<sup>-1</sup>) split in three doses. Sympal fertilizer was also added to all plots at the rate of 0.375 per plot (200 kg ha<sup>-1</sup>) as basal dressing. All the inputs were applied in the furrow and incorporated to a depth of 3 cm. The seeds were inoculated with filter mud-based inoculants (Section 2.4.3) using a solution of gum arabic (40%, w/v) as a sticker and planted immediately at spacing of 10 cm by 60 cm and 15 cm by 60 cm for bush beans (New Rosecoco) and climbing bean (Kenya Tamu) respectively as described in Section 2.4.4.

The first sampling was recovered at 48 days from planting and the second at crop maturity, 90 and 120 days from sowing for New Rosecoco and Kenya Tamu respectively as described in Section 2.4.6.

### 3.2.3 Statistical analysis

The MPNES program (Woomer *et al.*, 1990) was used to calculate the rhizobial populations in the soils. Analysis of variance (ANOVA) was used for evaluation of symbiotic properties of rhizobia. To compare treatment means, Fisher's protected LSD method was used at a significance level of 5%. The analysis was performed using GENSTAT statistical package (GenStat Release 6.1 Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK).

## 3.3 Results

### 3.3.1 Chemical characteristics of soils used in the greenhouse and field trials

The chemical characteristics of soils used in the greenhouse and field are indicated in Table 3.1. The soil pH was acidic and ranged from 5.2 to 5.7 in the three farms. The nutrient levels in the soils were relatively low particularly N and P. These nutrients were critically low, factors that affects biological nitrogen fixation.

Table 3.1: Characteristics of soils used in greenhouse and at field level (Butere and Nyabeda).

Site	pH H <sub>2</sub> O	C -----%-----	N	K ----- (ppm) -----	P
Greenhouse (soil from Butula)	5.5	1.86	0.08	73	4.9
Butere farm	5.2	1.5	0.24	101	3.9
Nyabeda farm	5.7	2.32	0.21	397	5.0

### **3.3.2 Greenhouse Experiment**

#### **3.3.2.1 Population of indigenous rhizobial**

The population estimate of indigenous rhizobia in soil was  $2.7 \times 10^3$ ,  $1.9 \times 10^3$  and  $2.1 \times 10^4$  per gram of soil from Butula, Nyabeda and Butere respectively as determined by the most-probable-number (MPN) method (Woomer *et al.*, 1990) using bush bean var. New Rosecoco as a test host.

#### **3.3.2.2 Plant biomass for bean varieties grown in soil collected from Butula at greenhouse level.**

Inoculation with rhizobia isolates gave significant ( $P < 0.001$ ) difference with respect to plant biomass in the two greenhouse experiments. Plant biomass was significantly ( $P < 0.001$ ) influenced by bean varieties and significant interactions between common bean and rhizobia isolates was observed at  $P = 0.021$  (Figure 3.1) and  $P = 0.028$  (Figure 3.2) in the first and second experiment respectively.

All rhizobia isolates outperformed the native rhizobia population in relation to plant biomass with Kenya Tamu in both experiments while 40% and 16% of the rhizobia isolates outperformed the native rhizobia populations on New Rosecoco in the first and second experiments respectively. These isolates were considered to be more competitive. Some rhizobia isolates classified as more competitive for bean variety Kenya Tamu were less competitive for bean variety New Rosecoco including NAK 8, 22, 67, 74, 83, 104, 157, 185, 186, 188, 191 and 208, suggesting as host-strain specificity.

Three of the indigenous rhizobia isolates outperformed commercial strain CIAT 899 and eight outperformed USDA 2667 with climbing bean variety Kenya Tamu. Six of the indigenous isolates outperformed CIAT 899 and two outperformed USDA 2667 with bush

bean variety New Rosecoco. Three rhizobial isolates (NAK 5, 186 and 191) outperformed both CIAT 899 and USDA 2667 with Kenya Tamu while NAK 5, 92 and 45 outperformed the two standard strains with New Rosecoco in relation to plant biomass. The indigenous rhizobia isolate NAK 5 outperformed CIAT 899 and USDA 2667 with both Kenya Tamu and New Rosecoco varieties in terms of plant biomass in the first experiment (Figure 3.1).

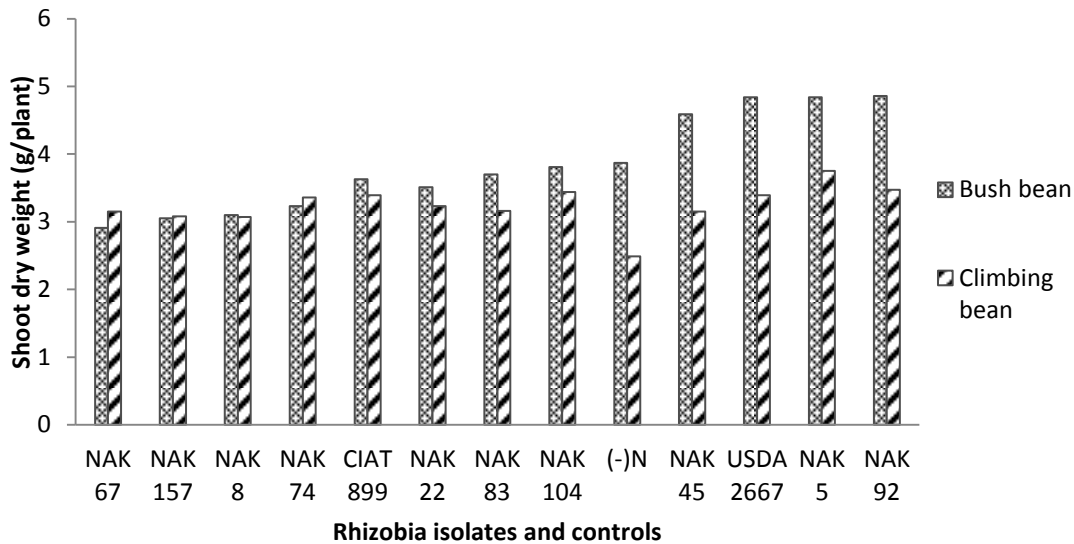


Figure 3.1: Performance of the ten isolates on the common bean varieties grown in potted Butula soil in the first greenhouse experiment.

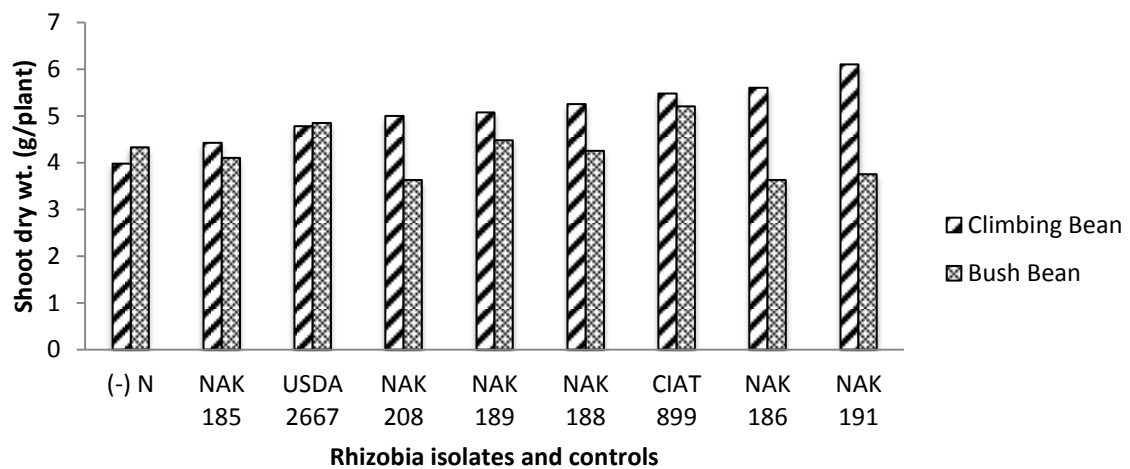


Figure 3.2: Performance of the six isolates on the common bean varieties grown in potted Butula soil in the first greenhouse experiment.



### **3.3.4 On farm field trials at Butula and Nyabeda**

#### **3.3.4.1 Effect of different rhizobia isolates on nodulation of two common bean varieties grown at Butere and Nyabeda.**

The nodule yield from Nyabeda was higher as compared to Butere. In Nyabeda, nodulation with the two bean varieties inoculated with different rhizobia isolates and the two standard strains (CIAT 899 and USDA 2667) was significantly different ( $p=0.001$ ) while at Butere, no significant difference was observed (Table 3.2). The nodule weight at Butere was  $7.4 \text{ kg ha}^{-1}$  and  $6.4 \text{ kg ha}^{-1}$  while at Nyabeda was  $19.9 \text{ kg ha}^{-1}$  and  $48.9 \text{ kg ha}^{-1}$  for New Rosecoco and Kenya Tamu respectively. NAK 5, NAK 92 and NAK 104 isolates performed better than the standard strains (CIAT 899 and USDA 2667) with Kenya Tamu variety on both Butere and Nyabeda farms. Rhizobia isolate NAK 92, NAK 67 outperformed CIAT 899 and USDA 2667 with New Rosecoco in both farms. Reference strains CIAT 899 and USDA 2667 performed poorly in terms of nodule weight on both farms as compared to the elite indigenous isolates (Table 3.2).

Table 3.2: Season 1 nodule dry weight for Kenya Tamu and New Rosecoco inoculated with best performing rhizobia isolates at Butere and Nyabeda.

Treatment	Nodule dry weight/plant (kg ha <sup>-1</sup> )			
	Kenya Tamu		New Rosecoco	
	Butere	Nyabeda	Butere	Nyabeda
+N	1.2	9.7	0	8.6
-N	4.4	38.4	12.6	22.5
NAK 5	6.5	60.6	8.9	18.3
NAK 45	14.6	41.4	5.9	12.7
NAK 67	13.1	46.0	4.7	24.4
NAK 92	4.6	96.5	7.8	35.0
NAK 104	9.4	68.5	15.9	18.2
NAK 157	3.9	58.2	11.6	25.3
USDA 2667	4.1	24.2	3.2	22.0
CIAT 899	2.2	45.7	3.0	12.1
LSD <sub>0.05</sub>	9.86	51.76	10.9	15.6
p-value	0.12	0.11	0.13	0.07
			Butere	Nyabeda
P-value for Rhizobia isolate			0.647	0.321
p-value for common bean variety			0.89	0.001
p-value for isolate* common bean variety			0.257	0.847
LSD <sub>0.05</sub>			8.7	33.8

### 3.3.4.2 Effect of rhizobia isolates inoculations on plant biomass

Inoculation with the rhizobial isolates did not significantly affect biomass production with both Kenya Tamu and New Rosecoco varieties in both season 1 and 2. There was also no interaction observed between rhizobia isolates and bean varieties on both Butere and Nyabeda farms in both seasons (Table 3.3 and 3.4). In season 1, the average biomass production at Butere was 487 kg ha<sup>-1</sup> and 793 kg ha<sup>-1</sup> while at Nyabeda was 956 kg ha<sup>-1</sup> and 1155 kg ha<sup>-1</sup> for Kenya Tamu and New Rosecoco respectively. Rhizobia isolate NAK 104 and NAK 45 performed better compared to CIAT 899 and USDA 2667 with both bean varieties at Nyabeda while in Butere three rhizobia isolates (NAK 45, 67 and 104) outperformed CIAT 899 and USDA 2667 with Kenya Tamu variety (Table 3.3). CIAT 899

performed poorly at Nyabeda while USDA 2667 performed poorly on both farms in season 1 (Table 3.3). In season 2, the average plant biomass production at Nyabeda for New Rosecoco and Kenya Tamu varieties were 795 kg ha<sup>-1</sup> and 988 kg ha<sup>-1</sup> while in Butere the average plant biomass yield was 1362 kg ha<sup>-1</sup> and 1025 kg ha<sup>-1</sup> for New Rosecoco and Kenya Tamu varieties respectively. Isolate NAK 191 gave higher biomass yield compared to both USDA 2667 and CIAT 899 at Nyabeda and Butere (Table 3.4).

Table 3.3: Season 1 plant biomass for Kenya Tamu and New Rosecoco inoculated with selected rhizobia isolates at Butere and Nyabeda.

Treatment	Plant biomass (kg ha <sup>-1</sup> )			
	Kenya Tamu		New Rosecoco	
	Butere	Nyabeda	Butere	Nyabeda
+N	679	1604 a	1483	1896 a
-N	374	1005 bc	552	1028 cd
NAK 5	266	828 bc	730	1000 cd
NAK 45	616	860 bc	806	1473 ab
NAK 67	786	984 bc	605	967 cd
NAK 92	340	783 bc	598	773 d
NAK 104	541	1213 ab	803	1196 bcd
NAK 157	463	908 bc	767	1241 bc
USDA 2667	365	585 c	614	1178 bcd
CIAT 899	493	795 bc	859	800 d
LSD <sub>0.05</sub>	478.9	466.4	573.3	427.3
p-value	0.460	0.012	0.107	<0.01

Means in a column followed by the same letter(s) are not significantly different (P=0.05) by Fisher's protected least significant difference test.

Table 3.4: Season 2 plant biomass for Kenya Tamu and New Rosecoco inoculated with selected rhizobia isolates at Butere and Nyabeda.

Treatment	Plant biomass (kg/ha)			
	Kenya Tamu		New Rosecoco	
	Butere	Nyabeda	Butere	Nyabeda
+N	1562	1332	1387	1406 a
-N	610	1061	1158	610 b
NAK 5	1411	1045	1126	605 b
NAK 45	930	776	1363	713 b
NAK 67	886	834	1142	761 b
NAK 92	758	1004	1655	685 b
NAK 104	1012	1032	1583	727 b
NAK 157	892	785	1126	780 b
NAK 186	971	1104	1429	686 b
NAK 191	1150	1042	1562	1209 a
USDA 2667	1130	956	1257	669 b
CIAT 899	987	884	1558	694 b
LSD <sub>0.05</sub>	566	353	670	375
p-value	0.098	0.141	0.710	0.002

Means in a column followed by the same letter(s) are not significantly different (P=0.05) by Fisher's protected least significant difference test.

### 3.3.4.3. Effect of different rhizobia isolates on yield of two bean varieties grown at Butere and Nyabeda

Grain yield production at Butere was higher compared to Nyabeda in both seasons though there were no significant differences observed with inoculation with the isolates in both sites (Table 3.5 and 3.6). In season 1, grain yield at Butere was 556 kg ha<sup>-1</sup> and 821 kg ha<sup>-1</sup> while at Nyabeda was 541 kg ha<sup>-1</sup> and 798 kg ha<sup>-1</sup> for Kenya Tamu and New Rosecoco respectively. In season 2, the grain yield at Butere was 457 kg ha<sup>-1</sup> and 539 kg ha<sup>-1</sup> and in Nyabeda was 388 kg ha<sup>-1</sup> and 308 kg ha<sup>-1</sup> for Kenya Tamu and New Rosecoco respectively. Rhizobial isolates

NAK 67, NAK 92 and NAK 104 outperformed the standard strains (CIAT 899 and USDA 2667) with Kenya Tamu in season 1 (Table 3.5).

Table 3.5: Season 1 grain yield for Kenya Tamu and New Roscoco inoculated with best performing rhizobia isolates at Butere and Nyabeda.

Treatment	Grain yield (kg ha <sup>-1</sup> )			
	Kenya Tamu		New Rosecoco	
	Butere	Nyabeda	Butere	Nyabeda
+N	754	867	1291	1153
-N	680	516	559	661
NAK 5	432	476	616	773
NAK 45	532	396	888	905
NAK 67	838	556	602	700
NAK 92	501	562	808	698
NAK 104	599	638	920	790
NAK 157	359	444	780	588
USDA 2667	376	508	960	703
CIAT 899	487	443	790	1013
LSD <sub>0.05</sub>	357	315	471	397
p-value	0.142	0.186	0.129	0.156

NAK 186 gave higher yield with both common bean varieties than CIAT 899 and USDA 2667 at Nyabeda (Table 3.6). The rhizobia isolate NAK 191 performed better than CIAT 899 and USDA 2667 with both varieties at Butere and Nyabeda in season 2 (Table 3.6). The rhizobia isolate NAK 67 outperformed both CIAT 899 and USDA 2667 with Kenya Tamu in both farms (Table 3.6). Six rhizobia isolates (NAK 5, 67, 92, 104, 186 and 191) outperformed the two standard rhizobia strains with Kenya Tamu at Nyabeda (Table 3.6). During season 1, the only isolate that outperformed the applied nitrogen treatment was NAK 67 with climbing bean at Butere, otherwise applied N consistently out-yielded inoculation, suggesting that the symbiotic capacity of these beans is relatively low (Table 3.5). In season 2, applied N consistently outperformed inoculation with climbing bean at both farms while NAK 5, 45, 67,

104, 186 and 191 outperformed applied N at Butere while NAK 157 and 191 outperformed applied N treatments at Nyabeda with New Rosecoco variety (Table 3.6).

Table 3.6: Season 2 grain yield for Kenya Tamu and New Rosecoco inoculated with selected rhizobia isolates at Butere and Nyabeda.

Treatment	Grain yield kg ha <sup>-1</sup>			
	Kenya Tamu		New Rosecoco	
	Butere	Nyabeda	Butere	Nyabeda
+N	689	508	435	467 bc
-N	277	464	439	221 ab
NAK 5	425	360	588	203 ab
NAK 45	388	224	611	218 ab
NAK 67	595	451	505	125 a
NAK 92	381	462	366	215 ab
NAK 104	498	429	635	205 ab
NAK 157	339	292	404	478 bc
NAK 186	326	439	635	409 bc
NAK 191	682	445	792	636 c
USDA 2667	381	279	428	274 ab
CIAT 899	501	312	635	251 ab
LSD <sub>0.05</sub>	375.63	258.98	334.71	278.11
p-value	0.348	0.407	0.310	0.024

Means in a column followed by the same letter(s) are not significantly different (P=0.05) by Fisher's protected least significant difference test.

### 3.4 Discussion.

#### 3.4.1 Characteristics of soil at Butula, Butere and Nyabeda

The soil from Nyabeda was more fertile than soils from Butere and Butula however, the nutrient levels in all soils were relatively low. The level N in the soils was low suggesting that nitrogen is limiting in the sites. Sympal, a fertilizer blend for legumes (0:23:15 plus 10% Ca, 4% S, 1% Mg and 0.1% Zn) was applied in these soil to counteract the nutrient deficiencies for improve BNF especially P that was relatively low. Report by Woome *et al.* (2014) indicated that application of sympal fertilizer in problem soils of Kenya, Rwanda and DR Congo improved bean and soybean yields by 6% to 15% despite its lower cost compared

to most P fertilizers. Low level of N in the soils stimulates biological N<sub>2</sub>-fixation since it suppresses nodulation of legumes as earlier reported (da Silva *et al.*, 1984; Herridge *et al.*, 1984). The soils also showed slight acidity a condition that negatively affects BNF. A study by Werner and Newton (2006) showed that soil acidity negatively affects nodulation and survival of rhizobia because it disrupts the communication process that need to occur for root hair infection, thereby limiting nodule development.

Results from MPN experiment show that all the soils in Butere, Nyabeda and Butula had high population of native rhizobia despite no history of inoculation. Presence of compatible native rhizobia in the soil could be an influence past legume (common bean) cropping system, contamination through dust or the rhizobia could be native to the soils. Previous study by Zengeni *et al.* (2006) reported the presence of great rhizobia diversity in most soils and an enhanced population size where compatible legumes are grown. Rhizobia are highly mobile as contaminants of dust and seed (Stepkowski *et al.*, 2005). Other studies (Meade *et al.*, 1985; Thies *et al.*, 1991) reported the presence of compatible native rhizobia in fields that have never been cultivated to a given host legume due to the presence of native legume species, transfer from adjacent fields, or as part of the native microbiological soil community.

#### **3.4.2 Performance of indigenous isolates in potted soil from Butula**

A large number of rhizobia isolates were collected from different ecological zones in Kenya and authenticated to be bean nodulating rhizobia in the greenhouse using sterile horticultural vermiculite held in pots. Variation in nodulation and plant biomass among the bioprospected isolates in sterile media successfully reduced the number of test isolates to 16 (Table 2.1) which were used in this study. This indicates the varying ability of the test isolates as symbiotic partners of common bean, a phenomenon reported in a similar study with soybean (Waswa *et al.*, 2014).

In this study, both Kenya Tamu and New Rosecoco bean varieties nodulated in the uninoculated treatment, which suggest that native rhizobia capable of nodulating the host were present in soil used for the experiment and they competed with inoculated strains (Corbin *et al.*, 1977). This confirms results from the MPN experiment which assessed the populations of compatible indigenous rhizobia in the soil from Butula. Meade *et al.* (1985) earlier indicated failure to achieve a response to inoculation with elite rhizobial strains is as a result of successful competition for nodule sites by native rhizobia.

Inoculation with different rhizobia isolates gave a significant ( $P < 0.001$ ) difference with respect to plant biomass in the two greenhouse experiments indicating the varying ability of the isolates as symbiotic partners of common bean. Other studies (Terpolilli *et al.*, 2008; Karaca and Uyanöz, 2012) noted that efficiency of nitrogen fixing symbioses can vary from those that fix little or no nitrogen to those that fix at levels equivalent to even greater than plants provided mineral N. Despite the high compatible rhizobial populations in the Butula soil, inoculation with most elite rhizobial isolates (Figure 3.1 and 3.2) improved biomass production with both bean cultivars. This confirms report by Giller (2001) that the presence of large population density of compatible native rhizobia does not preclude the possibility that responses to inoculation can be obtained if competitive and highly effective strains are introduced. A number of rhizobia isolates (NAK 5, NAK 92, 186 and 191) outperformed both standard strains (CIAT 899 and USDA 2667) with either Kenya Tamu or New Rosecoco in relation to plant biomass suggesting their superior performance in terms of effectiveness and competitiveness compared to the recommended commercial strains. Other studies (Appunu and Dhar, 2006 and Appunu *et al.*, 2008) also reported that indigenous rhizobia can be more effective than standard strains of foreign origin.



Specific interactions between bean varieties and rhizobia isolates were noted at  $P=0.021$  and  $P=0.028$  in relation to biomass production in the first and second experiment respectively. Different bean genotypes performed differently with rhizobia, for example NAK 92, NAK 186 and NAK 191 performed well in terms of plant biomass on New Rosecoco but did not perform well with Kenya Tamu suggesting host-strain specificity. This observation is supported by Tukamuhabwa *et al.*, (2012) and Gandanegara *et al.*, (1992). Caldwell and Vest, (1970) reported that at nodule formation, the legume may favor specific strains of *Rhizobium* to nodulate. *Rhizobium* spp. may therefore differ in their capacity to be selected by the plant host in nodulating competitiveness.

### **3.4.3 Performance of selected rhizobia isolates with Kenya tamu and new Rosecoco bean varieties grown in field trials at Butere and Nyabeda**

Optimal performance of the N-fixing symbiosis depends upon pre-selection of both symbiotic partners for adaptation to the target environment, that may in some form present a challenge to rhizobial survival or nodulation (Sessitsch *et al.*, 2002). In the field trials, inoculation with elite rhizobial isolates did not yield any significant difference in relation to nodulation, biomass and grain production in both farms suggesting the lack of response to inoculation. Failure of common beans to respond to *Rhizobium* inoculation has been reported in studies conducted in low-N sites in western Kenya (Masandu and Ogendo, 2002). Other studies have also reported the poor nodulation and little response in terms of  $N_2$ -fixation rates of beans (Graham *et al.*, 1982; Serraj and Sinclair, 1998 and Giller, 2001). In this study, the lack of response to inoculation in the two bean cultivars could be attributed to the soil acidity, the low mineral N and the presence of highly competitive resident rhizobia nodulating common bean. The soils in the two site had a low pH, a factor that could have affected the nodule formation and survival of inoculated strains as supported in a study by Werner and Newton

(2006). The high populations of compatible native rhizobia, as confirmed by MPN, in the field soils suggest that native rhizobia capable of nodulating the host were present and could have out-competed the inoculated strains for nodule occupancy. In their study, Cheminingwa *et al.* (2004) attributed lack of nodulation response to inoculation in grain legumes to the presences of highly competitive native rhizobia that restricted occupancy of the nodules by the inoculant strains. It is considered that legumes will often respond to inoculation where the rhizobial community is less than 100 cells g<sup>-1</sup> of soil (Thies *et al.*, 1991). Studies have also reported the difficulty and unlikely response to inoculation in soils with substantial populations of native rhizobia (Abaidoo *et al.*, 2007; Bloem and Law, 2001; Houngnandan *et al.*, 2000). The low level of soil N could have suppressed nodule and seedling development before onset of BNF process. Herridge *et al.* (1984) and Goi *et al.* (1993) have demonstrated that a moderate dose of starter-N is required by legume plants during the ‘nitrogen hunger period’ for nodule development and seedling growth thereby stimulating BNF under soils low in mineral N. Nonetheless, several of the elite isolates from a range of legume hosts and ecologies outperformed industry standard strains even in soils with relatively large native populations of rhizobia confirming reports by Appunu and Dhar (2006) and Appunu *et al.* (2008) that indigenous rhizobia can be more effective than standard strains of foreign origin .

There was a significant growth response to the application of N (+N) in this study suggesting that nitrogen was limiting and is essential for improved crop performance. In their study, Cheminingw’a *et al.* (2004) indicated that nitrogen is the most limiting nutrient to crop production in smallholder farms. The results also suggest that the symbiotic capacity of these beans is relatively low. However, some of the isolates were as superior in N<sub>2</sub> fixation and outperformed the +N treatment.

Yield varied between the two bean varieties at the two sites (Butula and Nyabeda) and this differences may be attributed to their differences in maturity time of the two bean cultivars and the prevailing weather during the two seasons. The two seasons received poorly distributed rains suggesting poor crop development. Bush bean variety (new Rosecoco) performed particularly well in the two seasons and this could be attributed to its early maturing time of 65 to 110 days from emergence to physiological maturing (Buruchara, 2007) as compared to 200 days among climbing varieties (Gomez, 2004).

### **3.5 Conclusion**

At the greenhouse level, isolates were effective and varied in their performance and showed interactions with bean varieties.

Poor response to inoculation with the bean cultivars in the field trials was due to highly competitive resident rhizobia.

### **3.6 Recommendation**

Rhizobial isolates (NAK 5, NAK 67 and NAK 92) have potential for fixing nitrogen and should be evaluated in different agro-ecological zones.

## CHAPTER 4

### INFLUENCE OF BIOCHAR AMENDMENT ON THE EFFECTIVENESS OF ELITE KENYAN RHIZOBIA NODULATING BEAN (*Phaseolus vulgaris* L.)

#### Abstract

This study examined the influence of biochar addition on the effectiveness of indigenous Kenyan rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in low fertility soils of West Kenya. Biochar was added at the rate of 2 t ha<sup>-1</sup> and effectiveness of native rhizobia isolates was determined by nodulation, biomass assessment and grain yield. A two-season field trial was conducted in two farms, Nyabeda and Butere, based in the western parts of Kenya using two common bean varieties, Kenya Tamu (Climbing bean) and New Rosecoco (bush bean) arranged as a Split-Plot in a Randomized Complete Block Design. In season 1 (LR-2013), the two bean cultivars were inoculated with rhizobia isolate (NAK 5) and a standard *commercial* strain (CIAT 899) with and without biochar amendment. During the short rains (2013-2014), the number of indigenous isolates were increased and included NAK 5, 45, 67, 92, 186 and 191. A non-inoculated control with and without mineral N and with and without biochar were also included in both experiments as a 2 × 2 matrix. Generally, treatments with biochar addition had greater biomass and grain yields than treatments without biochar in both seasons on the two farms. However, there were no significant differences and interactions observed in biomass production and grain yield from common beans inoculated with indigenous rhizobia isolates with or without biochar addition in both seasons in both Butere and Nyabeda. The N fertilized (+N) control with biochar addition outperformed the N fertilized without biochar, in relation to grain yield, in both Butere and Nyabeda in the two seasons. Most significant differences were between managements receiving mineral N and those receiving inoculant suggesting effective management of N fertilizer and little difference

between the candidate elite strains. While the effect of biochar addition was not significant in relation to inoculation, there are very strong trends suggesting benefits from adding 2 t ha<sup>-1</sup> biochar. Results emphasize the needs for long-term field studies to better understand the effects of biochar on BNF.

#### **4.1 Introduction**

According to Verheijen *et al.*, (2009) biochar is defined as a biomass that has been pyrolyzed in a zero or low oxygen environment that, owing to its inherent properties, consensus exists that application to soil at a specific site is expected to sustainably sequester carbon and concurrently improve soil functions under current and future management, while avoiding short- and long-term detrimental effects to the wider environment as well as human and animal health.

The interest on using biochar as soil amendment comes from studies of Amazonian soils (*terra preta*) where the presence of charcoal has led to significant improvements in soil quality and increases in crop yields (Lehmann, 2007; Novotny *et al.*, 2007; Lehmann and Joseph, 2009). The application of charcoal can increase soil pH and decrease the Al concentration of acid soils which are often the limitations to growth in tropical soils (Cochrane and Sanchez, 1980; Mbagwu and Piccolo, 1997). Charcoal has been shown to be a soil conditioner in many tropical and subtropical soils increasing exchangeable bases, cation exchange capacity, and nutrient availability, decreased soil bulk density and improving water holding capacity (Laird *et al.*, 2010; Liang *et al.*, 2006; Novak *et al.*, 2009).

Soils can be viewed as complex communities of organisms which are continually changing in response to soil characteristics and climatic and management factors, especially the addition of organic matter (Thies and Rillig, 2009). Addition of biochar to soils affects the abundance,

activity and diversity of soil biotic communities. Biochar addition to soils can stimulate microorganism activity in the soil, potentially affecting the soil microbiological properties (Hammes and Schmidt, 2009). Rather than supplying microorganisms with a primary source of nutrients, biochar is thought to improve the physical and chemical environment in soils, providing microbes with a more favourable habitat (Krull *et al.*, 2010). There are experimental evidence that soil microbial communities and their activity, which hold key roles in sustaining soil health and functioning, are directly affected by the addition of biochar to soils (Ogawa, 1994; Rondon *et al.*, 2007; Warnock *et al.*, 2007; Steiner *et al.*, 2008). It has been previously shown that biochar addition to soil increases N fixation by both free-living and symbiotic diazotrophs (Ogawa, 1994). Rondon *et al.* (2007) also found that biochar additions significantly increased BNF by rhizobia at all application rates (30, 60 and 90 grams per kilogram of soil). They also noted that the improvements in BNF and biomass productivity were significantly greater compared with normal productivity achieved by conventional fertiliser application in the absence of biochar.

The aim of this study is to investigate the influence of bio-char additions, as a soil amendment, on the effectiveness of rhizobia nodulating common beans (*Phaseolus vulgaris L.*) in low fertile soils of Western Kenya. It is hypothesized that bio-char improves biomass and grain yields of common beans due to decreased N availability, increased pH, favorable habitat for rhizobia, as well as nutrient availability thus improving BNF with inoculation.

## **4.2 Material and methods**

### **4.2.1 Rhizobia isolates**

The indigenous rhizobia isolates used in the field trials, their host legume and geographical origin are listed in Table 2.1 and included: NAK 5, 45, 67, 92, 186 and 191. The isolates were

grown in Yeast Extract Mannitol broth (Bohloul and Schmidt, 1970) and maintained on agar slants under refrigeration as described in Section 2.3.1.

#### **4.2.2 Biochar production**

Biochar was produced at the ICRAF (World Agroforestry Centre), Kisumu from bagasse (feedstock) using a temperature controlled kiln. Temperature was maintained at 350-500°C. Some physico-chemical analyses were performed on a subsample.

#### **4.2.3 Study area, soil sampling and chemical analysis**

A two-season field experiment was conducted in two fields in Western part of Kenya (Nyabeda and Butere) as described in section 2.2. The top soil (0–15 cm) from each field site was sampled before planting by sampling randomly eight cores per replicate using a 3.5 cm soil auger, they were bulked and sub-sampled. Chemical analysis of soils and biochar applied was done for nutrient composition as described by Okalebo *et al.* (2002).

#### **4.2.4 Experimental design and treatments**

The experiment was arranged as a Split-Plot in a Randomized Complete Block Design (RCBD) with four replicates. The bean cultivars were the main plots and the inoculation treatments were the sub-plots. Two bean cultivars, Kenya Tamu (climbing bean) and New Rosecoco (bush bean), were inoculated with indigenous Kenyan rhizobia isolates and a standard commercial *Rhizobium tropici* strain (CIAT 899) and comparisons made between treatments with and without biochar. A uninoculated control with and without mineral N and with and without biochar were also included in the experiments as a 2 × 2 matrix. Biochar was added at the rate of 2 t ha<sup>-1</sup>. 0.375 kg of CAN per plot equivalent to 65 kg N ha<sup>-1</sup> was applied in three split doses to the +N treatments. Sympal fertilizer (0-23-15 plus 10% Ca, 4% S,

1% Mg and 0.1% Zn) was also added to all plots at the rate of 0.375 per plot (250 kg ha<sup>-1</sup>) as basal dressing. All the inputs were applied in the furrow.

#### **4.2.5 Inoculant preparation**

Inoculants were prepared 21 days prior to planting as described in Section 2.4.2 and their quality tested as described in Section 2.4.3.

#### **4.2.6 Inoculating and planting of seeds**

The seeds were inoculated with inoculants using a solution of gum arabic (40%, w/v) as a sticker and planted immediately after inoculation at spacing of 10 cm by 60 cm and 15 cm by 60 cm for bush beans (New Rosecoco) and climbing bean (Kenya Tamu) respectively as described in Section 2.4.4

#### **4.2.7 Plant sampling**

The first sampling was carried out at 48 days after planting which coincided with 50% flowering of the plants. The second sampling was done at crop maturity, 3 months for bush bean (New Rosecoco) and 4 months for climbing bean (Kenya Tamu) from sowing. The first rows on either side of the plots were omitted to eliminate border effects. During the first sampling plants were carefully uprooted and samples were taken from an area of 0.3 m<sup>2</sup>. Roots were separated from the shoots and then soil was washed off in gently running water to ensure that roots and nodules remained intact then nodules were recovered and counted. Shoots and nodules were oven dried for 48 hours at 70 °C and dry weight noted. At harvest, bean grains were recovered from an area of 5.4 m<sup>2</sup>. Plant biomass and grain yield were recorded.



#### 4.2.9 Statistical analysis

The MPNES program (Woomer *et al.*, 1990) was used to calculate the rhizobial populations in the soils. Analysis of variance (ANOVA) was used for evaluation of symbiotic properties of rhizobia. To compare treatment means, Fisher's protected LSD method was used at a significance level of 5%. The analysis was performed using GENSTAT statistical package (GenStat Release 6.1 Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK).

#### 4.3 Results

##### 4.3.1 Chemical characteristics of soils and biochar used in the field level

Table 4.1 shows the chemical characteristics of the biochar used in the field experiment. The pH of this biochar was relatively high (pH=8.6). Biochar had an increased nutrient levels as shown in Table 4.1. The chemical characteristics of soils from the sites are presented in Table 3.1. The soil pH was acidic and ranged from 5.2 to 5.7 in the three farms. The nutrient levels in the soils were critically low.

Table 4. 1: The chemical characteristics of biochar used in the field trials.

Site	pH H <sub>2</sub> O	C -----%-----	N	K ------(ppm)-----	P
Biochar	8.6	2.1	0.336	507	92.2

### 4.3.2 Population of indigenous rhizobial

The population estimate of indigenous rhizobia in soils from field trials in Nyabeda and Butere are presented in Section 3.3.2.1. The population estimates were relatively high ranging from  $1.9 \times 10^3$  to  $2.1 \times 10^4 \text{ g}^{-1}$  of soil in Nyabeda and Butere respectively.

### 4.3.3 On-farm field trials in Butere and Nyabeda

#### 4.3.3.1 Effect of different rhizobia isolates inoculation and biochar addition on biomass production of two common bean varieties grown at Butere and Nyabeda.

Rhizobial isolate, NAK 5, with biochar amendment outperformed itself without biochar, in terms of biomass production, in both farms and seasons. NAK 5 with biochar addition also outperformed the N-fertilized treatment without biochar with Kenya Tamu bean variety at Butere in season 1.

Table 4.2: Season 1 plant biomass for Kenya tamu and new rosecoco inoculated with two rhizobia strains and with addition of biochar at Butere and Nyabeda.

Treatment	Plant biomass ( $\text{kg ha}^{-1}$ )							
	Nyabeda				Butere			
	Kenya tamu		New rosecoco		Kenya tamu		New rosecoco	
	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar
+ N	1302	1604 a	2735 a	1896 a	618	642	1611 a	1483
- N	1213	1005 b	1702 ab	1028 b	630	374	568 b	552
NAK 5	914	828 b	1370 b	1000 b	1220	266	945 ab	677
CIAT 899	895	796 b	1061 b	800 b	513	493	553 b	859
LSD <sub>0.05</sub>	531	412	1038	501	905	443	689	738
p-value	n.s	0.005	0.026	0.004	n.s	n.s	0.022	n.s

Means in a column followed by the same letter(s) are not significantly different ( $P=0.05$ ) by Fisher's protected least significant difference test

In season 2, NAK 67 and NAK 92 performed better with biochar than without in both farms (Table 4.3). Non-inoculated controls with (+N) and without nitrogen (-N) gave higher biomass yield with biochar addition compared to those without in both seasons. The standard strain (CIAT 899) with biochar also gave higher yield with both Kenya Tamu and New Rosecoco at Nyabeda and with Kenya Tamu at Butere compared to itself without biochar addition. NAK 5 and NAK 92 both with biochar amendment outperformed the +N treatment without biochar with New Rosecoco variety (Table 4.3). NAK 92 and CIAT 899 also performed better than +N all without biochar addition (Table 4.3).

Table 4.3: Season 2 plant biomass for Kenya tamu and new rosecoco inoculated with two rhizobia strains and with addition of biochar at Butere and Nyabeda.

Treatment	Plant biomass (kg ha <sup>-1</sup> )							
	Nyabeda				Butere			
	Kenya tamu		New rosecoco		Kenya tamu		New rosecoco	
	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar
+ N	1277	1332 a	1540 a	1407 a	1367	1562 a	1770	1387
- N	1010	1061 ab	921 bc	610 b	955	611 b	1578	1158
NAK 5	787	1045 b	603 c	606 b	995	1411 a	1652	1126
NAK 45	1040	776 b	1261 ab	713 b	1076	930 b	1303	1364
NAK 67	990	834 b	973 bc	761 b	1350	886 b	1333	1142
NAK 92	1033	1004 b	996 bc	685 b	1385	758 b	1759	1655
CIAT 899	1090	884 b	808 bc	694 b	1295	987 b	1054	1559
LSD <sub>0.05</sub>	404	285	517	361	721	413	786	834
p-value	n.s	0.014	0.03	0.003	n.s	0.001	n.s	n.s

Means in a column followed by the same letter(s) are not significantly different (P=0.05) by Fisher's protected least significant difference test.

Addition of biochar improved bean biomass yield compared to treatments with no biochar application in both seasons and farms. There were no significant differences observed among

the rhizobial isolates treatments with and without biochar in both seasons in relation to biomass production (Table 4.2 and 4.3).

#### 4.3.3.2. Effect of different rhizobia isolates inoculation and biochar addition on grain yield of two common bean varieties grown at Butere and Nyabeda.

Grain yield with treatments with biochar addition was greater than those without biochar application though there were no significant differences between the inoculated treatments. The N fertilized (+N) control with biochar addition outperformed the N fertilized without biochar in both seasons and farms. The grain yield with NAK 5 with biochar addition was higher compared to with no biochar addition in season 1 (Table 4.4).

Table 4. 4: Season 1 grain yield for Kenya Tamu and New Rosecoco inoculated with rhizobia isolates in soils amended with biochar at Butere and Nyabeda farms.

Grain yield (kg ha <sup>-1</sup> )								
Treatment	Nyabeda				Butere			
	Kenya tamu		New rosecoco		Kenya tamu		New rosecoco	
	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar
+ N	1217 a	867 a	1507 a	1153	860	755	1550 a	1291a
- N	675 b	516 b	1029 b	661	531	680	716 b	559 b
NAK 5	723 b	476 b	953 b	773	903	432	785 b	616 b
CIAT 899	524 b	443 b	1081 b	1013	461	487	913 b	790 b
LSD <sub>0.05</sub>	445	296	261	574	781	549	419	264
p-value	0.03	0.03	0.004	n.s	n.s	n.s	0.01	0.002

Means in a column followed by the same letter(s) are not significantly different (P=0.05) by Fisher's protected least significant difference test.

In season 2, NAK 67 with biochar addition in Butere farm performed better, in relation to grain production, than uninoculated with applied N (+N) and the standard strain CIAT 899 in both farms (Table 4.5). NAK 5, NAK 45, NAK 67, NAK 92 and CIAT 899 all with biochar

addition had higher grain yield production than the N fertilized control with no biochar in either or both of the farms and with either or both of the common bean varieties (Table 4.5).

Table 4. 5: Season 2 grain yield for Kenya Tamu and new Rosecoco inoculated with rhizobia isolates in soils amended with biochar at Butere and Nyabeda farms.

Grain yield (kg ha <sup>-1</sup> )								
Treatment	Nyabeda				Butere			
	Kenya tamu		New rosecoco		Kenya tamu		New rosecoco	
	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar	With Biochar	Without Biochar
+ N	632	508	632	467 a	1098	805	744	435
- N	374	464	335	221 b	726	387	760	439
NAK 5	256	360	273	203 b	843	535	699	588
NAK 45	649	202	449	218 b	345	498	643	611
NAK 67	329	451	529	138 b	1111	705	838	505
NAK 92	399	425	400	267 b	917	491	1090	366
CIAT 899	466	312	255	252 b	864	610	539	639
LSD <sub>0.05</sub>	310	205	264	140	595	511	371	350
p-value	n.s	n.s	n.s	0.006	n.s	n.s	n.s	n.s

#### 4.4 Discussion

Nitrogen fixation is reported to be affected by such factors as soil available N and effectiveness of the rhizobia-host association (Van Kessel and Hartley, 2000), soil pH, soil moisture, soil available P and plant management (Fosu *et al.*, 2004; Muza and Mapfumo, 1998; Peoples *et al.*, 1995; Thies *et al.*, 1995) and soil microbial populations (Dogbe *et al.*, 2000). In this study we focus on how management through biochar addition as a soil conditioner might be employed to reduce the detrimental effects of abiotic stresses or

competitive environments that are common in western parts of Kenya for nodulation of bean with elite native rhizobia.

Biochar used in the study was produced from bagasse (feedstock) and the temperature was maintained at 350-500°C. Chemical characteristics showed that it had a higher pH (8.6) and a higher nutrient levels suggesting an increase in nutrient level and higher pH in the soils. Biochar has been described as a possible means to improve soil fertility (Lehmann *et al.*, 2006; Sohi *et al.*, 2010) which have been attributed mainly by a pH increase in acid soils (Van Zwieten *et al.*, 2010) or improved nutrient retention through cation adsorption (Liang *et al.*, 2006). Some studies have reported increased microbial activity in soils amended with biochar (Steiner *et al.*, 2003; Steiner *et al.*, 2008). Ogawa (1994) also been noted that the physical structure of typical biochar products provides a secure environment for microbial colonies.

The soils collected from Nyabeda and Butere farms were generally low in nutrient fertility and were slightly acidic (Table 3.1). The soil pH ranges from strongly acid (4.6) to slightly acid (6.46) and moderately acid (5.25) to moderately alkaline (7.59) in Butere sub-county (Butere) and Rarieda sub-county (Nyabeda) respectively (GOK, 2014). Lower pH increases the solubility of Al, Mn, and Fe in soil causing toxicity to plants in excess by slowing or stopping of root growth. The soils are also characterized by low levels of nutrient content (GOK, 2014) which was also confirmed in this study. Sympal, a fertilizer blend for legumes (0:23:15 plus 10% Ca, 4% S, 1% Mg and 0.1% Zn) was applied in these soil thus alleviating nutrient deficiencies for improve BNF especially P that was relatively low as confirmed in a report by Woome *et al.* (2014).

The native rhizobial populations in the field soils were relatively high as determined using MPN technique. Population sizes ranging from 2 to 4200 cells per gram of soil were reported

in a study across nine African countries and the numbers were enhanced by previous cropping of the compatible legumes (Abaidoo *et al.*, 2007).

Inoculation with elite indigenous rhizobia isolates and reference strain did not significantly differ with or with no biochar addition. This could be attributed to the presence of large populations of highly competitive resident rhizobia adapted to the environment thus 'masking' inoculation. The adaptability of indigenous rhizobia to their environment results in high levels of saprophytic competence (Zengeni *et al.*, 2006). Studies have also reported the unlikely response to inoculation in soils with substantial populations of native rhizobia exceeding 100 g<sup>-1</sup> of soil (Abaidoo *et al.*, 2007; Bloem and Law, 2001; Houngnandan *et al.*, 2000; Theis *et al.*, 1991). The lack of response to inoculation could also be attributed to the lower application rate of biochar in this study of 1 g kg<sup>-1</sup> (2 t ha<sup>-1</sup>) compared to 30–90 g kg<sup>-1</sup> (Rondon *et al.*, 2007) and 100–200 g kg<sup>-1</sup> (Lehmann *et al.*, 2003). However, some rhizobial isolate such as, NAK 92 in biochar-amended soil did better compared to nitrogen (+N) treatments in both Butere and Nyabeda, in relation to grain yield and biomass production respectively suggesting an enhanced effectiveness and competitiveness of the isolate with biochar addition, consequently improving BNF. Evidence for improved BNF by bio-char was also provided for alfalfa (*Medicago sativa* L.) using the N difference and acetylene reduction assay methods (Nishio and Okano, 1991). The reason for the improved BNF are most likely a combination of factors related to nutrient availability in soil (Lehmann *et al.*, 2003) and stimulation of plant–microbe interactions (Nishio and Okano, 1991; Saito and Marumoto, 2002).

While the effect of biochar addition was not significant in relation to inoculation, its addition improved biomass production and grain yield of the two common bean varieties suggesting benefits from adding 2 t ha<sup>-1</sup> biochar. Such responses confirm earlier results with common

bean (*Phaseolus vulgaris* L.) (Rondon *et al.*, 2007), moong bean (*Vigna radiata* (L.) R. Wilczek], soybean [*Glycine max* (L.) Merr.], and pea (*Pisum sativum* L.) (Iswaran *et al.*, 1980), cowpea (*Vigna unguiculata* L.) and rice (*Oryza sativa* L.) (Nehls, 2002; Lehmann *et al.*, 2003). The greater biomass and grain yield reported in this study could largely be as a result of positive crop performance due to nutrient availability and raised pH from biochar addition. Niste *et al.* (2013) reported that legumes require a neutral or slightly acidic soil for growth. In their study Atkinson *et al.* (2010) reported that increases in soil pH are likely to influence P availability which is highly pH-dependent. In a study conducted by Rondon *et al.* (2007), the improved crop performance was largely an effect of elevated P, K, Mg, Ca, Mo, and B availability as well as higher pH with biochar addition. High rates of biochar addition in the tropical environment have been associated with increased plant uptake of P, K, Ca, Zn and Cu (Lehmann and Rondon, 2006).

Non-inoculated N-fertilized treatments with biochar had higher biomass and grain yield compared to those with no biochar, suggesting increased nitrogen use efficiency by the common beans. Similar results have been reported by Chan *et al.* (2007) who showed that, application of biochar and nitrogen fertilizer increased dry matter production by 266 per cent of the biochar-without-nitrogen treatment. Van Zwieten *et al.* (2010) found positive effects of biochar and nitrogen fertilisation on the growth of wheat, soybean and radish in an initially acidic, nutrient-poor Australian ferrosol.

#### **4.5 Conclusions**

Inoculated beans grown in soils amended with biochar gave higher biomass and grain yields which is attributed to nutrient availability and raised pH from biochar addition.



Grain and biomass production increased in the N-fertilized with biochar addition suggesting improved N-use efficiency.

#### **4.6 Recommendation**

There is need for long-term field studies to better understand the effects of biochar on BNF.

## CHAPTER 5

### DIVERSITY OF ELITE KENYAN RHIZOBIA ISOLATES NODULATING *Phaseolus vulgaris* L. AND *Glycine max.*

#### Abstract

A major strategy towards addressing soil fertility depletion is the conservation and sustainable use of rhizobia that are able to fix nitrogen in the soil in association with legumes. The identification of effective indigenous rhizobia strains which nodulate soybean (*Glycine max.*) and common bean (*Phaseolus vulgaris* L.) could be useful in the development of inoculant strains. Genetic diversity and phylogeny of the indigenous rhizobia strains was assayed using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the 16S-23S rDNA intergenic spacer region and 16S rRNA gene sequencing. Digestion of the six soybean nodulating rhizobial isolates with the restriction enzyme *MspI* produced two IGS (Intergenic spacer region) profiles that included I (NAK 84, 89, 117, 135 and 179) and II (NAK 128). *Bradyrhizobium elkanii* related strains predominated and accounted for the four strains identified as effective to soybean including NAK 84, 89, 117 and 135. A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis confirmed the greater relationship of indigenous strains of *Bradyrhizobium* to reference strains of *B. elkanii*, *B. japonicum* and *Bradyrhizobium spp.* Digestion of the seven bean nodulating rhizobial isolates with the restriction enzyme *MspI* produced three IGS profiles including I, II and III for NAK 104, 157 and 186 respectively. NAK 5, 45 and 67 were not restricted when digested with *Msp 1* enzyme. Alignments of partial sequences of five bean nodulating isolates (NAK 5, 45, 67, 92 and 104) with related 16S rRNA gene sequences in GenBank database revealed that the isolates belonged to different Genera. Only two isolates were closely related to the *Rhizobium* genus with NAK

92 closely related to *Rhizobium sp.* and NAK 104 to *Rhizobium tropici*. Two of the isolates (NAK 5 and 45) were closely related to the genus *Pseudomonas spp.* NAK 67 was closely related to *Paenibacillus spp.* A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis confirmed the greater relationship of indigenous strains of *Rhizobium* to reference strains of *Rhizobium sp.*, *Rhizobium tropici* and *Rhizobium phaseoli*. The diversity identified in *Bradyrhizobium* and *Rhizobium* populations in Kenyan soils represent a valuable genetic resource that has potential utility for the selection of more competitive and effective strains to improve biological nitrogen fixation and thus increase soybean and common bean yields at low cost

## 5.1 Introduction

The term rhizobia is used to describe a range of soil bacterial genera which include *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium* and *Azorhizobium* that are able to form symbiosis with plants predominantly legumes (Sprent, 2001; O'Hara *et al.*, 2003). Legumes-rhizobia symbiosis fix atmospheric N and because of this, they represent a significant model in agriculture for sustainable production (Robson, 1990; Reeves and Ewing, 1993; Brockwell *et al.*, 1995; Howieson *et al.*, 2000).

A number of *Rhizobium* species (fast growers) have been reported to form symbiotic association with common bean (*Phaseolus vulgaris L.*) including, *Rhizobium leguminosarum* bv. *phaseoli* (Jordan, 1984), *Rhizobium tropici* (Martinez- Romero *et al.*, 1991), *R. etli* bv. *phaseoli* (Segovia *et al.*, 1993; Souza *et al.*, 1994), *Rhizobium gallicum* and *Rhizobium giardinii* (Amarger *et al.*, 1997), *R. etli* bv. *Mimosa* (Wang *et al.*, 1999), *Rhizobium lustinaum* (Valverde *et al.*, 2006), *Rhizobium pisi* (Ramirez-Bahena *et al.*, 2008) and *Rhizobium alikisoli* (Lu *et al.*, 2009). Rhizobial strains forming effective symbiosis with soybean have been reported (slow-growing bradyrhizobia) and particularly the species *Bradyrhizobium*

*japonicum* (Jordan, 1982), *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992) and *Bradyrhizobium liaoningense* (Xu *et al.*, 1995). *Bradyrhizobium liaoningense* is very closely related to *B. japonicum* (Van Berkum and Fuhrmann, 2000). In addition, the moderately fast-growing *Mesorhizobium tianshanense* (Chen *et al.*, 1995) and the fast-growing *Sinorhizobium (Ensife) fredii* (Scholla and Elkan, 1984) and *Sinorhizobium xinjiangense* (Chen *et al.*, 1988) also nodulate soybean and as effective as bradyrhizobia in suitable ecological conditions (Albareda *et al.*, 2009).

A common genetic determinant for rhizobia is the presence of genes encoding nodulation and nitrogen fixation functions (*nod*, *nol*, *noe*, *nif* and *fix* genes). These genes are often carried on plasmids or other accessory elements, such as symbiotic islands, and properties encoded by them can be easily lost or gained (MacLean *et al.*, 2007). Initiation of nodule formation on compatible host plants results from a molecular dialogue between the host and the bacteria (Schultze and Kondorosi, 1998; Perret *et al.*, 2000; Spaink, 2000). Strain selection has previously and still is hampered by the fact that the genetic bases for competitiveness, symbiotic effectiveness and tolerance to environmental stresses are largely not known, irrespective of the knowledge of genes encoding nodulation and nitrogen fixation in rhizobia (Lindstorm *et al.*, 2010). A great diversity of indigenous rhizobia occurs in most soils and higher population where compatible legumes are cultivated and soils are fertile (Zengeni *et al.*, 2006). Rhizobia are very diverse genetically at both species and strain levels. A soil may contain various rhizobia species and various strains within specified species (Bala and Giller, 2001), and the same isolates may be found in other distant places (Abaidoo *et al.*, 2007). Plants belonging to family Leguminosae are very diverse ranging from annual grain legumes such as soybean to perennial trees such as *Sesbania*. The host-rhizobia symbiosis requires more precise matching to enable maximization of nitrogen fixation although there may be cross-inoculation of strains compatible with any given legume (Abaidoo *et al.*, 2007).

Polymerase Chain Reaction-Restriction Fragment Polymorphism (PCR-RFLP) analysis of the 16S-23 rDNA intergenic region and sequence analysis of the 16S rRNA gene are important tools in phylogenetic analysis of rhizobia. These molecular tools have been used in microbial taxonomy to determine inter and intra specific relationships (Abaidoo *et al.*, 2000; Doignon-Bourcier *et al.*, 2000; Sarr *et al.*, 2005). The PCR-RFLP analysis has been used to provide an insight of genetic diversity of indigenous *Bradyrhizobium* isolates nodulating cowpea (Krasova-Wade *et al.*, 2003) and promiscuous soybean varieties in Kenya (Wasike *et al.*, 2009).

The objectives of this study were (i) to assess the genetic diversity of elite indigenous rhizobial isolates in symbiotic association with common bean and soybean based on PCR amplification and restriction of the 16S-23S rDNA intergenic region and sequence analysis of the 16S rRNA gene and (ii) to determine the genetic relatedness of the indigenous rhizobial strains to the reference strains in the GenBank.

## **5.2 Materials and methods**

### **5.2.1 Rhizobial isolates, their origin and growth characteristic**

The 13 indigenous rhizobia isolates used in this study are presented in Table 5.1. These were selected through an empirical approach involving the stepwise collection, isolation and authentication, screening against reference strains for symbiotic effectiveness, assessment of their competitive abilities and evaluation of their performance under a range of field conditions (Waswa *et al.*, 2014). Six of the thirteen isolates formed the most effective symbiotic association with soybean with the rest forming an effective symbiosis with common bean. The isolates were maintained under refrigeration in Yeast Extract Mannitol Agar as described in Section 2.1.

Table 5. 1: Indigenous rhizobia isolates, the host they were isolated, their growth characteristic and geographical area they were collected

CODE	Geographical area (County)	Ecological zone	Host	Cultivated/wild	Growth characteristic	Legume symbiont
NAK 5	Embu	Montaine	<i>Macroptilium atropurpureum</i>		Fast	<i>Phaseolus vulgaris</i> L.
NAK 45	Taita	Montaine	<i>Macroptilium atropurpureum</i>		Fast	<i>Phaseolus vulgaris</i> L.
NAK 67	Mt. Elgon (Busia)	Montaine	<i>Phaseolus vulgaris</i>	Wild	Fast	<i>Phaseolus vulgaris</i> L.
NAK 84	Bungoma	Midlands	<i>Glycine max</i>	Cultivated	Slow	<i>Glycine max</i>
NAK 89	Mumias	Midlands	<i>Glycine max</i>	Cultivated	Slow	<i>Glycine max</i>
NAK 92	Mumias	Midlands	<i>Phaseolus vulgaris</i>	Cultivated	Fast	<i>Phaseolus vulgaris</i> L.
NAK 104	Butula	Midlands	<i>Phaseolus vulgaris</i>	Cultivated	Fast	<i>Phaseolus vulgaris</i> L.
NAK 117	Teso	Upper midlands	<i>Glycine max</i>	Cultivated	Slow	<i>Glycine max</i>
NAK 128	Bungoma	Midlands	<i>Glycine max</i>	Cultivated	Slow	<i>Glycine max</i>
NAK 135	Bungoma	Midlands	<i>Glycine max</i>	Cultivated	Slow	<i>Glycine max</i>
NAK 157	Kwale	Coastal plain	<i>Phaseolus vulgaris</i>	Cultivated	Fast	<i>Phaseolus vulgaris</i> L.
NAK 179	Ramisi	Coastal plain	<i>Eriosema</i> sp.	Wild	Slow	<i>Glycine max</i>
NAK 186	Kikuyu (Kiambu)	Escarpment forest	<i>Sesbania sesban</i>	wild	Fast	<i>Phaseolus vulgaris</i> L.
CIAT 899*						
USDA 110*						

\* Standard isolates

Source: N2Africa rhizobium Database (Woomer *et al.*, 2013)

### **5.2.2 Total genomic DNA extraction from liquid bacterial cultures**

The rhizobia isolates and the two reference strains (USDA 110 and CIAT 899) were cultured seven days prior to DNA extraction as described in Section 2.5.1. DNA was extracted from the liquid cultures of the isolates as described in Section 2.5.2. Two methods were used to determine genetic diversity of the indigenous rhizobial isolates in this study, 1). Restriction of the 16S-23S rDNA intergenic region after PCR and 2) 16S rRNA gene sequencing.

### **5.2.3 PCR-RFLP analysis of the 16S-23S rDNA intergenic spacer region**

The intergenic region between the 16S and 23S rDNA from the 13 DNA products of the rhizobial isolates was amplified by PCR (Polymerase Chain Reaction) with two primers: FGPS 1490-72; 5'-TGCGGCTGGATCCCCTCCTT-3' (Normand *et al.*, 1996) derived from the 3' end of the 16S rDNA corresponding to positions 1,521-1,541 of *E. coli* and from the 5' end of the 23S rDNA (FGPL 132-38; 5' CCGGGTTTCCCCATTCGG-3') corresponding to positions 114-132 of *E. coli* (Ponsonnet and Nesme, 1994) (Section 2.5.3.1). Aliquots (10 µl) of PCR products were digested with the restriction endonuclease (enzyme) *Msp* I as described in Section 2.5.3.2. The restriction fragments were separated by horizontal electrophoresis in 1 X TBE buffer with 3% (w/v) agarose Sigma® (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) pre-stained with 3.5 µl of ethidium bromide. The gels were run at 100 V for 3 hours and photographed under UV illumination with Gel Doc (BIO-RAD, USA) software. Isolates that had identical restriction fragment profile were classified into the same genotypic/intergenic spacer (IGS) group.

### **5.2.4 Sequence analysis of the 16S rRNA gene**

PCR was performed for the 13 DNA products of the isolates as described in Section 2.5.4.1. Primers used for amplification of the 16S rRNA included the forward primer 27F

(5'AGAGTTTGATCCTGGCTCAG3') corresponding to positions 27–46 of the *E.coli* of the 16S rRNA gene sequence and the reverse primer 1492R (5'TACGGCTACCTTGTTACGACTT 3') corresponding to positions 1,525–1,506 of *E.coli* (Lane, 1991). High pure PCR product kit version 15, (Roche, 2010) was used for purification of PCR product as described in Section 2.5.4.2. Purified PCR products were sequenced for the DNA region coding for the 16S rRNA gene in an ABI 377 (PE-Applied Biosystems sequence analyzer) at the Segolip unit of the BeCA hub, ILRI. The generated sequences were submitted to the GenBank database through BLAST (Basic Local Alignment Search Tool) to search for significant 16S rRNA alignments. A phylogenetic tree was constructed based on the partial 16S rRNA gene sequences of the soybean and common bean nodulating isolates from the GenBank. The sequences of the rhizobial strains were aligned pairwise and compared to type strains in the GenBank database. A dendrogram was inferred with Neighbour-Joining Algorithm (Saitou and Nei, 1987) using ClustalX software (Thompson *et al.*, 1997).

## **5.3 Results**

### **5.3.1 Soybean**

#### **5.3.1.1 PCR-RFLP Analysis of the 16S-23S rDNA intergenic spacer region**

Single IGS PCR products were obtained from the six indigenous rhizobial isolates and reference strain (USDA110). Digestion of the six soybean nodulating rhizobial isolates with the restriction enzyme *Msp* I produced two IGS (Intergenic spacer region) profiles that included I (NAK 84, 89, 117, 135 and 179) and II (NAK 128). IGS group I was the most dominant constituting 5 (83%) of the six analyzed soybean nodulating isolates while group II only represented NAK 128. The reference strains (USDA 110) produced a different IGS profiles from its corresponding soybean nodulating rhizobial isolates (Figure 1).





Figure 5. 1: IGS groups obtained from Msp 1 restricted products of indigenous rhizobial isolated nodulating soybean. Key: M-100 bp ladder; C- USDA 110

### 5.3.1.2 Sequence analysis of the 16S rRNA gene

After sequencing, four of the isolates (NAK 84, 89, 117 and 135) generated sequence data while two (NAK 128 and NAK 179) did not. The partial sequences of the 16S rRNA gene of the four isolates were deposited in the GenBank and given accession numbers (Table 5.2). Alignments of partial sequences of these isolates with related 16S rRNA gene sequences in GenBank database revealed that the isolates were all closely related to the *Bradyrhizobium* genus. The four soybean nodulating isolates were closely related to *Bradyrhizobium elkanii* and *Bradyrhizobium sp.* strains (Table 5.2).

Table 5.2: PCR-RFLP profiles and sequence analysis of 16S rDNA of 4 isolates nodulating soybeans.

Isolate ID	IGS Group	Accession number	Species affiliation	sequence length bp	% Similarity	NCBI sequences with greatest similarity
NAK 84	I		<i>Bradyrhizobium elkanii</i> strain NBRC 14791	434	96	NR_112927
NAK 89	I		<i>Bradyrhizobium elkanii</i> strain NBRC 14791	642	98	NR_112927
NAK 117	I		<i>Bradyrhizobium elkanii</i> strain USDA 76	482	96	NR_117947
NAK 135	I		<i>Bradyrhizobium elkanii</i> strain MH3	442	97	AB931152

A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis confirmed the greater relationship of indigenous strains of *Bradyrhizobium* to reference strains of *B. elkanii*, *Bradyrhizobium japonicum* and *Bradyrhizobium* sp.. NAK 89 and 117 are more closely related compared to NAK 84 and 135, with the latter (NAK 135) being more closely related to the two (Figure 5.2).

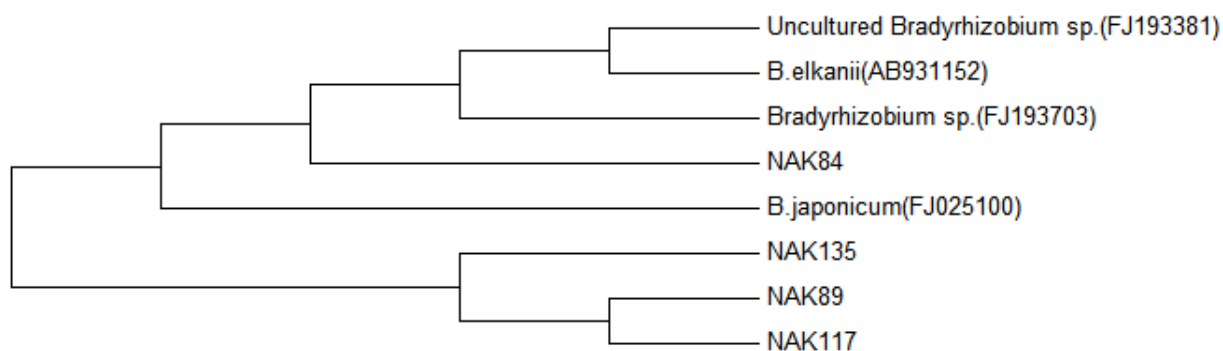


Figure 5.2 : Phylogenetic relationship between experimental and GenBank reference strains.

### 5.3.2 Common bean

#### 5.3.2.1 PCR-RFLP Analysis of the 16S-23S rDNA intergenic spacer region

Digestion of the seven bean nodulating rhizobial isolates with the restriction enzyme *Msp* I produced three IGS (Intergenic spacer) profiles including I, II and III for NAK 104, 157 and 186 respectively (Figure 5.3). Common bean nodulating isolates including NAK 5, 45 and 67 produced a single band indicating that there was no restriction with *Msp* I enzyme.

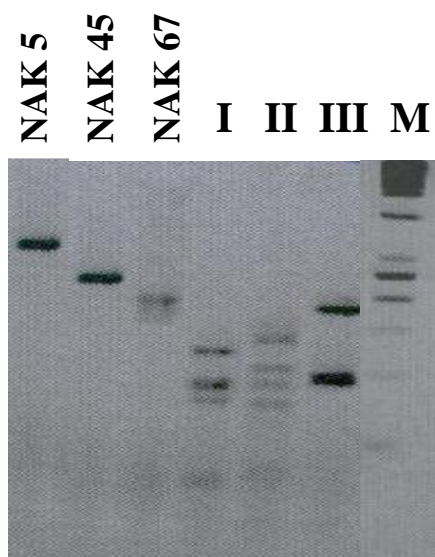


Figure 5.3 : IGS groups obtained from *Msp* I restricted products of indigenous rhizobial isolated nodulating common bean. Key: M-100 bp ladder

#### 5.3.2.2 Sequence analysis of the 16S rRNA gene

PCR product of NAK 157 isolate did not generate sequence while NAK 186 generated sequence data with no significant similarity to deposited GenBank sequence data. Partial sequences of the 16S rRNA gene of five common bean nodulating rhizobial isolates were deposited in the GenBank through BLAST and given accession numbers (Table 5.3). Alignments of partial sequences of these isolates with related 16S rRNA gene sequences in GenBank database revealed that the isolates belonged to different genera (Table 5.3). Only

two isolates were closely related to the *Rhizobium* genus with NAK 92 closely related to *Rhizobium sp.* and NAK 104 to *Rhizobium tropici*. Two of the isolates were closely related to the genus *Pseudomonas*. NAK 5 was closely related to *Pseudomonas putida* and NAK 45 to *Pseudomonas spp.* NAK 67 was closely related to *Paenibacillus polymyxa*. A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis confirmed the greater relationship of indigenous strains of *Rhizobium* to reference strains of *Rhizobium sp.*, *Rhizobium tropici* and *Rhizobium phaseoli*. The *Pseudomonas* reference strain constitute an outside group in the phylogenetic tree (Figure 5.4).

Table 5. 3: PCR-RFLP profiles and sequence analysis of 16S rDNA of 6 isolates nodulating common beans.

Isolates	Species affiliation	sequence length	% Similarity	NCBI
				sequences with greatest similarity
<b>NAK 5</b>	<i>Pseudomonas putida strain KT2440</i>	753	99	NR_074596
<b>NAK 45</b>	<i>Pseudomonas spp.</i>	1490	98	NR_025103
<b>NAK 67</b>	<i>Paenibacillus polymyxa strain E681</i>	1519	98	NR_103922
<b>NAK 92</b>	<i>Rhizobium sp. NR 3-02</i>	1469	96	KM253110
<b>NAK 104</b>	<i>Rhizobium tropici strain CPAO 1135</i>	1436	98	EF054882

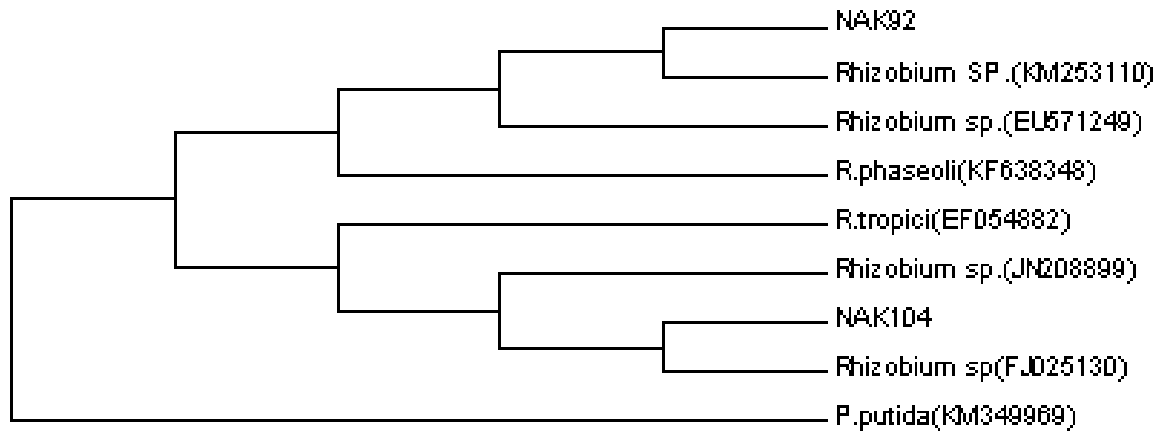


Figure 5. 4: Phylogenetic relationship between experimental and GenBank reference strains

## 5.0 Discussion

The indigenous rhizobial isolates used in this study formed effective symbiosis with soybean (Waswa *et al.*, 2014) and common bean. They were recovered from nodules of wild and cultivated legume hosts growing in different agro-ecological zones indicating the residence of indigenous rhizobia in Kenyan soils. The selection of the indigenous isolates isolated from different Kenyan soils under different land use and in different agroecological zones indicates their adaptability to the local environment resulting to high levels of saprophytic competence (Zengeni *et al.*, 2006) , which is an advantage for strain selection. Their presence in the different areas of origin nodulating various legumes could be attributed to past legume cropping systems, inoculation with commercial strains, contamination through dust and anthropogenic activities or the strains could be native to the sites. Previous study (Zengeni *et al.*, 2006) has reported the presence of great rhizobia diversity in most soils and an enhanced population size where compatible legumes are grown and the soil is fertile. Meade *et al.* (1985) and Thies *et al.* (1991) also reported the presence of compatible resident rhizobia in fields that have never been cultivated to a given host legume due to the presence of native legume species, transfer from adjacent fields, or as part of the native microbiological soil community. Rhizobia are highly mobile as contaminants of dust and seed. For example,

Stepkowski *et al.* (2005) have demonstrated that many of the contemporary lupin and serradella nodule occupants in farmland in South Africa and Western Australia derive from contaminant European strains imported to those continents unintentionally, probably in the preceding 200 years. Mulongoy and Ayanaba (1986) have reported the presence of *Bradyrhizobium japonicum* in some African soils even though soybean was not commonly grown.

Five of the superior strains nodulating soybean were isolated from non-inoculated soybean growing in farmers' fields, and only NAK 179 was isolated from *Eriosema spp.* NAK 179 recovered from the nodules of *Eriosema spp.* performed well on the soybean variety (SB19) reaffirming that TGX is more promiscuously nodulated (Sanginga *et al.*, 2000). In their study, Abaidoo *et al.* (2000) concluded that bradyrhizobia nodulating promiscuous soybean are diverse and genetically distinct from those nodulating North American soybeans. Isolates NAK 84, 89, 117, 128 and 135 were collected from same agroecological zone of west Kenya (Table 5.1) which is a soybean-growing region while NAK 179 was isolated from the Coastal plains. The growth characteristic (Table 5.1) and comparison of our 16S rRNA gene sequences with those deposited in the NCBI gene bank (Table 5.2) showed that soybean forms symbiosis with slow-growing rhizobia of genera *Bradyrhizobium* and all the strains were closely related to strain *Bradyrhizobium elkanii*, a main rhizobial partner of soybean (Kuykendall *et al.*, 1992). The phylogenetic trees show that there is adequate genetic variation among the indigenous strains of *Bradyrhizobium*.

The rhizobial isolates that formed effective symbiosis with common beans were isolated from different host legumes including *Macroptilium atropurpureum*, *Phaseolus vulgaris* and *Sesbania sesban* (Table 5.1). This indicates the promiscuous nature of common beans to nodulate with a wide range of *Rhizobium* strains and the cross-inoculation of strains

compatible with various legumes (Abaidoo *et al.*, 2007). Comparison of 16S rRNA gene sequence with those deposited in the GenBank and phylogenetic tree analysis indicated that two of the isolates nodulating common bean belongs to the genus *Rhizobium* being closely related to *Rhizobium spp* (NAK 92) and *Rhizobium tropici* (NAK 104). Soils of Kenya have been reported to harbor bean-nodulating rhizobia similar to *R. leguminosarum*, *R. tropici*, *R. phaseoli* and *R. etli* (Anyango *et al.*, 1995). Presence of *Rhizobium tropici* in Kenyan isolates confirms results by Pinto *et al.* (2007) who showed that the majority of very effective rhizobia isolated from common bean field-grown plants were *R. tropici*.

The other three isolates, two of them were closely related to the genus *Pseudomonas* and one to genus *Paenibacillus*. Since these bacteria are non-symbiotic, their presence in nodules suggests the acquisition of nodulation genes from rhizobia strains in the soils enabling the bacteria to effectively nodulate the host legume. Their isolation also suggest that these strains were internal or external contaminants of the nodules and since they were predominantly fast-growing, they grew faster than rhizobia during the isolation process. Silva *et al.* (2007) studied the effects of different *Paenibacillus macerans*, *Paenibacillus durus*, *Paenibacillus polymyxa* and *B. pumilus* strains on the symbiosis between *Bradyrhizobium* and cowpea, and showed that these strains stimulated nodulation and improved nitrogen fixation efficiency. In their study, Shiraishi *et al.* (2010) revealed that *Pseudomonas sp.* and *Burkholderia sp.* formed nodules on black locust and that their symbiotic genes were highly similar with those of rhizobial species and attributed this to horizontal gene transfer. MacLean *et al.* (2007) reported that genes encoding nodulation and nitrogen fixation are often carried on plasmids or other accessory elements, such as symbiotic islands, and properties encoded by them can be easily lost or gained. Other reports (Sullivan and Ronson, 1998) have described the transfer of symbiotic DNA in discrete units termed ‘symbiosis islands’ from legume inoculants to soil bacteria. Nandasena *et al.* (2006) have subsequently described how the

transfer of a symbiosis island from inoculant mesorhizobia to soil bacteria resulted in the rapid evolution of ineffective strains in the soil. These bacteria are non-symbiotic living in the rhizosphere and have been identified as PGPR (Plant growth-promoting rhizobacteria). Their superior performance in the previous study can be attributed to the positive effect they exert on plants through various mechanisms. Previous studies have reported that most of the bacteria which are effective in the field belong to phosphate solubilizing microorganisms (Pisney and Azcon, 1987; Ming and Alexander, 1988). In their study, Rasi poor and Ali Asgarzade (2002) investigated the role of four phosphates solubilizing bacteria, *P.flourescens*, *P.putida* and *Aeromonas hidrophyla* with *Bradyrhizobium japonicum* on soya nodulation and performance. The results showed that *P.putida* treatment had the most effect on soya nodulation, nitrogen fixation and phosphorus absorption, comparing with the other treatments only with *Bradyrhizobium japonicum* and concluded that these bacteria enhanced phosphorus-absorption.

## **5.1 Conclusion**

At the greenhouse level, isolates were effective and varied in their performance and showed interactions with bean varieties.

Poor response to inoculation with the bean cultivars in the field trials was due to highly competitive resident rhizobia.

## **5.2 Recommendation**

Rhizobial isolates (NAK 5, NAK 67 and NAK 92) have potential for fixing nitrogen and should be evaluated in different agro-ecological zones.



## CHAPTER 6

### GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

#### 6.1 Discussion

The empirical approach used in this study to identify elite rhizobia strains involved the stepwise collection, isolation and authentication of native rhizobia, the screening of the isolates against reference strains for symbiotic effectiveness, the assessment of their competitive abilities and the evaluation of their performance under a range of field conditions. The approach was a success since a large number of isolates were evaluated and this number was systematically scaled down to a few, highly effective and competitive rhizobial isolates compared to industry standard strains (USDA 2667 and CIAT 899). Three hundred and eighty rhizobia isolates were collected and isolated, authenticated and tested for effectiveness on climbing bean (*Phaseolus vulgaris L.*) var. Kenya Tamu in greenhouse pots containing sterile vermiculite. Variation in nodulation and plant biomass among the isolates was applied to reduce the number of test isolates to 16 which were used in this study. Effectiveness of the selected 16 rhizobial isolates were compared to two strains widely used commercial strains (USDA 2667 and CIAT 899) in potted greenhouse soil. A number of rhizobia isolates (NAK 5, NAK 92, 186 and 191) outperformed both standard commercial strains (CIAT 899 and USDA 2667) with the two bean varieties. Specific interactions between bean varieties and rhizobia isolates were also observed. Lack of response to inoculation in the field trials was attributed to acidity, the low N and the presence of highly competitive resident rhizobia. However, isolates NAK 5, NAK 67 and NAK 92 showed potential for fixing nitrogen with beans.

The study also focused on how management through biochar addition as a soil conditioner might be employed to reduce the detrimental effects of abiotic stresses or competitive environments that are common in Western Kenya for nodulation of bean with elite native rhizobia. Lack of response to inoculation with biochar amendment could be attributed to the presence of large populations of highly competitive resident rhizobia adapted to their environment and the lower application rate of biochar in this study ( $2 \text{ t ha}^{-1}$ ) though positive crop performance was recorded which was associated with improved nutrient availability.

A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis confirmed the greater relationship of indigenous strains of *Bradyrhizobium* to reference strains of *B. elkanii*, *B. japonicum* and *Bradyrhizobium spp.*. A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis confirmed the greater relationship of indigenous strains of *Rhizobium* to reference strains of *Rhizobium sp.*, *Rhizobium tropici* and *Rhizobium phaseoli*. The diversity identified in *Bradyrhizobium* and *Rhizobium* populations in Kenyan soils represent a valuable genetic resource that has potential utility for the selection of more competitive and effective strains to improve biological nitrogen fixation and thus increase soybean and common bean yields at low cost.

## 6.2 Conclusions

Poor response to inoculation with the bean cultivars in the field trials was due to highly competitive resident rhizobia. However, a number of isolates outperformed the standard commercial strains and the N-fertilized treatment.

Inoculated beans grown in soils amended with biochar gave higher biomass and grain yields which is attributed to nutrient availability and raised pH from biochar addition.

Grain and biomass production increased in the N-fertilized with biochar addition suggesting improved N-use efficiency.

The BLAST and phylogenetic tree analysis show that soybean forms symbiosis with slow-growing rhizobia of genera *Bradyrhizobium* and all the strains were closely related to strain *Bradyrhizobium elkanii*. There is adequate genetic variation among the indigenous strains of *Bradyrhizobium* isolated from different agroecological zones though NAK 128 and NAK 179 did not generate sequence data after sequencing.

NAK 92 was closely related to *Rhizobium spp* and NAK 104 to *Rhizobium tropici*. There is adequate genetic variation among the two indigenous strains of *Rhizobium* isolated from different agroecological zones.

NAK 5 and NAK 45 nodulating bean were related to the genus *Pseudomonas* and NAK 67 to genus *Paenibacillus*. NAK 157 isolate did not generate sequence data while NAK 186 generated sequence data with no significant similarity to deposited GenBank sequence data.

### **6.3 Recommendations**

Rhizobial isolates (NAK 5, NAK 67 and NAK 92) have potential for fixing nitrogen and should be evaluated in different agro-ecological zones and different soil types.

There is need for long-term field studies to better understand the effects of biochar on BNF.

The bean nodulating isolates, belonging to other genera other than *Rhizobium* should be re-evaluated for genetic compability test.

Superior genetic tools should be employed to show the genetic diversity of both bean and soybean noduating isolates at the strain level.

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**APPENDIX 1: N-free Nutrient Solution (Broughton and Dillworth, 1970)**

Stock solution	Element	M	Form	MW	g/l	M
1	Ca	1000	CaCl <sub>2</sub> ·2H <sub>2</sub> O	147.03	294.1	2.0
2	P	500	KH <sub>2</sub> PO <sub>4</sub>	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	MgSO <sub>4</sub> ·7H <sub>2</sub> O	246.5	123.3	0.5
	K	250	K <sub>2</sub> SO <sub>4</sub>	174.06	87.0	0.5
	Mn	1	MnSO <sub>4</sub> ·H <sub>2</sub> O	169.02	0.338	0.002
4	B	2	H <sub>3</sub> BO <sub>3</sub>	61.84	0.247	0.004
	Zn	0.5	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	287.56	0.288	0.001
	Cu	0.2	CuSO <sub>4</sub> ·5H <sub>2</sub> O	249.69	0.100	0.0004
	Co	0.1	CoSO <sub>4</sub> ·7H <sub>2</sub> O	281.12	0.056	0.0002
	Mo	0.1	Na <sub>2</sub> MoO <sub>2</sub> ·2H <sub>2</sub> O	241.98	0.048	0.0002

For each 10 liters of full strength culture solution, 5 milliliter each of solutions 1 to 4 was taken, then added to 5.0 liters of water, and then dilute to 10 liters. One normal NaOH was used to adjust the pH to 6.6-6.8. For plus N control treatments, KNO<sub>3</sub> (0.05%) was added giving an N concentration.