IMPROVING BIOLOGICAL NITROGEN FIXATION IN COMMON BEAN (*Phaseolus vulgaris*) VARIETIES GROWN WITHIN EASTERN KENYA

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DECLARATION

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

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DEDICATION

This work is dedicated to my daughter Deborah and my loving wife Halima who have sacrificed all they have to see me excel in my studies. To my loving parents, I salute you; your selfless devotion towards my studies will always be remembered.

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ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
CRD	Complete Randomized Design
KALRO	Kenya Agricultural Livestock and Research Organization
NN	Nodule Number
RDW	Root Dry Weight
SAS	Statistical Analysis System
SDW	Shoot Dry Weight

ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is the third most valuable food crop in Kenya and the main source of proteins to smallholder farmers. However, its production is constrained by insufficient nitrogen (N) in the soil exacerbated by acidic soil conditions and phosphorus (P) fixation. Majority of smallholder farmers are resource limited hence apply negligible amount of inorganic fertilizers. Therefore, enhancing biological nitrogen fixation (BNF) by bean germplasms grown by smallholder farmers using low-cost native rhizobia adapted to local agro-climatic conditions is imperative. Thus, this study was aimed at assessing the efficiency of biological nitrogen fixation in selected common bean varieties grown in Eastern Kenya. The questionnaires were used to identify the common bean varieties grown in Eastern Kenya. The native rhizobia were isolated from the root nodules of (MAC 13 and Mac 64) bean varieties grown as trap cultures in Eastern Kenya. Isolation of native rhizobia was done on Yeast extract Mannitol agar (YEMA) supplemented with Congo red dye and Bromothymol blue (BTB) for characterization. A greenhouse bioassay was carried out at Kenyatta University where ten common bean varieties widely cultivated in Eastern Kenya were grown and either inoculated with a consortium of native rhizobia, exotic *Rhizobium*, a mix of native consortium and exotic rhizobia, or left without inoculation (control). The experiment was set up as a completely randomized design with three replications per treatment. The crop was sampled after four weeks and examined for nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), root dry weight (RDW) and shoot nutrients namely; nitrogen (N), P, and potassium (K). The inoculated bean plants produced a significantly higher nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), as well as shoot N and P contents than non-inoculated bean plants. The highest significant SDW and N content were achieved in bean plants inoculated with a mix of native and exotic rhizobia, while the highest significant NN, NDW, and P content were realized in bean plants inoculated with native rhizobia. Among the ten common bean varieties, Kabuu produced the highest significant NDW, SDW, N and P content as compared to other varieties. These results demonstrate a key potential of native rhizobia inoculants in promoting BNF and form an important step towards the development of cost effective rhizobial cultures. Further studies should elucidate the performance of the native rhizobia inoculants used here under field conditions.

CHAPTER ONE

INTRODUCTION

1.1 Background

Common bean (*Phaseolus vulgaris* L.) is one of the most valued sources of protein cultivated by both commercial and smallholder farmers in sub-Sahara Africa (Mundi, 2012). The crop is among the most important sources of proteins worldwide because it is cheap and readily available (Duke, 2012). Equally, the rhizobia bacteria that invade the root nodule of bean plants can fix atmospheric nitrogen converting it to ammonia, which is utilized by the bean plant to synthesize proteins. The cultivation of beans has increased over decades due to the nutritive, economic value and other domestic uses such as animal feed. All these diverse uses of beans have made the plant to be common not only in Africa but also in the entire world (Nanyunja *et al*., 2015).

Common bean is an exceptional source of fiber that reduces cholesterol in the body. Besides lowering the cholesterol levels, the fiber content also prevents the rapid rising of blood sugar levels after meals thus preferable to people with diabetes (Câmara *et al*., 2013). When taken with rice, they offer a nearly cholesterol free and quality meal. Beans are as also an outstanding source of molybdenum that is an important component of sulfite oxidase enzyme, which detoxifies sulfites from the body. Beans can also elevate body energy by replacing the iron stores in the body. This replacement of iron is crucial in menstruating females who are susceptible to iron deficiency (Messina, 2014).

In Eastern Kenya, beans are the major source of protein. The bean plant hosts the rhizobia bacteria that have the ability to replenish soil fertility by fixing nitrogen from the atmosphere into the soil. According to Bothe *et al*. (2006), recovery of about 280 kg of nitrogen in one hectare has been achieved in common bean. This nitrogen fixation ability in common bean can be a crucial advantage in Eastern Kenya where continued cultivation has led to low soil fertility. The poor soils in Eastern Kenya are because of continued land cultivation to meet the food demand for the ever-escalating human population. On the other hand, fertilizers are rarely available or too expensive for farmers (Neugschwandtner *et al*., 2015). Besides, excessive use of nitrogen fertilizers may lead to environmental pollution and increased soil acidity (Padgett and Minnich, 2008).

Nitrogen is one of the key factors limiting plant growth as well as development (Kant *et al*., 2008). Biological nitrogen fixation (BNF) is the process where soil microorganisms fix atmospheric nitrogen by symbiotic or non-symbiotic means. The symbiotic BNF process is a mutual relationship between legume plants and rhizobia bacteria that stimulates the growth of root nodules in bean plants. This advantageous interaction between legumes such as beans and rhizobia bacteria for BNF is the best alternative since it makes use of energy from photosynthesis and is ecologically friendly. For efficient BNF and enhanced legume production, proper soil inoculation and appropriate soil microbes are required (Neugschwandtner *et al*., 2015).

1.2 Problem statement and justification

Kenya has undoubtedly witnessed rising trends in bean production in the past decade. The quantity of bean production has increased steadily from 4 million tons since 2005 to the present 6 million tons (Nanyunja *et al*., 2015). The increase is mainly due to the adoption of diverse bean varieties, the use of chemical fertilizers and the expansion in the area under bean cultivation to meet the population demand (Nanyunja *et al*., 2015). Despite the concerted efforts, the expected output level of the crop in Eastern Kenya is yet to be attained due to inadequate nitrogen and soil acidic conditions worsened by phosphorus fixation.

Smallholder farmers in Eastern Kenya usually grow the local bean varieties in every planting season. Besides, past studies on common bean in Eastern Kenya have not focused the efficiency of BNF in local bean varieties grown in the region. Therefore, there is a necessity to improve the productivity of local beans through enhanced BNF. This study aims to improve the efficiency of nitrogen fixation in the ten common bean varieties grown in Eastern Kenya by use of *Rhizobium* bacteria. The effect of native rhizobia, exotic rhizobia, and a co-inoculation of indigenous and exotic *Rhizobium* on nitrogen fixation in common beans varieties grown in Eastern Kenya will be evaluated.

1.3 Research questions

i. Do different bean varieties grown by smallholder farmers in Eastern Kenya differ in nodulation and nitrogen fixation?

ii. Do native and exotic rhizobia differ in nodulation and nitrogen fixation efficiency of common bean?

iii. Does increase in rhizobia isolates diversity enhance nodulation and nitrogen fixation in common bean?

iv. Does inoculation of *Rhizobium* increase shoot biomass production and shoot N, P, and K content of common bean varieties grown in Eastern Kenya?

1.4 Hypotheses

i. Different bean varieties grown by smallholder farmers in Eastern Kenya differ in nodulation and nitrogen fixation.

ii. Indigenous and exotic *Rhizobium* isolates differ in nodulation and nitrogen fixation efficiency of common bean varieties.

iii. Increasing *Rhizobium* isolates diversity enhances nodulation and nitrogen fixation in common beans.

iv. *Rhizobium* inoculation increases shoot biomass production and shoot N, P, and K contents of common bean varieties grown in Eastern Kenya.

1.5 Objectives

1.5.1 General objective

To assess the efficiency of biological nitrogen fixation in common bean varieties grown in Eastern Kenya inoculated with native, commercial rhizobia, or a combination of both.

1.5.2 Specific objectives.

i. To determine nodulation and nitrogen fixation of bean cultivars grown by smallholder farmers in Eastern Kenya.
ii. To determine the efficiency of indigenous and exotic rhizobia in nodulation and nitrogen fixation ability of common bean varieties grown by in Eastern Kenya.
iii. To determine whether increasing *Rhizobium* isolates diversity increases nodulation and nitrogen fixation in common bean.
iv. To examine the effect of rhizobia inoculation on shoot biomass production, shoot N, P, and K contents of common bean varieties grown in Eastern Kenya.

1.6 Significance of the study

This study was aimed at improving the efficiency of nitrogen fixation in common bean varieties in Eastern Kenya. A large-scale adoption of this mode of bean cultivation by use of *Rhizobium* bacteria will lead to increased production of domestically produced beans. The importation of beans could then reduce substantially and the millions of foreign exchange spent to purchase beans could be invested in the local agricultural economy.

CHAPTER TWO

LITERATURE REVIEW

2.1 Common beans

Common bean (*Phaseolus vulgaris* L.) is one of the herbaceous legume plants that belong to Leguminoseae family, Papilionoideae subfamily, Phaseoleae tribe, Phaseolinae sub-tribe and Phaseolus section (Lackey, 1977). According to Davis (1997), common bean is primarily grown for the green fleshy pod and the dry bean. About 130 green bean varieties differ in color, taste, and size of the pod. Green bean is the third most common garden plant after tomato plant and pepper (Gentry, 1969). According to Graham and Vanice (2003), the major production and consumption of common bean is that of dry bean as compared to garden bean. For years evolution, natural selection and mutation have brought striking changes regarding the physiology and morphology of the wild type that originates from the Middle America and the Andes resulting to the present characteristics in common bean cultivars (Back, 1996). The distribution of wild species extends from the Northern part of Mexico to the Northern Argentina (Kaenig *et al*., 1990).

According to Gepts and Debouck (1991), the evolutionary studies of common bean plant have pointed to new world origin even though the specific region of domestication of common bean is not known. According to Islam *et al*. (2002), comparative studies between wild and the presently cultivated common bean in Middle of America continent and the Andes show variations in the type of phaseolin, the bracteole size, as well as seed lectin and size. The genotypes from America show an “S” phaseolin, big

and oval lectins and bracteoles and less large seeds (Lambrecht *et al*., 2015). However, the genotypes from the Andes have a "T" phaseolin type, small and triangular bracteoles without lectins but with larger seeds. The "B" phaseolin was found in Colombia in the North Andes in small common bean cultivars (Singh *et al*., 2007).

According to Meng and Ma (2002), the three domestication centers are postulated to be the origin of the present common bean. The Middle America produce the small seeded common bean of fewer than 25 g/100 seeds, and the medium seed containing the common bean of less than 30 to 50 g/100 seeds that contain "S" phaseolin pattern. The other two are found in South America where one gives rise to the large seeded of above 40 g/100 seeds and the other small seeded cultivars containing a "B" phaseolin pattern (Rodiño *et al*., 2003). Common bean has shown varying characteristics to different climatic conditions. Likewise, common bean cultivars have been reported to show different growth habits (Cardona, 2004).

The determinate varieties are dwarf with less number of nodes in the stem with terminal inflorescent (Debouck,1991).The indeterminate bush varieties have upright stems with less branching while the indeterminate prostrate cultivars have relatively weak stems with profuse branching (Debouck,1991). The indeterminate prostrate cultivars have long and twisted thin stems with less branching (Chaturvedi *et al*., 2011). According to Alpern (1992), the Portuguese are the ones who took the common bean to Africa, Europe, and the Old world. This spread may have made common bean to be one of the most diverse crop in the globe regarding acreage and production (Rooney *et al*., 2007).

Dry bean serves as the prime protein-producing subsistence crop in America, Asia as well as Africa. According to Ahloowalie *et al*. (2004), in Canada, the plant is primarily grown for commercial export hence contributing to about 90 million US dollars annually to the economy. The main limiting factors in the cultivation and production of common bean are poor soil fertility, drought and diseases both in the tropics areas and in the subtropics (Graham and Vanice, 2003).

2.1.1 Common bean characteristics

Common bean plant is the main legume consumed globally (Broughton *et al*., 2003). According to Cardona (2004), common bean varieties vary greatly in color, shape, size, and fibrousness of the young pods. The bean crop is a polymorphic herbaceous plant with two main plant types. The erect, herbaceous bush type is usually 20 to 60 cm tall while the climbing twining vines being 2 to 5 m long (Duke, 2012). Common bean has a taproot containing several adventitious roots. The bush bean stems are slender with many branches. The climbing beans have prostrate stems with either green or purple trifoliate leaves and contain long and green petioles. The leaflets are 3 to 11 cm broad and 6 to 15 cm long with axillary or terminal inflorescences (Rubatzky and Yamaguchi, 1997).

The arrangement of flowers is in pairs or solitary along the rachis. Once pollination has taken place, each flower in common bean produces one pod. The pods are long and slender sometimes striped with black, yellow, green or purple color (Atanaf *et al*., 2013). The pods can be flat, cylindrical, curved, or straight 1 to 1.5 cm wide with the

length extending up to 20 cm; each pod contains 4 to 12 seeds. The seeds are kidney-shaped, 0.5 to 2 cm long with different colors ranging from green, white, red, purple, gray or black depending on the variety (Chen *et al*., 2004).

2.1.2 Common beans nutritional value

Common bean seeds are rich in protein, starch, and fiber (Messina, 2014). The abundance of the main dietary ingredients makes bean plant a major crop to both human and livestock (Bravo, 1998). According to Champ (2002), the presence of anti-nutritional compounds, however, lowers the likely feeding value, especially in mono-gastric animals. According to Alayande *et al*. (2012), the stems and pods have less protein with a composition of 8 % and 4 % dry matter (DM) respectively. The leaves of common bean have a relatively high amount of protein of 20 % DM. The haulms of common beans have 6 to 11 % DM protein and high fiber content as well (Sagarbieri, 1989).

Like other legumes, bean plant straws have an enhanced nutritive value as compared to the cereal straws because of the high protein content and low fiber (De Leeuw, 1997). According to Yasmin *et al*. (2008), there are several compounds in common bean plant such as trypsin-alpha amylase, chymotrypsin, lectins and saponins, which are considered anti-nutritional. The composition and activity of these anti-nutritional compounds differ in various genotypes. Studies have shown that uncooked navy beans have double the amount of trypsin inhibition as compared to the red kidney bean but with the lectin activity being half (Dhurandhar and Chang, 1990).

The anti-nutritional compounds have been found to be toxic in mono-gastric organisms. For instance, the undercooked bean lectins in many cases have caused food poisoning in humans. Therefore, one is advisable to cook these beans properly before feeding the mono-gastric livestock like pigs in order to avoid food poisoning (Swetman *et al*., 2008). Treatments such as cooking beans at the temperature above 100^0C significantly reduce the lectin content. Biological processes such as germination, pancreatin treatment and ensiling have been confirmed to reduce the dangerous anti-nutritional compounds (Soetan and Oyewole, 2009). Heating, extruding and autoclaving have also been reported to reduce the anti-nutritional compounds in common bean (Soetan and Oyewole, 2009).

2.1.3 Common bean agronomic characteristics

Common bean grows better in warm subtropical and temperate conditions (Teixeira *et al*., 2013). The crop can also thrive in tropical regions (Zahran, 1999). According to Beaver *et al*. (2003), under very high humid conditions, the crop fails to develop due to fungal diseases though such conditions have been found to favor cowpea. The plant grows well starting from sea level to heights of 3000 m above sea level with the average annual rainfall required being between 300 to 4300 mm (Boserup, 2005). Heavy rainfall and hot season makes the flowers and pods to drop and increase the occurrence of diseases (Van Zyl and Lorentz, 2004). The optimum temperature range is between 15^0C to 24^0C with the least mean daily temperature required for growth being l0°C.

According to Romani *et al*. (2003), the crop, however, can grow at temperatures of up to 35^0C, but the production of seeds is significantly affected leading to reduced yields and the intensification of the fiber content of the pod. Beans have a slight tolerance to frost; however, when the temperature falls below 10^0C, growth may stop since the cold conditions may affect the different stages of its growth (Źróbek-Sokolnik, 2012). Less humidity during the maturation stage is of great benefit for seed preservation. The optimum soil pH for proper growth of common bean ranges from 4 to 9 though the soil should have high organic content apart from being well-drained. The crop cannot withstand sandy soil, acidic soil, calcareous and waterlogged soils although some genotypes have been reported to do well in standing water. The plant has been reported to grow best in silt-loam, sandy-loam or clay-loam soils and is sensitive to minerals such as Boron, Aluminum, Manganese as well as high levels of sodium (Lucy *et al*., 2004).

2.1.4 Common bean production in Kenya

The bush bean and the climbing bean are two bean varieties grown in Kenya. According to (Kariuki *et al*., 2012), the production of conventional beans annually is nearly 420,000 metric tons and this production is an equal of 199,743,000 million US dollars. According to Nekesa *et al*. (1999), the contribution of common bean in the manufacture of animal feeds and the consumption in the human diet in Kenya is high. The use of beans per capita in Kenya is 14 kg per year (Wortmann, 1998). In Kenya, climbing bean is more productive since it requires less cultivation space and is more resistant to diseases compared with the bush type (Okello, 2005). One climbing bean has the

capacity of producing up to 100 pods as compared to 25 pods that are produced by bush bean in a single season (Lambrecht *et al*., 2015).

One hectare of climbing bean can produce 4 to 5 tons of beans, and this high yield has led to the extensive adoption of the climbing bean variety in Kenya where farming land is scarce (Kelly and Cichy, 2012). Consequently, the continuous increase in population on the existing land has led to the massive acceptance of the climbing bean. Common bean has been found to grow excellently in the highlands of Kenya and Midland regions particularly in Embu, Kisii, Kericho, Meru and the entire western Kenya except Busia (Schroeder *et al*., 2013).

The climbing bean when growing occupies more space vertically than the ground space, and this minimizes the occurrences of diseases since they are well aerated. Contrary to the bush bean, the climbing bean is not intercropped with other crops like maize and potatoes and is harvested after the appearance of the first pod and the harvest continues until the last pod (Willey and Osiru, 1972). It takes 86 days for the climbing bean to mature and the harvest starts after maturity onwards, unlike the climbing beans, the harvest in bush bean is after 86 days and production ceases just after harvest (Graham and Rosas, 1977).

2.2 *Rhizobium* and nitrogen fixation

The earth is made up of nearly 80 % nitrogen gas, which is unutilized by a majority of living things. Both animals and plants can die because of nitrogen deficiency (White,

2012). Therefore, it is important that nitrogen be made available to synthesize proteins, nucleic acids, amino acids and other nitrogenous cell organelles that are essential for life (Hungaria *et al*., 2003). Biological nitrogen fixation occurs when the rhizobia bacteria invade the plant roots inducing a nodule (Dhar *et al*., 2015). The bacteria in the nodule then reduce atmospheric nitrogen forming ammonia thus supplying the plant with nitrogenous compounds by either α-Proteobacteria or Rhizobiaceae nitrogen fixing bacteria (Hart *et al*., 2003).

The legume plant gains the capability to grow in nitrogen deficiency soils while the bacteria obtains sugars and a protected niche in which they multiply and finally escape back to the nearby soil when the nodule bursts (Tambalo *et al*., 2015). In legumes, the nitrogen-fixing bacteria live in root nodules where nitrogen fixation occurs. This mutual association between the bean plant and the bacteria is symbiotic and contributes less than 5lb of nitrogen per acre annually. The other non-symbiotic free-living nitrogen-fixing bacteria include lichen and blue-green algae. The free-living non-symbiotic bacteria contribute about 25 to 75 lb of biological nitrogen per acre annually (Felker *et al*., 1980).

2.2.1 Characteristics of legume nodules

The initial stage of BNF is the formation of root nodules because of rhizobia bacteria multiplying in the cortex cells of the root (Novak, 2010). After a period of 2 to 3 weeks, small white or gray visible nodules appear as a sign of rhizobia bacterial infection. As the size of nodules increases, they turn red or pink in color an indication that the process

of nitrogen fixation has started. The pink color is because of leghemoglobin that is responsible for controlling the flow of oxygen in bacteria (Heritage *et al*., 1999). The nodules in perennial legumes have a fingerlike shape and have the ability to fix nitrogen in the entire growing season as long as there are favorable growth conditions. The nodules for annual legumes such as beans are round, short lived and usually replaced throughout the growing season (Vance, 1998).

The nodules that have ceased to fix nitrogen usually turn green. Therefore, the pink nodules, which are a sign of nitrogen fixation, should be dominating at the middle of the growing season. However, if green, white or even gray nodules prevail in the growing season, then it means little or no nitrogen fixation is taking place. The low nitrogen fixation may be because of ineffective rhizobia or reduced plant nutrition (Reed and Walker, 1991). Stress factors like water availability and temperature are hard for the farmer to control. However, nourishment stress can be adjusted by fertilizer application on the crop. Inadequate biological nitrogen fixation can be corrected by proper irrigation, fertilization and inoculation practices (Hayat *et al*., 2008).

2.2.2 Characteristics of rhizobia

Rhizobia bacteria can grow in the laboratory by use of culture media. When seen under the microscope the rhizobia appear as short rods measuring 1.2 to 3.0 microns in length and 0.5 to about 0.9 microns wide (Somasegaram and Hoben, 2012). Besides, these bacteria form colonies that are spherical and elevated with smooth edges when observed under low power microscope. According to Tambalo *et al*. (2015), the bacteria can

move from place to place by use of distinctive thread-like structures known as flagella. When carrying out Gram staining technique, pink colored Gram-negative rods are usually observed (Somasegaram and Hoben, 2012). Rhizobia are quite simple to culture in the laboratory. The bacteria grow best in oxygen and use very simple compounds such as amino acids and carbohydrates. With just an exception of some strains, a majority of rhizobia bacteria only fix nitrogen within their legume host plants. A few strains of rhizobia need vitamins to grow (Joseph *et al*., 2003).

The ideal growth conditions for a majority of rhizobia occur at a temperature of between 25-30^{0}C, and the pH of 6 or 7. In spite of their typical aerobic metabolism, several micro-aerophiles can grow sufficiently at oxygen concentrations less than 0.01 atmospheres (Bergersen, 1961). *Rhizobium phaseoli* and *Rhizobium trifolii* nodulate beans and clovers plants respectively. They are acid producers and fast growing and produce turbidity in broth media within three days. They have an average doubling rate time of two hours (Hungria *et al*., 2000). The rhizobia bacteria cells contain either rod shapes or pleomorphic shapes; with the diameter of 0.6 to 0.9 microns while the length being 1.2 to 3.0 microns. They are motile with a majority having peritrichous flagella ranging from 2 to 6 flagellates. They grow on different carbohydrates, however; they best grow in mannitol or sucrose. Bacteria in this group infect legumes that grow in temperate regions (Segovia *et al*., 1993).

The strains of *Bradyrhizobium japonicum* and *Rhizobium lupini* nodulate soybeans and lupins respectively. They are alkali producing slow-growing rhizobia. This class of

rhizobia needs 3 to 5 days before the broth media develops a moderate turbidity (Beynon, 1980), on average, these microbes take 6 hours to double when favorable conditions are present. Besides, they grow well when carbon is obtained from pentoses. The members of this class are typically rod-shaped, with single polar flagella and nodulate legume plants in the tropical areas. Rhizobia bacteria do not produce endospores; the encounters of uneven Gram-staining depend on the culture age. Bacteria cells from new cultures and young nodule bacteroids display an even Gram-staining whereas older bacteria cells display unstained regions in the cell producing a banded appearance. The areas, which appear unstained, have been recognized as polymeric hydroxybutyric acid granules, which are usually large and when observed under the phase contrast microscope, the granules are refractive hence the reason for unstained regions (Hungria *et al*., 2000).

2.2.3 Taxonomy of rhizobia

Rhizobia microorganisms have the ability to fix biological nitrogen through the roots nodules or stems of legume crops. These microorganisms are of economic and agricultural importance since they are a potential input of nitrogen into the soil that is under cultivation. There are two main classes of Proteobacteria rhizobia namely beta and alpha-Proteobacteria with the majority of them falling under the Rhizobiales order (Maheshwari, 2010). The group of Proteobacteria comprises of a variety of non-symbiotic bacteria (Laguerre *et al*., 2001). The rhizobia bacteria are grouped into 17 genera including *Shinella, Microvirga, Devosia, Mesorhizobium, Ochrobactrum, Cupriavidus, Azorhizobium, Methylobacterium, Bradyrhizobium, Ensifer, Burkholderia,*

Herbaspirillum, Phyllobacterium, Ralstonia, Pseudomonas and *Rhizobium*. Through studies, the diversity of rhizobia bacteria has revealed significant information on specific genes that are well adapted to some environments. The genes important for the beneficial symbiosis interaction process with plants might be more closely connected with the legume plant than the life forms of microorganisms. These genes are obtained through bacterial conjugation as opposed to from a typical predecessor (Hirsch *et al*., 2001).

2.2.4 Symbiotic biological nitrogen fixation

A German agronomist Hermann Hellriegel was the first to discovered BNF (Dakora, 2008). Biological nitrogen fixation occurs when nitrogen from the atmosphere is transformed to ammonia by an enzyme known as nitrogenase in the reaction ($N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$). The reduction of nitrogen into ammonia requires ATP energy for the oxidation of sugars and other compounds (Canfield *et al*., 2010). The host plant in the process of photosynthesis synthesizes the sugars that undergo oxidation. One gram of nitrogen fixes 1 to 20 grams of carbon in photosynthesis an indication that BNF requires extra energy that can be put to use in the production of an additional photosynthetic product (Thies and Grossman, 2006).

According to Giller (2001), the formation of nodules, the ATP synthesis as well as the plant growth requires phosphorus. Biological nitrogen fixation as well requires electrons. The sources of electrons differ with organisms. Sources such as ferredoxin, ademine, and flavododins are protein in nature and are highly reductive. Nitrogenase is

an enzyme, which is used in BNF. When the availability of oxygen is low, compartmentation usually occurs in cyanobacteria. In *Azotobacter*, the low oxygen content is recognized by active respiration of the bacteria while in rhizobia the presence of low oxygen tension is identified by little or lack of leghaemoglobin (Raina *et al*., 1988). In rhizobia bacteria, the host plant synthesizes globine while the *Rhizobium* synthesizes the heme. Both the rhizobia and the host plant make Leghaemoglobin. The enzymes liable for nitrogenase action are susceptible to destruction in the presence of oxygen (Moling and Bisseling, 2015). Due to this reason, most of the bacteria stop producing the enzyme when oxygen is present. A majority of nitrogen-fixing bacteria exists in anaerobic conditions, and when oxygen is present, the nitrogen-fixing bacteria are bound to a protein called leghemoglobin (Ott *et al*., 2005).

2.3 Limitations of biological nitrogen fixation

According to Olivares *et al*. (2013), the actual estimation of biological nitrogen fixation is soil is problematic. However, a farmer can observe some characteristics that will allow confirmation if nitrogen content is enough in common field legumes. Slow growth and light green leaves in newly planted common bean plants is an indication of inadequate nitrogen fixation. In the field, reduced nitrogen fixation is because of stress and poor nutrition in plants, which leads to lack of native soil rhizobia responsible for BNF. Two to 3 weeks after sowing small nodules should appear in legume plants. However, if the nodules are too small or very few, then a small amount of nitrogen fertilizer should be applied to enhance crop production (Hardarson, 1993).

Several natural components can influence the number of rhizobia bacteria found in the soil. According to Dakora *et al*. (2008), the most significant factor is rainfall. Areas with inadequate rainfall usually have fewer numbers of native rhizobia. The decrease in numbers is because the *Rhizobium* bacteria survive better in moist soils as compared to waterlogged soils (Kant *et al*., 2008). Additional conditions like the soil pH and temperature additionally are essential. In the case of extreme soil temperature of above 35^0C and very low pH, the *Rhizobium* bacteria will die. The rhizobia prefer a soil temperature of about 25°C to 30°C and soil pH of 6.0 to 6.8 (Bordeleau and Prévost, 1994).

2.4 Measuring nitrogen fixation

The fixation of nitrogen can be determined by the utilization of acetylene reduction assay, which is a short-term method, and the analysis of soluble nitrogen in the xylem (Unkovich *et al*., 2008). In acetylene reduction method, the enzyme nitrogenase speeds up the reduction of both nitrogen and acetylene. In acetylene reduction assay, the whole plant or detached nodules are placed in a vessel having 10 % acetylene (Williams and Carpenter, 1998). The amount of ethylene gas usually expressed in micromoles is then determined by the utilization of gas chromatograph. This approach displays a precise and instantaneous measure of nitrogenase activity present.

For long-term estimation of nitrogenase activity, measurement of daily and seasonal changes must be done to establish the actual activity. However, variations usually occur due to the variable temperature, humidity and light intensity. The main problems

associated with this method is that there is always need of standardizing the produced ethylene with the exact rates at which nitrogen fixation occurs, this is because the often used ratio of 3:1 acetylene to nitrogen is sometimes not valid (Biswas *et al*., 2000). Secondly, the activity of nitrogenase in some nodules declines significantly once the nodules have been detached from the plant. Lastly, the method is not applicable to plants that have long roots since it is hard to collect the root nodules. However, there is always a possibility of doing the acetylene reduction assay in situ by confining the plant to open-ended chambers (Mattia *et al*., 2006).

The latter method of estimating biological nitrogen fixation is considered as a medium term because it involves events that proceed beyond one hour in a plant. In this method, nitrogen is transported into the leaves as ureides, allantoic acid, asparagine and glutamine, these solutes contain nitrates and organic compounds of nitrates found in the roots. The estimation of fixed nitrogen is determined by correlating nitrogen fixed in forms of allantoic acid, ureides, glutamine and asparagine and nitrogen derived from the soil by analyzing plant cell sap. The above methods are easy inexpensive and non-destructive (Unkovich *et al*., 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

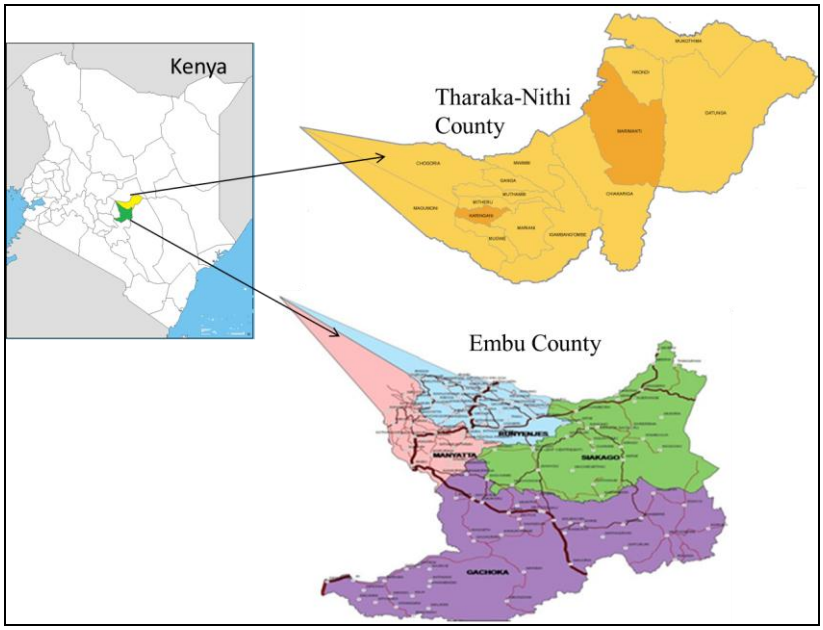

Figure 3.1: Map of Kenya showing Embu and Tharaka Nithi counties

3.2 Bean variety identification and collection of seeds

Ten common bean cultivars were identified by conducting an in-person interview with farmers in Eastern Kenya (Fig 3.1). Sixty farmers were interviewed, 32 households in Embu County and 28 households in Tharaka Nithi County. The two counties were chosen due to their high production of diverse common bean cultivars in both upper and low midland agro-ecological zones. Based on farmers' interview, the preferred and grown bean cultivars were; Kasango, Mwitemania brown, Mwitemania white, Karoyo, Muviki and Gacere and four non-climbers; Rose cocoa, Geturu, Kabuu and Kayiero. Correct identification of these bean varieties was done at Kenya Agricultural Livestock and Research Organization (KALRO) in Embu. Quantities of 500 grams of healthy

untreated seeds of each cultivar harvested by the interviewed farmers in the previous season prior to the interview were collected randomly. The collected seeds were used as the main treatments in the greenhouse.

3.3 Soil characterization and Analysis

Soil samples from four farms; two in each County were collected prior to the start of long rains of March. The sampling of soil was carried out diagonally from 20 locations in each of the selected farms by making a cut out section of 20 cm deep using a spade. The spade was sterilized before making each cut out with 5 % NaClO solution and then rinsed in three changes of sterile water after which it was dried with a sterile cloth. The obtained soil samples were mixed thoroughly to make a composite sample, which after drying was sieved through a 2 mm diameter strainer to make a homogenous composite soil that was used in the greenhouse.

A sample of 1kg of the obtained composite soil was analyzed for soluble salts in the laboratory by the use of both physical and chemical methods. Walkley-Black technique established the carbon content. Both Ca and Mg ions were assessed by the use of atomic absorption spectrophotometry while Bray-I technique assessed soil phosphorus. The soil texture characteristics ware estimated through sieving 100 g of soil through a brass sieve of different pores size to separate clay, silt, coarse and fine sand. In establishing soil pH, 5 g of soil sample was dissolved in 100 ml of distilled water and then left for a period of 1 hour to mix. The supernatant was then used to measure the pH of the soil.

The homogeneous composite soil that was used in the greenhouse was sterilized by heating the soil in the oven at a temperature of 80^0C overnight (Robert, 1993).

3.4 Preparation of yeast mannitol broth

The yeast mannitol broth was made by combining 1 gram of baker's yeast, 0.5 g K_2HPO_4, 10 g of Mannitol, 0.2 g $MgSO_4$, 0.1 grams of NaCl, as well as 1 gram of $CaCO_3$ so as to give the broth the pH of 6.8. The ingredients were suspended in a liter of distilled water, and then heated to boil and then mixed well. The Mannitol media were then autoclaved at a temperature of 121°C and pressure of 15 atmospheres for 15 minutes (Corry *et al*., 2011).

3.5 Field trap cultures and isolation of indigenous strains

The trap cultures of native rhizobia were set in four selected farms in both counties of Embu and Tharaka Nithi using MAC 64 and MAC 13 bean varieties from Kenya Seed. The MAC 64 and MAC 13 bean varieties were used because they are compatible with the native rhizobia in Eastern Kenya. Quality seeds of both MAC 13 and MAC 64 were selected and planted in each farm after tilling. The bean varieties were supplied with phosphorus by applying Triple superphosphate (46.0% P_2O_5) fertilizer at rates of 50 kg ha^{-1}. Thirty days after emerging, 10 bean plants from every farm were randomly sampled and harvested by making a 15 cm radius circle around the plant with a cut out section of 20 cm deep using a spade. The clump was then lifted slowly and soil carefully removed to abstain from removing secondary roots from the plant. The root nodules were detached along with small few roots and washed in sterile water to remove the soil particles. Nodules were packed in sampling vials containing desiccated

silica gel and cotton wool and transported to the research laboratory for the isolation of rhizobia.

In the lab, nodules were surface sterilized by wrapping them in a muslin cloth containing 90% alcohol for 1 minute. The nodules were then subjected to 6 changes of sterilized water after which the small roots were removed. The obtained nodules were then crushed to release the bacteria by a sterile glass rod in a watch glass containing 0.5 ml of sterile water. To ensure that proper sterilization was performed, the plates containing YEMA complemented with Congo red. One plate was streaked with a loop full of sterile water (control), second plate was streaked with water used for the sixth change of the root nodules and third plate was streaked with a loop full of the nodule exudates. The plates that had colonies were picked and subjected to further purification. If a culture had many colonies, the colonies were aseptically transferred to separate plates of CR- YEMA and treated as separate isolates (Kneen and LaRue, 1983).

3.5.1 Verification of isolated rhizobia

Typical rhizobia were recognized by Gram staining, morphological appearance and the production of acidity or alkalinity in YEMA with bromothymol blue. In Gram staining, rhizobia bacteria were obtained from the plate by a sterilized wire loop; a smear was then made on a glass slide containing a drop of normal saline. The slide containing the smear was then stained with crystal violet and left for 1 minute to react. Excess crystal violet solution was then rinsed with water; the excess water was then drained. Iodine solution was then added to the slide and left for one minute to react. Excess iodine was drained off and ethanol was added to decolorize the iodine. To remove excess iodine,

the slide was rinsed with water after which safranin was added, left for one minute after which the slide was rinsed with water. The slide was then observed for Gram-negative rods under the microscope by use of the oil immersion objective (Cooper and Rao, 2006).

The growth characteristics of rhizobia were identified by using three methods. In the first method, YEMA with Congo red was prepared, after which it was inoculated with rhizobia and incubated at a temperature of 28^0C. The identity of rhizobia was recognized by weakly absorbing the dye a characteristic which is not found in any other agro-bacteria. In the second method, acid production was tested by use of plate culture; 1 ml of Bromothymol blue was carefully added to 10 ml of YEMA and left to solidify. A loop-full of rhizobia was streaked on the media and incubated at 28^0C for 2 days. The production of yellow coloration was detected, an indication of rhizobia bacteria. In the last method, the rhizobia bacteria were cultured in plates containing peptone glucose agar, then incubated at 28^0C for 48 hours. The absence of bacteria growth was a clear indication of the presence of *Rhizobium* bacteria. The lack of growth was because rhizobia bacteria prefer mannitol as the carbon source and not peptone (Arora *et al*., 2001).

3.5.2 Inoculum preparation

Pure isolates of native rhizobia, exotic and a mix of native and exotic rhizobia were aseptically transferred into three different identical conical flasks having 100 ml of YEM broth. The rhizobia isolates were then incubated in a rotary shaker at 28^0C, 7 days

in advance of planting time. After 7 days, the three bacteria isolates revealed moderate turbidity in YEMB a clear characteristic of viable rhizobia (Corry *et al*., 2011).

3.6 Greenhouse bioassays

The greenhouse experiment was set as a completely randomized design (CRD) with 10 common bean varieties as the main treatment and four rhizobia inoculants as a sub-treatment. The ten bean varieties included Kabuu, Gacere, Geturu, Muviki, Mwitemania brown, Mwitemania white, Kasango, Kayiero, Karoyo and Rose cocoa. Rhizobia inoculants included a consortium of native rhizobia, exotic rhizobia (from MEA, Kenya), and a mix of native consortium and exotic rhizobia. Four treatments were set where ten common bean varieties were grown in sterilized soil inoculated with a consortium of native rhizobia (T1), exotic strain (T2), a mix of native and exotic rhizobia (T3), and a control, with no inoculation (T4). Each treatment was then replicated 3 times with 2 plants in each pot. The experimental research was conducted to examine the influence of different rhizobia inocula on the growth of ten common bean varieties.

3.7 Sterilization and pre-germination of seeds

Uniform seeds of the ten common beans were surface disinfected by submerging them in 95 % ethanol for 15 seconds to eliminate air and waxy material after which they were submerged in a sterile flask containing 3 % NaClO for 3 minutes. The seeds were then washed in six changes of sterile water. The bean seeds were then left in the last change of sterile water for four hours until they completely soaked up. The bean seeds were again washed in two changes of sterile water. A short time later, they were aseptically

transferred with a sterilized forceps onto the surface of a 2 % water agar petri-dish and incubated at a constant temperature of 25°C until they developed a radical of about 1cm long (Elfeel, 2012).

3.8 Planting and inoculation of seeds

Three holes were made in the soil medium each one centimeter deep. The water agar pre-germinated seeds were aseptically picked with a sterilized forceps and sown one seed for every hole. The sown seeds were then inoculated with an accurate quantity of 1ml of broth having 10^9 *Rhizobium* microbes by using a 1ml micro-pippete. After 5 days of emergence, the seedlings were thinned to two uniform plants for each pot. During thinning, the disturbing of soil was avoided by cutting the plant rather than direct uprooting of the plant.

3.9 Crop maintenance and harvesting

Plants were irrigated 2 times a week until sampling time, throughout this time the plant growth and leaf color were frequently noted for any abnormality. Throughout the study period, the highest temperature recorded during the day was 33^0C while the lowest was 24^0C. After 28 days, the three replicates of bean varieties from each treatment were randomly selected and separated into the shoots, roots and nodules with each treatment being kept in separate bags. The bean plant samples were then dried in an oven at a temperature of 70^0C until a constant weight was attained (Singh *et al*., 2014). The samples were then analyzed for nitrogen (N), potassium (K), and phosphorus (P).

3.10 Biomass measurements and determination of shoot nutrients (N, P, and K) content

3.10.1 Shoot nitrogen (%N)

The dry weights of roots, nodules and shoots were measured and recorded. The Kjedahl procedure established the shoot nitrogen content. The method involved the conversion of organic nitrogen into measurable ammonium cations by acid digesting the milled sub-samples by using hydrogen peroxide - sulphuric acid. The ammonium ions were obtained by first adding sodium hydroxide to the digest to make it a strongly basic solution. The highly volatile ammonia was then collected in a solution of boric acid indicator by steam distillation. The distilled ammonia gas was then titrated with boric acid by standardizing 0.005M sulphuric acid after which the end point was indicated by the pink color. The total percentage nitrogen was then calculated by the formulae: percentage N = (mL of the sample that was titrated - mL of the black titer) x the acid normality x 1.401/g sample (Clark, 2013).

3.10.2 Shoot phosphorus (ppm P)

The shoot phosphorus was determined by using colorimetric procedure where acidified ammonium molybdate was added to the milled plant tissues. The plant phosphate reacted with ammonium molybdate forming a complex compound of ammonium molydiphosphate. The amount of molybdiphosphorus present was then proportionally equated with the blue color intensity and the spectrophotometer measured the absorbed light at 660nm where the reading on the screen was converted into parts per million (Boone, 2007).

3.10.3 Shoot potassium (ppm K)

The soluble plant K was determined in a neutral extract of ammonium acetate. The function of ammonium cation was to provide an accurate and sharp separation of potassium (K) from the exchange complex. The extracted tissue was then atomized in the flame where the electrons in the atom where excited releasing wavelengths with a unique wavelength. The emitted radiations were then passed to a photocell through a filter emitting electrons that generate an electric current. The electric current created by the electrons was then measured on the photometric galvanometer. The amount of electric current created was relative to the quantity of Potassium (K) ions available in the shoot extra (Jones, 2001).

3.11 Data analyses

The greenhouse data were tested for homogeneity of variance by Bartlett test before analyses. The percentage data were arcsine ($\sqrt{x}$) transformed, whereas other data were log (x+1) transformed wherever it was necessary to achieve the expectations of ANOVA. The data reported in tables and graphs was as well back transformed. Two-way ANOVA analyzed data from greenhouse experiment as a completely randomized design. Pearson correlation coefficient was used to find out the relationship between growth parameters and root nodulation. Wherever applicable, post hoc test was executed using Tukey's HSD test ($P < 0.05$). All statistical analyses were performed using the general linear model (GLM) procedure of the Statistical Analysis System (version 9.0) (SAS Institute Inc., Cary, NC, USA).

CHAPTER FOUR

RESULTS

4.1 Distribution of common bean varieties

The most popular common bean variety among farmers who were interviewed was Kabuu with a frequency distribution of 55 while the least cultivated variety was Karoyo with a frequency of 6 (Table 4.1). Among the climber varieties, Mwitemania brown and Mwitemania white were the most prevalent varieties grown by farmers in eastern Kenya with percentage distribution of 31 and 26 respectively (Fig 4.2). Kabuu and Rose cocoa non-climbers were the most preferred cultivars (Fig 4.1) with percentage distribution of 46 and 26 respectively. The least cultivated bean variety among the climber bean variety was Karoyo with a frequency distribution of 6 while the least cultivated variety among the non-climbers was Kayiero (Table 4.1) with a frequency distribution of 14.

Table 4.1: Distribution of different varieties of common bean grown in selected places of Eastern Kenya

	Tharaka Nithi		Embu County		Total Distribution	
Variety	TUM	TLM	TUM	TLM	Frequency	%
Kabuu	16	11	14	14	55	19
MBRN •	15	10	15	12	52	18
MWHT •	14	9	11	11	45	15
Rose cocoa	7	8	10	6	31	11
Gacere •	8	7	5	7	27	9
Muviki •	8	11	5	2	26	9
Geturu	3	6	8	3	20	7
Kasango •	3	4	4	3	14	5
Kayiero	2	4	5	3	14	5
Karoyo •	1	3	1	1	6	2

KEY: TUM, Tharaka Nithi Upper Midland; TLM, Tharaka Nithi Lower Midland; EUM, Embu Upper Midland; ELM, Embu Lower Midland; ●, Climbing bean; MBRN, Mwitemania brown; MWHT, Mwitemania white.

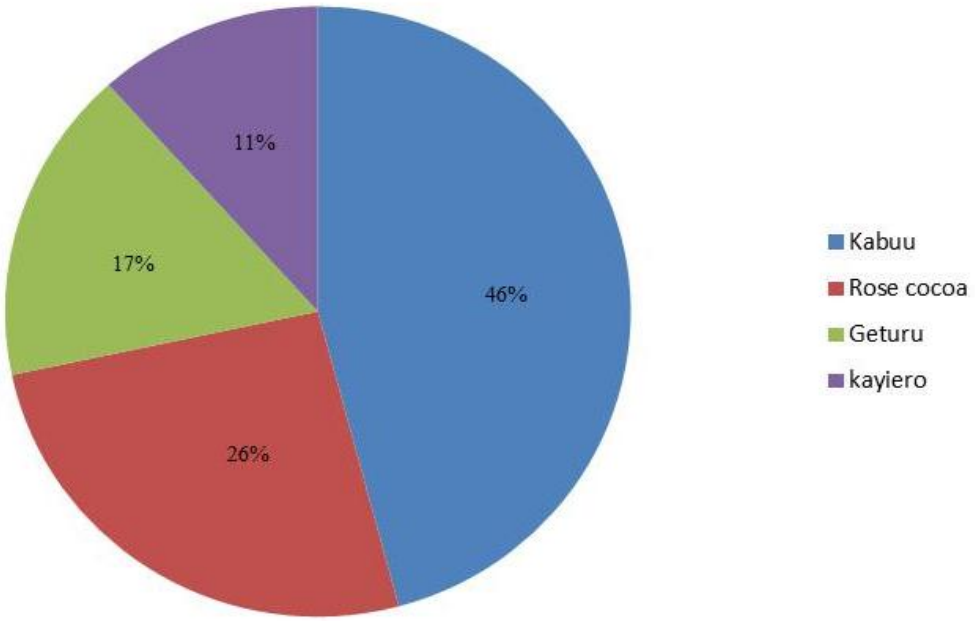

Figure 4.1: Percentage distributions of the non-climber varieties in Eastern Kenya.

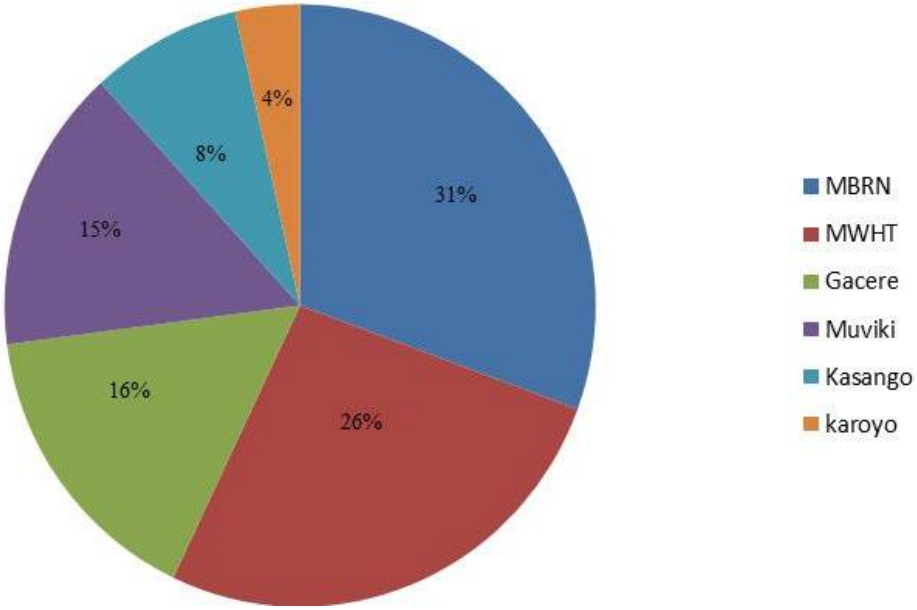

Figure 4.2: Percentage distributions of the climber varieties in Eastern Kenya.

4.2 Soil characterization

The soil used for the greenhouse bioassay was sandy clay loam with the percentage composition of sand, silt, and clay being 65%, 12%, and 23% respectively. The soil pH was slightly acidic while the soil nutrients ranged from moderate to high with phosphorus showing the highest nutrient value (Table 4.2).

Table 4.2: Soil pH and nutrient

Sample Description	pH	%N	%OC	cmol/kg			P (ppm)
				K	Ca	Mg	
Soil Nutrient level	5.93	0.24 Mod	2.8 Mod	2.7 Mod	9.1 Mod	3.65 High	143.5 High

4.3 Common bean nodulation growth parameters

4.3.1. Nodule number

Inoculation with different rhizobia isolates significantly ($P < 0.0001$) affected common bean nodulation with native rhizobia recording the highest number of nodules at an average of 68.73±6.18 per plant. Inoculation with the mix of native + exotic rhizobia and that of exotic rhizobia produced an average of 58.87 ±6.28 and 44.20±4.65 nodules per plant respectively. The control, with no inoculation, was the least producing no nodules (Fig 4.5). Inoculation with rhizobia significantly ($P < 0.0001$) affected varieties with Kabuu recording the highest number of nodules 74.42±14.94 while Rose cocoa produced the least number of nodules 17.83±4.54. Moreover, a significant ($P < 0.0001$) interaction between inoculation and common bean variety was shown (Table.4.3). Kasango, Geturu, Karoyo, Kayiero, Muviki, and MWHT varieties recorded the most

significant number of nodules in native rhizobia (Fig 4.3). Gacere and Kabuu varieties, recorded the highest significant number of nodules in the mix of native + exotic rhizobia, the varieties of MBRN, and Rose cocoa recorded no significant difference in the rhizobia treatments (Fig 4.4).

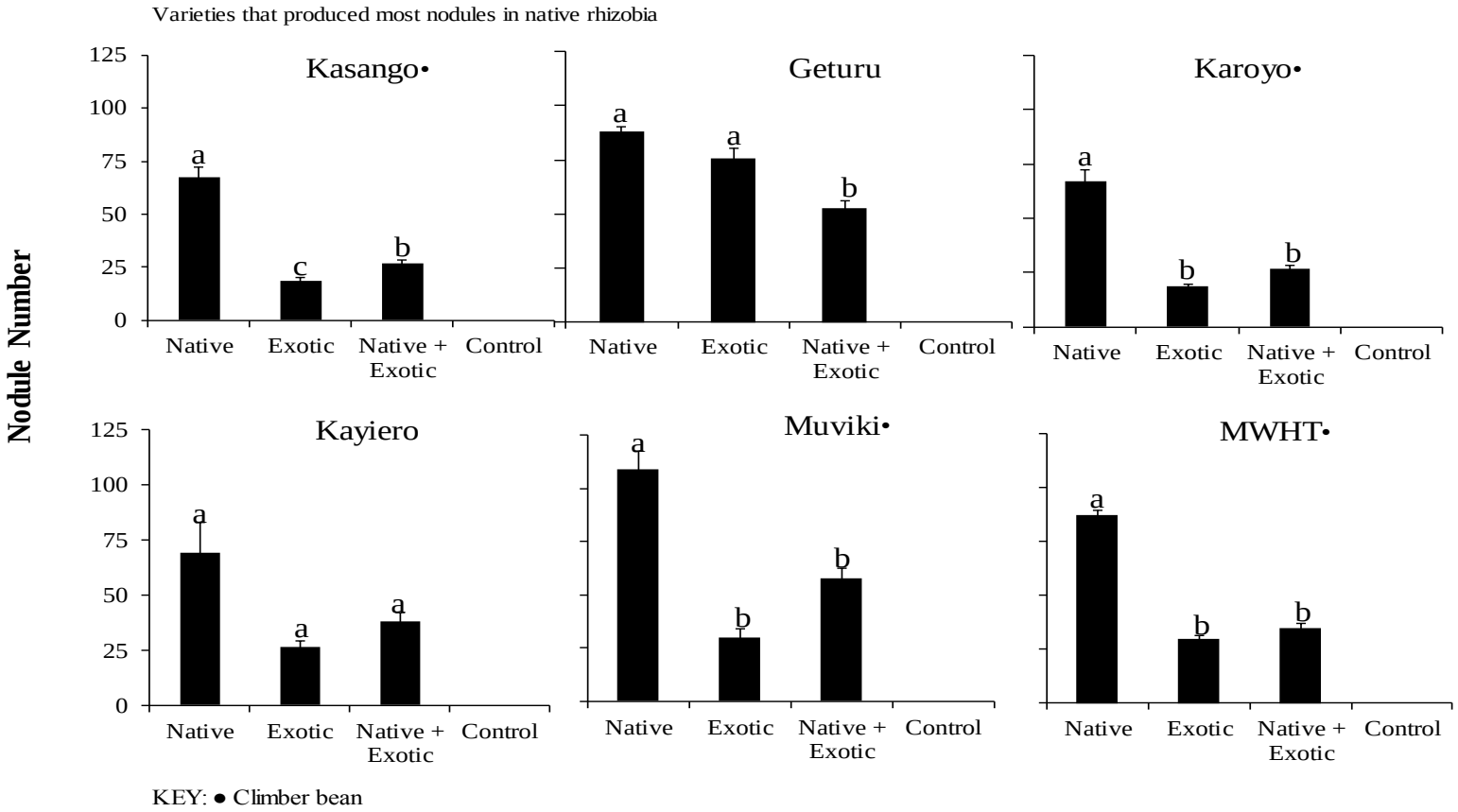

Figure 4.3: Common bean varieties with the highest significant nodule number in native rhizobia. Different letters indicates a significant difference in NNO at P< 0.05 (Tukey's HSD test).

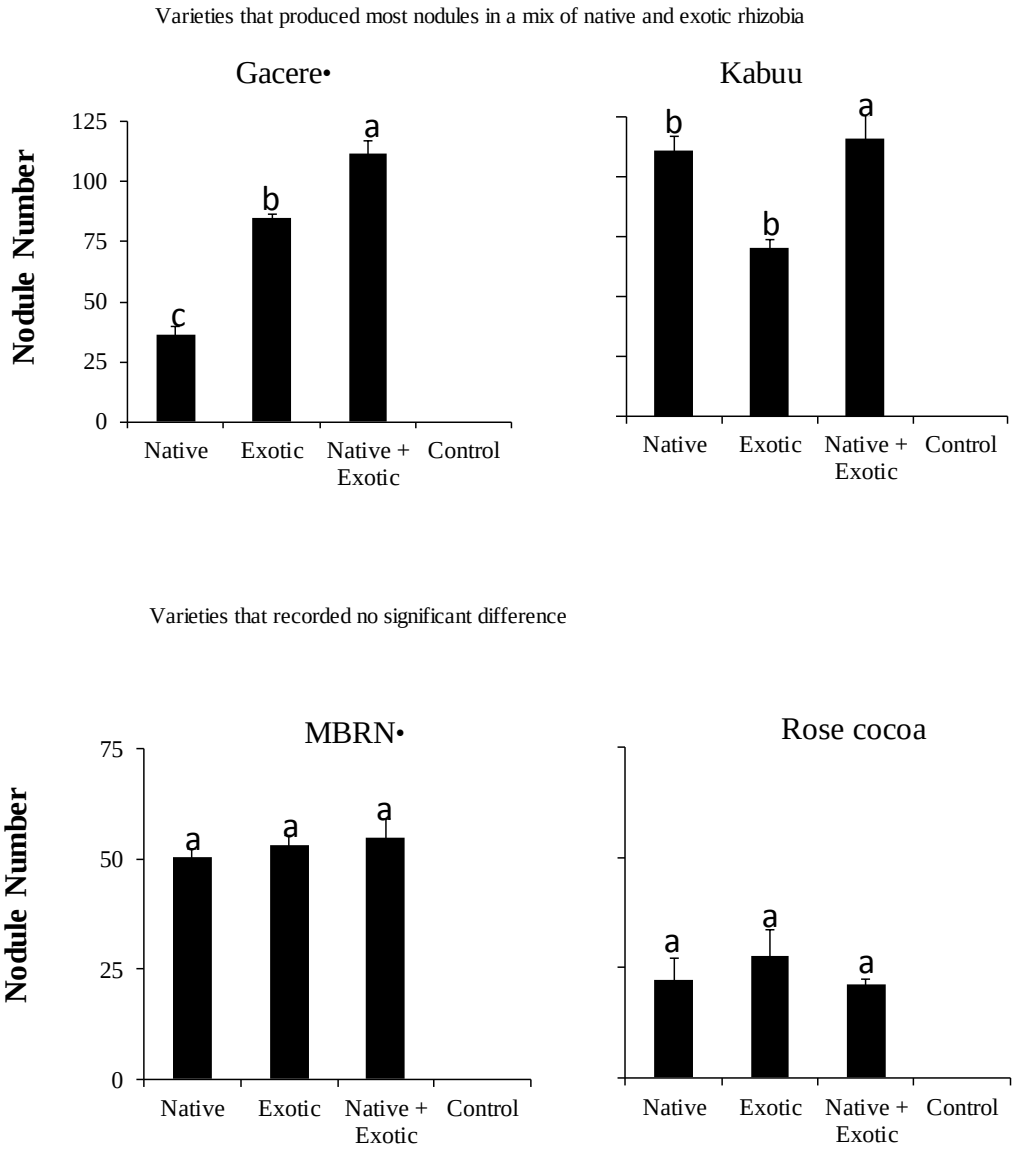

Figure 4.4: Common bean varieties with the highest significant nodule number in a mix of native + exotic rhizobia and the bean varieties that showed no significant difference in the rhizobia treatments. Different letters indicates a significant difference in NNO at $P< 0.05$ (Tukey's HSD test).

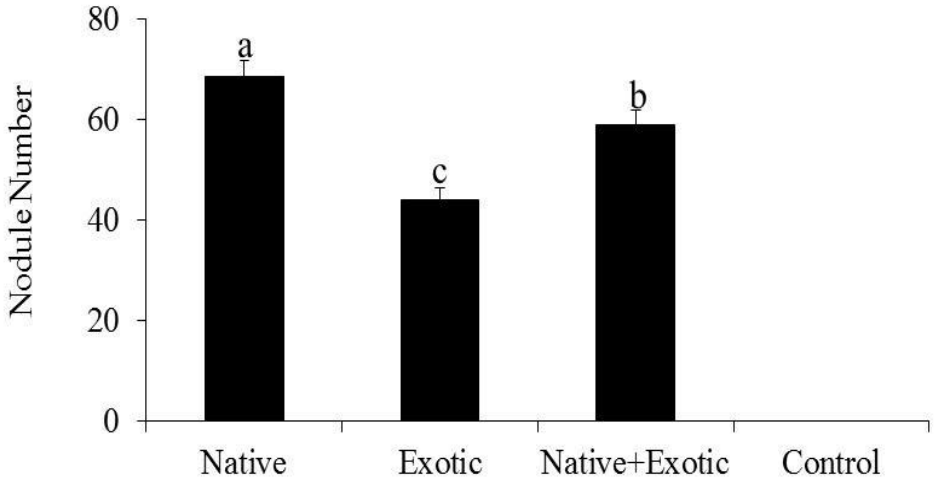

Figure 4.5: Nodule numbers for the ten common bean varieties in native, native + exotic, and exotic rhizobia. Different letters indicates a significant difference in NNO at P< 0.05 (Tukey's HSD test).

4.3.2 Nodule dry weight

Inoculation with different rhizobia isolates significantly ($P < 0.0001$) affected common bean nodule dry weight with native rhizobia recording the highest nodules dry weight at an average of 0.08±0.006 g plant^{-1}. Inoculation with exotic rhizobia and a mix of native + exotic rhizobia produced an average of 0.05±0.0049 g plant^{-1} and 0.06±0.007 g plant^{-1} respectively. The control, with no inoculation was the least producing no NDW (Fig.4.8). Rhizobia inoculation significantly ($P < 0.0001$) affected varieties with Kabuu recording the highest NDW 0.076±0.014 g plant^{-1} while Rose cocoa produced the least NDW 0.022±0.006 g plant^{-1}. Moreover, a significant ($P < 0.0001$) interaction between inoculation and common bean variety was recorded (Table.4.3). Geturu, Karoyo, Kayiero, Muviki, and MWHT varieties produced the highest significant NDW in native rhizobia (Fig. 4.6). Kasango, Gacere, and Kabuu varieties recorded the highest significant nodule dry weight in the mix of native + exotic rhizobia (Fig.4.7).

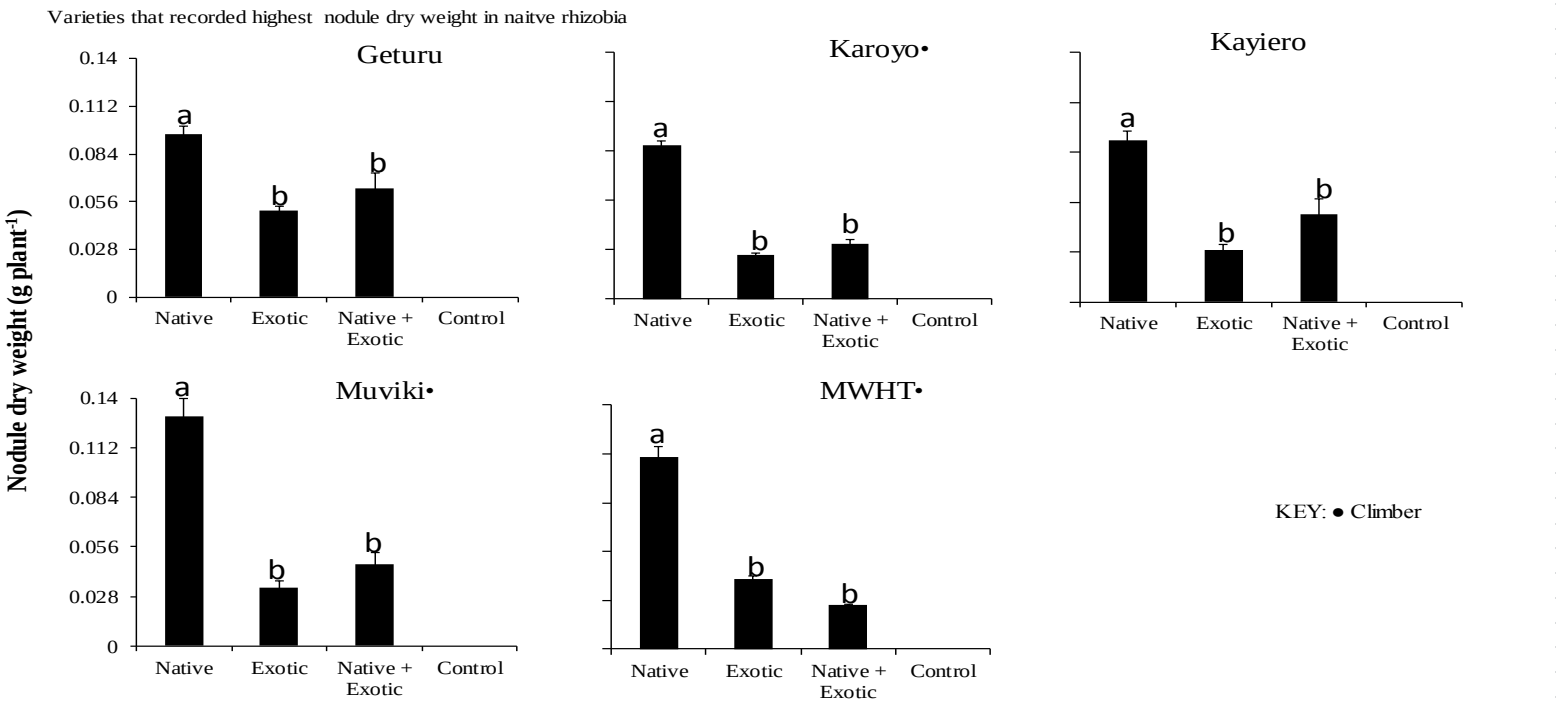

Figure 4.6: Common bean varieties with the highest significant nodule dry weight in native rhizobia. Different letters indicates a significant difference in NDW at P< 0.05 (Tukey's HSD test).

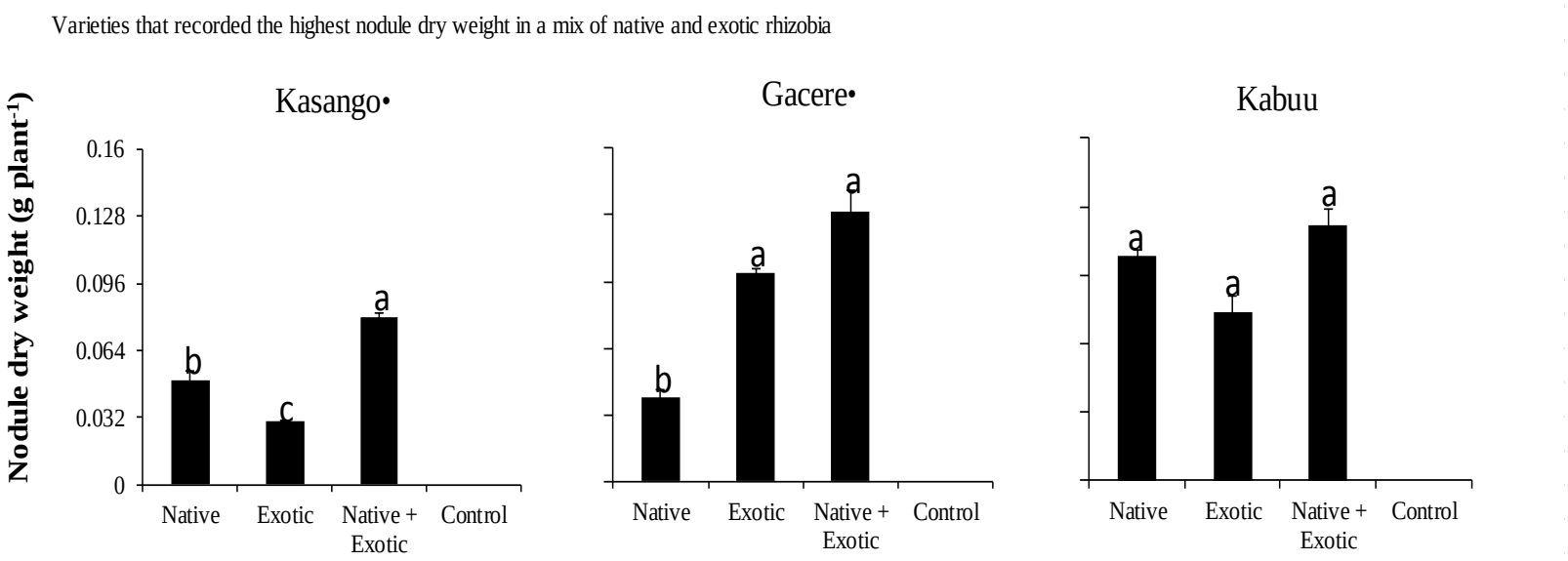

Figure 4.7: Common bean varieties with the highest significant nodule dry weight in a mix of native + exotic rhizobia. Different letters indicates a significant difference in NDW at P< 0.05 (Tukey's HSD test).

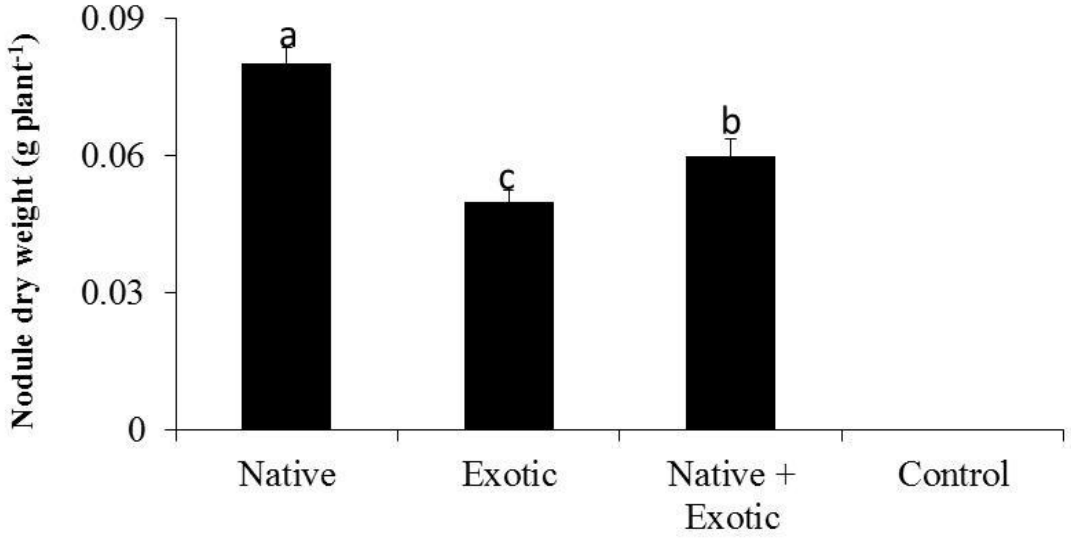

Figure 4.8: Nodule dry weight for the ten common bean varieties as affected by native, native + exotic, and exotic rhizobia. Different letters indicates a significant difference in NDW at P< 0.05 (Tukey's HSD test).

4.4 Shoot dry weight

Inoculation with different rhizobia isolates significantly (P < 0.0001) affected common bean shoot dry weight with a mix of native + exotic rhizobia recording the highest shoot dry weight at an average of 2.37±0.089 g plant^{-1}. Inoculation with exotic rhizobia and native rhizobia produced an average of 2.08±0.068 g plant^{-1} and 2.15±0.079 g plant^{-1} respectively. The control, with no inoculation, was the least producing an average of 1.67± 0.048 g plant^{-1} (Fig 4.11). Rhizobia inoculation significantly (P < 0.0001) affected varieties with Kabuu recording the highest SDW at an average of 2.72±0.16 g plant^{-1} while Rose cocoa and Karoyo produced the least SDW at an average of 1.86±0.082 g plant^{-1} and 1.84±0.062 g plant^{-1} respectively. Moreover, there was a significant (P < 0.0001) interaction between inoculation and common bean variety (Table 4.3). Kasango, Gacere, Kabuu, MBRN, Kayiero, Muviki, and MWHT varieties produced the highest significant SDW in a mix of native + exotic rhizobia (Fig 4.9).

Rose cocoa recorded the highest shoot dry weight in exotic rhizobia while Geturu and Karoyo varieties recorded no significant difference in shoot dry weight (Fig 4.10).

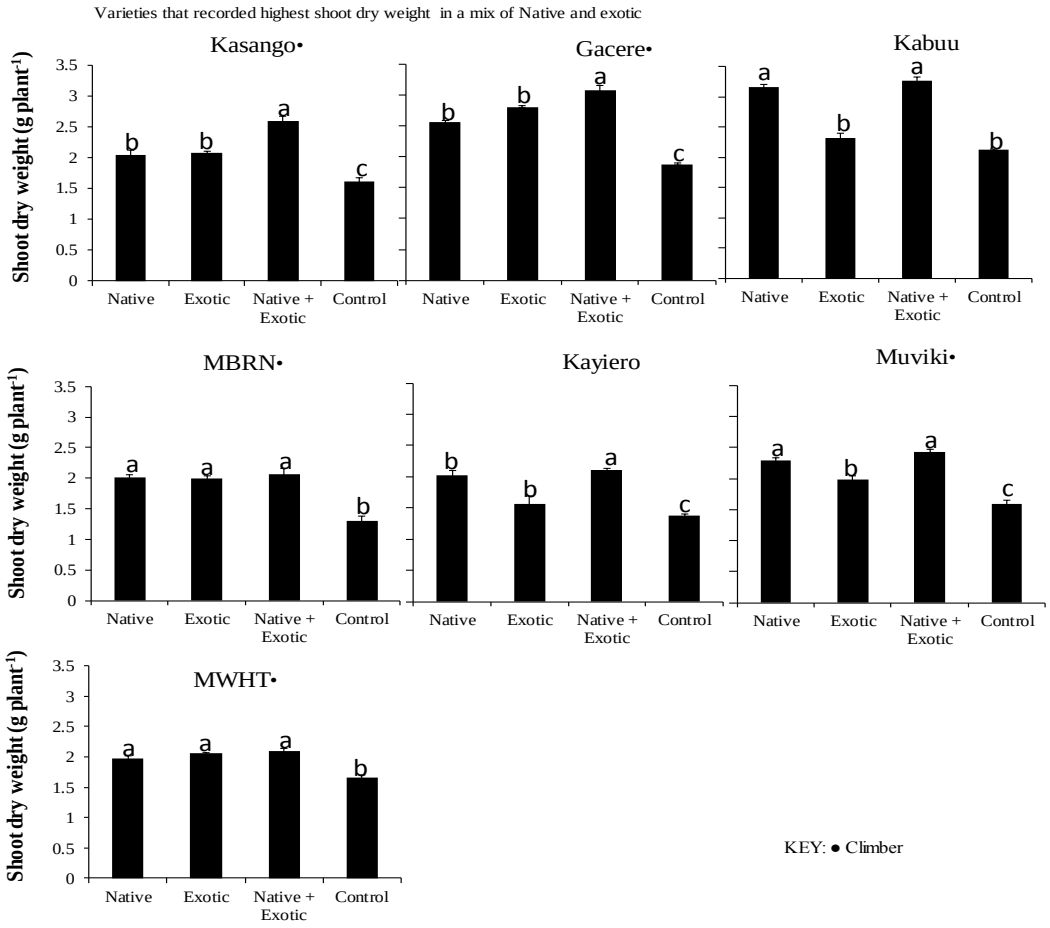

Figure 4.9: Common bean varieties with the highest significant shoot dry weight following inoculation with mix of native and exotic rhizobia. Different letters indicates a significant difference in SDW at P< 0.05 (Tukey's HSD test).

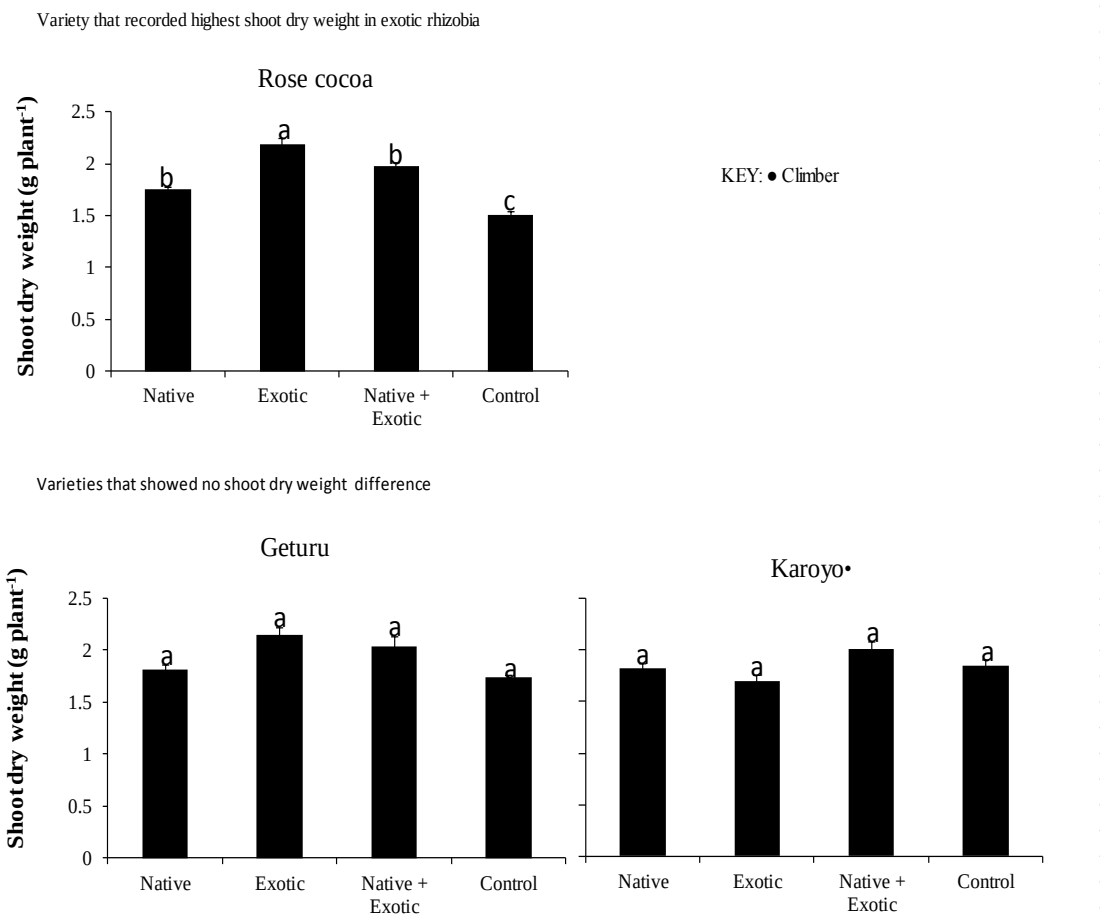

Figure 4.10: Common bean variety with the highest significant shoot dry weight in exotic rhizobia and the varieties that recorded no significant difference in SDW in the rhizobia treatments. Different letters indicates a significant difference in SDW at P< 0.05 (Tukey's HSD test).

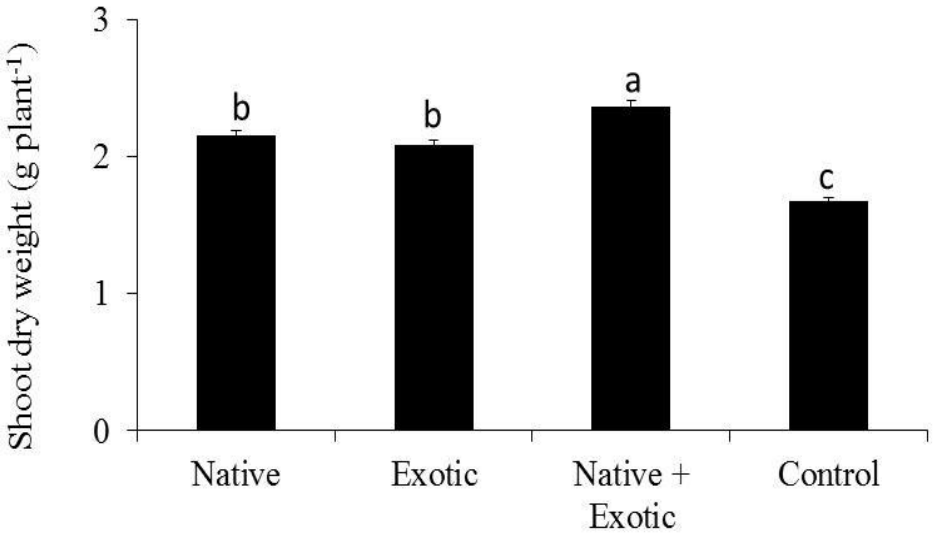

Figure 4.11: Shoot dry weight for the ten common bean varieties in native, native + exotic, exotic rhizobia and control. Different letters indicates a significant difference in SDW at P< 0.05 (Tukey's HSD test).

4.5 Root dry weight

Inoculation with different rhizobia isolates revealed no significant difference (P = 0.6439). Inoculation with all rhizobia inocula produced a RDW of more than 0.5 g plant^{-1}. The control produced the least RDW with an average of 0.52 ± 0.041 g plant^{-1}. A significant (P < 0.0001) difference was however revealed in varieties with Rose cocoa and Muviki producing the highest RDW at an average of 0.82 ±0.049 g plant^{-1} and 0.72 ±0.061 g plant^{-1} respectively. The least RDW was recorded in Mwitemania White (MWHT) variety at an average of 0.40 ±0.056 grams per plant. Interaction between inoculation and common bean varieties recorded no significant difference (P < 0.4734) (Table 4.3). Kasango, Geturu, Kabuu, Karoyo, Kayiero, Muviki, MWHT, and Rose cocoa recorded no significant difference in RDW. However, Gacere and MBRN variety recorded the highest root dry weight in the mix of native + exotic and exotic rhizobia respectively (Fig 4.12).

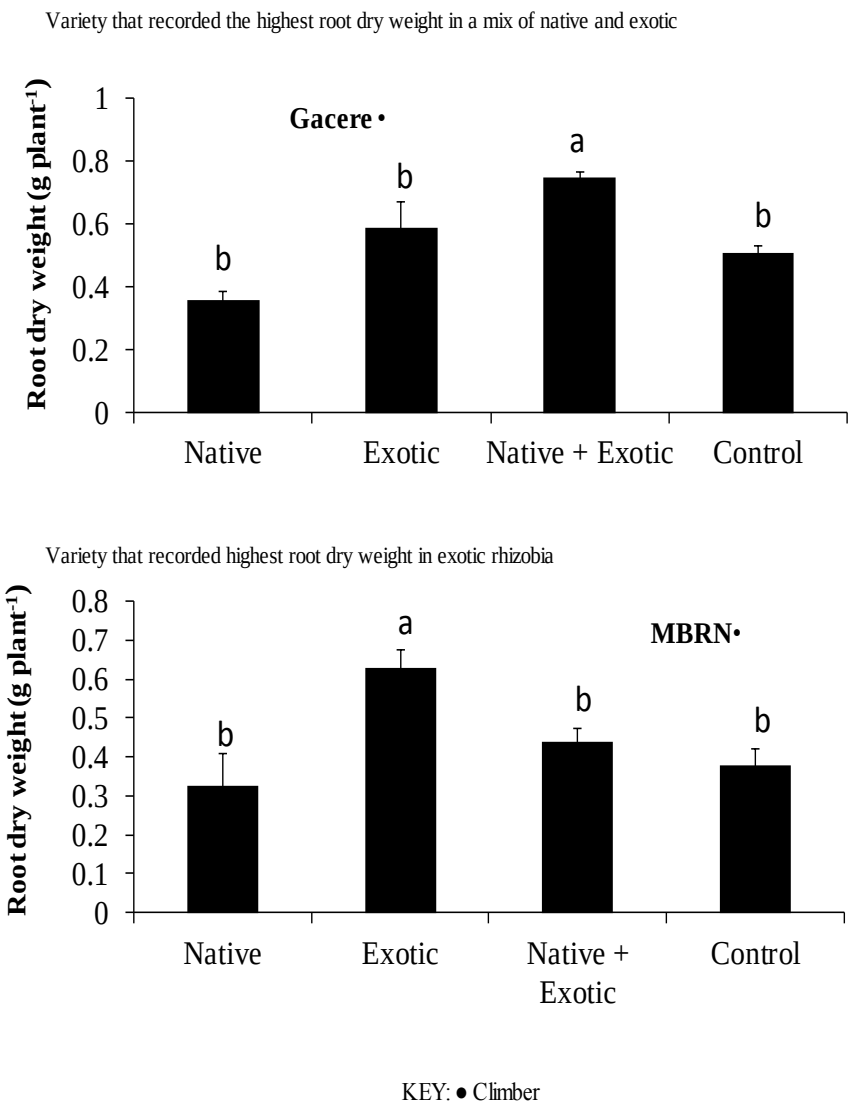

Figure 4.12: Common bean varieties that recorded the highest significant root dry weight in the mix of native and exotic rhizobia and in exotic rhizobia respectively after rhizobia inoculation. Different letters indicates a significant difference in RDW at P< 0.05 (Tukey's HSD test).

Table 4.3: Two-way-anova (mean ± SE) for nodule number (NNO), nodule dry weight (NDW), shoot dry weight (SDW), and root dry weight (RDW) as affected by rhizobia inoculation and common bean variety. Means within a column followed by different letter(s) are statistically different (Tukey's HSD test)

Rhizobia inoculation	NNO	NDW(g)	SDW(g)	RDW(g)
MIX	58.87 ±6.28b	0.0635±0.007b	2.365±0.089a	0.5734±0.0353a
EXT	44.20±4.65c	0.0484±0.0049c	2.082±0.068b	0.5668±0.0398a
NTV	68.73±6.18a	0.0803±0.006a	2.146±0.079b	0.5340±0.0432a
CONTROL	0	0	1.666± 0.048c	0.5234± 0.0413a
Variety				
KABU	74.42±14.94a	0.0757±0.014a	2.715±0.159a	0.682±0.0304ab
GACE●	58.25±13.32b	0.0691±0.015a	2.581±0.138a	0.554±0.0565bc
GETU	54.08±10.47b	0.0526±0.011b	1.932±0.073bc	0.535±0.0722cd
MUVI●	49.17±12.86bc	0.0522± 0.015b	2.074±0.106b	0.721±0.0614a
MBRN●	39.58±7.24cd	0.0455±0.008bc	1.849±0.108cd	0.443±0.0574cd
MWHT●	37.92±9.56cd	0.0438±0.013bc	1.942±0.062bc	0.401±0.0558d
KASA●	36.58±8.49d	0.0417±0.009bc	2.085± 0.115b	0.466± 0.0349cd
KAYI	33.50±9.61d	0.0421±0.011bc	1.765±0.114d	0.462±0.0318cd
KARO●	28.17±7.78de	0.0359±0.009c	1.843±0.062cd	0.415±0.0435cd
ROSE	17.83±4.54e	0.0218±0.006d	1.859±0.082cd	0.816±0.0486a
P value for the main treatments and their interaction				
Rhizobial inoculation	<0.0001	<0.0001	<0.0001	0.6439
Variety	<0.0001	<0.0001	<0.0001	<0.0001
Rhizobial inoculation x Variety	<0.0001	<0.0001	<0.0001	0.4734

KEY: MIX, combination of native and exotic rhizobia; EXT, exotic rhizobia; NTV, native rhizobia; KABU, Kabuu; GACE, Gacere; GETU, Geturu; MUVI, Muviki; MBRN, Mwitemania brown; MWHT, Mwitemania white; KASA, Kasango; KAYI, Kayiero; KARO, Karoyo; ROSE, Rose cocoa; ●, climber beans.

4.6 Common bean shoot nutrients

4.6.1 Percentage shoot nitrogen (N)

Inoculation with different rhizobia isolates significantly ($P < 0.0001$) affected shoot nitrogen in common bean when compared with control. The mix of native + exotic rhizobia recorded the highest shoot nitrogen at an average of 3.40 ±0.08 percent (Fig 4.13). The least shoot percentage nitrogen was recorded in control at an average of 2.21±0.06 percent. A significant difference ($P < 0.0001$) was also revealed in varieties with Kabuu producing the highest percentage nitrogen at an average of 3.22 ±0.20 percent. Kayiero produced the least shoot percentage nitrogen at an average of 2.19±0.12 percent. Moreover, there was a significant ($P < 0.0006$) interaction between inoculation and common bean variety (Table 4.4).

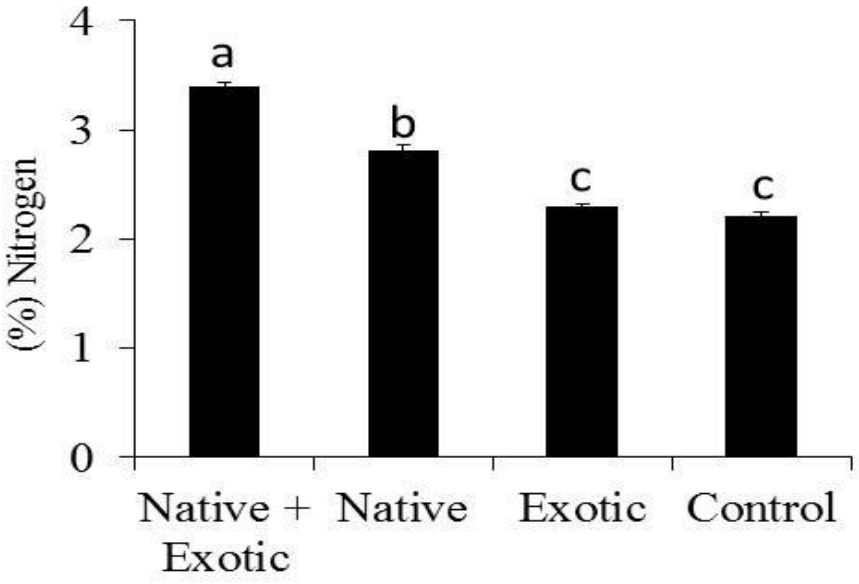

Figure 4.13: Shoot Percentage Nitrogen for the ten common bean varieties as affected by native, native + exotic, exotic rhizobia and control. Different letters indicates a significant difference in % nitrogen at $P< 0.05$ (Tukey's HSD test).

4.6.2 Shoot potassium (K)

Inoculation with different rhizobia isolates revealed a significant difference ($P < 0.0001$) in shoot potassium. The control produced the highest amount of potassium at an average of 5093.97±312.22 ppm. The native and mix of native + exotic produced an average of 4196.9±181.05 ppm and 4084.57±157.95 ppm respectively while Exotic rhizobia produced the least amount of K at an average of 3943.83±179.67 ppm (Fig.4.14). A significant difference ($P < 0.0001$) was also revealed in varieties with Kabuu showing the highest amount of potassium at an average of 6267.92±400.39 ppm. Moreover, there was a significant ($P < 0.0001$) interaction between inoculation and common bean variety (Table 4.4).

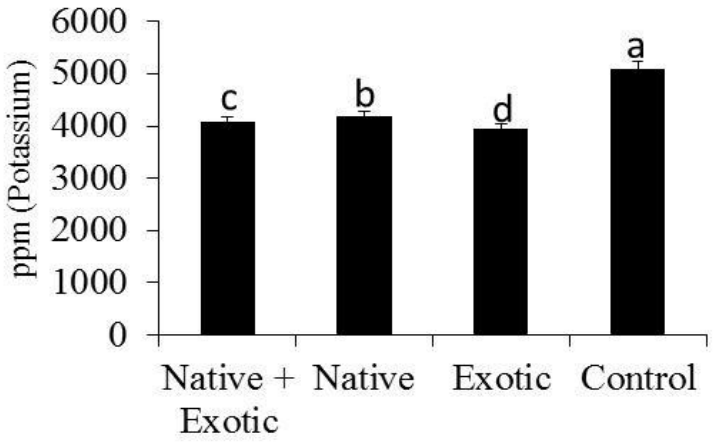

Figure 4.14: Shoot (ppm K) in the ten common bean varieties as affected by native, native + exotic, exotic rhizobia and control. Different letters indicates a significant difference in ppm potassium at $P< 0.05$ (Tukey's HSD test).

4.6.3 Shoot phosphorus (P)

Inoculation with different rhizobia isolates revealed a significant difference ($P < 0.0001$) in shoot phosphorus with native rhizobia showing the highest amount of

phosphorus at an average of 9126.70±291.12 ppm. The mix of native + exotic rhizobia and exotic *Rhizobium* recorded an average of 9000.13±588.92 ppm and 8304.27±196.0 ppm respectively (Fig 4.15). A significant difference ($P < 0.0001$) was also revealed in varieties with Kabuu producing the highest amount of phosphorus at an average of 11740.92±671.99 ppm. The least amount of phosphorus was recorded in Kayiero at an average of 6654.25±219.27 ppm. Moreover, there was a significant ($P < 0.0001$) interaction between inoculation and common bean variety (Table 4.4).

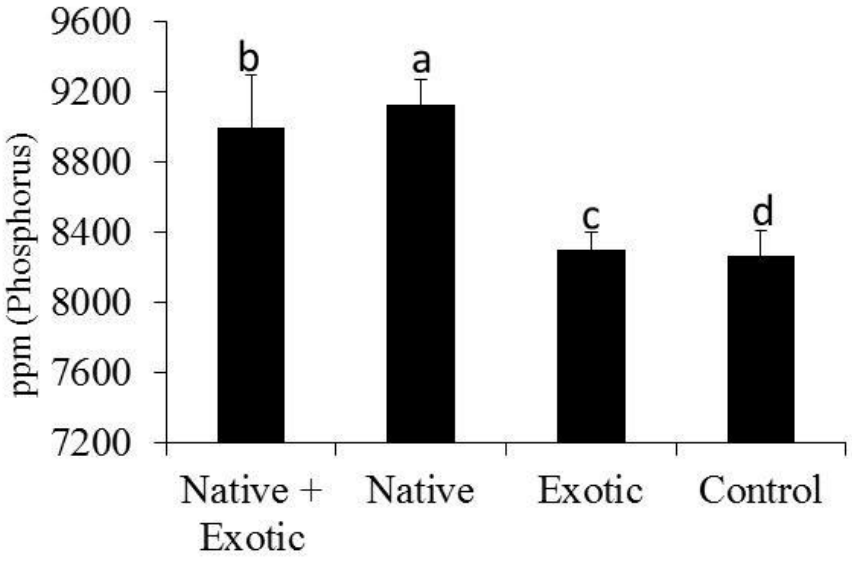

Figure 4.15: Shoot (ppm P) in the ten common bean varieties as affected by native, native + exotic, exotic rhizobia and control. Different letters indicates a significant difference in ppm phosphorus at $P< 0.05$ (Tukey's HSD test).

Table 4.4: Two-way-anova (mean ± SE) for percent shoot nitrogen (%N), ppm potassium (ppmK), and ppm phosphorus (ppmP) as affected by rhizobia inoculation and common bean variety. Means within a column followed by different letter(s) are statistically different (Tukey's HSD test)

Rhizobia inoculation	N (%)	K (ppm)	P (ppm)
MIX	3.398±0.08a	4084.57±157.95c	9000.13±588.92b
NTV	2.811±0.11b	4196.9±181.05b	9126.70±291.12a
EXT	2.295±0.04c	3943.83±179.67d	8304.27±196.01c
CONTROL	2.214±0.06c	5093.97±312.22a	8263.00± 293.59d
Variety			
KABU	3.216±0.20a	6267.92±400.39a	11740.92±671.99a
MUVI●	3.096±0.18ab	4881.58±198.99c	9378.75±782.00c
GACE●	2.938±0.17bc	5103.50±180.75b	10277.58±489.67b
KASA●	2.859±0.16cd	3602.83±194.54e	9095.50±175.74d
MWHT●	2.688±0.17de	3903.58±70.05d	9004.58±199.59e
GETU	2.63±0.15e	4900.92±462.28c	8154.92±148.95g
ROSE	2.522±0.14ef	3924.92±233.43d	8483.83±332.41f
MBRN●	2.387±0.14fg	3191.58±105.39f	6918.83±360.07i
KARO●	2.273±0.11gh	3609.08±318.51e	7026.08±190.66h
KAYI	2.187±0.12h	3912.25±224.42d	6654.25±219.27j
P value for the main treatments and their interaction			
Rhizobial inoculation	<0.0001	<0.0001	<0.0001
Variety	<0.0001	<0.0001	<0.0001
Rhizobial inoculation x Variety	<0.0006	<0.0001	<0.0001

KEY: MIX, combination of native and exotic rhizobia; EXT, exotic rhizobia; NTV, native rhizobia; KABU, Kabuu; GACE, Gacere; GETU, Geturu; MUVI, Muviki; MBRN, Mwitemania brown; MWHT, Mwitemania white; KASA, Kasango; KAYI, Kayiero; KARO, Karoyo; ROSE, Rose cocoa; ●, climber beans.

4.7 Effect of rhizobia on relative increase in SDW, Nitrogen, Phosphorus, and potassium

4.7.1 Relative increase in shoot dry weight

Inoculation with different rhizobia isolates revealed a significant difference ($P < 0.0001$) in response of the ten common bean genotypes. Kayiero and Muviki variety responded to the native rhizobia when they were compared to the control. Rose cocoa responded to exotic rhizobia when it was compared with the control. Kabuu, Gacere and Kasango variety responded to the mix of native + exotic rhizobia while MWHT an MBRN responded to all rhizobia isolates. The genotypes of Geturu and Karoyo were non responsive to either of the rhizobia (Fig 4.16).

4.7.2 Relative increase in % N (Nitrogen)

The shoot nutrient analysis revealed a significant difference ($P<0.0001$) in percentage nitrogen with all the ten bean varieties producing the highest percentage shoot nitrogen in the mix of native + exotic rhizobia when compared to the control. Muviki variety recorded the highest increase while the genotype of Kabuu produced highest percentage nitrogen in both native rhizobia and the mix of native + exotic rhizobia (Fig 4.17).

4.7.3 Relative increase in ppm of K (Potassium)

The shoot potassium analysis revealed a significant difference ($P<0.0001$) in the increase in K among the ten bean genotypes. The genotypes of Geturu, Mwitemania brown, Kayiero and Muviki responded to native rhizobia. Mwitemania white, Gacere and Karoyo variety responded to exotic rhizobia while Gacere and Karoyo variety

responded to the combination of native + exotic rhizobia. Kabuu variety was responsive irrespective of the rhizobia (Fig 4.18).

4.7.4 Relative increase in ppm of P (Phosphorus)

Shoot analysis on phosphorus indicated a significant difference ($P<0.0001$) in the increase in phosphorus in the ten common bean varieties. Geturu, MBRN, Kayiero, MWHT variety responded to native rhizobia. Karoyo and Rose cocoa variety responded to both native and exotic rhizobia. The genotypes of Gacere, Kabuu and Muviki responded to the combination of native + exotic rhizobia while Kasango variety was responsive irrespective of the rhizobia (Fig 4.19).

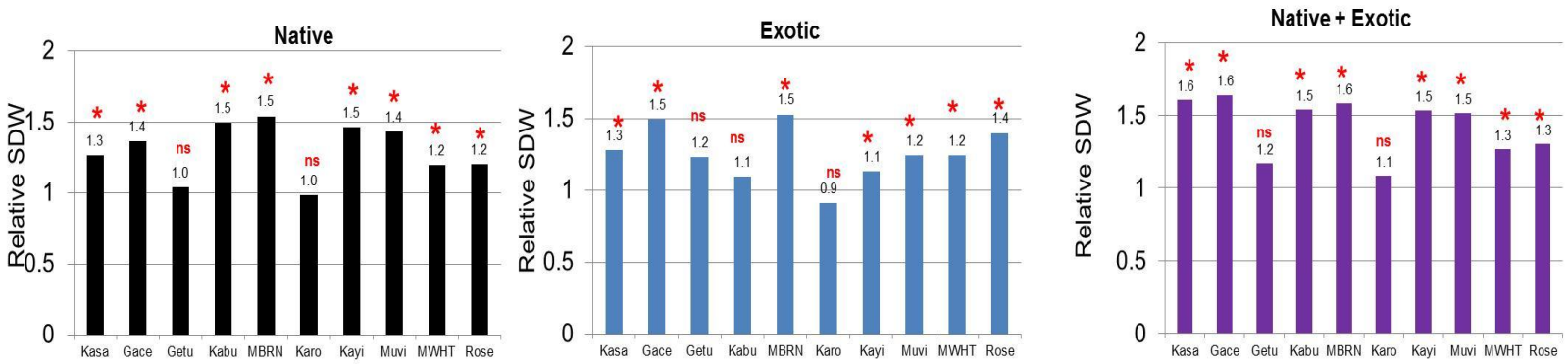

Figure 4.16: Relative increase in SDW in native, native + exotic, and exotic rhizobia**.** * indicates shoot dry weight changes under certain treatments is significant from control at P<0.05(Tukey's HSD test). ns indicated no significant difference from control.

Responsive to native
1. Kayiero
2. Muviki

Responsive to exotic
1. Rose cocoa

Responsive to mix
1. Kabuu
2. Gacere
3. Kasango

Responsive irrespective of Rhizobia
1. Mwitemania brown
2. Mwitemania white

Non responsive genotypes
1. Geturu
2. Karoyo

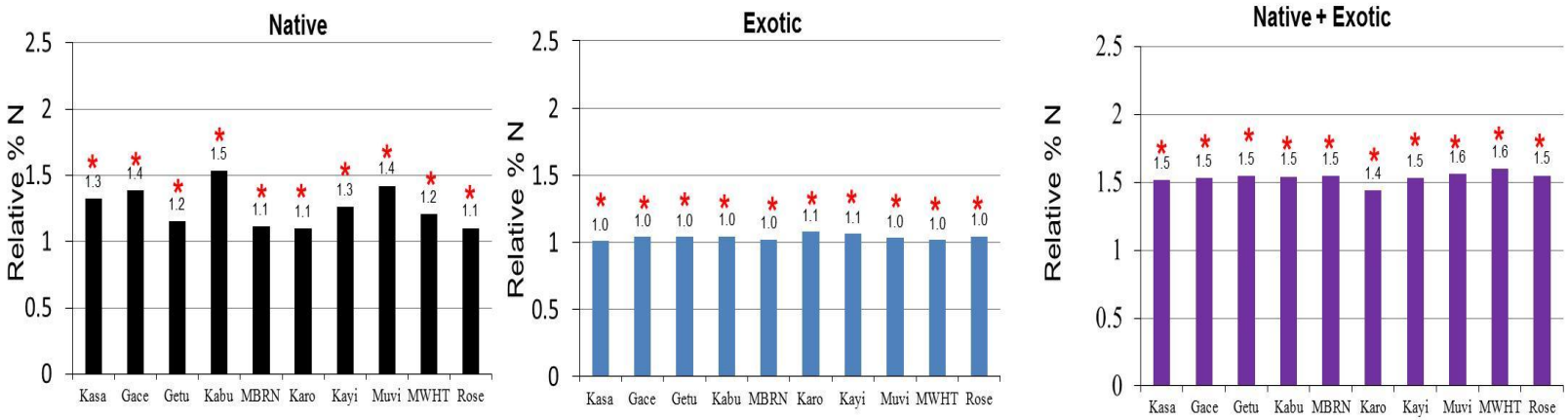

Figure 4.17: Relative increase in % nitrogen in native, native + exotic, and exotic rhizobia. * indicates shoot percentage nitrogen changes under certain treatments is significant from control at $P<0.05$(Tukey's HSD test).

<u>Responsive to mix</u>
1. Kasango
2. Gacere
3. Geturu
4. Mwitemania brown
5. Karoyo
6. Kayiero
7. Muviki
8. Mwitemania white
9. Rose cocoa
10. Kabuu

<u>Responsive to both native and mix of native + exotic Rhizobia</u>
1. Kabuu

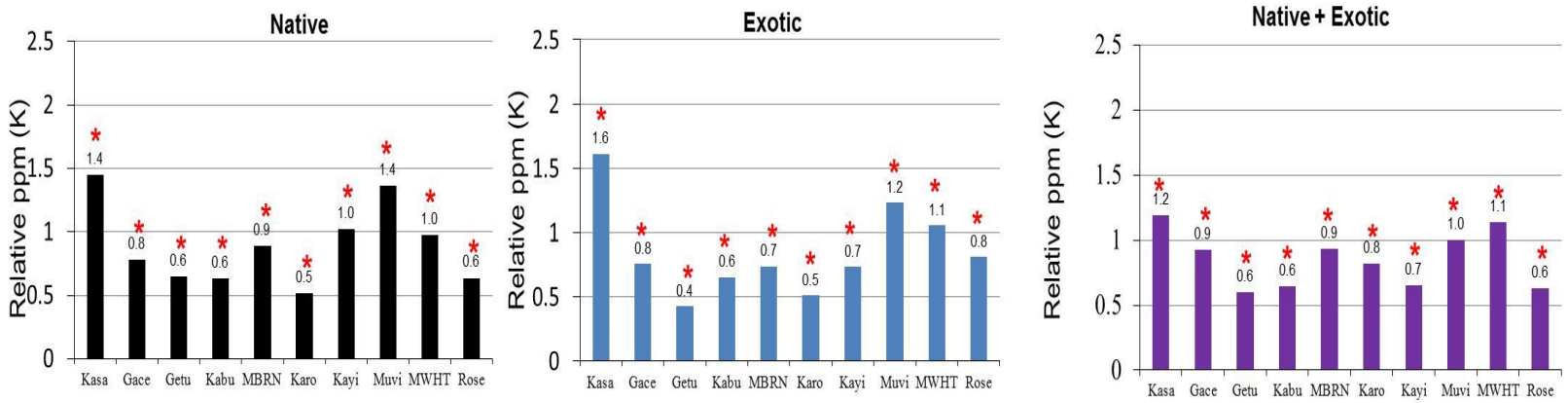

Figure **4.18:** Relative increase ppm of potassium in native, native + exotic, and exotic rhizobia. * indicates ppm (K) changes under certain treatments is significant from control at P<0.05(Tukey's HSD test).

Responsive to native
1. Geturu
2. Mwitemania brown
3. Kayiero
4. Muviki

Responsive to exotic
1. Mwitemania white
2. Rose cocoa
3. Kasango

Responsive to mix
1. Gacere
2. Karoyo

Responsive irrespective of Rhizobia
1. Kabuu

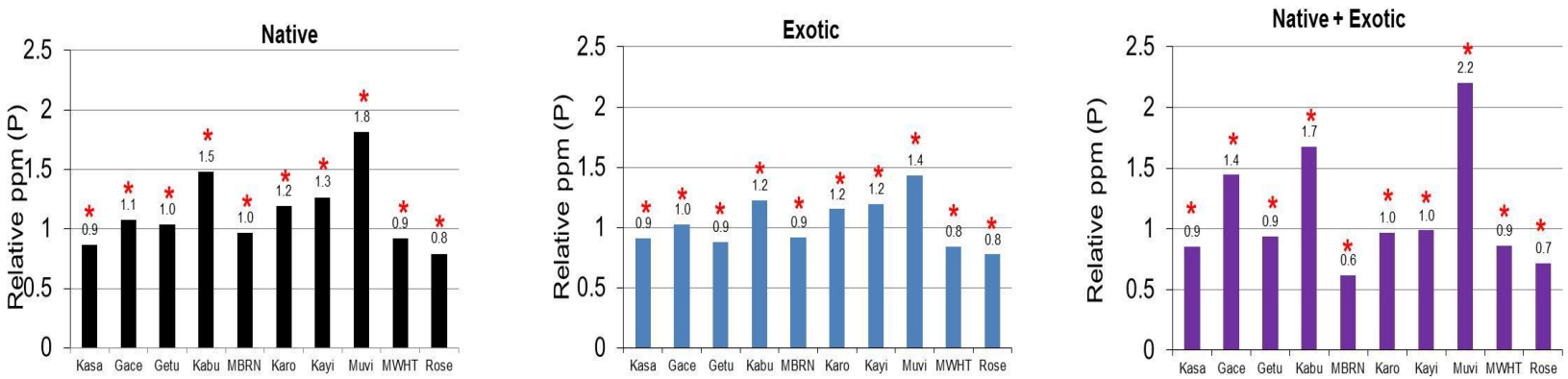

Figure 4.19: Relative increase ppm of phosphorus in native, native + exotic, and exotic rhizobia. * indicates ppm of phosphorus changes under certain treatments is significant from control at P<0.05(Tukey's HSD test).

Responsive to native
1. Kayiero
2. Mwitemania white
3. Geturu
4. Mwitemania brown

Responsive to exotic and Native
1. Rose cocoa
2. Karoyo

Responsive to mix
1. Kabuu
2. Gacere
3. Muviki

Responsive irrespective of Rhizobia
1. Kasango

4.8 Correlation between nodule dry weight and SDW, N, P, and K

There was a positive correlation between nodule dry weight and shoot dry weight (R^2 = 0.7361, P<0.0001), where an increase in nodule dry weight resulted to increased shoot dry weight (Fig.4.20). Moreover, a positive (R^2 = 0.4702, P<0.0001) correlation between nodule dry weight and percentage nitrogen was observed (Fig.4.21). Likewise there was a positive correlation between nodule dry weight and ppm Phosphorus (R^2 = 0.4888, P<0.0001) (Fig.4.22). Nodule dry weight and ppm K similarly had a positive correlation (R^2=0.6525, P<0.0001) (Fig.4.23).

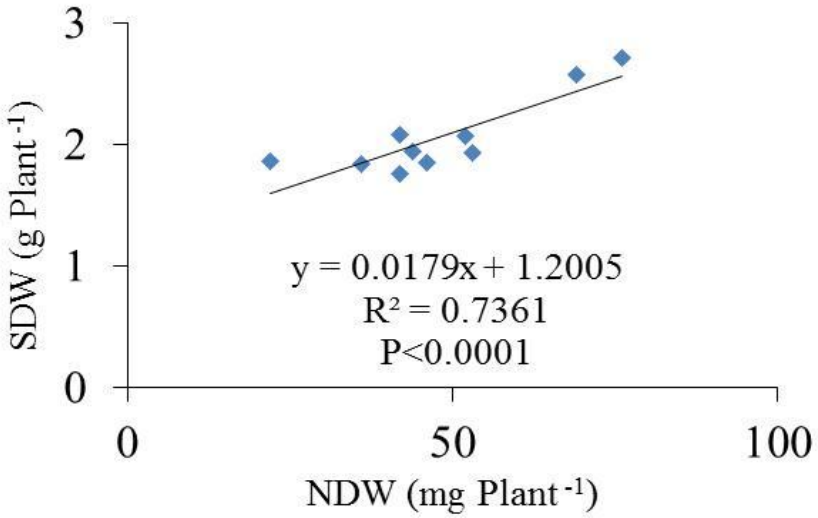

Figure 4.20: Relationship between NDW and SDW following *Rhizobium* inoculation of common bean varieties grown in Eastern Kenya.

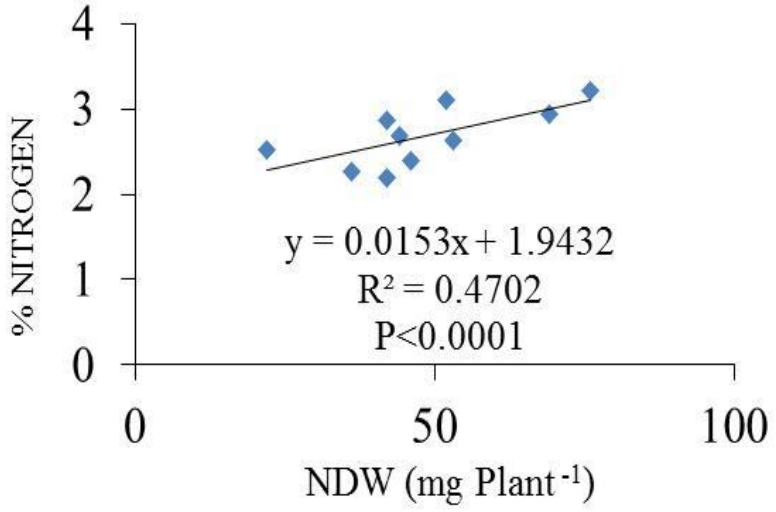

Figure 4.21: Relationship between NDW and % N following *Rhizobium* inoculation of common bean varieties grown in Eastern Kenya.

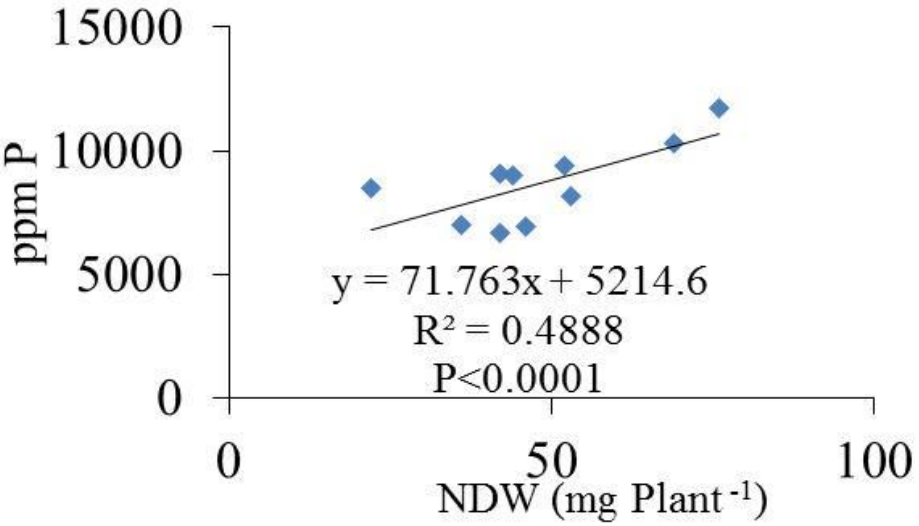

Figure 4.22: Relationship between NDW and ppm of Phosphorus (P) following *Rhizobium* inoculation of common bean varieties grown in Eastern Kenya.

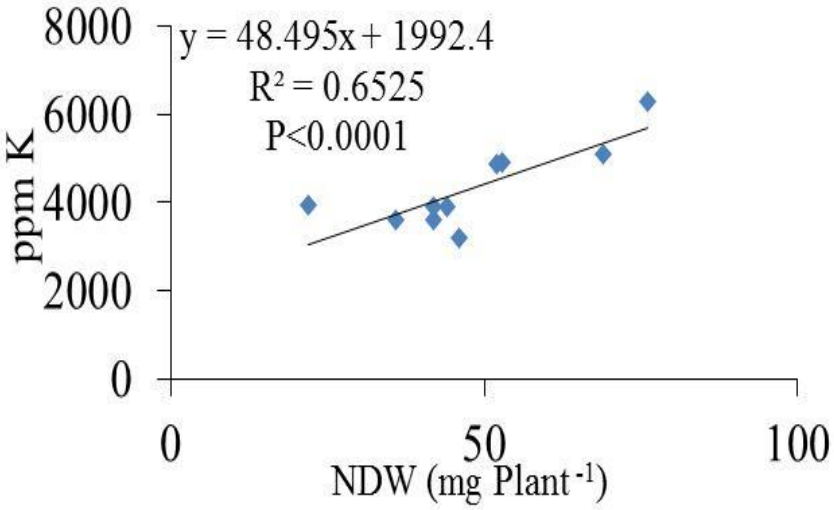

Figure 4.23: Relationship between NDW and ppm of Potassium (K) following *Rhizobium* inoculation of common bean varieties grown in Eastern Kenya.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 The physical and chemical properties of soil used in the greenhouse

The composite soil analysis on physical as well as chemical properties revealed that the Eastern Kenya soil was rich in nutrient with phosphorus being the highest. According to Whitehead (2000), the high amount of phosphorus is crucial to the proper development of leaves and dry matter production in plants. The soil analysis also revealed that the soil was less acidic with pH of 5.9. Russell (2002) suggests that bean plants require slightly acidic or neutral top soil to appropriately grow specifically when relying on symbiotic biological nitrogen fixation. The presence of nutrients such as calcium and magnesium were also an indication of suitable soil to use in the greenhouse since most plant tissues contain these essential elements (Hart *et al*., 2003).

5.1.2 Effect of rhizobia inoculation on nodulation

In nodule number production, all *Rhizobium* inoculation revealed a significant difference with a consortium of native rhizobia producing the most number of nodules ($P \leq 0.05$). These results suggested that native rhizobia are well adapted to the native bean cultivars as compared to the exotic and the mix of native + exotic (Hungaria *et al*., 2000). Among genotypes, inoculation with native rhizobia produced the highest number of nodules in average. Inoculation with native and mix of native + exotic produced the highest nodule number in Kabuu, while inoculation with exotic rhizobia produced the highest nodules in Gacere bean variety, the result relates to the work done by (Mhamdi

et al., 2002) who found that different bean genotypes prefer certain rhizobia with exotic rhizobia being as good as native rhizobia.

5.1.3 Effect of rhizobia inoculation on nodule dry weight

For nodule dry weight, all rhizobia inoculation displayed a significant difference with the consortium of native rhizobia producing the highest number of NDW ($P \leq 0.05$). These results relate to the work done by (Romdhane *et al*., 2007) who found out that native rhizobia are well adapted to the native bean genotypes and can compete effectively in root colonization as compared to the exotic rhizobia. Among genotypes, inoculation with native rhizobia produced the highest number of NDW in average. Inoculation in native rhizobia produced the highest nodule dry weight in Muviki, while inoculation with exotic and combination of native + exotic rhizobia produced the highest nodules in Gacere bean variety, these results relates to the study done by (Triplett and Sadowsky, 1992), Who found that different bean genotypes prefer certain rhizobia with exotic rhizobia being as good as native rhizobia.

5.1.4 The impact of rhizobia inoculation on common bean shoot dry weight

For shoot dry weight, all *Rhizobium* inoculation revealed a significant difference with the combination of native + exotic rhizobia producing the highest SDW ($P \leq 0.05$). These findings are related to the study carried out by Zablotowicz *et al.* (1991) who found that increasing rhizobia diversity increase shoot dry weight in bean plants. Among genotypes, inoculation with a mix of native + exotic rhizobia produced the highest SDW on average. Inoculation with the native and a mix of native + exotic rhizobia

produced the highest SDW in Kabuu, while inoculation with exotic rhizobia produced the highest SDW in Gacere bean variety; the result suggested that increased rhizobia diversity increased SDW. This as well indicates that for a higher shoot dry weight in common bean the indigenous rhizobia are not efficient and that there is need to introduce other rhizobia strains. This relates to the work done by Hungaria *et al*. (2003) who observed that for high shoot dry weight in bean plants proper combination of *Rhizobium* have to be identified to enhance more competitiveness in effective production of SDW.

5.1.5 Effect of rhizobia inoculation on root dry weight

In root dry weight, all rhizobia treatments revealed no significant difference. Among genotypes, inoculation with native and exotic rhizobia produced the highest RDW in average with Rose cocoa and Muviki varieties producing the highest RDW in native rhizobia. The result suggests that native rhizobia increase effectively the RDW on common bean. The results are similar to the work done by Kellman (2008) who observed that native rhizobia have high competitive ability in the production of RDW.

5.1.6 Effect of rhizobia inoculation on shoot nitrogen

Shoot nitrogen analysis revealed that all rhizobia inocula showed a significant difference with the combination of native + exotic rhizobia producing the highest percentage nitrogen ($P \leq 0.05$). These results are similar to the report by Singleton and Tavares (1986) that increases in rhizobia diversity increase shoot nitrogen in bean plants. Among genotypes inoculation with a mix of native + exotic rhizobia produced

the highest percentage nitrogen on average. Inoculation in the native, exotic and a mix of native + exotic rhizobia produced the highest percentage nitrogen in Kabuu; the result suggests that increase in rhizobia diversity increase percentage nitrogen. This also indicates that for higher shoot nitrogen content in common bean the indigenous rhizobia are not effective and that there is necessity to introduce other rhizobia strains. This relates to the work done by Maingi *et al*. (2001) who observed that for high shoot nitrogen content in bean plants proper combination of *Rhizobium* have to be identified to enhance more effective fixation of biological nitrogen.

5.1.7 Effect of rhizobia inoculation on shoot phosphorus

Shoot phosphorus analysis showed that all plants inoculated with rhizobia showed a significant difference in the phosphorus content with native rhizobia producing the highest phosphorus content ($P \leq 0.05$). These findings are similar to the study by Khan *et al.* (2009) who stated that native rhizobia increase shoot phosphorus in bean plants. Among genotypes, inoculation with native rhizobia produced the highest phosphorus shoot content on average. Inoculation in the native, exotic and a mix of native + exotic rhizobia produced the highest shoot phosphorus in Kabuu with the variety producing the highest phosphorus content in the mix of native + exotic rhizobia. The result suggests that increase in rhizobia diversity increase phosphorus content in shoots of bean plants. This also indicates that for higher shoot phosphorus content in common bean proper variety response should be screened and that there is need to introduce other rhizobia strains. This relates to the work done by Ramaekers *et al*. (2010) who observed that for

high shoot phosphorus content in bean plants, variety and effective *Rhizobium* have to be identified to improve shoot phosphorus in common bean.

5.2 Conclusions

i. Kabuu bean variety responded better to inoculation than other bean varieties.

ii. The common bean varieties grown by smallholder farmers responded differently in nodulation and nitrogen fixation.

iii. Consortium of native rhizobia enhanced nodulation in common bean varieties grown in Eastern Kenya.

iv. Increasing rhizobia isolates diversity enhanced nitrogen fixation in common bean varieties grown in Eastern Kenya.

v. Rhizobia inoculation increased shoot biomass, N and P in common bean varieties grown in Eastern Kenya.

5.3 Recommendations

i. For high bean yield, farmers in Eastern Kenya should adopt Kabuu bean variety.

ii. The consortium of native rhizobia should be used to enhance BNF and SDW in common bean.

iii. The effective use of rhizobia multistrain is necessary if increased yield and sustainable agriculture is to be realized.

iv. Field experiments need to be done to investigate the effectiveness of different rhizobia isolates on common bean.

v. The inoculation of efficient and compatible rhizobia should be screened further through multiple greenhouse bioassays.

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